Igh-6−/− (B-Cell-Deficient) Mice Fail To Mount Solid Acquired Resistance to Oral Challenge with Virulent *Salmonella enterica* Serovar Typhimurium and Show Impaired Th1 T-Cell Responses to *Salmonella* Antigens

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In the present study we evaluated the role of B cells in acquired immunity to *Salmonella* infection by using gene-targeted B-cell-deficient innately susceptible mice on a C57BL/6 background (*Igh-6*−/−). *Igh-6*−/− mice immunized with a live, attenuated *aroA Salmonella enterica* serovar Typhimurium vaccine strain showed impaired long-term acquired resistance against the virulent serovar Typhimurium strain C5. *Igh-6*−/− mice were able to control a primary infection and to clear the inoculum from the reticuloendothelial system. However, *Igh-6*−/− mice, unlike *Igh-6*+/+ C57BL/6 controls, did not survive an oral challenge with strain C5 at 4 months after vaccination. Transfer of immune serum did not restore resistance in *Igh-6*−/− mice. Total splenocytes and purified CD4+ T cells obtained from *Igh-6*−/− mice 4 months after vaccination showed reduced ability to release Th1-type cytokines (interleukin 2 and gamma interferon) upon in vitro restimulation with serovar Typhimurium soluble cell extracts compared to cells obtained from *Igh-6*+/+ C57BL/6 control mice. Therefore, the impaired resistance to oral challenge with virulent serovar Typhimurium observed in B-cell-deficient mice, which cannot be restored by passive transfer of *Salmonella*-immune serum, may be in part due to a reduced serovar Typhimurium-specific T-cell response following primary immunization.

Salmonella infections are still a serious health problem worldwide. The recent emergence of multidrug-resistant *Salmonella* strains calls for a more rational approach to the treatment of the disease and increases the need for the rapid development of safer and more effective vaccines. A better understanding of the protective mechanisms of acquired immunity to *Salmonella* would undoubtedly be beneficial for improving vaccination strategies.

The mouse model is commonly used to study the mechanisms of natural resistance and acquired immunity to *Salmonella*. Early bacterial growth in mice is controlled by the *Nramp (Ity)* gene, expressed in macrophages (43), and is suppressed by a T-cell-independent host response which requires production of tumor necrosis factor alpha (TNF-α), interleukin 12 (IL-12), IL18, and gamma interferon (IFN-γ) as well as granuloma formation and the production of nitric oxide (10, 15, 18, 19, 25, 27, 28, 34, 35, 41, 42). Clearance of bacteria from the tissues requires functional CD4+ T cells (10), is under the control of genes within and outside the H-2 complex, and can leave long-lasting specific immunity to rechallenge (reviewed in references 14 and 24).

Mice immunized with protective live attenuated *Salmonella* vaccines mount antibody responses against lipopolysaccharide and protein antigens in addition to acquiring Th1-type T-cell memory (8). The relative importance of T- and B-cell-associated immunity in long-term vaccine-induced acquired protection against virulent salmonellae has been intensively investigated and much debated (reviewed in references 6, 14, and 24). Members of our group and others have demonstrated that both CD4+ and CD8+ T cells, with the contribution of TNF-α and IFN-γ, are needed for the expression of full acquired resistance to *Salmonella* (7, 23, 41). A role for B-cell-dependent humoral immunity in vaccine-induced protection has also been demonstrated (4–6, 11).

It is now becoming increasingly clear that antibodies or T cells alone can confer only a moderate level of protection against salmonellosis, especially when innately susceptible animals are challenged with fully virulent salmonellae. Passive administration of immune serum or B cells alone can protect innately resistant mice against virulent salmonellae or susceptible mice against moderately virulent organisms (5, 6, 11). In such host-parasite combinations, passively administered antibody can reduce the number of organisms in a moderate, but lethal, challenge to sublethal levels such that humoral immunity alone can confer protection (5). Adoptive transfer of immune T cells can protect mice against infection with very low doses of virulent salmonellae or against moderately virulent microorganisms (7, 37). However, adoptive transfer of both immune serum and immune T cells is absolutely required to protect innately susceptible mice against highly virulent salmonellae (26), indicating that neither humoral nor cell-mediated immunity is dispensable for resistance to virulent salmonellae in *Nramp*s/s animals.

In addition to antibody production, B cells display a wide range of functions within the immune system, including antigen presentation and production of cytokines (9, 33). Epstein-Barr virus-transformed B cells have been successfully used to present *Salmonella* antigens to T cells (40). Recent reports indicate that gene-targeted B-cell-deficient mice display in-

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creased susceptibility to infections by some agents but not by others (1, 3, 13, 17, 21, 22, 44, 45). In murine models of infection with *Chlamydia trachomatis*, lymphocytic choriomeningitis virus, and *Bordetella pertussis*, a role for B cells in the initiation and persistence of T-cell responses has been demonstrated (13, 22, 45).

In the present study, we evaluated the role of B cells in long-term acquired immunity to virulent *Salmonella enterica* serovar Typhimurium in mice vaccinated with live, attenuated serovar Typhimurium strain SL3261 (*aroA*).

