INFECTION AND IMMUNITY, Nov. 1996, p. 4424–4432 Vol. 64, No. 11
0019-9567/96/$04.00+0

Control by *H-2* Genes of the Th1 Response Induced against a Foreign Antigen Expressed by Attenuated *Salmonella typhimurium*

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Received 25 April 1996/Returned for modification 21 June 1996/Accepted 9 August 1996

Attenuated salmonellae represent an attractive vehicle for the delivery of heterologous protective antigens to the immune system. Here, we have investigated the influence of the genetic background of the host which regulates the growth and elimination of *Salmonella* cells on the cellular response induced against a foreign antigen delivered by an *aroA Salmonella* strain. We have tested $CD4^+$ T-cell responses (cell proliferation and cytokine production) in various mouse strains following immunization with *Salmonella typhimurium* SL3261 expressing a high level of the recombinant *Escherichia coli* MalE protein. We were able to detect a $CD4^+$ T-cell response against the recombinant MalE protein only in a restricted number of mouse strains, whereas all mice produced good levels of anti-MalE immunoglobulin G antibodies. The *Ity* gene did not play a major role in these differences in T-cell responses, since both *Ity*-resistant and -susceptible strains of mice were found to be unresponsive to MalE delivered by recombinant salmonellae. In contrast, when B10 congenic mice were used, a correlation was established between MalE-specific T-cell unresponsiveness and *H-2* genes. The discrepancies described in this paper in the ability of various strains of mice to develop an efficient Th1 response against a recombinant antigen displayed by a live *Salmonella* vaccine underscore the difficulties that can be encountered in the vaccination of human populations by such a strategy.

Immunization with live attenuated pathogens represents a widespread vaccine strategy to induce protection against the corresponding virulent pathogen (63). A new step of this strategy is to use these attenuated microorganisms as vectors for recombinant heterologous antigens to vaccinate against other microorganisms (11, 19). For several years, a large number of investigators have focused on bacterial vectors to induce both humoral and cellular immunity against various antigens. Vaccine strains of *Salmonella typhi*, the typhoid fever disease agent, have been widely developed to express heterologous protective antigens, and recombinant attenuated *Salmonella typhi* has been tested for safety and immunogenicity in humans (21, 30, 53, 54, 58).

Many antigens have also been introduced into *aroA Salmonella typhimurium* strains which were shown to elicit immune responses against these antigens in mice (1, 3, 8, 10, 20, 55). In some cases, protective immunity was induced in inbred mice against various pathogens (16, 24, 48, 56, 64). An extension of this same strategy using attenuated *Salmonella* strains expressing defined B- and T-cell determinants to stimulate a more-targeted immune response is also under investigation (43, 44, 60–62). In most of these studies, *Salmonella* strains expressing foreign antigens were usually tested in a single mouse strain, without considering the interaction of the strain with its host which is controlled by various genes. In a few cases, studies on protection against *S. typhimurium* afforded by attenuated salmonellae were indeed performed with several mouse strains (14, 31). Effectively, the induction of delayed-type hypersensitivity reactions following immunization with attenuated *Salmonella* strain SL3235 was variable among mouse strains of the C3H lineage, despite the fact that the experimental vaccine strain induced protection against challenge by virulent salmonellae in all cases (32).

Recently, using various strains of mice, we showed that the *Ity* gene, which is implicated in the bacterial growth or elimination rate (7, 52), can modulate the antibody response induced against a foreign antigen expressed by recombinant *aroA S. typhimurium* SL3261 (18). Moreover, this study also emphasized the importance of the amount of heterologous antigen expressed by the bacteria upon induction of immune responses against this antigen. Indeed, strong differences in the ability to develop an antibody response to the foreign antigen were observed between various strains of mice depending on their *H-2* genes and were shown to be related to the ability of each strain of mice to raise antibodies after immunization with low doses of antigen (18).

In the present study, we aimed to investigate these phenomena at the T-cell level. The T helper function mediated by $CD4^+$ T cells can be divided into two distinct subsets, Th1 and Th2, characterized by different cytokine producing patterns: Th1 cells secrete gamma interferon (IFN-γ), interleukin 2 (IL-2), and tumor necrosis factor beta, while Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13 (40). The immune regulation of these two subsets is mediated by different cytokines, with IL-4 favoring the forthcoming of Th2 cells and IL-12 potentiating Th1 cells (51). *Salmonella* infection was shown to trigger Th1-like cytokine production (5, 49). Moreover, the delivery of foreign antigens by live recombinant salmonellae preferentially

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induces the development of the Th1 subset of $CD4^+$ T cells (6, 57, 64).

Using attenuated *S. typhimurium* SL3261 (26) expressing the recombinant *Escherichia coli* MalE protein, we have analyzed the $CD4^+$ T-cell response in various strains of mice following immunization with these live bacteria. The *E. coli* MalE protein is widely used to produce antigens as fusion proteins, which can be expressed in bacterial strains (25). Moreover, the MalE protein is a suitable vector for expressing antigenic peptide fragments. Using this approach, we successfully induced immune responses against B- and T-cell determinants derived from different viruses, such as hepatitis B virus, poliovirus, or human immunodeficiency virus, after immunization with chimeric MalE proteins either as purified molecules or expressed by live bacteria, such as *E. coli* or attenuated *S. typhimurium* (9, 33–37).

The results presented here show that strong differences exist in the capacity of various strains of mice to develop a Th1 immune response against the recombinant MalE protein delivered by live salmonellae. Although antibody responses were elicited by such recombinant bacteria expressing high levels of MalE in all strains of mice, we were unable to detect any in vitro T-cell response against MalE in some strains of mice. These variations were shown to be controlled by the genetic background of the mice. Moreover, the results of this study clearly demonstrate that the *Ity* gene does not play an important role in such a lack of a T-cell response but that the differences in T-cell responses were associated with *H-2* genes. These results therefore indicate that independent of the induction of the humoral immune response, the induction of efficient Th1 responses against recombinant antigens expressed by live attenuated salmonellae can be impaired depending on the genetic background of the immunized animals.

