INFECTION AND IMMUNITY, Mar. 1992, p. 791–797
0019-9567/92/030791-07$02.00/0

Vol. 60, No. 3

# Natural Killer Cells Mediate Protection Induced by a *Salmonella aroA* Mutant

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Received 5 August 1991/Accepted 10 December 1991

**We have previously shown that an avirulent strain of *Salmonella typhimurium*, SL3235, blocked in aromatic synthesis, confers high levels of resistance to challenge with virulent *Salmonella* as early as 3 days postvaccination. In the present studies, it was found that immunization with SL3235 resulted in high levels of natural killer (NK) cell activity in the spleens and peritoneal cavities of C3H/HeJ mice, as measured by cytotoxicity against YAC-1 targets. NK cell activity was at its maximum 2 to 4 days after immunization and was ablated by in vivo or in vitro treatment with anti-asialo $GM_1$. In vivo treatment with anti-asialo $GM_1$ during the first week after immunization with SL3235 depleted NK cell activity and markedly increased mortality in mice challenged with a virulent *Salmonella* strain. These results are compatible with a role for NK cells as one important component in the resistance against virulent *Salmonella* infection induced by a live, attenuated vaccine.**

Attenuated *Salmonella* strains, which are blocked in aromatic synthesis (*aroA* mutants), were developed by Hoiseth and Stocker (13). They have been shown to be avirulent and to confer very high levels of protection on both innately hypersusceptible and resistant mice (7, 13, 17). *Salmonella* strains of this class are of interest, as they are being considered as vaccine candidates for typhoid fever in humans (22) and *Salmonella* gastroenteritis in cattle (32) and as carrier organisms to deliver cloned genes of other microbes (4, 6, 30). Studies in our laboratory on the mechanisms of immunity to typhoid fever, using a mouse model of vaccination with the *aroA* mutant strain *Salmonella typhimurium* SL3235, have shown that protection is evident as early as 3 days after immunization and lasts for at least 7 months (17).

There are a number of reports in the literature which suggest a role for natural killer (NK) cells in host defense against bacteria (3, 8, 23, 25, 38), fungi (12, 14, 26), and parasites (10, 11). We have considered the hypothesis that the vaccine-induced protection against a virulent *Salmonella* strain at 3 days postvaccination may be mediated by NK cells. The results presented here show that the *aroA* mutant strain of *S. typhimurium*, SL3235, induces high levels of NK cell activity in the spleen and peritoneal cavities of immunized mice, which peaks between 2 and 4 days postinfection, and that this activity is mediated by an asialo $GM_1^+$ cell. In vivo treatment of SL3235-immunized mice with anti-asialo $GM_1$ to deplete NK cell activity markedly reduced the protective capacity of the vaccine. These findings are consistent with the hypothesis that NK cells are important in the early phases of induction of immunity against systemic *Salmonella* infection.

## MATERIALS AND METHODS

**Mice.** Male and female C3H/HeJ mice were purchased from Jackson Laboratory (Bar Harbor, Maine) at 6 to 8 weeks of age and were acclimatized for at least 1 week before use. Mice received fresh water and Purina Mouse Chow ad libitum.

**Bacteria.** Strain SL3235, a smooth, avirulent, auxotrophic derivative of *S. typhimurium*, was isolated and characterized by Hoiseth and Stocker (13). *S. typhimurium* W118-2, which is virulent for C3H/HeJ mice (50% lethal dose [$LD_{50}$], <10 cells) was used as the challenge organism.

**Immunizations.** Log-phase cultures of *S. typhimurium* SL3235 were harvested and diluted in sterile nonpyrogenic saline (Abbott Laboratories, North Chicago, Ill.) to the desired bacterial concentrations as estimated by Petroff-Hausser counts, as previously described (17). Mice received 0.5 ml of bacteria or saline intraperitoneally (i.p.).

**Culture medium.** Complete medium was RPMI 1640 (GIBCO) containing 2.0 g of $NaHCO_3$ per liter, 10% fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Cells were harvested in medium with only 1% serum.

