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Moderate Immunodeficiency Does Not Increase Susceptibility to *Salmonella typhimurium aroA* Live Vaccines in Mice

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Salmonellae carrying appropriate mutations in genes of the aromatic biosynthesis pathway are effective as live vaccines in animals, and they are candidate typhoid vaccines for human use. They are also very effective as carriers of recombinant antigens from other pathogens to the immune system, eliciting circulatory, secretory, and cell-mediated immunity to foreign antigens. Their attenuation is believed to be due to their requirement for the metabolites *p*-aminobenzoic acid and 2,3-dihydroxybenzoate, which are not available in mammalian tissues. Immunosuppression (e.g., acquired immunodeficiency syndrome) is a major contraindication to the use of live vaccines. If the avirulence of Aro mutants is largely due to their auxotrophy, they should not be markedly more invasive in immunosuppressed animals. We report that wild-type *Salmonella typhimurium* M525 of intermediate virulence was much more invasive in sublethally irradiated BALB/c mice than in normal BALB/c mice, whereas sublethal irradiation had little if any effect on the invasiveness of an *S. typhimurium aroA* vaccine strain apart from a delay in its clearance from the reticuloendothelial system. *xid* mutant CBA/N mice carry an X-linked B-cell functional defect which results in immunoglobulin G3 agammaglobulinemia, and they are known to be more susceptible to salmonellae in late stages of the infection. We found that whereas male (CBA/N × BALB/c)F_1 mice (immunodefective) were more susceptible to wild-type *S. typhimurium* C5 than female littermates (immunocompetent), there was no difference in the response to the *S. typhimurium aroA* vaccine strain. The results indicate that moderate immunosuppression does not markedly enhance susceptibility to *S. typhimurium aroA* live vaccines.

Salmonellosis is a major worldwide health problem. There is an increasing interest in the development of live attenuated salmonella vaccines since they provide better protection than the whole-cell killed vaccines, probably because of their ability to induce cellular immunity in addition to humoral immunity (6, 13).

Hoiseth and Stocker (7) constructed *Salmonella typhimurium* strains carrying defined, nonreverting deletions in the *aroA* gene, rendering them avirulent by making them dependent for growth on the aromatic compounds *p*-aminobenzoic acid and 2.3-dihydroxybenzoate, which are not found in mammalian tissues. They were found to be safe and effective live vaccines for mice and, together with Aro mutants of *Salmonella typhi*, are currently being considered for use as live vaccines in domestic animals and humans. They have also been found to be very effective as recombinant vaccines carrying heterologous antigens derived from bacteria, viruses, and parasites (4, 5).

These vaccines could be used in human populations, some members of which may be immunocompromised as a result of being at extremes of age, malnourished, or suffering from concurrent disease or from an immunosuppressive disorder, e.g., acquired immunodeficiency syndrome. Immunosuppression is a major contraindication to the use of live vaccines. However, Aro mutants should not be markedly more invasive in moderately immunosuppressed hosts if their attenuation is due to their auxotrophy.

Immunosuppression is known to increase the susceptibility of mice to wild-type salmonellae. Sublethal irradiation reduces the 50% lethal dose (LD_{50}) in oral and intravenous (i.v.) challenge; irradiated mice cannot suppress bacterial growth in the reticuloendothelial system at the end of week 1 of the infection (3). The X-linked *xid* defect also makes mice more susceptible to salmonellae but in the later stages of the infection (16, 17). *xid* causes a B-lymphocyte functional defect with low serum immunoglobulin M and immunoglobulin G3 and is expressed in F_1 male mice from crosses of homozygous CBA/N females with male mice from other inbred strains (20–22). The virulence of Aro$^-$ salmonellae was explored in these two models.

MATERIALS AND METHODS

Mice. BALB/c mice were bred in this department or in the Wellcome Research Laboratories (Beckenham, Kent, United Kingdom) from breeders originally purchased from Harlan Olac Ltd. (Blackthorn, Bicester, United Kingdom). Male mice 3 to 6 months of age were used. (CBA/N × BALB/c)F_1 male and female mice were bred in this department from breeders purchased from Harlan Olac Ltd. Mice used were 3 to 6 months of age.

Irradiation. Mice were whole-body X-irradiated with 350 rads at 48 rads/min from a 240-keV source 24 h before infection (9).

Bacteria. *S. typhimurium* SL3261 (7), M525 (10), and C5 (10) (i.v. LD_{50} for BALB/c mice, $\sim10^7$, $\sim10^5$, and $<10^2$, respectively) were grown in stationary tryptic soy broth (Oxoid Ltd., London, England) cultures overnight at 37°C, and aliquots were snap frozen and stored in liquid nitrogen. For use, vials were rapidly thawed and diluted in phosphate-buffered saline (pH 7.2) to the desired inoculum strength, which was checked by pour plates.

