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Delayed-Type Hypersensitivity and Immunity to *Salmonella typhimurium*

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Studies were carried out to correlate immunity and expression of delayed-type hypersensitivity (DTH) in mice of the C3H lineage immunized with an avirulent strain of *Salmonella typhimurium* (strain SL3235). This strain belongs to a class of *aroA*$^-$ organisms which are being considered as vaccine strains for humans and veterinary use. In a systematic study, the relationship between the mouse strain and the immunizing dose of strain SL3235 on the development of protective immunity and DTH was examined. It was found that in hypersusceptible C3H/HeJ and C3HeB/FeJ mice, several doses of strain SL3235 afforded protection against intravenous challenge doses as high as 1,300 50% lethal doses. Despite these significant levels of immunity to challenge, mice of these two strains never mounted significant DTH responses following immunization with the doses of strain SL3235 tested, which spanned 3 orders of magnitude. Nonresponsiveness was not due to antigen overload, as all of the mouse strains were comparably colonized with strain SL3235 at the time of DTH elicitation. Further, it was found that the ability of responsive C3H/HeNCrlBR mice to display DTH was dependent on the immunizing dose of strain SL3235 and that a dosage could be found that resulted in increased resistance to challenge in these mice without a concomitant display of DTH. Thus, while both induction of protective immunity and DTH were vaccine dosage dependent in the responsive mouse strain (C3H/HeNCrlBR), DTH was a less sensitive measure of protective immunity than survival. Vaccine dosages ranging over three orders of magnitude failed to yield positive footpads to the *Salmonella* elicitin in the nonresponsive mice. The data suggest that caution should be observed in interpreting *Salmonella* DTH tests that are used as screens of immune status to typhoid fever in humans, as the extent of discordance between immunity and DTH in humans is unknown.

Salmonella infection has been shown to result in the development of delayed-type hypersensitivity (DTH) in the mouse model of typhoid fever (5, 12). While correlations have been observed between the development of DTH and immunity to *Salmonella* spp. in some mice (5), certain mouse strains have been found which do not demonstrate DTH following *Salmonella* infection (11, 19, 20). However, the relationship between development of immunity to *Salmonella* challenge and DTH responsiveness in nonresponsive mouse strains has not been fully investigated. Such studies have practical as well as theoretical import, because of current interest in developing an assay for DTH in humans to assess the immune status and efficacy of a typhoid vaccine (strain Ty21a) currently being tested in field trials.

In previous studies, we have shown that intraperitoneal inoculation of an avirulent strain of *Salmonella typhimurium*, strain SL3235, induces high levels of protection against *Salmonella* challenge in inherently hypersusceptible C3H/HeJ and C3HeB/FeJ mice (7, 15). In subsequent experiments it was found that following intravenous (i.v.) inoculation with strain SL3235 these mice do not develop significant DTH responses, whereas similar vaccinating protocols in inherently salmonella-resistant C3H/HeNCrlBR and CD-1 mice did result in significant DTH responses (14).

In the present study we describe a systematic study of the effect of various i.v. immunizing doses of strain SL3235 on the ability of different mouse strains in the C3H lineage to develop immunity against challenge with virulent organisms and to display DTH responsiveness. We found that hypersusceptible C3H/HeJ and C3HeB/FeJ mice did not develop DTH responses at any of the immunizing doses of strain SL3235 tested, although many of the doses were protective. A correlation between DTH responsiveness and protection did exist at the higher immunizing doses in innately resistant C3H/HeNCrlBR mice; however, at the lowest dose of strain SL3235, protection was obtained in this strain in the absence of DTH responsiveness. The presence or absence of DTH did not correlate with the degree of splenic colonization by strain SL3235 at 21 days after vaccination in the three mouse strains tested.