**Cell preparations.** Peritoneal cells were nonelicited, resident cells. Mice were exsanguinated by decapitation, and 8 to 10 ml of harvesting medium was injected into the exposed peritoneal cavity. Peritoneal fluid was withdrawn through the anterior abdominal wall with a 20-gauge needle. Fluids from four to six mice were pooled, and a sample was taken for differential and total cell counts. The remainder was centrifuged in polypropylene tubes (50 ml; Falcon; Becton Dickinson, Cockeysville, Md.) for 10 min at 1,200 rpm (300 × *g*) and washed twice with complete medium. Spleens from three to five mice were teased gently in cold culture medium, and the cells were pooled. The erythrocytes were lysed by hypotonic disruption. Cells were resuspended in complete medium to the desired cell concentration. Adherent cells were depleted in some experiments by incubation at 37°C for 2 h in plastic petri dishes.

**Antibodies.** Anti-asialo $GM_1$ was from WAKO Chemicals USA, Inc. (Dallas, Tex.). Normal rabbit serum was obtained from Rockland Inc. (Gilbertsville, Pa.). The anti-asialo $GM_1$ and the normal rabbit sera were diluted in RPMI 1640 medium and filter sterilized. The appropriate dilutions were determined prior to use.

**Asialo $GM_1$ cell depletions.** For in vitro cell depletions, $10^7$

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TABLE 1. Role of NK cells in *Salmonella* immunity: protocol

| Group | Day 0 | | Day 3 (challenge)[d] | Day 5 (passive serum) |
|---|---|---|---|---|
| | Immunization | Passive serum | | |
| A (Saline) | Saline | None | *S. typhimurium* W118-2 | None |
| B (SL3235) | SL3235[a] | None | *S. typhimurium* W118-2 | None |
| C (NRS) | SL3235[a] | Normal rabbit serum[b] | *S. typhimurium* W118-2 | Normal rabbit serum[b] |
| D (AsGM) | SL3235[a] | Anti-asialo $GM_1$[c] | *S. typhimurium* W118-2 | Anti-asialo $GM_1$[c] |

[a] Mice received $5 \times 10^5$ SL3235 cells i.p.
[b] Mice received 0.2 ml of normal rabbit serum (1:10) i.v.
[c] Mice received 0.2 ml of anti-asialo $GM_1$ (1:10) i.v.
[d] Mice received either 500 $LD_{50}$ (experiment 1) or 3,000 $LD_{50}$ (experiment 2) i.p.

cells per ml were treated for 45 min at 4°C with anti-asialo $GM_1$ (1:200) in RPMI medium with 1% fetal bovine serum. Cells were centrifuged, and the pellet was resuspended in a 1:12 dilution of Low-tox-M rabbit complement and incubated at 37°C for 45 min. Cells were washed three times and resuspended to the desired concentrations. For in vivo cell depletions, mice were injected intravenously (i.v.) with 0.2 ml of either normal rabbit serum (1:5 to 1:10 dilution) or anti-asialo $GM_1$ (1:5 to 1:10 dilution). Efficiency of asialo $GM_1$ cell depletion was assessed by measuring peritoneal or spleen cells for cytotoxic activity against the NK cell-sensitive YAC-1 target cell line.

**$^{51}$Chromium ($^{51}$Cr) release assay for NK cell activity.** Peritoneal cells or spleen cells at concentrations of $5 \times 10^6$, $2.5 \times 10^6$, $1.25 \times 10^6$, and $6.25 \times 10^5$ cells per ml were plated at 0.1 ml per well in 96-well microtiter plates (3596; Costar, Cambridge, Mass.) in complete medium. YAC-1 cells were labeled overnight with $^{51}$Cr (as sodium chromate, 250 Ci/mg; ICN Radiochemicals, Irvine, Calif.), washed, and resuspended to $5 \times 10^4$/ml, and 0. 1 ml was added to the appropriate wells for a final concentration of $5 \times 10^3$ cells per well. This yielded an effector-to-target-cell ratio of 100:1, 50:1, 25:1, and 12:1, respectively. Experimental groups were tested in quadruplicate. The plates were spun down for 5 min at 400 rpm ($30 \times g$) at the beginning of the assay to promote cell contact and at the end of a 4-h incubation to obtain cell-free supernatants. Cytotoxicity was measured as the release of $^{51}$Cr from target cells at the end of the 4-h incubation by removing 0.1 ml of supernatant from each well and counting its radioactivity for 1 min on an automatic gamma spectrometer (Gamma Trac II 1191). For measurement of total incorporated counts, tumor cells were lysed with 0.1 ml of 0.5% sodium dodecyl sulfate, and the radioactivity of 0.1 ml of the supernatant was counted. Spontaneous release was determined as the amount of $^{51}$Cr released in wells with target cells only and was always less than 15%. Results were expressed as percent specific $^{51}$Cr release, as calculated by the following formula: % specific $^{51}$Cr release = [(mean cpm experimental release − mean cpm spontaneous release)/(mean cpm total releasable counts − mean cpm spontaneous release)] × 100. Standard deviations of replicates were less than 10% and are not reported. Representative experiments are reported, but all experiments were done three or more times unless noted.