LD_{50} determinations. Groups of three to six mice were infected i.v. in a tail vein with 0.2 ml of serial 10-fold dilutions of the culture in phosphate-buffered saline. Mortality was recorded over 4 weeks, and the LD_{50} was determined

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TABLE 1. LD_{50} values (log_{10} CFU) of *S. typhimurium aroA* SL3261 and *S. typhimurium* M525 injected i.v. in irradiated and control BALB/c mice and *S. typhimurium aroA* SL3261 and *S. typhimurium* C5 in male (immunodefective) and female (immunocompetent) (CBA/N × BALB/c)F_1 mice

Mouse strain	Log_{10} LD_{50} of salmonella strain:		
	SL3261	M525	C5
BALB/c			
Control	7.6	5.3	
Irradiated	7.2	≤1.8	
(CBA/N × BALB/c)F_1			
Female	6.5		3.3
Male	6.5		1.4

by the method of Reed and Muench (18). Results are expressed as log_{10} CFU.

Enumeration of viable organisms in liver and spleen homogenates. Mice were infected i.v. with a sublethal dose of the desired strain. At intervals, groups of three to four mice were sacrificed and livers and spleens were homogenized separately in 10 ml of distilled water with a Colworth stomacher (A. J. Seward, London, England) as previously described (8). Viable counts were determined with a Colworth droplette (A. J. Seward, London, England) or by pour plates in tryptic soy agar (Oxoid) when counts of $<10^3$ organisms were expected (8). Viable counts are expressed as geometric means of log_{10} CFU per whole organ ± 1 standard error of the mean in groups of three to four mice per point.

RESULTS

LD_{50} determinations. Table 1 shows the LD_{50} values of *S. typhimurium* M525 (wild type, intermediate virulence strain) and SL3261 (*aroA* vaccine strain) in sublethally irradiated BALB/c mice. Irradiation caused a marked increase in susceptibility to M525, with a pronounced drop in the LD_{50}. All irradiated mice died in 8 days. In contrast, irradiation had little if any effect on the virulence of the *aroA* SL3261 vaccine strain.

Table 1 also shows the LD_{50}s of SL3261 and the virulent C5 strain in *xid* mice. The immunodefective male (CBA/N × BALB/c)F_1 mice were, as expected (16, 17), much more susceptible to the virulent C5 strain than their immunocompetent female littermates, although time to death was longer than with irradiated BALB/c mice (2 to 3 weeks). On the other hand, there was no difference in the LD_{50} of the *aroA* SL3261 strain for these mice.

