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Recombinant *Salmonella typhimurium* Strains That Invade Nonphagocytic Cells Are Resistant to Recognition by Antigen-Specific Cytotoxic T Lymphocytes

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To address the question of whether *Salmonella*-infected nonphagocytic cells could serve as target cells for recognition by antigen-specific, major histocompatibility complex class I-restricted cytotoxic T lymphocytes (CTL), four recombinant *Salmonella typhimurium* constructs that expressed full-length, or fragments of, influenza A virus nucleoprotein (NP) were made. The bacteria were shown to infect Chinese hamster ovary (CHO) cells. Appropriate major histocompatibility complex restriction molecules, HLA-B27 and H-2 D^b, were transfected into CHO cells, which were then infected with recombinant *S. typhimurium* and used as targets for NP-specific CTL. The cells in which NP was expressed by intracellularly replicating bacteria were not lysed by NP-specific CTL, although they were killed when appropriate influenza A virus or peptides were used. Thus, *S. typhimurium* bacteria within nonphagocytic cells were resistant to CTL recognition. In contrast to these results, mice infected with recombinant *S. typhimurium* that expressed fragments of NP in the periplasm were primed for NP-specific CTL responses. The results indicate that CTL responses specific to *Salmonella* antigens can be generated, but the bacteria may be safe from the CTL attack once they have entered the nonphagocytic cells.

Salmonella typhimurium is the agent responsible for salmonellosis in mice, a disease analogous to typhoid fever caused by *Salmonella typhi* in humans (1). Acquired immunity against *Salmonella* infection is known to be manifested by the synergistic effects of activated macrophages, cytophilic antibodies, and delayed-type hypersensitivity (DTH) (3, 6, 7, 9, 15, 16, 19, 22, 23, 28, 29). While *Salmonella* antigen-specific, $CD4^+$ helper T cells play an important role in the activation of macrophages, the production of antibodies, and the induction of DTH (5, 12, 32, 33), the function of major histocompatibility complex (MHC) class I-restricted, $CD8^+$ cytotoxic T lymphocytes (CTL) in the anti-*Salmonella* responses of the host has not been specifically investigated. A T cell recognizes a foreign antigen through its T-cell receptor (TCR), which is specific for a particular MHC class I (for $CD8^+$ T cell) or class II (for $CD4^+$ T cell) molecule plus a short peptide (usually a nonamer for MHC class I molecules) derived from the antigen (26, 30). It is clear that intracellular and extracellular antigens represent very different challenges to the immune system: peptides derived from intracellular antigens are presented to $CD8^+$ T cells by MHC class I molecules (26, 36), and peptides derived from extracellular antigens are generally presented to $CD4^+$ helper T cells by MHC class II molecules (13, 30); activation of CTL leads to lysis of the antigen-presenting cells, and activation of helper T cells leads to secretion of a large array of lymphokines. As *S. typhimurium* is a facultative intracellular pathogen (10, 15, 20), it is of interest to ask whether there is a role for CTL in protection against *Salmonella* infection. *S. typhimurium* is capable of multiplying within nonphagocytic parenchymal cells, e.g., hepatocytes and epithelial cells in the intestine (10, 15, 20). Unlike macrophages, however, these cells are not capable of killing and digesting the invading bacteria. It is possible that these nonphagocytes provide a safe haven for the invading salmonellae, protecting them from the hostile intracellular environment of activated macrophages and the humoral elements of the host. However, if any intracellular bacterial antigen gains access to the cytoplasm, it is likely to be degraded and derivative peptide fragments could associate with class I MHC molecules to stimulate a CTL response, which could then lyse the infected cells.

To address these questions, we have set up a model system which takes advantage of the ability of *S. typhimurium* to invade and survive within CHO cells that express either transfected HLA-B27 or H-2 D^b, and we have made constructs of *S. typhimurium aroA* mutants that express either the full-length nucleoprotein (NP) of influenza A virus (A/NT/60/68) in an inclusion body or fragments of NP in the periplasm. We were able to explore the recognition of NP in bacterium-infected cells by an HLA-B27-restricted CTL line and an H-2 D^b-restricted clone. We demonstrate that these recombinant *S. typhimurium*-infected CHO cells appear to be resistant to specific CTL lysis in vitro, although some of these recombinant bacteria could prime CTL responses in vivo.

MATERIALS AND METHODS

Plasmids. All plasmids were maintained in *Escherichia coli* HB101 (Table 1). Plasmid pSV2gpt/Db10 was constructed by inserting a 10-kbp *Hin*dIII fragment, including the H-2 D^b gene, from cosmid B1.1 (24) into expression vector pSV2gpt (27). Insertion of a 6.7-kbp *Eco*RI fragment, including HLA-B2705 gene, from genomic clone cd2.6 (37) into pUC13 resulted in plasmid pB27.13. Plasmid pG418/neo containing the G418 resistance gene was kindly provided by G. Brownlee (Sir William Dunn School of Pathology, Oxford, United Kingdom).