**Mitogenicity assay.** Spleen cells were prepared and tested in 96-well tissue culture plates for the mitogenicity assay as previously described (21).

**Plaque-forming cell responses.** Splenic plaque-forming cell responses were determined on cells obtained from normal or anti-asialo $GM_1$-treated animals by using the Cunningham slide modification of the Jerne hemolytic plaque assay (1).

**Experimental design of protection studies.** Protection experiments were done in mice which were treated with anti-asialo $GM_1$ to deplete NK cell activity in vivo. Table 1 summarizes the treatment each group received during the initial 5 days of the experiment. Group A (Saline) received saline i.p. on day 0. Group B (SL3235), group C (NRS), and group D (AsGM1) were immunized i.p. on day 0 with live SL3235. In addition, on day 0, group C (NRS) received normal rabbit serum i.v. (a control for the anti-asialo $GM_1$ serum), and group D (AsGM1) received anti-asialo $GM_1$ i.v. On day 3 after immunization, all groups were challenged with a lethal dose of virulent *Salmonella* strain W118-2. Two days after challenge (day 5), groups C (NRS) and D (AsGM1) received a second dose i.v. of normal rabbit serum or anti-asialo-$GM_1$, respectively. For clarity, these group designations are used in the figure legends for the protection study. NK cell activity in the spleen and peritoneal cavity was determined on day 3, prior to the virulent challenge, and at various time points after challenge by sacrificing three mice per time point from each of the groups. Survival curves were determined for an additional 10 mice in each group for 60 days after challenge.

**Statistics.** Tests of statistical significance employed a weighted linear regression of percent specific release for the effector-to-target-cell ratios of controls and experimental groups. These were analyzed for significance of slope by analysis of variance. Individual group means were tested for significance by the Student $t$ test at the level $P < 0.01$. NK cell activity was considered significant for a given set of experimental conditions if $P < 0.01$ at all effector-to-target-cell ratios. Calculations employed the computer program of Tallarida and Murray (35).

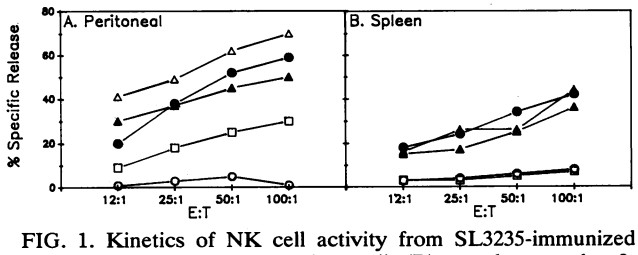


FIG. 1. Kinetics of NK cell activity from SL3235-immunized mice. Peritoneal cells (A) and spleen cells (B) were harvested at 2, 4, 7, and 10 days postinjection from mice immunized with $5 \times 10^5$ SL3235 cells and incubated with $^{51}$Cr-prelabeled YAC-1 target cells. Cytotoxicity was estimated by measuring $^{51}$Cr release at 4 h and expressed as percent specific release. ○, saline; ●, day 2; △, day 4; ▲, day 7; □, day 10. E:T, effector-to-target-cell ratio.

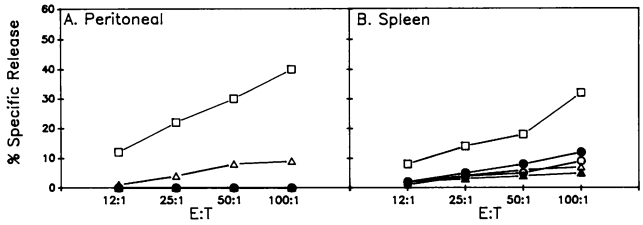


FIG. 2. NK cell activity from mice immunized with various doses of SL3235. Peritoneal cells (A) and spleen cells (B) from mice immunized 2 days previously with doses of SL3235 ranging from $10^2$ to $10^5$ cells per mouse were incubated with $^{51}$Cr-prelabeled YAC-1 cells, and cytotoxicity was measured at 4 h. Results are expressed as percent specific release. $P < 0.01$ compared with saline for $10^5$ cells (Student $t$ test). ○, saline; ●, $10^2$ cells; △, $10^3$ cells; ▲, $10^4$ cells; □, $10^5$ cells. E:T, effector-to-target-cell ratio.