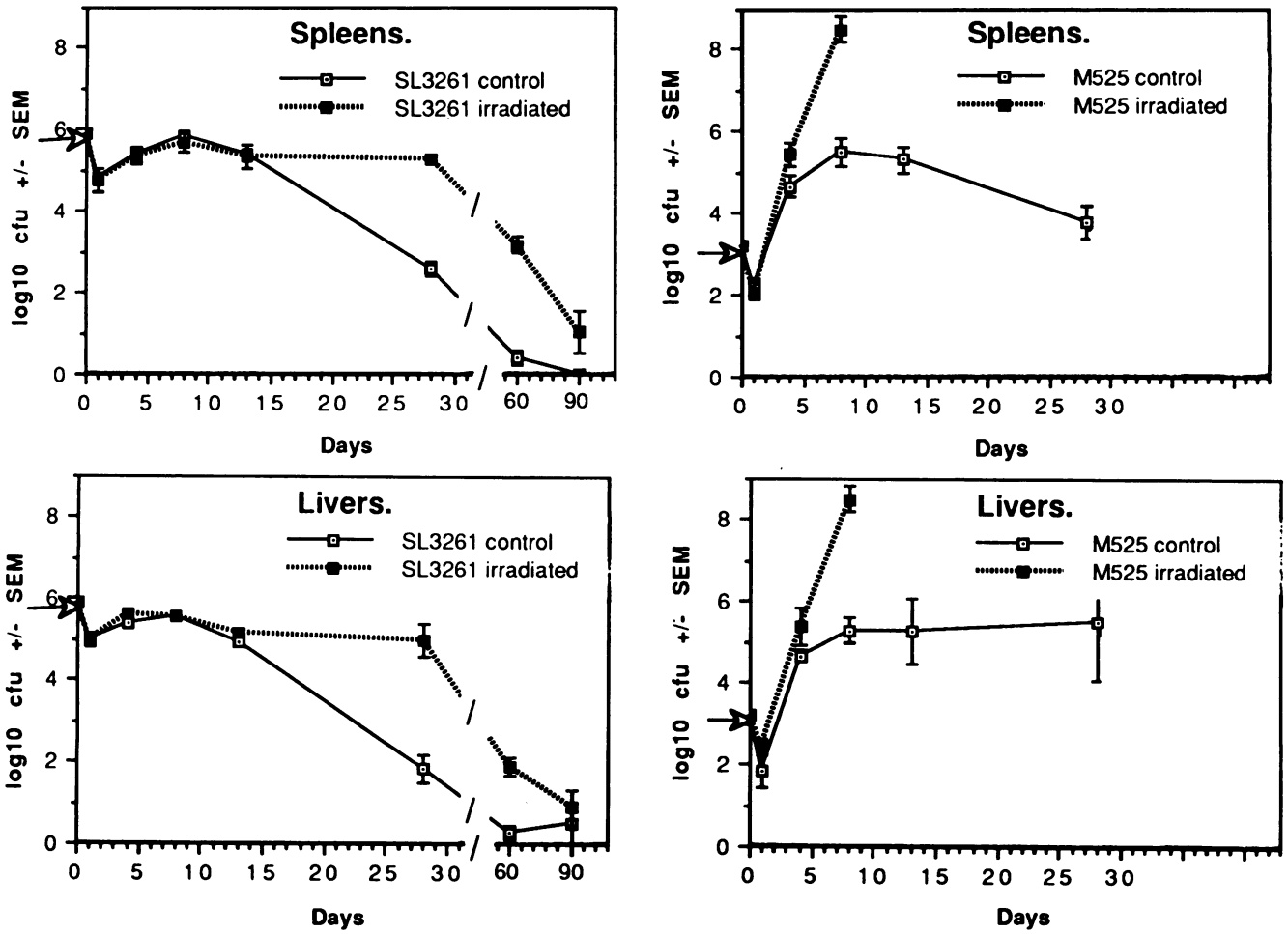

FIG. 1. Viable bacterial counts in liver and spleen of irradiated (■) and control (□) mice after i.v. inoculation of 10^6 *S. typhimurium* SL3261 or 10^3 *S. typhimurium* M525. Arrow indicates the inoculum dose. SEM, Standard error of the mean.

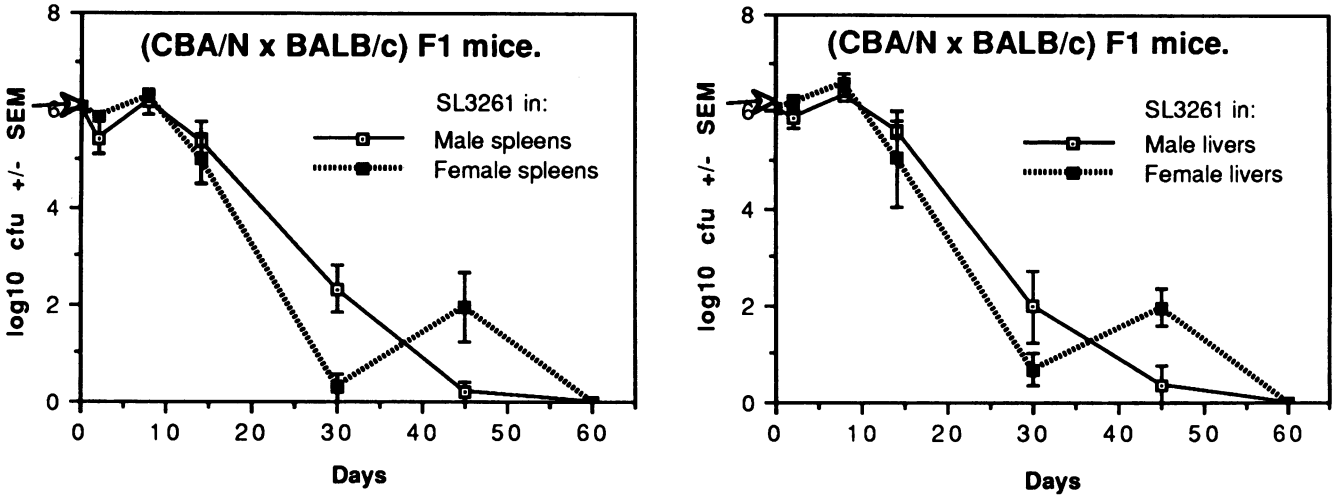

FIG. 2. Viable bacterial counts in liver and spleen after i.v. administration of 10^6 *S. typhimurium* SL3261 in male (immunodefective) (□) and female (immunocompetent) (■) (CBA/N × BALB/c)F_1 mice. Arrow indicates the inoculum dose. SEM, Standard error of the mean.