MATERIALS AND METHODS

Animals. C57BL/6 mice were purchased from Harlan Olac Ltd., Blackthorn, Bicester, United Kingdom. B-cell-deficient mice on a C57BL/6 background homozygous for a targeted mutation in the gene for immunoglobulin heavy chain 6 (*Igh-6*) (20) were bred either at BⓍK Universal Ltd. (Hull, United Kingdom) or at the Imperial College animal unit from breeder mice originally obtained from The Jackson Laboratory, Bar Harbor, Maine. Age- and sex-matched groups were used when over 8 weeks old.

Bacteria. *S. enterica* serovar Typhimurium strain SL3261 is an *aroA* attenuated live vaccine strain (12) with an intravenous (i.v.) 50% lethal dose (LD_{50}) for C57BL/6 mice of ca. 10^7 CFU. *S. enterica* serovar Typhimurium strain C5 is a virulent strain with an i.v. LD_{50} for C57BL/6 mice of <10 CFU (26) and an oral LD_{50} for C57BL/6 mice of ca. 10^7 CFU (unpublished data). Strain C5 *rfa* is an O-rough derivative of strain C5 kindly provided by B. A. D. Stocker, Stanford University, Stanford, Calif. For i.v. inoculation, bacteria were grown at 37°C as stationary-phase overnight cultures in Luria-Bertani (LB) broth (Difco). Aliquots were snap frozen and stored in liquid nitrogen. The inoculum was diluted in phosphate-buffered saline (PBS) and injected into a lateral tail vein. For oral inoculations, bacteria were grown as described above, harvested by centrifugation, resuspended in sterile PBS, and administered intragastrically to mice lightly anesthetized with fluoroalothane by using a gavage tube (26). The numbers of viable bacteria in the inocula were checked by pour plating on LB agar plates.

Bacterial enumeration in organ homogenates. Mice were killed by cervical dislocation. Spleens and livers were aseptically removed and homogenized in a Colworth stomacher in 10 ml of cold distilled water (15). Viable counts were performed by using pour plates of LB agar.

Collection of sera and passive transfer. For serum transfer, the donor mice were sample bled from a lateral tail vein or exsanguinated under anesthesia by cardiac puncture. The sera were collected and used fresh or stored at −20°C. For passive transfer, immune serum from vaccinated (i.v. with ca. 5×10^5 CFU of strain SL3261) and subsequently reinfected (orally with strain C5) C57BL/6 donors was injected i.v. or intraperitoneally (i.p.) into *Igh-6*$^{-/-}$ recipient mice, 2 h before challenge and at various times thereafter (days 1, 3, and 6 in experiment A and days 1, 3, 4, 6, and 12 in experiment B). Each mouse received 0.5 ml of immune serum at each time point. Sera injected 2 h before challenge were collected from donor mice (and stored at −20°C) 2 days before the designated challenge date for the recipients; sera to be transferred at any other time points were collected on the same day of the serum transfer (days 1, 3, and 6 in experiment A and days 1, 3, 4, 6, and 12 in experiment B).

Preparation of T-cell antigens. Antigens for T-cell assays were prepared as described previously (8). Briefly, an overnight, stationary-phase culture of strain C5 *rfa* in LB broth was pelleted, washed once in PBS containing 5 mM EDTA, and washed once more in PBS. The suspension was sonicated on ice, and cellular debris were removed by centrifugation at 13,000 × *g*. The supernatant (C5SE) was sterile filtered through a 0.22-μm-pore-size filter (Sartorius, Epsom, Surrey, United Kingdom) and stored at −70°C. The protein concentration was determined by using the bicinchoninic acid kit (Pierce Biochemicals, Rockford, Ill.) according to the instructions of the manufacturer. Alkali-treated antigen (C5SENaOH) was prepared from C5SE by addition of NaOH up to 0.25 M; the mixture was incubated at 37°C for 3 h before it was neutralized with HCl and sterile filtered through a 0.22-μm-pore-size filter.

T-cell assays. Unless otherwise stated all tissue culture reagents were purchased from Sigma Chemical Co. (Poole, Dorset, United Kingdom). Splenocytes for T-cell assays were prepared as described previously (8). Briefly, mice were sacrificed by cervical dislocation and single cell suspensions were prepared. Cells were washed once in RPMI 1640 medium and incubated in Gey's solution to lyse the erythrocytes. Leukocytes were washed twice more and resuspended in RPMI 1640 medium supplemented with 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2 mM glutamine, 2×10^{-5} M β-mercaptoethanol, 1 mM HEPES, and 10% heat-inactivated fetal calf serum (FCS). For in vitro stimulation, cells were dispensed at a concentration of 4×10^5/well in round-bottom 96-well plates (Corning Glass Works, Corning, N.Y.) in 200 μl and triplicates were incubated with the relevant concentration of antigen (1.25 μg of concanavalin A [ConA] per ml, 20 μg of C5SENaOH per ml, and 1.2 μg of C5SE per ml). Preliminary titration experiments using cells from naive C57BL/6 mice determined the highest concentration of T-cell antigens which would not exert an inhibitory effect on cell proliferation or IL-2 release from mouse splenocytes in response to an optimal dose of ConA. Within the noninhibitory range, the optimal stimulatory

concentrations of C5NaOH (20 μg/ml) and C5SE (1.2 μg/ml) were determined by titration experiments by using spleen cells of C57BL/6 mice immunized 4 months earlier with 5×10^5 CFU of strain SL3261.