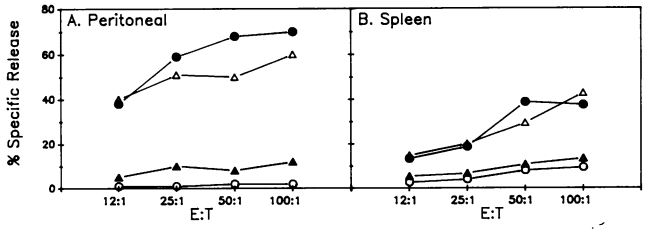


FIG. 4. In vitro depletion of NK cells with anti-asialo $GM_1$ serum. Peritoneal cells (A) and spleen cells (B) from mice immunized 2 to 4 days previously with $5 \times 10^5$ SL3235 cells were incubated with complement (△) or anti-asialo $GM_1$ and complement (▲) in vitro and tested for cytotoxicity against $^{51}$Cr-prelabeled YAC-1 target cells. Cytotoxicity was estimated by measuring $^{51}$Cr release at 4 h and expressed as percent specific release. ○, saline; ●, SL3235. E:T, effector-to-target-cell ratio.

## RESULTS

**Induction of NK cell activity by *S. typhimurium* SL3235.** A single i.p. injection of live SL3235 was found to induce significant levels of peritoneal and splenic NK cell activity in C3H/HeJ mice as measured by a standard $^{51}$Cr release cytotoxicity assay against YAC-1 target cells. The level of NK cell activity was consistently higher in the peritoneal cavity than in the spleen. Time course studies showed that NK cell activity was detectable as early as 1 day (data not shown) after SL3235 injection and peaked from 2 to 4 days postinjection (Fig. 1). Activity was still significant at days 7 and 10 in the peritoneal cavity but had returned to baseline levels in the spleen by day 10. Based on these kinetic results, all further studies were done at 2 to 4 days after injection of SL3235.

Dose-response studies showed that $10^5$ SL3235 cells were needed to induce splenic and peritoneal NK cell activity (Fig. 2). In addition, the lytic capacity of total cells in the spleen or peritoneal cavity was compared with that of macrophage-depleted populations. Removal of adherent cells (Fig. 3) actually increased the activity of peritoneal cells but did not significantly alter spleen NK cell activity.

**Depletion of NK cell activity with anti-asialo $GM_1$.** Cell depletion studies were done in vitro and in vivo with anti-asialo $GM_1$. Spleen and peritoneal cells from mice immunized with SL3235 were collected and treated in vitro with anti-asialo $GM_1$ and complement, or complement alone, and then assayed for NK cell activity. Depletion of asialo $GM_1^+$ cells abrogated the NK activity of cells obtained from both the peritoneal cavity (Fig. 4A) and spleen (Fig. 4B). Complement treatment alone had no effect.

Anti-asialo $GM_1$ was administered in vivo to determine the effect of treatment on induction of NK cell activity by SL3235. Groups of mice received SL3235 (i.p.), SL3235 (i.p.) plus anti-asialo $GM_1$ (i.v.), or SL3235 (i.p.) plus normal rabbit serum (i.v.). The normal serum was a control for the anti-asialo $GM_1$ rabbit serum. Mice given SL3235 and anti-asialo $GM_1$ had no detectable peritoneal or splenic NK cell activity, whereas the mice given SL3235 and normal rabbit serum had levels of peritoneal and splenic NK cell activity comparable to those of the SL3235 group (Fig. 5).