MATERIALS AND METHODS

Mice. Six- to 8-week-old female inbred mice were used in all experiments. BALB/c, C57BL/6, and C3H/HeOuJ mice were from Iffa Credo (L'Abresle, France), C57BL/10, B10.D2, B10.G, and B10.Br mice were from Olac Ltd. (Blackthorn, Bicester, England).

Plasmids, bacterial strains, and growth conditions. The *AroA S. typhimurium* strain SL3261 (26) was a gift from B. A. D. Stocker. It was used as a recipient for the different plasmids. The SL3261 ΔMalE strain corresponding to the SL3261 strain with a deletion in the *S. typhimurium malE* gene was constructed as follow. The *zja-122* marker is 30% cotransducible with the *malB* region (which contains the *malE* gene). A P22 lysate made on strain TA5053 (*zja-122*::Tn*10* Δ*hisF645*) (50) was used to transduce TA5121(Δ*malE682*) for tetracycline resistance. Among the tetracycline clones, a maltose-negative strain was kept. A P22 lysate made on this strain was used to transduce SL3261. Maltose-negative colonies were selected among tetracycline-resistant clones on eosin-methylene blue-maltose plates (39) with 10 μg of tetracycline per ml.

The *E. coli malE* gene was expressed constitutively on a multicopy plasmid derived from pBR322 under the control of the *ptac* promoter. Two constructions (plasmids pTE and pNTE) leading to different expression levels were used. Comparison of the levels of expression of *malE* cloned on these two plasmids was described previously (18). The quantification method of the MalE protein uses a monoclonal antibody (MAb) directed against the *E. coli* MalE which does not recognize the *S. typhimurium* MalE (MAb 56.5) (unpublished data). SL3261(pTE) expressed 4.7 ng of *E. coli* MalE per 10^6 bacteria, and SL3261(pNTE) expressed 15.7 ng of *E. coli* MalE per 10^6 bacteria.

For immunization, SL3261(pTE), SL3261(pNTE), SL3261, and SL3261 ΔMalE bacterial strains were cultured for 16 h at 37°C, without shaking, in a closed bottle of L broth (39) containing 100 μg of ampicillin per ml. Bacteria were collected by centrifugation and resuspended in phosphate-buffered saline (PBS) at the required concentration. Bacteria were prepared the day of immunization and estimated by the optical density of the suspension at 600 nm (1 $OD_{600} = 5 \times 10^8$ bacteria per ml). The viable bacterial count and the plasmid stability were confirmed by plating suitable dilutions on L agar plates with or without 100 μg of ampicillin per ml.

Reagents. MalE protein was purified by affinity chromatography of *E. coli* ED9(pPD1) as described previously (37). Purified protein was concentrated by ammonium sulfate precipitation, resuspended in PBS, and desalted on Sephadex G-25 (Pharmacia Inc.) in PBS. The concentration of the protein was determined by UV absorbance. Concanavalin A (ConA) was purchased from Sigma Chemical Co. (St. Louis, Mo.). GK1.5 anti-CD4 and H35.17.2 anti-CD8 MAbs were prepared from ascitic fluids as described previously (17).

T-cell proliferation assay. Mice were immunized intraperitoneally (i.p.) with 10^6 live bacteria on days 0 and 21. Five to 10 weeks later, spleen cells were removed and single-cell suspensions were prepared and cultured in RPMI 1640 medium (Seromed, Munich, Germany) supplemented with 10% fetal calf serum, antibiotics, 2 mM L-glutamine, and 5×10^{-5} M 2-mercaptoethanol. Spleen cells (10^6 per well) were plated onto 96-well microtiter plates (TPP, Trasadingen, Switzerland) with serial dilutions of MalE protein or with medium alone. After 3 days at 37°C, the cells were pulsed for 18 h with [^{3}H]thymidine ([^{3}H]TdR) (NEN, Boston, Mass.) and then harvested onto fiberglass filters (Wallac Oy, Turku, Finland) with an automated cell harvester. Incorporated radioactivity was measured by scintillation counting. Results were expressed as mean counts per minute from duplicate or triplicate culture wells. Standard deviations were less than 15% of the mean. Results are representative of three to four experiments.

Assay of cytokine production. Spleen cells were prepared as in the T-cell proliferation assay. Supernatants were harvested at 24 and 48 h for the IL-2 assay and at 48, 72, and 96 h for the other cytokines. IL-2 was measured by using the IL-2-dependent CTLL cell line. The levels of IL-4, IL-5, and IFN-γ were determined by a enzyme-linked immunosorbent assay sandwich (ELISA) using, respectively, BVD4-1D11, TRFK5, and R4-6A2 (Pharmingen, San Diego, Calif.) as capture antibodies and appropriate secondary biotinylated anticytokine-specific MAbs (BVD6-24G2, TRFK4, and XMG1.2; Pharmingen). The binding of the second MAb was detected by streptavidin-horseradish peroxidase (Amersham, Les Ulis, France) and *O*-phenylenediamine (Sigma) and hydrogen peroxide (Sigma) as the substrate. All assays were standardized with recombinant murine cytokines. Recombinant IL-2 and IL-4 were obtained from Genzyme, whereas IL-5 and IFN-γ were purchased from Pharmingen. In the time course of cytokine production in supernatants after in vitro antigenic stimulation, only the time point corresponding to the maximum cytokine level was shown, i.e., at 24 h for IL-2, at 48 h for IFN-γ, and at 96 h for IL-4 and IL-5. All cytokine levels were expressed in picograms per milliliter, and results are representative of three to four experiments. This assay was capable of detecting 250 pg of IFN-γ per ml, 62 pg of IL-4 per ml, 125 pg of IL-5 per ml, and 300 pg of IL-2 per ml.