CD4$^+$ T cells were positively enriched by using magnetic beads coated with anti-mouse CD4 antibodies (Miltenyi Biotec, Camberley, Surrey, United Kingdom). The CD4$^+$ cells were >94% pure as assessed by flow cytometry. Gamma-irradiated (3,000 rads) total spleen cells from age- and sex-matched naive C57BL/6 mice were used as feeder cells in the in vitro assays.

For IL-2 and IFN-γ measurements, the supernatants were harvested at 24 and 48 h, respectively, aliquoted, and stored at −70°C.

IL-2 and IFN-γ ELISA. IL-2 and IFN-γ were measured by capture enzyme-linked immunosorbent assay (ELISA) with antibody pairs and cytokine standards purchased from Pharmingen (Becton Dickinson Ltd., Cowly, Oxford, United Kingdom).

For IFN-γ concentration determinations, 96-well ELISA plates (Maxisorp Nunc Immuno plate, Nunc, Roskilde, Denmark) were coated overnight at 4°C with 50 μl of a capture rat anti-mouse IFN-γ immunoglobulin G1 (IgG1) monoclonal antibody (clone R4-6A2) per well in 0.1 M NaHCO$_3$ buffer (pH 9.5) at 2 μg/ml. After blocking with PBS supplemented with 10% FCS at 37°C for 1 h, supernatants from T-cell assays were loaded in 50 μl in triplicate and the plates were incubated at 37°C for 2 h. Serial twofold dilutions of recombinant IFN-γ ranging from 20 ng/ml to 40 pg/ml were included as standards. Biotinylated rat anti-mouse IFN-γ IgG1 monoclonal antibody (clone XMG1.2; 100 μl/well) at 1 μg/ml in PBS–10% FCS was then added for 1 h at 37°C, after which 100 μl of peroxidase-labelled streptavidin per well at 2.5 μg/ml (Sigma) in PBS–10% FCS was added for 45 min at room temperature. *ortho*-Phenylenediamine (1 mg/ml in 0.2 M Na$_2$HPO$_4$–0.1 M citrate buffer) in the presence of H$_2$O$_2$ was used to develop the plates. The reaction was stopped by adding 15 μl of 3 M H$_2$SO$_4$ per well. The optical density was read at 490 nm. IFN-γ values were determined by comparison with the standard curve. We considered 80 pg/ml the lower limit of sensitivity of our IFN-γ ELISA.

For IL-2 measurement, the OptEIA set (Pharmingen) was used according to the instructions provided by the manufacturer, the only modification being in the use of *ortho*-phenylenediamine as the substrate. The sensitivity of the IL-2 ELISA is ≥7 pg/ml.

Statistical analysis. Student's *t* test was used to determine the significance of differences between controls and experimental groups. Differences between experimental groups were considered significant for *P* values of <0.05.

RESULTS

Infection of *Igh-6*$^{-/-}$ mice with *S. enterica* serovar Typhimurium strain SL3261. *Igh-6*$^{-/-}$ mice and C57BL/6 control mice were infected i.v. with 5×10^5 CFU of strain SL3261. Spleen and liver counts of viable bacteria obtained thereafter showed that the courses of the infection in B-cell-deficient and control animals were very similar (Fig. 1). The initial bacterial growth was efficiently controlled and suppressed by both groups of mice, followed by a steady decline in the numbers of bacteria in the tissues, with low bacterial numbers being detectable on day 40 of the infection. The microorganisms were cleared from the spleen and liver by day 60 as assessed by pour plating of the whole-organ homogenate.

Thus, B cells are not required for control of the growth of an aromatic-dependent *S. enterica* serovar Typhimurium *aroA* strain. B cells are also dispensable for the clearance of *Salmonella* from the reticuloendothelial system (RES), which is known to require the contribution of T cells (10, 39).

Protection against oral challenge with virulent strain C5 in *Igh-6*$^{-/-}$ mice immunized with strain SL3261. Groups of seven *Igh-6*$^{-/-}$ mice and seven C57BL/6 control mice were immunized with strain SL3261 as for Fig. 1. A similar number of age- and sex-matched naive mice for each strain were used as unimmunized controls. Four months after vaccination, all mice were challenged orally with ca. 2.5×10^9 CFU of strain C5. As expected, all naive mice of either strain succumbed to the challenge with the virulent strain C5 within 8 or 9 days after infection. No deaths were observed among the immunized C57BL/6 mice; conversely, six of seven immunized *Igh-6*$^{-/-}$ mice died within 11 days of challenge (Fig. 2). Two repeat experiments gave similar results.