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TABLE 1. Summary of the plasmids used to prepare recombinant SL3261

Plasmid	Derived from	Expression	Location
pNP-2	pAT158	Full-length NP	Inclusion body
p12	pFV1	LTB–NP-364-382	Periplasm
p14	pFV1	LTB–NP-377-392	Periplasm
pKOP1	pKK223	NP-362-498	Periplasm

Construction of plasmid pFV1 and its usage of expressing foreign epitopes as fusion proteins with heat-labile enterotoxin B (LTB) has been described elsewhere (21). Two pairs of oligonucleotides coding for either NP-377-393 (5′-GATC AGTACTCTGGAACTGCGTAGTCGTTACTGGGCTATC CGTACCCGTTCTGA-3′ and 3′-TCATGAGACCTTGACG CAATGACCCGATAGGCATAAGCAAGACTGATCAGT C-5′) or NP-364-382 (5′-GATCCAGATCGCTTCTAACGAA AACATGGACGCTATGGAGACCTC-3′ and 3′-GTCTAGC GAAGATTGCTTTTGTACCTCCGATACCTCTGAGCTG AGACCTTGACGCACTGATC-5′) of influenza A/NT/60/68 virus were synthesized, and the annealed oligonucleotides were kinased and cloned into the *Bgl*II-*Spe*I site of pFV1, forming plasmids p12 and p14, respectively.

Phage mp18 containing the full-length OmpA gene (25) was kindly provided by C. Higgins, ICRF Laboratories, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, United Kingdom. This sequence (450 bp) was amplified by the polymerase chain reaction with M13 mp18-specific forward and reverse sequencing primers (New England Biolab, Bishop's Stortford, United Kingdom). A 101-bp *Asp*718 and *Bam*HI fragment of the polymerase chain reaction product, including the sequence coding for the first 26 amino acid residues of OmpA, was cloned into pUC18 cut with the same enzymes, forming plasmid pOP1.

To construct plasmid pKOP1, a 491-bp *Hin*dIII (blunt ended) and *Bam*HI fragment of DNA coding for the C-terminal third of the NP of influenza A/NT/60/68 virus was purified from plasmid pTKNP2 (35), and this was used to replace an *Eco*RI and *Bam*HI fragment (11 bp) of pOP1, giving rise to plasmid pONP1. Finally, the *Nde*I (blunt ended) and *Bam*HI fragment (569 bp) of pONP1 was transferred to a prokaryotic expression vector pKK223 (Pharmacia LKB Biotechnology, Milton Keynes, United Kingdom) digested with *Sma*I and *Bam*HI. The predicted protein sequence encoded by plasmid pKOP1 would be the first 26 amino acid residues from the N terminus of OmpA (leader sequence) followed by 136 residues (362 to 498) of the C-terminal NP molecule. All enzyme manipulations were carried out according to the manufacturers' instructions (Boehringer Mannheim Biochemica and New England Biolabs.)

Bacterial strains and manipulation of *S. typhimurium aroA* mutant SL3261. *S. typhimurium* SL3261 *aroA* His$^-$ was obtained from B. A. D. Stocker, Department of Medical Microbiology, Stanford University, Calif. *S. typhimurium* LB5010 (F′ *gal*) *galE* r$^-$ m$^+$ was obtained from the *Salmonella* Stock Center, University of Calgary, Alberta, Canada. Salmonella-specific bacteriophage p22HT105/1 *int* was obtained from Ian Charles, Department of Cell Biology, The Wellcome Research Laboratories, Kent, United Kingdom.

Transduction of plasmid pNP-2 (18) into SL3261 and the expression of full-length NP as a fusion protein has been described earlier (34). Plasmids p12, p14, and pKOP1 were similarly introduced into SL3261 by using phage p22 transduction. They were transformed into *S. typhimurium* LB5010. p22 lysates of the transformed LB5010 were used to infect SL3261, and the positive transductants of SL3261 were selected on L broth agar plates supplemented with ampicillin (100 μg/ml) and EGTA [5 mM; ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]. The plasmids used to prepare recombinant SL3261 are listed in Table 1.

Viruses and synthetic peptides. Influenza A virus Puerto Rico/34 (PR8) was kindly provided by S. Russell of the Department of Virology, The Wellcome Research Laboratories, Kent, United Kingdom. Influenza A virus H17 was prepared by F. Gotch, Institute of Molecular Medicine, John Radcliffe Hospital. Influenza A virus peptides, 380 to 393 and 365 to 379, were synthesized with an Applied Biosystems synthesizer by J. Rothbard, ICRF Laboratories.

Monoclonal antibodies and polyclonal antisera. Monoclonal antibodies (MAb) specific for mouse H-2 D^b (281485) and the NP of influenza A viruses (4.7.18) were prepared as ascites. MAb ME1, specific for the α1 domain of the HLA-B27 molecule, was prepared as ascitic fluid by S. Ellis, Institute of Molecular Medicine, John Radcliffe Hospital. Rabbit antisera against H and O antigens of *S. typhimurium* were provided by C. Ashton at the Department of Bacteriology, John Radcliffe Hospital. Rabbit anti-NP (34) and anti-LTB (21) sera were as described. Polyclonal antipeptide p365-379 and p380-393 of NP were prepared by immunizing rabbits subcutaneously with synthetic peptides coupled to bovine serum albumin (Sigma, Dorset, United Kingdom) in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). Peripheral blood was collected 3 weeks later.