Analysis of antibody response. Bacterium-immunized mice were bled 1 week after the last boost and the day before the T-cell response assays. Individual mouse serum samples were tested for antibodies against MalE protein or whole *Salmonella* extract by ELISA as described previously (18). Goat anti-mouse immunoglobulin G (IgG) peroxidase conjugate (Sigma) and *O*-phenylenediamine were used to detect antibodies. The negative control consisted of naive mouse serum diluted 100-fold. ELISA antibody titers were determined by linear regression analysis plotting dilution versus A_{492}. The titers were calculated to be the $\log_{10}$ highest dilution which gave twice the absorbance of normal mouse serum diluted 1/100. Titers were given as the arithmetic mean ± standard deviation of the $\log_{10}$ titers. Statistical analysis was performed by Student's *t* test. *P* values of less than 0.05 were considered significant.

RESULTS

Induction of a $CD4^+$ T-cell response against MalE protein expressed by attenuated *S. typhimurium*. We previously showed that the antibody response against antigens delivered by attenuated *S. typhimurium* (strain SL3261) was under the control of the genetic background of the mouse strains (18). Indeed, while C57BL/6 and BALB/c mice developed a good anti-MalE antibody response after priming mice with SL3261 expressing the *E. coli* MalE protein [SL3261(pTE)], C3H/He failed to do so. The level of MalE expressed by SL3261(pTE) was shown to be critical for this difference, since the lack of antibody response was overcome by priming C3H/He with SL3261(pNTE), which expressed a fourfold-higher level of MalE.

Here, to analyze further this phenomenon, we compared the MalE-specific $CD4^+$ T-cell responses induced in BALB/c, C57BL/6, and C3H/He mice by SL3261 expressing MalE. We first performed a kinetic analysis of the proliferative response induced in BALB/c mice against the recombinant MalE protein expressed by SL3261(pNTE) to determine the optimal experimental conditions to study $CD4^+$ T-cell immune responses. BALB/c mice were injected i.p., once (day 0) or twice (days 0 and 21), with 10^6 live SL3261(pNTE) bacteria, and the proliferation of spleen cells in response to in vitro stimulation by purified MalE protein was analyzed at different time points (Fig. 1A). After one injection of the recombinant salmonellae, a significant MalE-specific proliferative response could be de-

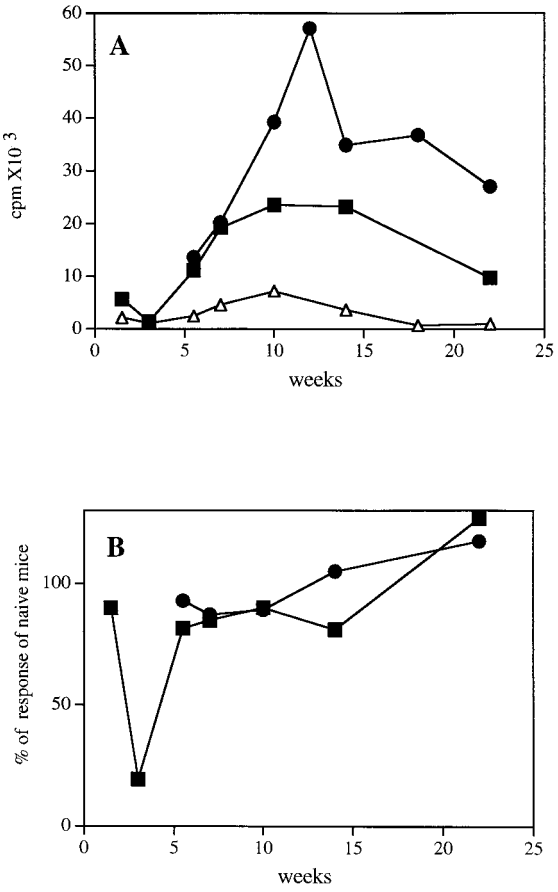

FIG. 1. Kinetics of proliferative response to MalE protein of BALB/c mice immunized with SL3261(pNTE). BALB/c mice received one (day 0) (■) or two (days 0 and 21) (●) i.p. injections of 10^6 live SL3261(pNTE) bacteria, whereas control mice were left untreated (△). At different time points, spleen cells from these mice were stimulated in vitro with 100 ng of MalE protein per ml (A) or 2.5 μg of ConA per ml (B). Proliferation was assayed by [^{3}H]TdR incorporation.

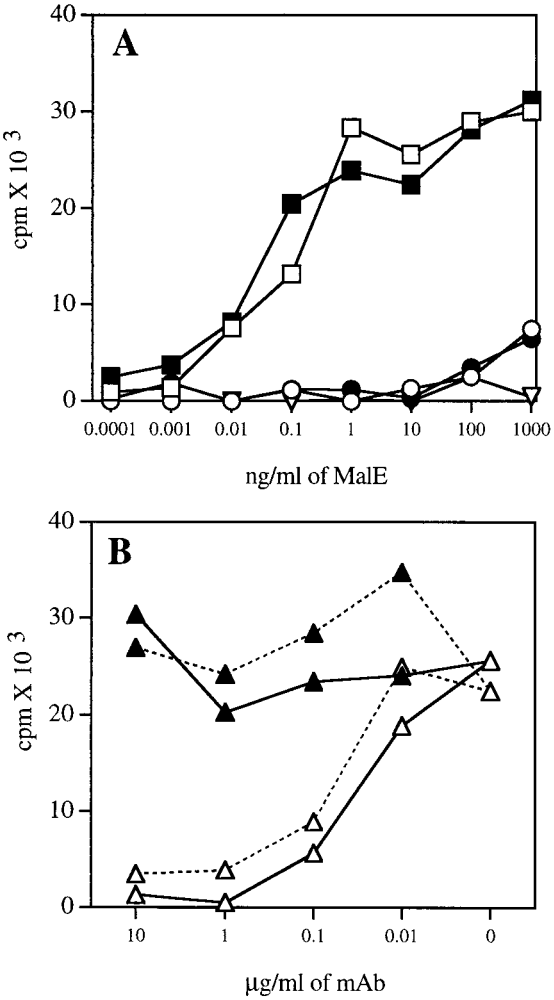

FIG. 2. Recombinant *Salmonella*-induced MalE-specific CD4$^+$ T-cell responses in BALB/c mice. (A) Two mice per group were injected i.p. twice (days 0 and 21) with 10^6 live SL3261(pNTE) (□), SL3261(pTE) (■), SL3261 (○), or SL3261 ΔMalE (●) bacteria or were left untreated (▽). Five weeks later, pooled spleen cells from each group were incubated with serial dilutions of MalE. (B) Blocking experiments were performed by adding serial dilutions of GK1.5 anti-CD4 (△) or H35.17.2 anti-CD8 (▲) MAb to spleen cells from mice immunized with SL3261(pNTE) (——) or SL3261(pTE) (– – – –) before incubation with 10 ng of MalE per ml. [^{3}H]TdR incorporation was assessed on day 4.