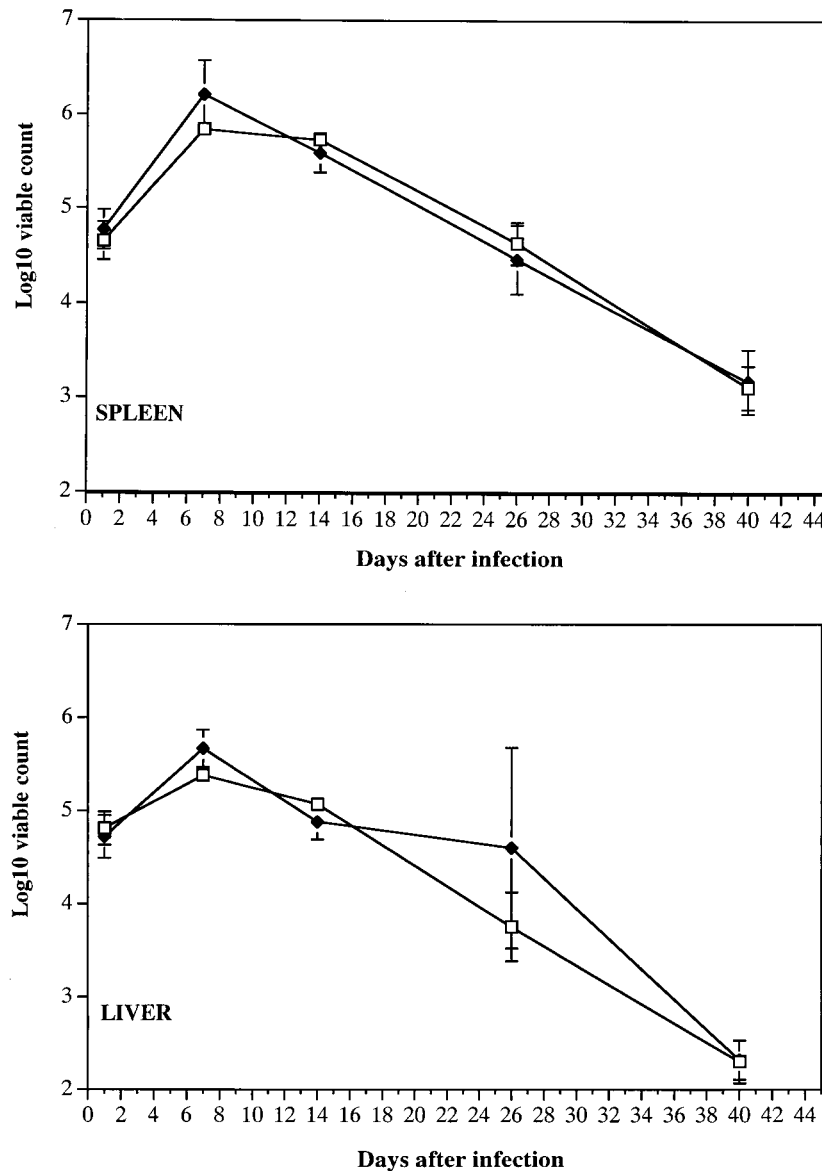Thus, B-cell-deficient mice have an impaired ability to de-

FIG. 1. *Igh-6*$^{-/-}$ mice (closed diamonds) and C57BL/6 mice (open squares) were infected i.v. with 5×10^5 CFU of serovar Typhimurium strain SL3261. Spleen and liver counts of viable bacteria were obtained thereafter. Results are means $\pm$ standard deviations from groups of four mice.

velop long-term acquired resistance against oral challenge with *S. enterica* serovar Typhimurium strain C5.

Effect of transfer of immune serum on resistance to virulent oral challenge in *Igh-6*$^{-/-}$ mice immunized with the attenuated strain SL3261. We have previously shown that both T cells and antibodies are required for optimal vaccine-induced protection against oral challenge with virulent *S. enterica* serovar Typhimurium in innately susceptible mice (26). Although the lack of *Salmonella*-specific antibody could be the only cause of the impaired resistance to reinfection seen in B-cell-deficient mice, other B-cell-dependent functions could also play a role in the development of long-term vaccine-induced acquired immunity to rechallenge with virulent salmonellae. To investigate this, we attempted to restore resistance to oral challenge in B-cell-deficient mice by passive transfer of immune serum. Previous work established that immune serum in the presence of immune T cells can protect innately susceptible

mice against oral challenge with virulent serovar Typhimurium (26).