Transfection of CHO cells. CHO cells were maintained in R10 (RPMI 1640 [Flow Laboratory] supplemented with 10% heat-inactivated fetal calf serum and penicillin-streptomycin [100 μg/ml] [GIBCO, Middlesex, United Kingdom]). Subconfluent monolayers of CHO cells grown in 50-ml flasks were cotransfected with plasmid pB27.13 (5 μg) and pG418/neo (0.5 μg) by the calcium phosphate-mediated transfection method with G418 (1.2 mg/ml) (Sigma) resistance as a selection marker. HLA-B27-positive CHO cells were purified by using MAb ME1-treated Dynabeads coated with goat anti-mouse immunoglobulin G (IgG) (Dynal Ltd., Wirral, United Kingdom). For transfection of CHO cells with plasmid pSV2gpt/Db10, a similar strategy was adopted, except that the selection marker was mycophenolic acid (2.5 μg/ml) resistance and MAb 281485, specific for H-2 D^b, was used for the purification of D^b-positive transfectants. Both transfectants expressed high levels of surface antigens of the transfected genes as determined by fluorescence-activated cell sorter (FACS) analysis.

Infection of CHO cells with *S. typhimurium aroA* mutant SL3261. Overnight cultures of recombinant SL3261 were washed in RPMI 1640, and the resuspended bacteria were added to subconfluent monolayers of CHO cells (bacteria/cell ratio, 100:1) either in 50-ml flasks or on 6-well Costar plates. They were cocultured for 2 to 3 h at 37°C with gentle shaking every 10 min. The cells were washed three or four times at the end of the incubation, and RPMI 1640 medium containing gentamicin (50 μg/ml) and 10% fetal calf serum was provided for further incubation at 37°C in 5% CO_2.

Staining of the intracellular bacteria in CHO cells. The infected CHO cell monolayers were washed three times with phosphate-buffered saline before fixation with 95% alcohol for 15 min and being air dried. The *Salmonella* bacteria were then stained by using rabbit anti-*Salmonella* sera, followed

by alkaline phosphatase-labelled swine anti-rabbit IgG (Dako Ltd.), and visualized by using a fast red substrate as described by Boenisch (4).

Immunoprecipitation and Western blots. CHO cells (5×10^6), infected with recombinant SL3261 36 h earlier or with PR8 virus 1 h earlier, were harvested and resuspended in 1 ml of methionine-free RPMI 1640; after 3 h, 100 μCi of [^{35}S]methionine (Amersham, Aylesbury, United Kingdom) was added, and the incubation at 37°C was continued for another 4 h. Labelled cells were washed and lysed in 1 ml of lysis buffer (1% Nonidet P-40, 1 mM EDTA, 50 mM Tris-HCl [pH 7.4]) for 20 min in the presence of phenylmethylsulfonyl fluoride (10 μg/ml; Sigma) on ice. The lysates were precleared by centrifugation at 5,000 × g for 30 s. Goat anti-mouse IgG-coated Dynabeads (2×10^7) were treated with MAb 4.7.18, specific for NP, for 2 h at 4°C before washed and resuspended in 100 μl of lysis buffer. The prepared beads (25 μl) were added to the supernatant (0.5 ml) of the cell lysates and incubated together for 1 h in a cold room, with continuous slow rotation. The beads, after washes, were then resuspended in 50 μl of sodium dodecyl sulfate (SDS) loading buffer, boiled, and centrifuged, and the supernatant was loaded onto an SDS–10% polyacrylamide gel. After electrophoresis, the gel was fixed, treated with Amplify (Amersham), dried, and autoradiographed. For detection of the NP in the insoluble components of the cell lysates, the pellets were boiled in SDS loading buffer, electrophoresed on SDS-polyacrylamide gels, and autoradiographed similarly. For Western blot (immunoblot), transfer of protein bands to nitrocellulose was performed immediately after the electrophoresis by using a Bio-Rad Transblot apparatus. Transferred NP was detected with rabbit anti-NP serum, followed by alkaline phosphatase-coupled swine anti-rabbit IgG (Dako), and visualized by using a chromogenic substrate (BCIP/NBT [5-bromo-4 chloro-3-indolylphosphate toluidinium/nitroblue tetrazolium]; Sigma).

Immunoelectron microscopy. Pellets of cells infected with recombinant SL3261 were divided into two portions, with one portion being fixed in 4% glutaraldehyde in 0.1 M phosphate buffer and processed for routine electron microscopy examination. The portions for immunoelectron microscopy were fixed for 2 h in 1% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer, dehydrated in ethanol, and embedded in LR white (London Resin Company Ltd., Hants, United Kingdom). Sections were etched with a saturated aqueous solution of sodium metaperiodate, and nonspecific binding was blocked with 1% bovine serum albumin. Sections were then incubated sequentially with rabbit anti-NP sera (1:10) for 2 h, mouse anti-rabbit IgG (Sigma) (1:50) for 30 min, and either goat anti-mouse IgG conjugated to 10-nm gold (BioCell, Cardiff, United Kingdom) or protein A conjugated to 5-nm gold (BioCell) (1:25) for 30 min. Sections were lightly stained with uranyl acetate prior to examination with a Jeol 100CX electron microscope. Controls were treated with an irrelevant primary antibody (rabbit anti-*Toxoplasma* antibody) in place of rabbit anti-NP antibody.

Preparation of periplasmic extracts and GM1 ELISA. Overnight cultures of recombinant SL3261 in 10 ml of L broth medium supplemented with ampicillin (100 μg/ml) were washed and resuspended in 1 ml of spheroplast buffer (20% sucrose, polymyxin B [1,000 U/ml], lysozyme [1 mg/ml], 1 mM EDTA, 30 mM Tris-HCl [pH 7.4]) for 20 min on ice. The spheroplasts were centrifuged, and the supernatant was kept at −80°C until used. The GM1 enzyme-linked immunosorbent assay (ELISA) was done as described previously (21).