MATERIALS AND METHODS

Mice. Female mice that weighed 19 to 21 g were used in all experiments. Inbred C3H/HeNCrlBR mice were purchased from Charles River Breeding Laboratories, Inc., Wilmington, Mass. These mice are relatively resistant to *Salmonella* infection (50% lethal dose [LD_{50}] of 10^3 cells) (6). C3H/HeJ and C3HeB/FeJ animals were obtained from Jackson Laboratory, Bar Harbor, Maine; these strains are hypersusceptible to *Salmonella* infection, with LD_{50}s approaching 1 cell (6). All of the mice were housed in plastic cages containing Absorb-Dri for bedding. Purine Mouse Chow and fresh water were available ad libitum.

Organisms. *S. typhimurium* W118-2, which is virulent for mice, was originally obtained from Samuel Formal, Walter Reed Army Institute of Medical Research, Washington, D.C., and has been maintained in our laboratory for many years (6). *S. typhimurium* SL3235, a smooth, avirulent, *aroA*$^-$ derivative, was kindly provided by Bruce A. D. Stocker, Department of Medical Microbiology, Stanford University, Stanford, Calif. (10). Lyophilized organisms

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were hydrated and grown to log phase as previously described (6, 7).

Preparation of *Salmonella* DTH-eliciting antigen. The antigen for eliciting footpad responses was prepared by using the method of Collins and Mackaness, as previously described (5, 14). This antigen, which was prepared from a cell-free culture supernatant of strain SL3235, contained 1 μg of protein per 52.6 μg (dry weight), as determined by the method of Lowry et al. (17). Eliciting doses were based on protein content.

Immunization. The mice were immunized i.v. with a single injection of live organisms suspended in 0.1 ml of saline. The controls received 0.1 ml of saline i.v. The number of organisms injected was approximated by Petroff-Hauser direct counts and was verified by duplicate spread plates.

Measuring DTH responses. Mice were tested at 3 weeks after immunization for the presence of DTH by measuring footpad swelling as previously described (14). Briefly, the eliciting antigen suspended in 0.04 ml of saline was injected into the right hind footpad, and saline alone was injected into the left hind footpad as a control. Footpad thickness was measured with dial gauge calipers (Schnelltaster, Kröplin, German Democratic Replublic) prior to elicitin injection and at 3, 6, 24, 30, and 48 h after injection. Triplicate measurements of both elicitin- and saline-injected footpads were made and averaged at each time point. The average measurement for the saline-injected footpad was subtracted from that for the elicitin-injected footpad to yield the difference in footpad thickness for each mouse. The mean difference in footpad thickness and standard deviation for each group of mice were then calculated. The significance of footpad swelling was determined by using the Student *t* test (9).

Protection studies. To assay the protection induced by strain SL3235 against virulent *Salmonella* challenge, mice were injected intraperitoneally with strain W118-2 suspended in 0.5 ml of saline 3 weeks after i.v. immunization with strain SL3235. Deaths were scored for 60 days after challenge.

Colonization. Following footpad testing, spleens were removed from the mice and individually homogenized with a Tekmar Tissumizer (Tekmar Co., Cincinnati, Ohio) in a total volume of 5 ml of sterile, distilled water; 0.1 ml of the homogenate was then plated onto eosin methylene blue agar to determine the number of colony-forming units of strain SL3235 per spleen.

RESULTS

To assess the effect of vaccine dosage on induction of footpad reactivity, groups of C3H/HeNCrlBR, C3HeB/FeJ, and C3H/HeJ mice were given graded immunizing doses of strain SL3235 (19 mice were used per dose for each mouse strain). Elicitation of footpads was carried out on three mice of each strain 3 weeks postvaccination by using 2.5 μg of antigen, parameters which have been shown previously to be optimal (14). Figure 1 shows that for the C3H/HeNCrlBR mice, the footpad responses were dose dependent, with a threshold at an immunizing dose of 9×10^3 SL3235 cells. Neither of the hypersusceptible mouse strains (C3H/HeJ and C3HeB/FeJ) developed significant footpad responses at any of the immunizing doses tested (Fig. 2 and 3).