Groups of *Igh-6*$^{-/-}$ and C57BL/6 mice were immunized as for Fig. 1, and parallel groups of naive mice of both strains were used as unimmunized controls. Six mice per group were used in experiment A, and eight mice per group were used in experiment B. All mice were challenged orally with ca. 5×10^9 CFU (experiment A) or 1×10^9 CFU (experiment B) of virulent strain C5 4 months after the strain SL3261 vaccination. In each experiment, a parallel group of similarly immunized and challenged C57BL/6 mice were used as donors for adoptive transfer of serum. Figure 3 shows that all naive mice from either strain died within 9 days postchallenge in both experiments. Six of six and seven of eight immunized C57BL/6 mice survived the oral challenge in experiments A and B, respectively. Six of six immunized B-cell-deficient animals died within 11 days postinfection in experiment A, and seven of eight
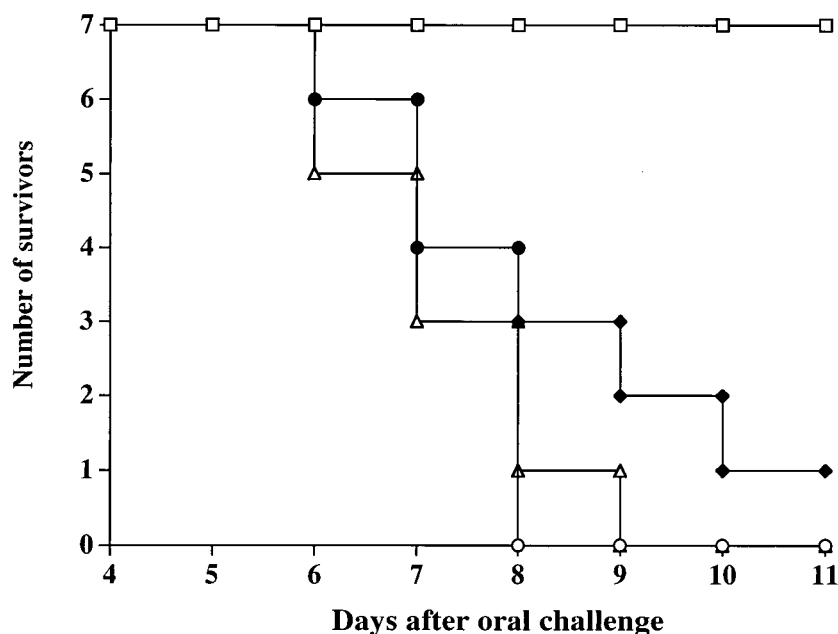
FIG. 2. *Igh-6$^{-/-}$* mice (closed diamonds) and C57BL/6 mice (open squares) were immunized with serovar Typhimurium strain SL3261 as for Fig. 1. Age-matched naive *Igh-6$^{-/-}$* mice (open triangles) and naive C57BL/6 mice (open circles) were used as unimmunized controls. Four months after vaccination, all mice were challenged orally with ca. 2.5×10^9 CFU of virulent serovar Typhimurium strain C5.

B-cell-deficient mice died within 18 days in experiment B. One additional group of *Igh-6$^{-/-}$* mice in each experiment received immune serum from immunized (with strain SL3261) and re-infected (with strain C5) C57BL/6 donors 2 h before challenge and at various times thereafter (days 1, 3, and 6 in experiment A and days 1, 3, 4, 6, and 12 in experiment B). Despite the transfer of immune serum, six of six B-cell-deficient mice died within 12 days after challenge in experiment A and six of eight B-cell-deficient mice died within 21 days in experiment B.

Thus, adoptive transfer of immune serum does not restore acquired immunity in vaccinated B-cell-deficient mice, indicating that antibody production alone does not appear to be the only function of B cells in salmonellosis.

T-cell responses in *Igh-6$^{-/-}$* mice. Splenocytes from immunized and naive C57BL/6 mice and *Igh-6$^{-/-}$* mice taken 4 months after vaccination with strain SL3261 were restimulated in vitro with optimal concentrations of crude C5SE or detoxified C5SENaOH soluble extract prepared from the O-rough derivative C5 *rfa*. Figure 4 shows that IL-2 and IFN-γ production from splenocytes of immunized C57BL/6 mice was significantly higher than cytokine release by spleen cells of *Igh-6$^{-/-}$* mice upon restimulation with antigen. Cells from naive mice of either strain produced undetectable levels of IL-2 and very low levels of IFN-γ. Spleen cells from *Igh-6$^{-/-}$* mice and C57BL/6 mice produced similar amounts of IL-2 and IFN-γ in response to in vitro stimulation with the T-cell mitogen ConA (not shown). Three repeat experiments gave similar results.

Results similar to the ones obtained with whole splenocyte populations were obtained when CD4$^+$ T cells from C57BL/6 and *Igh-6$^{-/-}$* mice were restimulated in vitro with the detoxified *Salmonella* soluble cell extract in the presence of irradiated feeder cells (splenocytes) prepared from naive C57BL/6 mice (results not shown).

Thus, total spleen cells and purified CD4$^+$ T cells from *Igh-6$^{-/-}$* mice showed impaired ability to produce Th1-type cytokines in response to in vitro restimulation with *Salmonella* soluble cell extracts.

DISCUSSION

In the present paper we report that *Igh-6$^{-/-}$* mice immunized with a live, attenuated, aromatic-dependent *S. enterica* serovar Typhimurium *aroA* vaccine strain exhibited an impaired long-term acquired immunity against virulent *Salmonella* microorganisms. *Igh-6$^{-/-}$* mice were able to control a primary infection and to clear the inoculum from their RESs. However, *Igh-6$^{-/-}$* mice, unlike C57BL/6 controls, did not survive an oral challenge with virulent strain C5 4 months after vaccination. Transfer of immune serum did not restore resistance in *Igh-6$^{-/-}$* mice. Total splenocytes and purified CD4$^+$ T cells from *Igh-6$^{-/-}$* mice showed a reduced ability to release the Th1-type cytokines IL-2 and IFN-γ upon in vitro restimulation with serovar Typhimurium soluble extracts.