CTL lines. The HF-CTL lines, specific for NP-380-393, restricted by HLA-B27 (17), and the mouse H-2 D^b-restricted, 1968 NP-365-379-specific CTL clone (14) were prepared as described previously.

Infection of mice with recombinant SL3261. C57BL/6 mice of both sexes were purchased from Olac Ltd. (Bicester, United Kingdom). Mice, aged 8 to 12 weeks, were infected intraperitoneally with various live recombinant SL3261 (10^6 to 5×10^6/ml, 100 μl). About 10% of the mice died a few days after such infection; those which survived were sacrificed for their spleens 4 to 5 weeks later.

Preparation of spleen cells for CTL assays. Spleens taken from the mice infected with recombinant SL3261 were homogenized, and erythrocytes were lysed. The remaining cells were resuspended in R10. These cells (10^7 in 5 ml of medium) were stimulated with syngeneic spleen cells (4×10^6 in 5 ml of R10), pulsed with peptide p365-379, in 50-ml flasks for 5 days before being tested for NP peptide-specific cytotoxicity.

^{51}Cr-release cytotoxicity assay. Bacterium-infected transfectant CHO cells (2×10^6) were harvested 36 h after the infection and labelled with 100 μCi of ^{51}Cr (Amersham) for 90 min at 37°C in a total volume of 200 μl of serum-free RPMI 1640. Standard 5- to 6-h ^{51}Cr-release assays were then performed with killer cell HF-CTL and clone F5 as described previously (17, 35, 36).

For sensitization of CHO transfectant cells by using influenza A virus or peptides, the experimental procedure was done as described previously (17, 36).

Experimental wells were dispensed in duplicate; controls, medium alone (spontaneous release), and 5% Triton X-100 (maximum release) were dispensed in quadruplicate. The percentage of specific lysis is calculated as follows: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100.

RESULTS

Infection of CHO cell culture monolayers by attenuated *aroA* mutant of *S. typhimurium*. The ability of *aroA* mutant of *S. typhimurium* SL3261 to infect CHO cells was assessed. An overnight culture of SL3261 was washed and added to subconfluent monolayers of CHO cells and cultured for 2 to 3 h before fresh medium containing gentamicin was provided for further incubation. Infected cells were stained for bacteria, by using rabbit anti-*Salmonella* sera followed by alkaline phosphatase-coupled swine anti-rabbit IgG, at different time points of the incubation. This staining method could not determine whether SL3261 had entered the cells during the initial infection period; a number of bacteria were shown to adhere to the surface of the CHO cells (Fig. 1A). Intracellular multiplication of the bacteria was evident 24 h later (Fig. 1B), resulting in the lysis of some of the infected cells at 48 to 72 h (Fig. 1C). Ultrastructural examination of CHO cells at 36 h postinfection showed that intracellularly replicating bacteria were located within membrane-bound vacuoles of the host cells (Fig. 2A).

SL3261-pNP-2 was unable to sensitize transfectant CHO cells for recognition by NP-specific CTL. The transduction of plasmid pNP-2 into SL3261 (forming SL3261-pNP-2) and the expression of the NP molecule has been described earlier (34). To determine whether SL3261-pNP-2 infection of HLA-B27-transfectant CHO cells (CHO/B27) sensitized them for CTL recognition in vitro, the cells were infected