To measure protection by the i.v. route, 12 animals of each mouse strain were taken from groups given different immunizing doses of strain SL3235 and were challenged with virulent *Salmonella* W118-2 cells at one of two different doses. As Table 1 shows, strain SL3235 given i.v. was capable of conferring significant protection on all three

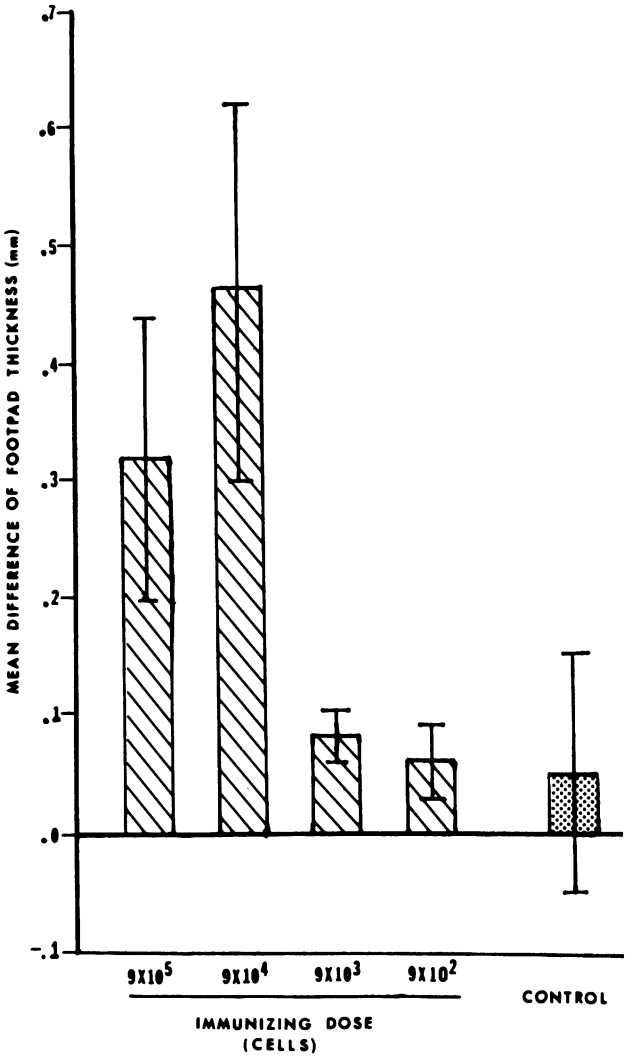

FIG. 1. Footpad swelling responses at 24 h after elicitation of C3H/HeNCrlBR mice immunized 3 weeks previously with graded doses of strain SL3235 (cross-hatched bars) or saline (dotted bar). The responses of the mice immunized with 9×10^5 and 9×10^4 cells were significantly different from the responses of the controls ($P < 0.05$).

mouse strains. For C3H/HeJ and C3H/HeNCrlBR mice, higher immunizing doses conferred greater protection. A dose-response effect was less obvious in C3HeB/FeJ Mice. An examination of the correlation between protection and footpad responsiveness showed that for C3H/HeJ and C3HeB/FeJ mice, many immunizing doses conferred protection in the absence of any positive footpad reaction. In the C3H/HeNCrlBR mice, an immunizing dose of 9×10^3 SL3235 cells induced significant protection (83% survival), but not footpad reactivity. Thus, even in this responder strain, protection could be separated from DTH, as measured by footpad swelling.

Antigen overload has been shown by Collins (4) to lead to footpad anergy. To test whether antigen overload could be the cause of anergy in the hypersusceptible mouse strains infected with strain SL3235, spleen weights and the number of SL3235 cells in the spleens of the three different mouse strains were compared in the remaining animals of each group at the time of DTH elicitation (Table 2). It was found