**Effect of anti-asialo $GM_1$ on immune functions.** In vivo administration of anti-asialo $GM_1$ 4 days prior to harvest of spleen cells did not appreciably diminish the capacity of T cells to give mitogenic responses to concanavalin A or phytohemagglutinin or of B cells to give mitogenic responses to lipopolysaccharide (Table 2). Further, spleen cells of anti-asialo $GM_1$-treated animals gave normal responses to sheep erythrocytes. C3H/HeJ mice received either no treatment or 0.2 ml of a 20% sheep erythrocyte suspension i.p. These mice had 24 ± 20 and 616 ± 80 plaque-forming cell responses per $10^6$ spleen cells, respectively. Two groups also received sheep erythrocytes plus either 0.2 ml of normal rabbit serum i.v. or 0.2 ml of anti-asialo $GM_1$ i.v. 4 days prior to assay. These groups had 696 ± 32 or 624 ± 78 plaque-forming cell responses per $10^6$ spleen cells, respectively. (Responses were determined from quadruplicate determinations ± standard deviation. Spleen cells from three animals

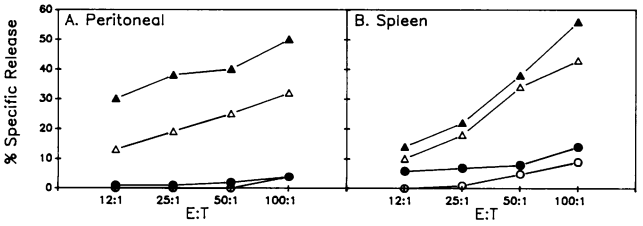


FIG. 3. NK cell activity of mice immunized 7 days previously with $5 \times 10^5$ SL3235 cells. NK cell activity of total or nonadherent peritoneal cells (A) or spleen cells (B) was tested for cytotoxicity against $^{51}$Cr-prelabeled YAC-1 target cells from either saline controls or SL3235-immunized mice. Cytotoxicity was estimated by measuring $^{51}$Cr release at 4 h and expressed as percent specific release. ○, saline; ●, saline (nonadherent); △, SL3235; ▲, SL3235 (nonadherent). E:T, effector-to-target-cell ratio.

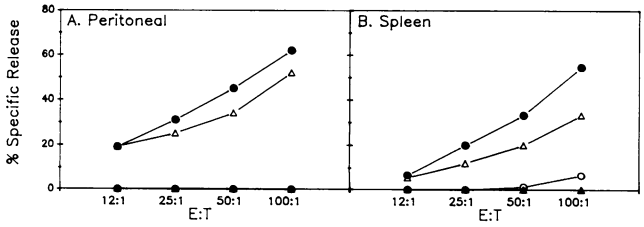


FIG. 5. In vivo depletion of NK cells with anti-asialo $GM_1$ serum. Groups of mice were immunized with saline (○) or $5 \times 10^5$ SL3235 cells (△, ▲, and ●) i.p. Two groups of mice were also given either normal rabbit serum (△) or anti-asialo $GM_1$ (▲) i.v. at the time of immunization. Peritoneal cells (A) and spleen cells (B) from all groups were tested 2 days later for cytotoxicity against YAC-1 targets. Results are expressed as percent specific release. E:T, effector-to-target-cell ratio.

TABLE 2. Effect of in vivo anti-asialo $GM_1$ treatment on T- and B-cell responses

| Treatment in vivo[a] | Mitogen (cpm ± SD)[b] | | | |
|---|---|---|---|---|
| | None | ConA | PHA | LPS |
| None | 2,904 ± 220 | 87,455 ± 3,212 | 78,702 ± 6,435 | 69,813 ± 2,625 |
| Normal rabbit serum | 2,869 ± 434 | 98,347 ± 6,084 | 97,293 ± 2,905 | 64,473 ± 3,719 |
| Anti-asialo $GM_1$ | 2,359 ± 324 | 79,525 ± 3,875 | 60,832 ± 773 | 67,576 ± 1,027 |

[a] C3H/HeJ mice received no treatment, 0.2 ml of normal rabbit serum i.v., or 0.2 ml of anti-asialo $GM_1$ i.v., 4 days prior to assay.
[b] Counts per minute ± standard deviation were determined from triplicate cultures. ConA, concanavalin A; PHA, phytohemagglutinin; LPS, lipopolysaccharide.

were pooled for responses.) These results show that administration of anti-asialo $GM_1$ does not ablate normal function of antigen-presenting cells, T helper cells, or B cells.