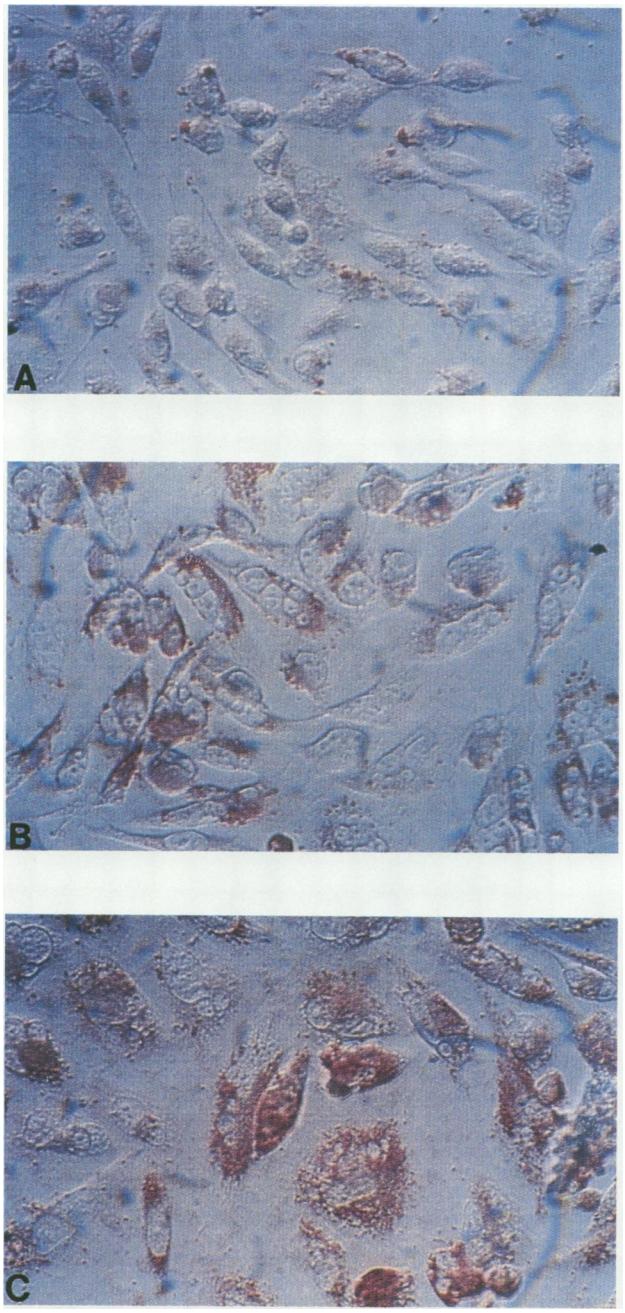

FIG. 1. Replication of SL3261 within CHO cells. Monolayers of CHO cells, infected with SL3261, were cultured at 37°C for 1 (A), 24 (B), or 48 (C) h in the presence of gentamicin before fixation with alcohol. *S. typhimurium* bacteria were stained by using rabbit anti-*S. typhimurium* sera, followed by alkaline phosphatase-labelled swine anti-rabbit IgG, and visualized by using a fast red substrate.

with SL3261-pNP-2 and used, 36 h later, as target cells in ^{51}Cr-release cytotoxicity assays. Effector cells were the HLA-B27-restricted HF-CTL line, which recognized the NP-380-391 epitope of influenza A virus. CHO/B27 served as efficient target cells, for HF-CTL recognition, when infected with influenza A virus PR8 or pulsed with synthetic peptide p380-393 of NP (Fig. 3). However, NP-specific lysis of the cells infected with SL3261-pNP-2 by HF-CTL was not detectable (Fig. 3). Similarly, SL3261-pNP-2-infected H-2 D^b transfectant CHO cells (CHO/D^b) were not lysed by the H-2 D^b-restricted CTL clone, F5, which recognized the NP 365-379 epitope of influenza A/NT/60/68 and A/H17 viruses (see below).

Detection of NP synthesis in CHO cells infected with SL3261-pNP-2. It was important to exclude the possibility that failure of HF-CTL and F5 to recognize transfectant CHO cells infected by SL3261-pNP-2 might be due to down regulation of NP expression by the bacteria once they had entered the host cells. Figure 4 shows a representative immunoprecipitation experiment, with anti-NP MAb 4.7.18, which demonstrates NP synthesis in CHO/B27 cells infected with either SL3261-pNP-2 (36 h earlier) or influenza PR8 virus (1 h earlier). PR8 virus-infected cells showed a large amount of newly synthesized NP in the cytoplasm; however, no NP was detected in the cells infected by either SL3261 or SL3261-pNP-2 (Fig. 4A). A possible explanation for this was that although the intracellularly replicating SL3261-pNP-2 made NP, it failed to reach the cytosol of the host cells remaining in the bacteria, which were not lysed by the lysis buffer. As shown in Fig. 4B, this was the case: when the pellets of the cell lysate, including the bacteria released from the infected cells, were boiled in SDS loading buffer and loaded on an SDS-polyacrylamide gel, an unique protein band at 57 kDa was present in the sample from SL3261-pNP-2-infected cells. This band was confirmed as NP of influenza A virus by Western blotting with polyclonal rabbit anti-NP serum (Fig. 4C). Synthesis of NP by intracellular bacteria was further confirmed by immunoelectron microscopy. Using this technique, it was shown that, in intracellular SL3261-pNP-2 bacteria, NP was specifically located within the bacterial inclusion bodies and that there was no specific labelling of the nuclear region of the bacteria or the host cell cytoplasm (Fig. 2B and C).

Expression of different NP fragments in the periplasmic space of SL3261. The above results implied that the inability of SL3261-pNP-2 to sensitize transfectant CHO cells for CTL recognition was because NP was expressed and retained in an inclusion body in the intracellular bacteria. Therefore, three constructs were designed to express fragments of the NP molecule in the periplasmic space of SL3261. Two were derivatives of the vector pFV1 (12), which expresses foreign epitopes as fusion proteins with the nontoxic B subunit of the LTB of enterotoxigenic *E. coli*. LTB binds to the ganglioside receptor, GM1, which can be used in an assay to detect the recombinant products. Two pairs of oligonucleotides encoding epitopes NP-377-392 (HLA-B27 restricted) and NP-364-382 (H-2 D^b restricted) of A/NT/60/68 virus were synthesized and cloned into the *Bgl*II-*Spe*I site of pFV1, forming plasmids p14 and p12, respectively (Fig. 5). The plasmids were transduced into SL3261, and the periplasmic extracts were tested for expression of LTB-epitope fusion proteins by GM1 ELISA. As illustrated in Fig. 6, the GM1-binding components in the periplasmic extracts from SL3261-p12 and SL3261-p14 were also recognized by anti-LTB as well as by anti-peptide antibodies, indicating that correct fusion proteins between LTB and NP epitopes were expressed in the periplasm. The fourth construct (pKOP1) utilized a prokaryotic expression vector, pKK223, to express the C-terminal third fragment of NP, residues 332 to 498, in the periplasm of SL3261. To direct the fragment into the periplasmic space of the bacteria, a DNA fragment of 79 bp encoding the first 26 amino acid residues of OmpA was added to the 5′ end of the NP-332-498 gene (Fig. 5). The plasmid was transduced into SL3261, and the expression of NP-332-498 in the periplasm was demon-