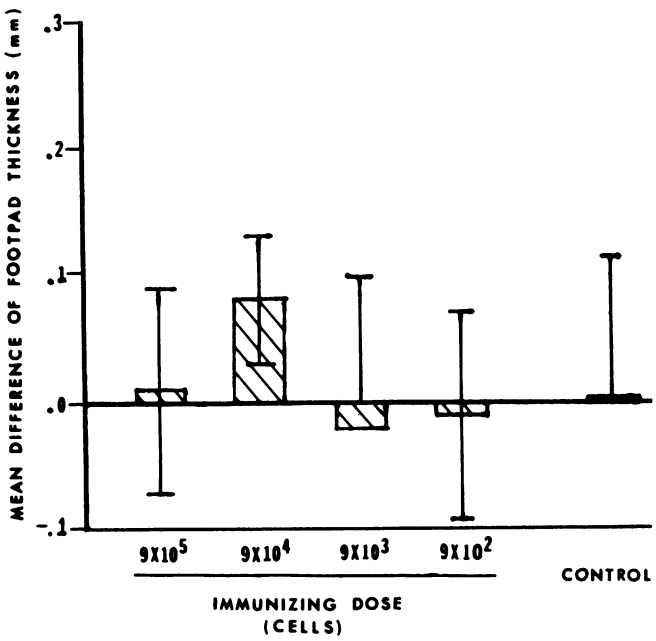

FIG. 2. Footpad swelling responses of C3H/HeJ mice. See the legend to Fig. 1. None of the responses of the SL3235-immunized mice was significantly different from the response of the controls.

that DTH-nonresponsive, hypersusceptible C3H/HeJ and C3HeB/FeJ mice had levels of splenic colonization comparable to those of the DTH-responsive C3H/HeNCrlBR mice and that most of the animals harbored low numbers of SL3235 (less than 10^3 CFU) in their spleens. The intrastrain variations in spleen weights and bacterial load were as great as the differences between the strains. Thus, the failure of C3H/HeJ and C3HeB/FeJ mice to produce a DTH response to strain SL3235 is not attributable to anergy induced by antigen excess. Furthermore, we concluded that the dose dependency of expression of DTH in the C3H/HeNCrlBR mice (Fig. 1) was also not related to antigenic burden at the time of footpad testing 21 days postvaccination.

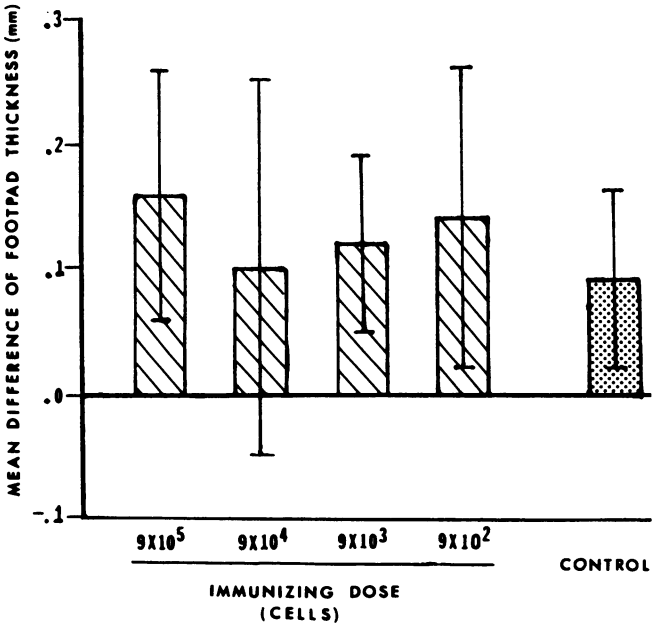

FIG. 3. Footpad swelling responses of C3HeB/FeJ mice. See the legend to Fig. 1. None of the responses of the SL3235-immunized mice was significantly different from the response of the controls.

TABLE 1. Protection and DTH responses of mice of the C3H lineage following immunization with graded doses of *S. typhimurium* SL3235

Mouse strain	Strain SL3235 immunizing dose (no. of cells)[a]	Survivors/total (%) at W118-2 challenge dose[b]: 1.34×10^2 LD_{50}	Survivors/total (%) at W118-2 challenge dose[b]: 1.34×10^3 LD_{50}	DTH response[c]
C3H/HeNCrlBR	9×10^5	5/6 (83)[d]	3/6 (50)	+
	9×10^4	6/6 (100)	2/5 (40)	+
	9×10^3	5/6 (83)	0/6 (0)	−
	9×10^2	3/6 (50)	0/6 (0)	−
	0	0/7 (0)	ND[e]	−
C3H/HeJ	9×10^5	6/6 (100)	5/6 (83)	−
	9×10^4	5/6 (83)	5/6 (83)	−
	9×10^3	3/6 (50)	1/6 (16)	−
	9×10^2	0/6 (0)	0/6 (0)	−
	0	0/6 (0)	ND	−
C3HeB/FeJ	9×10^5	5/6 (83)	3/5 (60)	−
	9×10^4	4/6 (67)	3/6 (50)	−
	9×10^3	3/6 (50)	5/6 (83)	−
	9×10^2	4/6 (67)	2/6 (33)	−
	0	0/6 (0)	ND	−