tected 5 weeks after immunization. This response persisted at the same level for 10 weeks and then slowly decreased after that point. No MalE-specific proliferative response was found in untreated naive BALB/c mice. Mice which received a boost injection on day 21 displayed an increased MalE-specific proliferative response which was still detectable 6 months after the first injection of salmonellae. The analysis of spleen cell responses to ConA showed that no proliferative response to this T-cell mitogen could be detected until 4 weeks after the administration of salmonellae. Such a nonspecific suppression following attenuated *Salmonella* infection was previously shown to be mediated by activated macrophages (2). Interestingly, no suppression of proliferative response was demonstrated after the boost on day 21 (Fig. 1B).

To assess the specificity of the proliferative response against the *E. coli* MalE protein, BALB/c mice were immunized twice (days 0 and 21) with SL3261 expressing the *E. coli* MalE protein under the pTE or pNTE promoter or with two control *Salmonella* strains corresponding to SL3261 with the wild-type *S. typhimurium malE* gene and to strain SL3261 with a deletion in the *S. typhimurium malE* gene (SL3261 ΔMalE). Both SL3261(pTE) and SL3261(pNTE) strains induced strong MalE-specific proliferative responses in BALB/c mice (Fig. 2A). By contrast, BALB/c mice injected with SL3261 or SL3261 ΔMalE or left untreated did not develop any proliferative response to MalE, showing the specificity of the response. Moreover, these results also demonstrate that the proliferative response was specific for the *E. coli* MalE protein and was not directed against the *S. typhimurium* MalE protein.

As shown in Fig. 2B, MalE-specific proliferative responses were blocked by GK1.5 anti-CD4 MAb but not by H35.17.2 anti-CD8 MAb, showing that the proliferative immune responses induced by SL3261(pTE) or SL3261(pNTE) were mediated by CD4$^+$ T lymphocytes.

Altogether, these data show that in BALB/c mice, the administration of two doses of attenuated SL3261 expressing MalE induces a strong and long-lasting CD4$^+$ T-cell response against the MalE protein.

SL3261(pNTE) induced an anti-MalE antibody response in C3H/He mice but failed to elicit an efficient anti-MalE CD4$^+$ T-cell response in this strain of mice. We next compared the CD4$^+$ T-cell responses induced by SL3261 expressing MalE in C57BL/6 and C3H/He mice, which were previously shown to

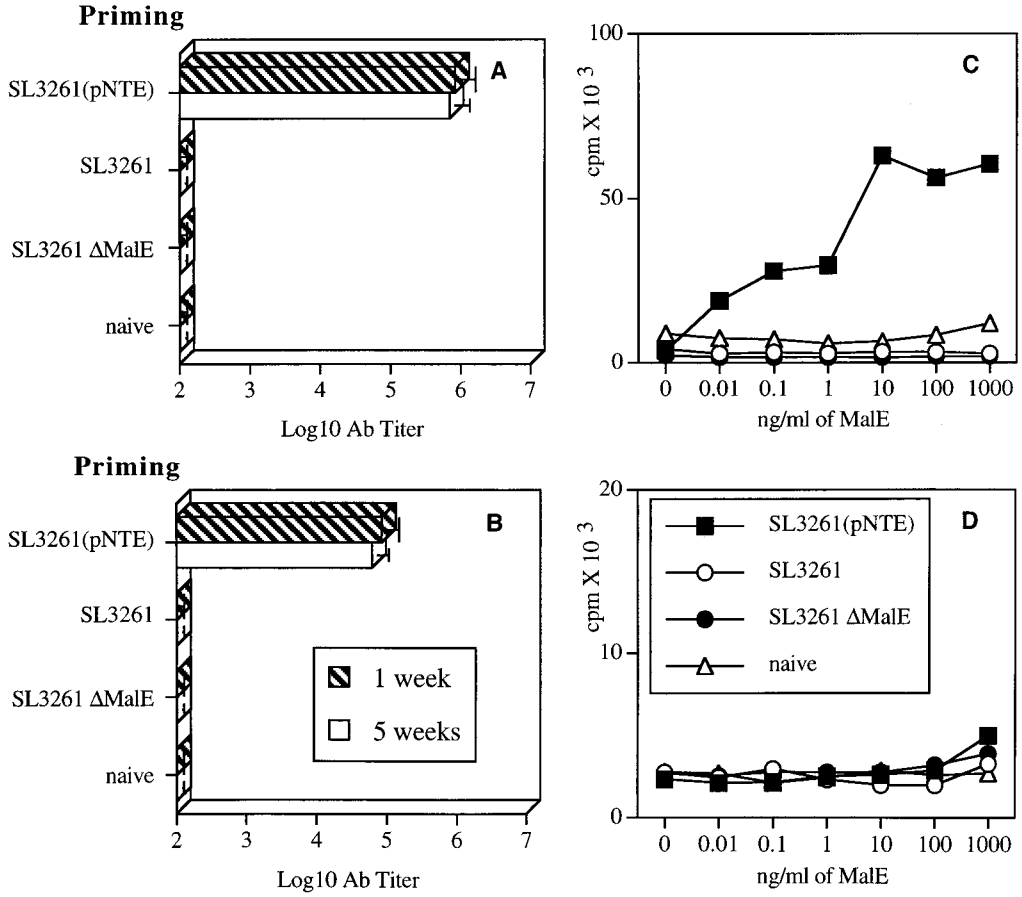

FIG. 3. Comparison of anti-MalE humoral and cellular immune responses in C57BL/6 and C3H/He mice following immunization with SL3261(pNTE). C57BL/6 (A and C) or C3H/He (B and D) mice received two i.p. injections of 10^6 SL3261 bacteria or derivatives as indicated or were left untreated. (A and B) Sera from four to five mice per group were collected 1 and 5 weeks after the boost and tested for anti-MalE IgG antibodies; results are expressed as means ± standard deviations of $\log_{10}$ individual titers. (C and D) Five weeks after the boost, spleen cells were removed and incubated with MalE protein, and proliferative responses were determined by [^{3}H]TdR incorporation.