**Protection studies.** Figure 6A shows that on day 3 postimmunization, mice in group B (SL3235) or C (NRS), which received SL3235 alone or in combination with normal rabbit serum, had significant levels of NK cell activity in their spleens and peritoneal cavities (see Table 1 for experimental details). In contrast, animals in group D (AsGM1), which received SL3235 plus anti-asialo $GM_1$, had no activity against the YAC-1 target cells, confirming the in vivo efficacy of anti-asialo $GM_1$ in reduction of NK cell activity. In addition, control mice in group A (Saline), which received saline on day 0, had no detectable NK activity at day 3, the time of virulent *Salmonella* challenge.

Experiments done on day 7 post-SL3235 vaccination (Fig. 6B), which is 4 days after challenge with the virulent organisms, showed that peritoneal NK cell activity remains elevated in groups B (SL3235) and C (NRS), with significant, although lower, levels of activity in the spleen. Again, no detectable NK cell activity was seen in the anti-asialo $GM_1$-treated mice, group D (AsGM1), in either the spleen or the peritoneum (Fig. 6B). It is notable that mice in group A (Saline) developed high levels of NK cell activity in both the spleen and peritoneum after inoculation of the virulent *Salmonella* strain (Fig. 6B). These unvaccinated mice were extremely ill, however, and by day 11 all of them had died (Fig. 7A). By day 10 (Fig. 6C), NK cell activity in groups B and C was returning to baseline levels. The results with group D (AsGM1) show that the anti-asialo $GM_1$ treatment rendered mice which had been immunized with SL3235 NK cell deficient at the time of virulent *Salmonella* challenge and for up to 1 week afterward. The immunized mice which received no treatment, group B (SL3235), and mice that received normal rabbit serum instead of anti-asialo $GM_1$, group C (NRS), responded to vaccination with high levels of NK cell activity during the same period.

To test the effect of NK cell depletion on the capacity of the vaccine to protect, we challenged 10 mice in each group with the virulent *Salmonella* strain. In the first experiment,

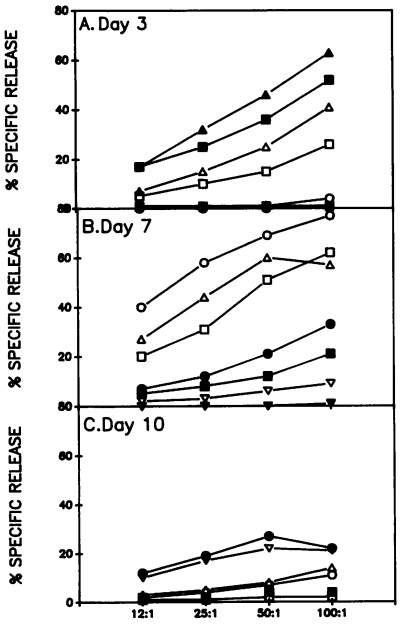


FIG. 6. NK cell activity at day 3 (A), day 7 (B), and day 10 (C) of a protection experiment. Spleen (open symbols) and peritoneal (closed symbols) cells from group A (●, ○, saline), group B (▲, △, SL3235), group C (■, □, SL3235 plus normal rabbit serum), and group D (▼, ▽, SL3235 plus asialo $GM_1$) were harvested and incubated with $^{51}Cr$-prelabeled YAC-1 cells. Cytotoxicity was determined at 4 h and is expressed as percent specific release.

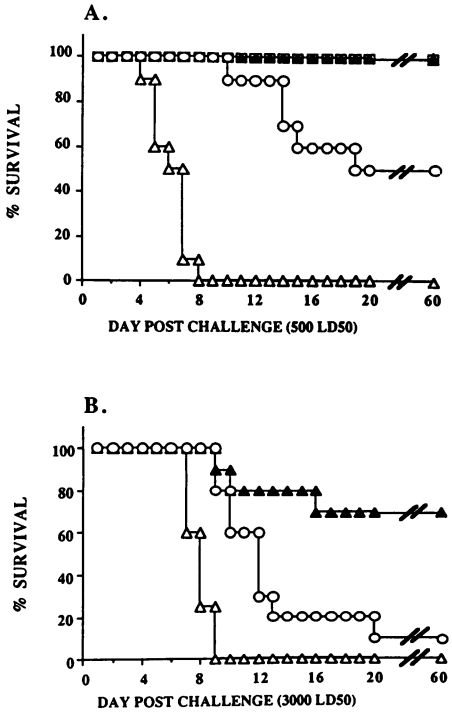


FIG. 7. Survival curves of C3H/HeJ mice challenged with 500 $LD_{50}$ (A) or 3,000 $LD_{50}$ (B) of *S. typhimurium* W118-2. Survival was recorded for 60 days after challenge in group A (△, saline), group B (□, SL3235), group C (▲, SL3235 plus normal rabbit serum), and group D (○, SL3235 plus asialo $GM_1$) (10 mice per group).