[a] Live organisms were suspended in 0.1 ml of saline and injected i.v. The controls received 0.1 ml of saline i.v.

[b] At 3 weeks after immunization, the mice were challenged intraperitoneally with *S. typhimurium* W118-2 suspended in 0.5 ml of saline. Deaths were scored for 60 days. The LD_{50} for strain W118-2 in C3H/HeNCrlBR mice is 10^3 cells, and the LD_{50}s for C3H/HeJ and C3HeB/FeJ mice are assumed to be 1 cell.

[c] At 3 weeks after immunization, the DTH responses were assessed by determining footpad swelling following injection of eliciting antigen as described in Materials and Methods.

[d] The numbers in parentheses are percentages.

[e] ND, Not done.

DISCUSSION

These results show that the induction of DTH responses by avirulent *S. typhimurium* SL3235 is dose and mouse strain dependent. Other investigators have also observed differences among mouse strains in their ability to develop DTH responses following infection with *Salmonella* spp. or other intracellular pathogens. However, the relationship between DTH and immunity and the effect of immunizing dose on the development of DTH in the *Salmonella* model has not been systematically investigated previously (1, 11, 12, 18, 19, 21).

In this study, immunization with strain SL3235 resulted in increased resistance to challenge with virulent *Salmonella* strains in the three mouse strains tested, but positive DTH responses, as assessed by footpad swelling, were only observed in inherently resistant C3H/HeNCrlBR mice. Innately hypersusceptible C3H/HeJ and C3HeB/FeJ mice failed to develop positive DTH responses following immunization with strain SL3235 even though they were protected against challenge. In the C3H/HeNCrlBR mice, development of DTH responsiveness was dependent on the immunizing dose of strain SL3235. Higher immunizing doses (9×10^5 and 9×10^4 cells) resulted in the development of both DTH and protection against *Salmonella* challenge, whereas lower immunizing doses (9×10^3 and 9×10^2 cells) induced increased resistance in the absence of positive DTH.

TABLE 2. Spleen weights and colonization of spleens by strain SL3235 in immunized mice

Strain SL3235 immunizing dose (no. of cells)[a]	Mouse no.	Spleen wt (g)[b]			No. of strain SL3235 CFU/spleen[c]		
		C3H/HeNCrlBR	C3H/HeJ	C3HeB/FeJ	C3H/HeNCrlBR	C3H/HeJ	C3HeB/FeJ
9×10^5	1	0.24	0.87	0.40	TNTC[d]	3.1×10^4	$<5 \times 10^1$
	2	0.34	0.33	0.66	2.5×10^2	5.5×10^2	TNTC
	3	0.13	0.61	0.50	$<5 \times 10^1$	6×10^3	TNTC
	4	0.29	0.72	ND[e]	2.6×10^3	1.3×10^3	ND
9×10^4	1	0.26	0.15	0.31	$<5 \times 10^1$	$<5 \times 10^1$	$<5 \times 10^1$
	2	0.24	0.31	0.22	5×10^1	2.5×10^2	$<5 \times 10^1$
	3	0.18	0.66	0.21	$<5 \times 10^1$	1.9×10^3	$<5 \times 10^1$
	4	0.20	0.22	0.28	$<5 \times 10^1$	3.5×10^2	$<5 \times 10^1$
9×10^3	1	0.20	0.14	0.17	$<5 \times 10^1$	$<5 \times 10^1$	$<5 \times 10^1$
	2	0.20	0.15	0.16	$<5 \times 10^1$	2×10^2	$<5 \times 10^1$
	3	0.19	0.12	0.19	$<5 \times 10^1$	$<5 \times 10^1$	$<5 \times 10^1$
	4	0.32	0.12	0.20	7×10^2	5×10^1	$<5 \times 10^1$
9×10^2	1	0.20	0.13	0.14	$<5 \times 10^1$	$<5 \times 10^1$	$<5 \times 10^1$
	2	0.54	0.16	0.14	1.9×10^3	2.5×10^2	$<5\times 10^1$
	3	0.26	0.14	0.16	$<5 \times 10^1$	$<5 \times 10^1$	$<5 \times 10^1$
	4	0.35	0.09	0.16	$<5 \times 10^1$	$<5 \times 10^1$	$<5 \times 10^1$