The control and eradication of *Salmonella* infection in the mouse model occur in a number of phases that involve distinct cellular and humoral mechanisms (reviewed in reference 24). The early bacterial growth in the RES is controlled by the *Nramp* gene. The suppression of bacterial growth in the tissues requires bone marrow-derived cells and a wide array of cytokines but does not need conventional T cells (15, 25, 28–30, 34, 35). CD4$^+$ T lymphocytes are essential for the clearance of the organisms from the RES and for the development of T-cell-dependent long-term immunity (24, 39). The role of B cells and antibody in host resistance to primary *Salmonella* infection has been studied with *xid* mice, which lack B1 cells and have a partial defect in a proportion of B2 cells (36). It has been shown that *xid* mice have increased susceptibility to i.p. infection with *S. enterica* serovar Typhimurium. *xid* mice were able to mount an IgM response to *Salmonella* although they displayed impaired, but not abolished, IgG production. Resistance in *xid* mice was restored by passive transfer of *Salmonella*-immune serum generated in immunocompetent mice, leading the authors to conclude that the diminished or delayed antibody response in *xid* mice was responsible for the increased susceptibility to infection. Nevertheless, the passive transfer of
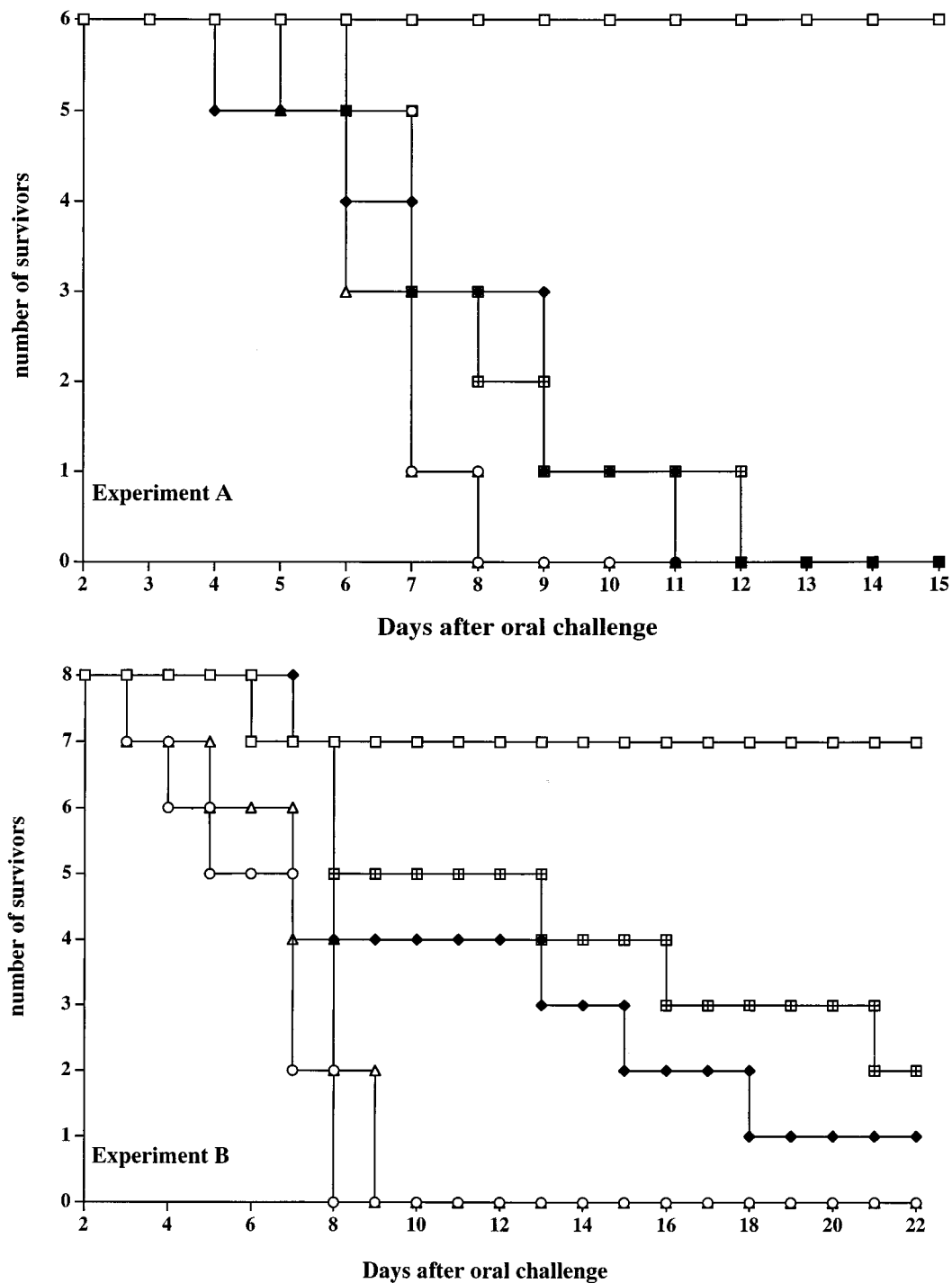
FIG. 3. *Igh-6*$^{-/-}$ mice (closed diamonds) and C57BL/6 mice (open squares) were immunized with serovar Typhimurium strain SL3261 as for Fig. 1. Age-matched naive *Igh-6*$^{-/-}$ mice (open triangles) and naive C57BL/6 mice (open circles) were used as unimmunized controls. One additional group of *Igh-6*$^{-/-}$ mice in each experiment (squares with bars) received immune serum from similarly immunized and orally reinfected C57BL/6 donors 2 h before challenge and at various times thereafter (days 1, 3, and 6 in experiment A and days 1, 3, 4, 6, and 12 in experiment B). All mice were challenged orally with serovar Typhimurium strain C5 (5 × 10^9 CFU [experiment A] or 1 × 10^9 CFU [experiment B]).