the challenge was 500 $LD_{50}$ (Fig. 7A). There were no survivors among the nonimmunized mice, group A (Saline). In group B (SL3235), which received SL3235, and group C (NRS), which received SL3235 and normal rabbit serum, there was a 100% survival rate. Mice which had been immunized with SL3235 but depleted of their NK cell activity, group D (AsGM1), had a significantly lower survival rate of 50%. The depletion of NK cells with anti-asialo $GM_1$ had no effect on survival of mice receiving SL3235 alone. These mice had a 100% survival rate and no signs of infection (data not shown). This result is not unexpected since SL3235 is an *aroA* mutant which cannot undergo significant replication in vivo because of its requirement for *p*-aminobenzoic acid, which is not available in mammalian tissue (13, 17).

In the second experiment, the challenge dose was increased to 3,000 $LD_{50}$. Again, group A (Saline) had no survivors. Group C (NRS) had a 70% survival rate, while in contrast, the anti-asialo $GM_1$-treated mice, group D (AsGM1), had only a 10% survival rate. The experiments shown in Fig. 6 and 7 demonstrate that treatment with anti-asialo $GM_1$ depletes NK cell activity in SL3235-immunized mice and leads to a significant reduction in survival against a virulent *Salmonella* challenge.

## DISCUSSION

Previous work in our laboratory has shown that *S. typhimurium* SL3235 confers high levels of protection against virulent *Salmonella* strains in hypersusceptible C3H/HeJ mice from 3 days to at least 7 months (17). The present study was undertaken to examine the potential role of NK cells in the mechanism of immunity induced in the first week after vaccination. The studies reported in the present communication examined the capacity of *Salmonella* vaccines injected in vivo to induce NK cell activity against YAC-1 cells in vitro. The results showed that immunization with the live, avirulent strain of *S. typhimurium* SL3235 induces significant levels of NK cell activity by 2 days after immunization in the spleen and peritoneal cavities of C3H/HeJ mice. This induced NK cell activity against YAC-1 targets is abrogated by in vitro or in vivo treatment with anti-asialo $GM_1$. The protection experiments show that treatment in vivo with anti-asialo $GM_1$ serum depletes NK cell activity in SL3235-immunized mice through the first 2 weeks after immunization and even after challenge with a virulent *Salmonella* strain. The SL3235-immunized mice, which are rendered NK cell deficient, become susceptible to virulent *Salmonella* infection. In addition, although virulent *Salmonella* infection induced NK cell activity in nonimmunized control mice (Fig. 6B, group A), the mice were already extremely ill by this time and died from the infection. These results support the hypothesis that NK cells are an important part of the defense mechanisms induced by vaccination with an avirulent *Salmonella* strain. The significant NK cell activity that is present at the time of virulent *Salmonella* challenge is consistent with a role for them in the early (3 days) resistance observed in SL3235-vaccinated mice (17).

Macrophages have been considered to be the essential effector cells in innate and vaccine-induced *Salmonella* resistance (17, 28), although a recent study suggests that they are not essential to host resistance (20). Work in our laboratory had found that *Salmonella* strain SL3235 was protective 3 days after immunization (17). However, we did not detect activated macrophages until 7 to 10 days after SL3235 administration (31). Therefore, the relative contribution of NK cells compared with macrophages was of interest. The present data would be compatible with an important role for NK cells in the first few days (2 to 4) after immunization with an attenuated vaccine and a role for macrophages after 1 week to 10 days.

Although the literature implicating a role for NK cells in mediating resistance to microbial infection is limited, it is provocative. There is evidence for at least four different mechanisms by which NK cells can augment resistance to infection. These include direct killing in vitro of paracocci (14), *Cryptococcus* species (12), *Toxoplasma* species (11), *Trypanosoma cruzi* (10), *Salmonella* species (8, 27), and *Escherichia coli* (8) or direct killing in conjunction with antibody by an antibody-dependent cell-mediated cytotoxicity for *Salmonella* species, meningococci, and *Shigella* species (23, 25, 27). NK cells can apparently also lyse mammalian cells harboring bacteria, as has been described for *Legionella* and *Shigella* species (3, 18, 25). Finally, *Salmonella* and *Shigella* species are reported to activate human NK cells to release alpha and gamma interferon (18, 36, 37) and thus may potentiate antimicrobial resistance by an immunoregulatory role.