[a] Cells were suspended in 0.1 ml of saline and injected i.v.
[b] Control animal spleen weights ranged from 0.10 to 0.20 g for each mouse strain.
[c] Spleens were homogenized in a total volume of 5 ml of sterile distilled water, and 0.1 ml of homogenate was plated onto eosin methylene blue agar plates.
[d] TNTC, Too numerous to count.
[e] ND, Not done.

The ability to mount a significant DTH response was not dependent on the degree of colonization by strain SL3235. Spleens from C3H/HeJ and C3HeB/FeJ mice were colonized with SL3235 to the same extent as spleens from the resistant mice. In C3H/HeNCrlBR mice, the levels of colonization of spleens were comparable at an immunizing dose of 9×10^3 SL3235 cells, which induced DTH, and at an immunizing dose of 9×10^2 SL3235 cells, a dose that did not induce DTH.

In addition to the present study showing a lack of DTH responses in strain SL3235-immunized C3H/HeJ and C3HeB/FeJ mice, we have also shown that spleen cells from these immunized mouse strains, as well as spleen cells from C3H/HeNCrlBR mice, are markedly suppressed to a panel of B- and T-cell mitogens (7, 16). The suppression is macrophage mediated. In a number of infections, DTH refractoriness and low mitogenic responses have frequently been associated with depressed cellular immunity and poor prognosis for the host (reviewed in reference 22). In contrast, we have found that despite a lack of DTH responsiveness, in some mouse strains, SL3235 induces both long-lasting, specific immunity against *Salmonella* sp. and transient, nonspecific resistance against *Listeria* sp., which are indications of intact cellular immunity and macrophage activation (7, 15).

The immunological mechanism which permits development of cellular immunity without a concomitant manifestation of DTH reactions is not clear at this time. Immunity does not seem to be humorally mediated, as it has been found that hyperimmune serum is not protective in C3H/HeJ mice (8) and only marginally protective in C3HeB/FeJ mice (8). Furthermore, sera of C3H/HeJ mice immunized with strain SL3235 also did not passively transfer immunity to naive C3H/HeJ mice (15). Expression of DTH to certain haptens has been shown to be under the control of complex regulatory suppressor circuits (2, 3, 13). Perhaps similar mechanisms are operative in the manifestation of DTH in strain SL3235-infected C3H/HeJ and C3HeB/FeJ mice, or else these strains are incapable of mounting a response to strain SL3235 antigens. We do not know yet whether these two mouse strains can give normal DTH responses to other antigens. One way to dissect whether the nonresponsiveness resides in the afferent or efferent arm of the immune system might be through cell transfer studies. However, attempts to demonstrate the presence of a sensitized T-cell population which could transfer immunity from strain SL3235-immunized C3H/HeJ mice have not been successful (15), although some evidence for a T-cell-sensitized population was obtained by using an assay in which peritoneal T cells responded to specific *Salmonella* antigens (PETLs) (15).

The present study shows that expression of DTH as assessed by footpad swelling following immunization with live, avirulent *S. typhimurium* SL3235 is mouse strain and dose dependent. Furthermore, DTH is a less sensitive measure of immunity than protection from infection. The data suggest that caution should be observed in interpreting *Salmonella* DTH tests used as screens of immune status to typhoid fever in humans, as the extent of discordance between immunity and DTH in humans is unknown.

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