serum from immunocompetent to *xid* mice was performed before the mice were infected with salmonellae. This protocol is known to confer a degree of protection to mice infected by the i.p. route due to a reduction in bacterial numbers very early in infection (before colonization of the RES) and is not rep-

resentative of the physiological production of protective antibodies, which certainly starts after infection (5). Furthermore, the *xid* mutation might have other effects on host resistance to infection independent of the lack of B1 cells (2). More work is needed to ascertain whether deficient antibody production is
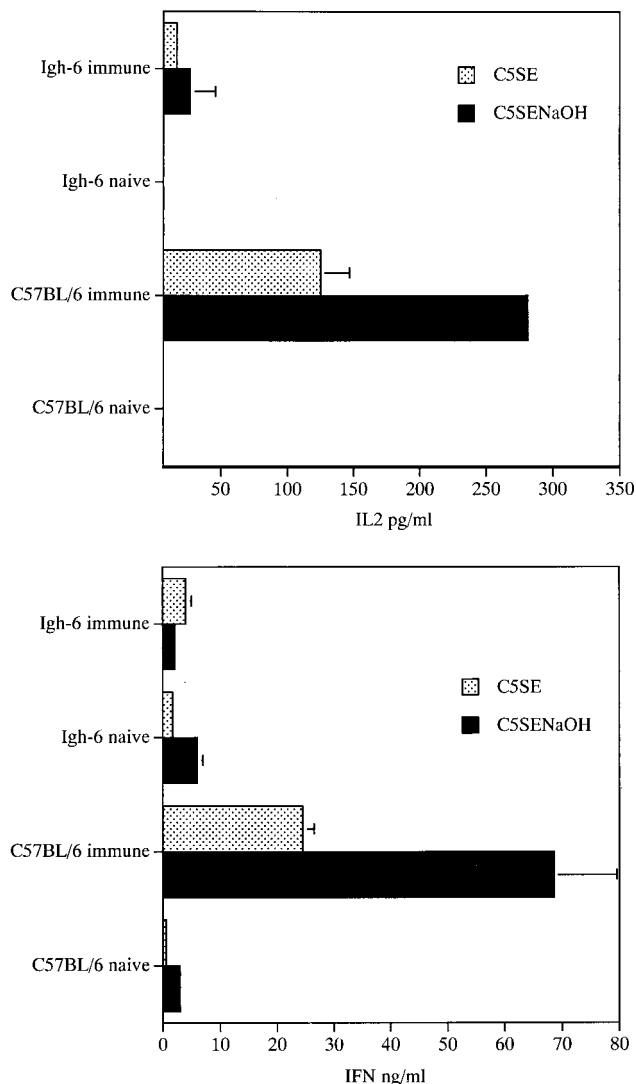
FIG. 4. Splenocytes were prepared from naive C57BL/6 mice and *Igh-6*$^{-/-}$ mice as well as from mice of either strain which had been vaccinated 4 months earlier with 5×10^5 CFU of serovar Typhimurium strain SL3261. The cells were restimulated in vitro with a crude (C5SE at 1.2 µg/ml) or detoxified (C5SENaOH at 20 µg/ml) *Salmonella* soluble extract. IL-2 and IFN-γ production was measured by ELISA. Values are means ± standard deviations of triplicate cultures.

the only cause of the impaired resistance to primary serovar Typhimurium infection seen in *xid* mice.

In the present study we used *Igh-6*$^{-/-}$ mice, which are B cell deficient and incapable of antibody production. We did not find differences in bacterial viable counts between wild-type and *Igh-6*$^{-/-}$ mice during the course of a primary infection with an aromatic-dependent strain of serovar Typhimurium. Members of our group previously reported that the growth of an *aroA* serovar Typhimurium strain is not exacerbated in *xid* mice compared to that in wild-type control animals (16). The results obtained in the present study confirm that B cells and antibody are not needed for the control and clearance of attenuated *aro* mutant salmonellae. It must be noted that aromatic-dependent serovar Typhimurium strains can grow in immunocompromised mouse strains and can cause lethal infections. *nu/nu* mice, T-cell receptor α/β$^{-/-}$ mice, IFN-γ$^{-/-}$ mice, mice treated with aminoguanidine (an inhibitor of induc-

ible nitric oxide synthase), *NOS2*$^{-/-}$ mice, and anti-IL-12-treated mice all succumb to infections with *aro* mutant *Salmonella* strains (10, 28, 31, 39, and our unpublished observations). The results shown in the present paper demonstrate that unlike other forms of immunodeficiency, the lack of B cells does not impair host resistance during a primary infection. We obtained similar results by infecting B-cell-deficient mice with wild-type *Salmonella* strains of intermediate virulence (results not shown), thus proving that the unimpaired resistance to primary infection seen in *Igh-6*$^{-/-}$ mice is not limited to the attenuated serovar Typhimurium *aroA* vaccine strain described in this study.