The present studies differ in design from other reports in the literature on NK cells and *Salmonella* species. Two groups have investigated the capacity of NK cell populations, enriched from the peripheral blood of nonvaccinated humans, to directly kill *Salmonella* species in vitro. Garcia-Penarrubia et al. (8) reported that NK cells have bactericidal activity against *Salmonella* species in vitro, whereas Tarkkanen et al. (37) found no direct salmonellacidal effect. In preliminary experiments, we have not found the NK cell-rich peritoneal or spleen populations to be directly salmonellacidal. In a different type of study, Nencione et al. (27) found that a variety of normal mouse tissues contained cells with natural bactericidal activity against *Salmonella* species, some of which was attributable to cells with an NK phenotype. The activity was strongest in the gut-associated lymphoid tissues and was augmented by antibody. In contrast, in the experiments reported here, we examined NK cell activity by capacity to lyse YAC-1 targets and found that vaccination with live *Salmonella* cells induced this activity in the spleen and peritoneal cavities of mice. These observations would be consistent with those of Tarkkanen et al. (37) and Klimpel et al. (18), who found, respectively, that glutaraldehyde-fixed and kanamycin-treated *Salmonella* species could enhance NK cell activity of cells in human peripheral blood against NK target cells.

To further define the role of NK cells in our system, we did depletion studies using antiserum to asialo $GM_1$. Asialo $GM_1$, a glycosphingolipid, is a cell surface marker found primarily on NK cells (15, 16). In some cases, this marker can also be expressed on other cell types, such as monocytes and some activated T cells (24, 33, 34, 39), although these cells have been shown to be less susceptible to killing in vitro by anti-asialo $GM_1$ plus complement than are NK cells (16, 24, 39). Asialo $GM_1$ was used in these studies because C3H/HeJ mice display the NK1.2 (19) allele of the NK-specific marker and there is no effective antibody to NK1.2 for in vivo depletions. However, several lines of evidence indicate that the observed NK activity is mediated by typical NK cells and is not due to T cells or macrophages. Removal of adherent cells did not decrease the NK cell activity but actually increased it. This could possibly be due to an enrichment of NK cells or, alternatively, the adherent cells could have been secreting prostaglandins, which have been shown to inhibit NK cell activity (5). In addition, the time

course of cytolytic activity against the YAC-1 target cells at 2 to 4 days post-SL3235 injection is consistent with NK cell activation. This is in contrast to the induction of tumoricidal macrophages by *Salmonella* sp., which is not evident until 7 to 10 days postvaccination (31). It was also found that depletion of T cells in vitro with anti-Thy1.2 and complement had no significant effect on NK cell activity levels (data not shown) and that in vivo treatment with anti-asialo $GM_1$ did not appreciably affect T-cell, B-cell, or macrophage function as assessed by mitogen responses and capacity to form plaque-forming cells to sheep erythrocytes (Tables 2 and see Results). Our interpretation of these results is that anti-asialo $GM_1$ treatment in vivo sensitizes SL3235-vaccinated mice to *Salmonella* infection by removing cells classically defined as NK cells (9, 29). The NK cell activity induced by vaccination with the avirulent *Salmonella* strain enhances the initial resistance to virulent *Salmonella* challenge. However, the possibility remains to be tested that other cell types including macrophage precursors (1, 2) may be affected by the anti-asialo $GM_1$ treatment in vivo and that these cells may have a role in protection against *Salmonella* sp.

The exact mechanism by which NK cells may mediate immunity to *Salmonella* species is also open for future study. More rigorous experiments are warranted to determine whether these cells can kill *Salmonella* cells directly or can damage host cells expressing *Salmonella* antigens. In addition, it would be interesting to determine whether cytokines, such as gamma interferon, secreted by NK cells could contribute to the activation of macrophages or the induction of specific protective immune responses by B and T cells against *Salmonella* species in later stages of immunity.

## ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-15613 from the National Institutes of Health.

We thank R. J. Tallarida (Department of Pharmacology, Temple University School of Medicine) for assistance in statistical analysis and Greg Harvey for typing the manuscript.

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