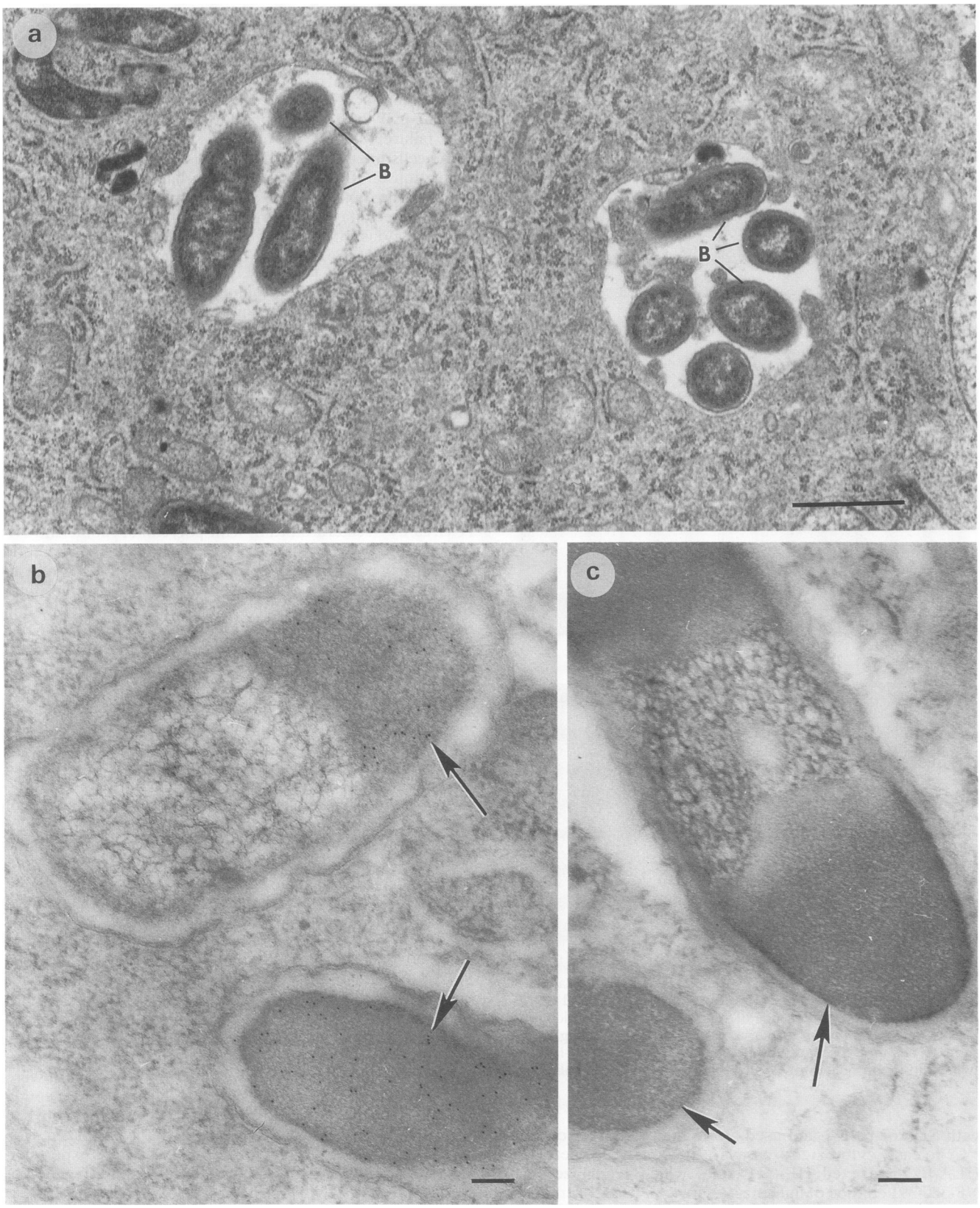

FIG. 2. Transmission electron microscopy of CHO cells infected with recombinant *S. typhimurium*. (a) Section through CHO cells showing a number of SL3261-pNP-2 bacteria (labelled B) located in membrane-bound vacuoles within the host cell cytoplasm, in a routinely processed sample. Bar, 1 μm. (b) Immunostained section of a CHO cell infected with SL3261-pNP-2 bacteria and exposed to rabbit anti-NP serum. Note the numerous 5-nm gold particles specifically located over the bacterial inclusion bodies (arrows). Bar, 100 nm. (c) Immunostained control. A serial section of that shown in panel b was similarly treated except for exposure to an irrelevant primary antibody (rabbit anti-*Toxoplasm* antibody). Note the absence of gold particles over the inclusion bodies (arrows) or other areas of the section. Bar, 100 nm.