Kinetics of infection. Figure 1 shows the growth of salmonellae in the liver and spleen of irradiated and control mice. The number of viable M525 bacteria recovered from the spleen and liver increased from day 1 to day 5 at approximately the same rate in irradiated and control mice. After day 5, further growth was suppressed (the plateau phase) in control mice, whereas it continued unchecked to lethal levels in irradiated mice. One of the irradiated mice infected with M525 had very high viable counts (~10^9) on day 4, and these results were not plotted; none of these mice survived beyond day 8. Two unirradiated mice infected with M525 died (days 1 and 15). In contrast, the only difference with the *aroA* SL3261 strain was in the late clearance phase; the Aro strain persisted longer in the reticuloendothelial system of the irradiated mice than in controls. All mice infected with SL3261 remained apparently healthy, as did four uninfected irradiated controls.

Figure 2 shows the growth curves obtained after i.v. infection of (CBA/N × BALB/c)F_1 male and female mice with a sublethal dose of *S. typhimurium* SL3261. The pattern of growth was similar in both. All mice remained healthy until the end of the experiment.

DISCUSSION

The present results indicate that moderate immunosuppression which is not in itself life threatening but which causes increased susceptibility to wild-type salmonellae does not seriously affect resistance to an *aroA* salmonella vaccine strain. Mice immunosuppressed by sublethal irradiation or by virtue of the *xid*-determined B-cell functional defect were more susceptible to wild-type salmonellae but not to the *aroA* strain. Whereas the wild-type strains were more virulent in immunosuppressed mice, both in terms of the LD_{50} and, for M525, invasiveness of host tissue, no such increase was seen for the *aroA* mutant. This is in keeping with the currently accepted view that the main reason for the attenuation of Aro strains may be linked to their requirement for nutrients not available in the host, rather than to an increased susceptibility to a particular host defense mechanism which, if reduced by immunosuppression, might reverse the attenuation of the strain. This could be an important safety factor when considering use of these mutants in the field.

Sublethal irradiation increases susceptibility to salmonellae in mice by ablating the plateau phase which normally occurs at the end of week 1 of a sublethal infection, coinciding with a marked reduction in peripheral blood leukocyte count (3). The precise mechanism responsible for the plateau phase in sublethal salmonella infections in mice is unclear, although it is unlikely to be T cell mediated (14, 15). Ablation of the plateau phase by radiation can be prevented by lead shielding of the hind legs of the mice during irradiation or by adoptive transfer of T-cell-depleted bone marrow, suggesting that the plateau phase requires an influx of bone marrow-derived radiation-sensitive cells (C. E. Hormaeche, P. Mastroeni, A. Arena, J. Uddin, and H. S. Joysey, Immunology, in press). Current data from other models such as *Listeria* infections (12) suggest that monocytes are probably important, but it is possible that other inflammatory cells (polymorphonuclear leukocytes) play a role in this first phase of resistance (2, 11, 19). One mechanism operating in the establishment of the plateau phase in *Listeria* infections in *scid* mice is tumor necrosis factor-mediated gamma interferon release from natural killer cells (1). Whatever the mechanism is in salmonellosis, its temporary suppression by irradiation did not enhance the invasiveness of the *aroA* strain but only retarded its clearance.

The *xid* B-cell defect causes low serum immunoglobulin M and immunoglobulin G3 levels, poor or absent responses to certain T-independent and T-dependent antigens, defective splenic proliferative responses to some B-cell mitogens, and increased susceptibility to in vitro tolerance induction (20–22). It also causes increased susceptibility to wild-type salmonellae, but this occurs later in the infection, with a slow progressive increase in bacterial load (17). Salmonella-infected *xid* mice are reported to die in weeks 2 and 3 (16, 17), which we confirmed. Again, there was no effect of the *xid* character on the clearance of the *aroA* mutant, suggesting that late clearance of the mutant is due to cellular rather than humoral mechanisms. The precise stage at which the *xid* defect operates is unclear and warrants further investigation.

The present results are encouraging when considering the potential use of live attenuated vaccines. Further work is needed to study the effect of immunosuppression induced by

various means on the clearance of these strains. The current results suggest that these strains may not be a major health hazard in immunocompromised populations.

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