differ in their abilities to develop an antibody response to MalE after SL3261(pTE) priming (18). Mice received two i.p. injections of either SL3261(pNTE), SL3261, or SL3261 ΔMalE. As observed previously, a good anti-MalE IgG antibody response was induced following the administration of SL3261(pNTE) in both C57BL/6 and C3H/He mice (Fig. 3A and B). No anti-MalE antibodies were elicited by control bacterial strains (SL3261 and SL3261 ΔMalE), whereas comparable anti-*Salmonella* antibody levels were induced in all groups of immunized animals (data not shown).

The anti-MalE CD4$^+$ T-cell responses of C57BL/6 and C3H/He mice were compared following administration of SL3261(pNTE) by analyzing the proliferative response of spleen cells after in vitro stimulation with serial doses of MalE. In C57BL/6, a strong anti-MalE T-cell proliferation was found in vitro, indicating that T cells specific for this antigen were efficiently primed in vivo by SL3261(pNTE), whereas no response was detected in control groups (Fig. 3C). Strikingly, spleen cells from C3H/He mice immunized with SL3261 (pNTE) did not proliferate after in vitro stimulation with MalE (Fig. 3D), despite the fact that a good anti-MalE antibody response was induced in these mice (Fig. 3B). A similar lack of proliferative response was obtained with SL3261(pTE), which expressed a lower MalE protein level than that of the SL3261 (pNTE) construct (data not shown). In these experiments, the lack of a detectable anti-MalE T-cell response in *Salmonella*-infected C3H/He mouse spleen cells was due neither to the incapacity of primed spleen T cells to respond to in vitro stimulation as shown by their ability to proliferate in response to ConA or salmonella extract nor to the inability of their spleen antigen-presenting cells to process and present antigen to T cells (data not shown).

Analysis of cytokine production by T lymphocytes primed with recombinant attenuated *S. typhimurium* SL3261(pNTE). The ability of SL3261(pNTE) to induce anti-MalE IgG antibodies in C3H/He mice strongly suggests that anti-MalE T helper cells were primed in vivo, despite the fact that no proliferative T-cell response could be characterized in vitro. One possibility for this would be that the activated T-cell subset belongs to a Th2 phenotype, characterized by an IL-4 cytokine production and associated with a weak antigen-specific T-cell proliferation (15). Therefore, to further analyze the anti-MalE CD4$^+$ T-cell response induced by recombinant *Salmonella* bacteria, we analyzed the cytokine production by anti-MalE-specific T cells following SL3261(pNTE) injection.

First, spleen cells from SL3261(pNTE)-primed BALB/c mice were assayed for IL-2, IFN-γ, IL-4, and IL-5 production following stimulation with MalE. As shown in Fig. 4, MalE-specific T-cell activation was characterized by the release of IL-2 and IFN-γ and the lack of detectable IL-4 and IL-5

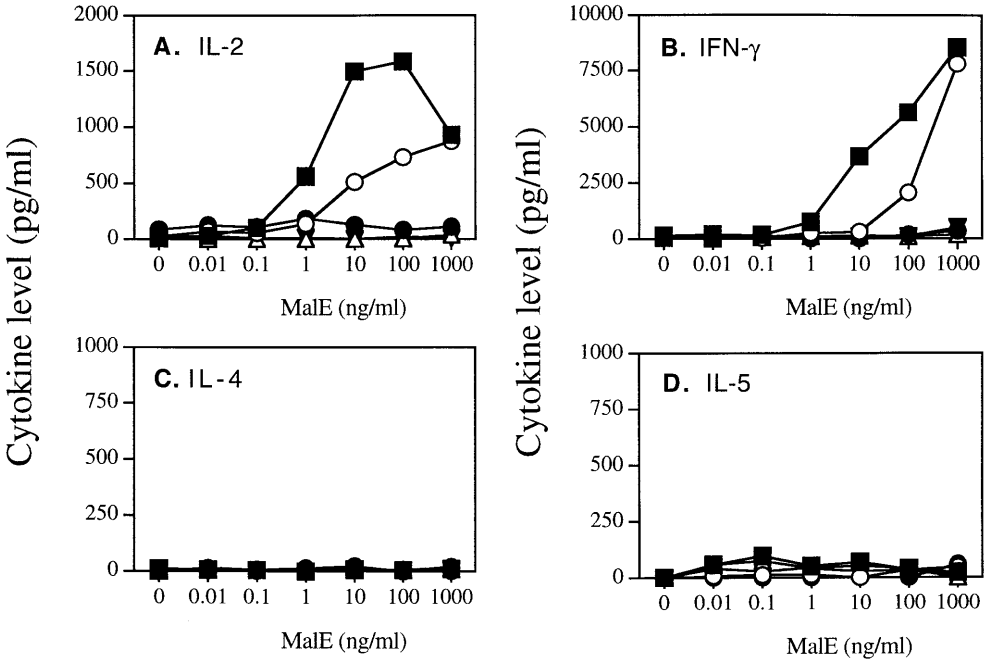

FIG. 4. BALB/c mice immunized with SL3261(pNTE) exhibit a Th1 response against MalE protein. BALB/c mice were immunized i.p. with 10^6 SL3261 bacteria or derivatives on days 0 and 21 or were left untreated. Five weeks later, spleen cells were removed and stimulated with serial doses of MalE protein. Cytokine production was assayed in the culture supernatant of duplicate culture wells. Symbols: ■, SL3261(pNTE); ○, SL3261(pTE); ●, SL3261 △, SL3261 ΔMalE; ▼, untreated.

production, corresponding to a Th1 response. This cytokine production pattern was abolished by preincubation of spleen cells with GK1.5 anti-CD4 MAb but not with H35.17.2 anti-CD8 MAb, showing that the response observed was due to $CD4^+$ T lymphocytes (data not shown). No cytokine production after in vitro stimulation with MalE was detected in cultures of spleen cells from mice primed with SL3261 and SL3261 ΔMalE or from untreated mice. These results are in agreement with previous studies showing that antigen-specific T-cell activation by salmonellae induced a Th1 phenotype response (6, 38, 57, 64).