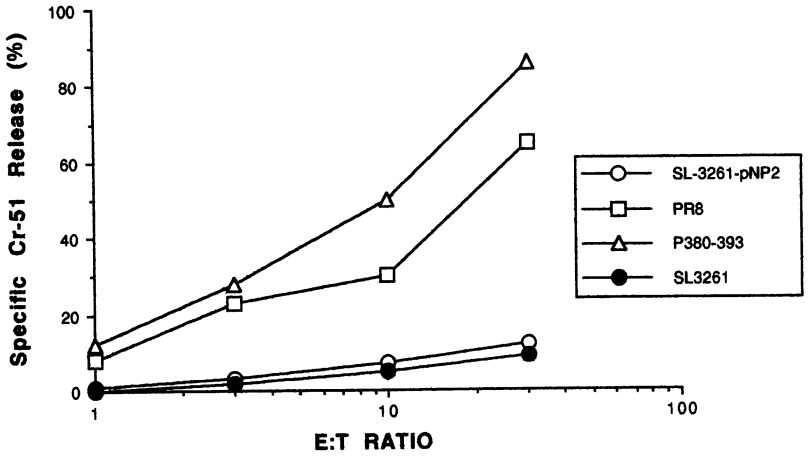

FIG. 3. SL3261-pNP-2 failed to sensitize CHO/B27 for recognition by HF-CTL. CHO/B27 cells, infected with either SL3261-pNP-2 or SL3261 36 h earlier, were labelled with ^{51}Cr as target cells (T) by using HF-CTL as effector cells (E). Healthy CHO/B27 cells, either pulsed with peptide p380-393 or infected with PR8 virus, were also labeled as controls. The results were expressed as the percentage of specific ^{51}Cr release. Spontaneous release of ^{51}Cr was less than 15% of the ^{51}Cr incorporated into the target cells.

strated by immunoprecipitation by using polyclonal rabbit anti-NP serum (data not shown).

Recombinant SL3261 expressing NP fragments in the periplasm did not sensitize target cells for CTL recognition. Both SL3261-p14 and SL3261-pKOP1 expressed fragments of NP including the HLA-B27-restricted epitope (380 to 391) in the periplasm of the bacteria, while SL3261-pNP-2 produced full-length NP in an inclusion body. Their abilities to sensitize CHO/B27 for HF-CTL recognition in vitro are compared in Table 2. SL3261-pKOP1- and SL3261-p14-infected cells were slightly more sensitive to HF-CTL lysis than were those infected by SL3261-p12 and SL3261-pNP-2, as might be expected, but the difference was minimal. SL3261-p12 and SL3261-pKOP1 also express the H-2 D^b-restricted

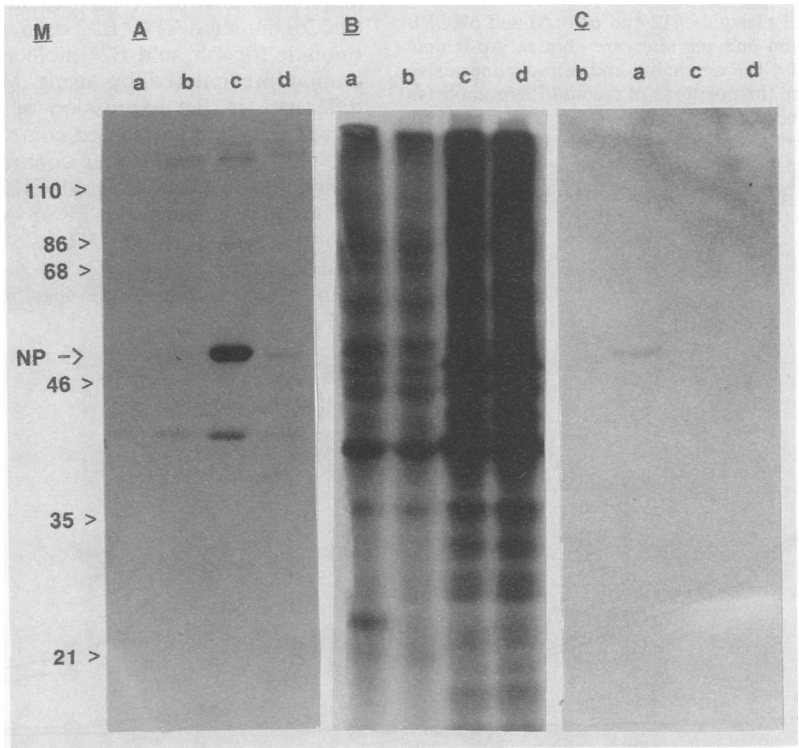

FIG. 4. Detection of NP synthesis in CHO/B27 cells infected with SL3261-pNP-2. CHO/B27 cells, infected either with SL3261-pNP-2 (lanes a) or SL3261 (lanes b) (36 h earlier) or with PR8 virus (lanes c) (1 h earlier), were labelled with [^{35}S]methionine for 4 h before lysis. Uninfected cells were also included as the negative control (lanes d). (A) The NP molecule in the lysate supernatant was immunoprecipitated by using MAb 4.7.18. (B) The pellets of the lysates were boiled in SDS loading buffer before being loaded onto an SDS–10% polyacrylamide gel and autoradiographed. (C) Samples used for panel B were also analyzed by Western blotting with rabbit anti-NP sera. Molecular size markers (in thousands) are indicated on the left.

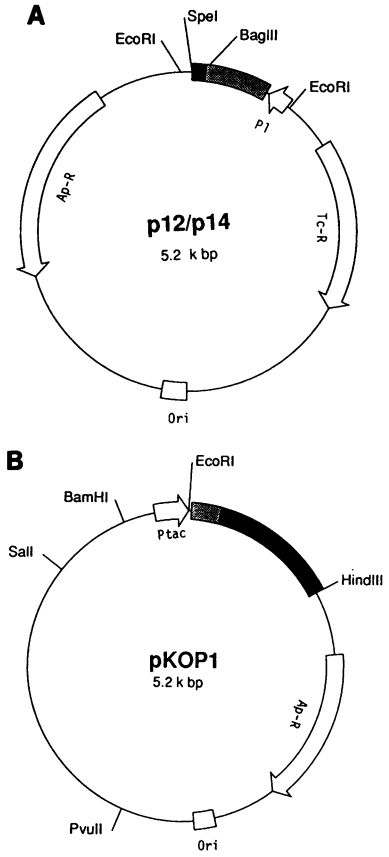

FIG. 5. Physical maps of plasmids p12 and p14 (A) and pKOP1 (B). Only relevant restriction enzyme sites are shown. Ap-R and Tc-R represent, respectively, the ampicillin and tetracycline resistance genes. Ptac and P1 are the positions of tac and P1 promoters, respectively. In panel A, the stippled section is the LTB gene and the black section is the insert coding for NP epitope. In panel B, the stippled section is the OmpA leader sequence gene and the black section is the NP-362-498 gene. Arrows indicate the direction of transcription.