In the mouse model, immunization with live attenuated aromatic-dependent *Salmonella* vaccines elicits good antibody responses against protein and polysaccharide determinants, long-lasting Th1-type immunological memory, and CD8$^+$ cytotoxic T cells (7, 23). Vaccination confers solid and long-lasting protection against oral challenge with virulent microorganisms which persists long after the initial serovar Typhimurium vaccine strain has been cleared from the RES (12, 14, 24). It has been shown that both humoral and cellular factors play a role in acquired immunity to salmonellae; the relative importance of either type of factor depends on the susceptibility of the mouse strain used and on the relative virulence of the bacterial challenge strain (5, 6, 26). When innately susceptible mice are challenged with virulent salmonellae, survival of the host requires the presence of antilipopolysaccharide antibodies, immune CD4$^+$ T cells and CD8$^+$ T cells, IFN-γ, TNF-α, IL-12, and granuloma formation (7, 23, 28, 41).

In the present paper we show that B cells are essential for the expression of full acquired immunity to virulent oral challenge. Immunized B-cell-deficient mice succumb to an oral challenge with virulent serovar Typhimurium strain C5. At the lowest challenge dose used (Fig. 3, experiment B) a proportion of immunized *Igh-6*$^{-/-}$ mice showed a delay in time to death compared to that of unimmunized controls, indicating that in the absence of B cells a low degree of protection is still present, although it is clearly insufficient to control the infection.

A role for B cells in acquired immunity to *Salmonella* could be due to the absolute requirement for antibodies in secondary resistance to salmonellae in innately susceptible mice (26). However, our present results indicate a more complex contribution of B cells to specific immunity to *Salmonella* infections. Antibody production alone does not appear to be the only function of B cells in salmonellosis. In fact, acquired resistance in *Igh-6*$^{-/-}$ mice could not be restored, even at the lowest challenge dose used, by passive transfer of large amounts of immune serum, despite the documented (6, 26) efficacy of adoptive transfer of antibodies in the presence of functional immune T cells. Noticeably, a marked reduction in the ability of mouse splenocytes from *Igh-6*$^{-/-}$ mice to produce Th1-type cytokines in response to antigen-specific (but not ConA-mediated) restimulation was observed. This indicates a significant impairment of Th1-type memory, which is known to be essential for vaccine-induced acquired resistance to virulent salmonellae (8, 23). The inadequate Th1-type memory in immunized *Igh-6*$^{-/-}$ mice would explain, at least in part, the increased susceptibility to rechallenge despite the transfer of immune serum. Evidence from other infection models also indicates that B cells are required for the establishment and/or persistence of long-lasting T-cell-mediated immunity. In fact, in some infections B cells are required for the initial establishment of cell-mediated immunity during a primary infection (e.g., malaria [21], tuberculosis [44], or lymphocytic choriomeningitis virus [13] or *C. trachomatis* infection [45]) or for

long-term persistence of solid T-cell immunity (e.g., in the *B. pertussis* mouse model [22]).

At this stage we cannot rule out a role for B cells in the modulation of CD8$^+$-T-cell functions. In fact, this T-cell subset is needed for the full expression of immunity to oral challenge with virulent serovar Typhimurium in vaccinated mice (23). We cannot rule out the possibility that in the absence of B cells (especially B1 cells) an impairment of gut humoral immunity might contribute to the reduced vaccine-induced resistance to oral challenge with virulent organisms seen in *Igh-6*$^{-/-}$ immunized mice. In fact, B1 cells have been shown to be important for IgA responses in the gut mucosa following oral immunization with serovar Typhimurium (38). Evidence for a protective role of IgA against very low oral doses of virulent *Salmonella* has been provided (32).

In primary *Salmonella* infections in the mouse model, clearance of the inoculum from the tissues requires functional CD4$^+$ T cells and the development of Th1-type T-cell immunity (10, 39). The results shown in this report indicate that *Igh-6*$^{-/-}$ mice can control and clear a primary infection (within 60 days after primary vaccination) despite their apparent inability to display antigen-specific long-lasting Th1-type T-cell immunity (at 4 months after immunization). It is possible that either (i) T-cell immunity to *Salmonella* is normally initiated but is short lasting in the absence of B cells, (ii) the lower degree of T-cell memory seen in *Igh-6*$^{-/-}$ mice is sufficient to control an infection with salmonellae of low virulence yet is insufficient for resistance against virulent organisms, or (iii) clearance of the inoculum from the tissues and resistance to rechallenge are mediated by different T-cell-dependent mechanisms. This is currently under investigation.

ACKNOWLEDGMENTS

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