We next tested the T helper cytokine response to MalE in C57BL/6 and C3H/He mice after administration of SL3261 (pNTE). C57BL/6 mice raised a strong Th1 response to MalE as shown by the production of IL-2 and IFN-γ (Fig. 5A and C) and the lack of IL-4 or IL-5 synthesis (data not shown). In C3H/He mice, neither IL-2- and IFN-γ-producing T cells (Fig. 5B and D) nor IL-4- and IL-5-producing T cells (data not shown) were detected in response to MalE.

Role of *H-2* genes in the $CD4^+$ T-cell response induced by SL3261(pNTE). In a previous study, we demonstrated that the minimal antigen dose required to elicit an antibody response to MalE did vary among strains depending on their *H-2* haplotype (18). Therefore, we postulated that *H-2* gene polymorphism may explain the differences observed between the C3H/He ($H\text{-}2^k$) nonresponder strain and the BALB/c ($H\text{-}2^d$) and C57BL/6 ($H\text{-}2^b$) responder strains in the $CD4^+$ T-cell response to MalE after SL3261(pNTE) immunization.

To test this hypothesis, we analyzed the anti-MalE immune response induced by SL3261(pNTE) in B10 congenic mice which differ only in their *H-2* genes. C57BL/10 ($H\text{-}2^b$), B10.Br ($H\text{-}2^k$), B10.G ($H\text{-}2^q$), and B10.D2 ($H\text{-}2^d$) mice received two i.p. injections (days 0 and 21) of SL3261(pNTE). The anti-MalE antibody response was analyzed 1 week after the boost and then 10 weeks after, just prior to the T-cell response assays. Results did not show any difference in the antibody titers between these congenic mice when measured 1 week after the boost injection, which was in good agreement with our previous demonstration that increasing the amount of MalE delivered by recombinant bacteria overcomes the *H-2* control of antibody response (18). However, 10 weeks later, the antibody responses of C57BL/10 and B10.D2 mice were significantly higher than those of B10.Br and B10.G mice ($P < 0.05$) (Fig. 6A). The lower anti-MalE antibody titers of B10.Br and B10.G mice suggest that the T-cell priming was less efficient in these strains of mice. This was confirmed by the analysis of the anti-MalE $CD4^+$ T-cell response induced by SL3261(pNTE). Indeed, no specific proliferative response was evidenced after culture of B10.Br and B10.G mouse spleen cells with MalE, in contrast to what was observed for C57BL/10 and B10.D2 mouse spleen cells (Fig. 6B). Likewise, IL-2 and IFN-γ were produced by spleen cells from infected C57BL/10 and B10.D2 mice but not by B10.Br or B10.G mouse spleen cells (Fig. 6C). In vitro stimulation with MalE did not reveal any IL-4 or IL-5 production by T cells in any B10 congenic mice. These results demonstrate that the ability of mouse strains to elicit an efficient Th1 cell response against MalE after immunization with recombinant attenuated salmonellae depends on their *H-2* genetic background.

DISCUSSION

In the present study, we have examined the ability of live attenuated *aroA S. typhimurium* as an antigen delivery system to induce a $CD4^+$ T-cell response in mouse strains of different genetic backgrounds. We used strain SL3261 of *S. typhimurium* expressing the *E. coli* MalE protein as an experimental model. The results presented here show dramatic differences between various mouse strains in their ability to mount a Th1 $CD4^+$ cell response against the MalE antigen after immunization with recombinant *S. typhimurium*. These results highlight the fact that the genetic background may impair the elicitation of an

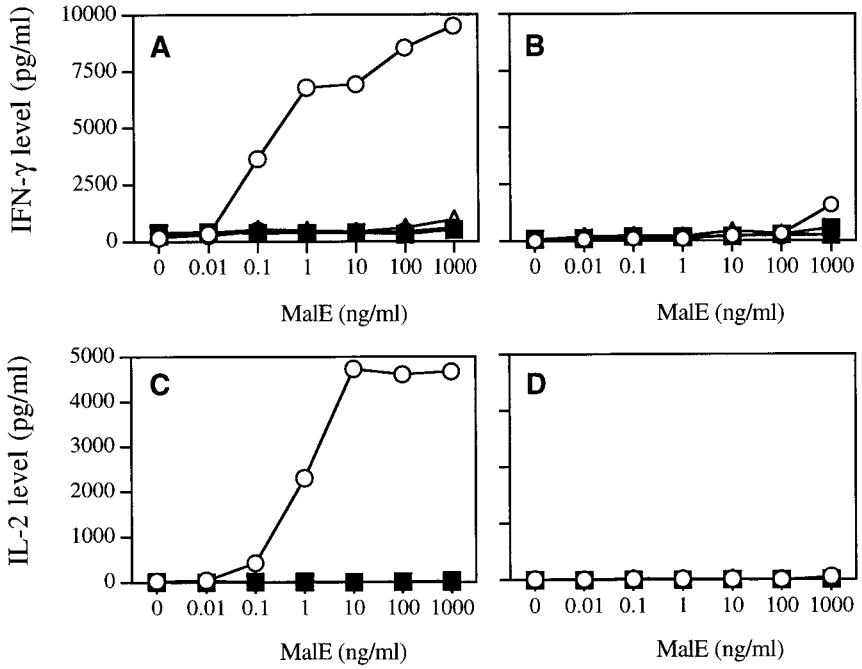

FIG. 5. Lack of Th1 response in C3H/He mice compared with C57BL/6 mice immunized with SL3261(pNTE). C57BL/6 (A and C) or C3H/He (B and D) mice were injected i.p. with 10^6 SL3261 bacteria or derivatives or were left untreated. Five weeks later, spleen cells were incubated with the MalE protein, and IFN-γ (A and B) and IL-2 (C and D) production in supernatants was assessed. Positive controls after ConA T-cell stimulation gave similar responses for all groups: IFN-γ levels for C57BL/6 ranged from 5,084 to 6,806 pg/ml, and those for C3H/He ranged from 3,479 to 4,702 pg/ml; IL-2 levels were >10,000 pg/ml for all groups. Results are expressed as the mean of duplicate culture wells and are representative of three experiments using four mice per group. Symbols: ○, SL3261(pNTE); ■, SL3261; ●, SL3261 ΔMalE; △, untreated.

efficient T-cell response against a recombinant protein antigen expressed by a *Salmonella* vaccine and indicate that *H-2* genes are responsible for this phenomenon.

Infection by salmonellae has been shown to promote a strong cellular immune response against *Salmonella* components, and *Salmonella* vectors were shown to represent a potent means of inducing a $CD4^+$ T-cell response against recombinant antigens. Indeed, *Salmonella* infection initiates a strong Th1 response via production of IL-12 and IFN-γ, which are required for bacterial killing activity of macrophages (5, 29), this antibacterial activity being inhibited by IL-4 (12). Incidentally, this phenomenon drives the T helper phenotype of the T-cell response when *S. typhimurium* is used as a delivery vehicle. This point is well illustrated by the protective effect of a *Salmonella* vaccine strain expressing *Leishmania major* gp63 in the *Leishmania* infection model (64), for which the Th1 response is critical for protection (22, 23). In our study, we clearly showed that the Th1 response elicited against MalE after immunization with recombinant SL3261 can be evidenced in vitro in C57BL/6 and BALB/c mice but was totally undetectable in C3H/He mice. The induction of anti-MalE IgG antibodies in all strains of mice suggests that an anti-MalE T-cell response was induced in vivo; however, it clearly appears that the efficiency of T-cell priming was strongly modulated.

The innate susceptibility or resistance of mice to *S. typhimurium* infection is governed by several genes (13, 27, 45–47), and differences in the genetic background between nonresponder and responder mouse strains may contribute to this heterogeneity through the growth and/or elimination rate of *S. typhimurium*. For instance, we showed that following i.p. immunization with SL3261 bacteria expressing MalE, the bacterial clearance was delayed by 3 to 4 weeks in susceptible (C57BL/6 × BALB/c)F_1 mice compared with that in resistant C3H/He mice, whereas after inoculation, the growth rates of SL3261 were comparable in both strains (18). Therefore, a rapid elimination of the vaccine strain could compromise the efficiency of T-cell priming. In our experiments, one of the loci of interest is represented by the *Ity* gene, which controls the growth rate of the bacteria in the early phase of infection (7, 27). Mice bearing the dominant Ity^r allele, such as C3H/He mice, were described as resistant to *Salmonella* infection, whereas the Ity^s allele conferred a susceptible phenotype to mouse strains such as BALB/c and C57BL/6. *H-2* genes were also shown to strongly contribute to bacterial clearance of salmonellae but during the late phase of the infection (28, 41, 42). Regarding *H-2* genes, the C3H/He ($H\text{-}2^k$) mice carry a resistant *H-2* haplotype, but BALB/c ($H\text{-}2^d$) and C57BL/6 ($H\text{-}2^b$) mice carry a susceptible haplotype. Therefore, in these strains of mice, it was not possible to distinguish between the role of *Ity* and *H-2* gene-associated resistance or susceptibility to *Salmonella* infection in the modulation of T-cell responsiveness.

Following immunization with SL3261(pNTE), the different patterns of Th1 response to MalE obtained in congenic B10 mice carrying the Ity^s allele but various *H-2* haplotypes demonstrated that the *Ity* gene was not responsible for the heterogeneity of T-cell responses against the recombinant antigen. In contrast, *H-2*-associated resistance or susceptibility to *S. typhimurium* infection correlated with low MalE responder strains (B10.Br [$H\text{-}2^k$] and B10.G [$H\text{-}2^q$] mice) and high MalE responder strains (C57BL/10 [$H\text{-}2^b$] and B10.D2 [$H\text{-}2^d$]) (18). Indeed, mice of $H\text{-}2^k$ and $H\text{-}2^q$ haplotypes were described to be resistant to *Salmonella* infection, while $H\text{-}2^b$ and $H\text{-}2^d$ mice were shown to be susceptible (28, 42). This correlation could suggest that the lack of a detectable T-cell response to MalE delivered by a live SL3261 vehicle is due to the high rate of

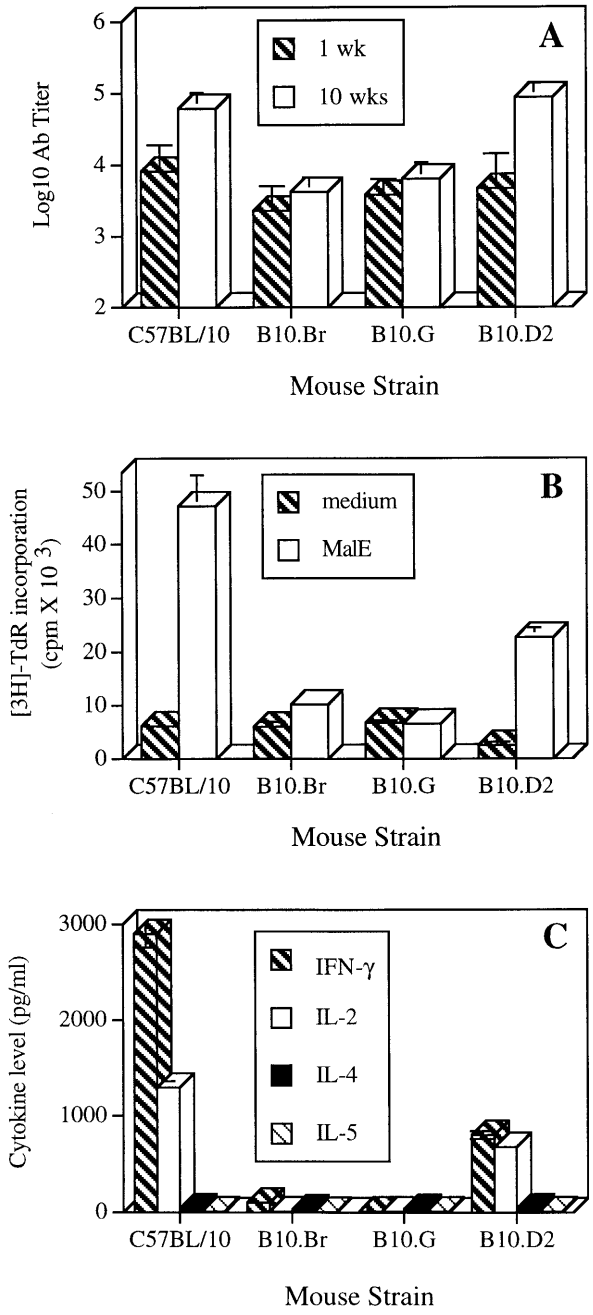

FIG. 6. Role of *H-2* genes in the Th1 response to recombinant MalE antigen expressed by attenuated *Salmonella* bacteria. B10 congenic mice received two i.p. injections (days 0 and 21) of 5×10^6 SL3261(pNTE) bacteria. (A) Four to six mice per group were analyzed for anti-MalE antibody (Ab) responses 1 and 10 weeks following the second injection of SL3261(pNTE). Ten weeks after the boost, spleen cells were removed and incubated with 100 ng of MalE per ml. (B) Proliferative responses were determined by [^{3}H]TdR incorporation. (C) Culture supernatants were assayed for IFN-γ, IL-2, IL-4, and IL-5 content.

bacterial clearance in mice and therefore is associated with the low survival of the *Salmonella* strain.

Because the CD4$^+$ T-cell response is also totally dependent on *H-2* genes through antigen presentation by major histocompatibility complex (MHC) class II molecules, it is difficult to discriminate between *H-2*-linked factors influencing MHC class II presentation and *Salmonella* clearance. However, several lines of evidence are in favor of a direct influence of the *H-2* genes on the presentation by MHC class II molecules.

First, in previous work, we have shown that the ability of various strains of mice to develop an antibody response following immunization with live SL3261 expressing MalE was highly dependent on the expression level of the recombinant antigen. Thus, by using different SL3261 constructs, a four- to fivefold increase of the expression level of MalE by the bacteria can turn a nonresponder strain into a responder strain with regards to the anti-MalE antibody response (18). This result was correlated with the threshold amount of soluble MalE protein required to elicit an antibody response in each strain of mice. This latter phenomenon was associated with *H-2* genes, as demonstrated in earlier works (59), and linked to the T-cell immune response (4).

Second, in the present study, we were not able to detect any anti-MalE T-cell response after SL3261(pNTE) priming in some mouse strains, but a T-cell priming probably occurred since anti-MalE IgG could be detected in these mice. Using overlapping synthetic peptides, we recently mapped the CD4$^+$ T-cell determinants of the MalE protein in different inbred mice of *H-2*^{k}, *H-2*^{d}, and *H-2*^{b} haplotypes, revealing, respectively, one, four, and seven determinants (34a). Interestingly, after SL3261(pNTE) immunization, we were not able to detect any in vitro T-cell response of spleen cells from *H-2*^{k} mice (C3H/He and B10.Br) for which there exists only one single T-cell determinant. To date, there is no evidence of a relationship between the number of immunogenic peptides in a protein antigen and its T-cell immunogenicity. However, a large number of immunogenic peptides could stimulate a larger set of T lymphocytes. In this case, one might suggest that the outcome of an efficient T-cell priming could be linked to the number of T-cell determinants present in the protein antigen. Therefore, the immunogenic characteristics of the MalE protein in *H-2*^{k} mice may contribute to the lack of a detectable anti-MalE T-cell response in C3H/He and B10.Br mice.

Finally, several studies have demonstrated the induction of T-cell responses to recombinant antigens expressed by an attenuated *S. typhimurium* vector in mice harboring a resistant *H-2*^{k}-associated phenotype (55, 61, 64), which are unresponsive in our model. Although it remains difficult to compare results from the literature obtained with different constructs and *Salmonella* strains, in terms of in vivo stability or bacterial clearance, these studies indicate that there are no particular constraints in the response of *H-2*^{k}-bearing mice to a *Salmonella* vaccine.

We cannot totally exclude a role of *H-2* genes in the T-cell responses to MalE related to the innate susceptibility or resistance of the host to *Salmonella* infection, but the elements discussed above argue in favor of a direct influence of *H-2* genes on the ability of each mouse strain to respond to the recombinant MalE protein. However, to clearly establish the exact role of *H-2* genes, identical experiments should be performed with the same *Salmonella* strains but expressing various foreign antigens. The data obtained with the *E. coli* MalE protein show that although *S. typhimurium* strains are a very potent means of inducing a cellular response, this delivery system was not sufficient to trigger an efficient T-cell response to the recombinant antigen in any strain of mice, whatever its *H-2* genetic background. Regarding the high degree of polymorphism of MHC genes in humans, our results underscore the problems that can be encountered in elaborating vaccines based on live bacteria expressing recombinant antigens.

ACKNOWLEDGMENTS

This work was supported by a grant from EEC (Biotechnology Program, contract PL 920349). R. Lo-Man was supported by ARC.

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Editor: A. O'Brien