TABLE 2. SL3261-NP constructs did not sensitize CHO/B27 for HF-CTL recognition[a]

Target cell[b]	Specific ^{51}Cr release (%) with:				
	PR8 virus (20 HAU)[c]	Peptide p380-393 at a concn (μg/ml) of[d]:			
		30	10	3	0
SL3261-pNP-2	42	65	34	18	12
SL3261-pKOP1	36	60	35	16	17
SL3261-p12	39	55	31	14	11
SL3261-p14	38	64	36	17	16
CHO/B27	41	85	57	24	12
CHO	10	6	5	7	6

[a] For the HF-CTL-killing assay, CHO and CHO/B27 cells were harvested and labelled with ^{51}Cr for 1 h in the absence or presence of PR8 virus or peptide p380-393 at different concentrations, as indicated in the table. Effector cells were HF-CTL at an effector cell/target cell ratio of 10:1. The supernatant was harvested 6 h later, and the results were expressed as the percentage of specific ^{51}Cr release. The spontaneous ^{51}Cr release was less than 15% of the maximum release.

[b] Target cells were CHO, CHO/B27, or CHO/B27 cells infected with recombinant SL3261 as listed in this column.

[c] HAU, hemagglutinating units.

[d] Final concentration in the culture.

epitope (365 to 379) of NP in the periplasm of the bacteria; it was also possible to repeat the experiment by using CHO/D^b cells and mouse CTL clone F5, but with very similar results (Fig. 7). To exclude the possibility that the infection of transfectant CHO cells by *S. typhimurium* might disrupt the expression of HLA-B27, H-2 D^b, or accessory molecules and inhibit their ability to present antigens to the killer cells, SL3261-infected CHO/B27 cells were labelled with [^{35}S]methionine for 4 h, and B27 molecules in the cell lysate were immunoprecipitated by using MAb ME1. No significant difference in the expression of HLA-B27 by bacteria-infected cells and uninfected controls was observed (data not shown). This was further confirmed by the experiment in which the bacteria-infected CHO/B27 cells could present viral antigen (influenza A/PR8/34 virus) as well as exogenous peptide p380-393 to HF-CTL as efficiently as uninfected cells (Table 2).

In vivo induction of NP-specific CTL in mice by priming

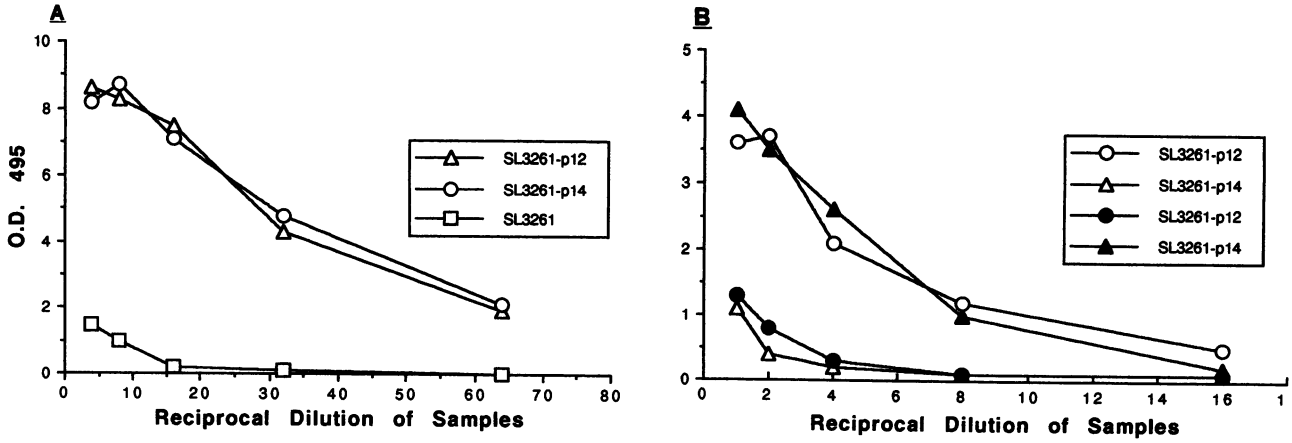

FIG. 6. GM1 ELISA analyses of LTB fusion protein expression in the periplasm. Periplasmic extracts, prepared from SL3261-p12, SL3261-p14, SL3261-pKOP1, SL3261-pNP-2, and SL3261, were diluted, added to GM1-coated 96-well plates, and treated either with rabbit anti-LTB (A) or with anti-p365-380 (B) (closed symbols) or anti-p380-393 (B) (open symbols) sera. Peroxidase-labelled swine anti-rabbit IgG was then added and developed by using DAB substrate. The results were expressed as optical density at 495 hm (O.D. 495).

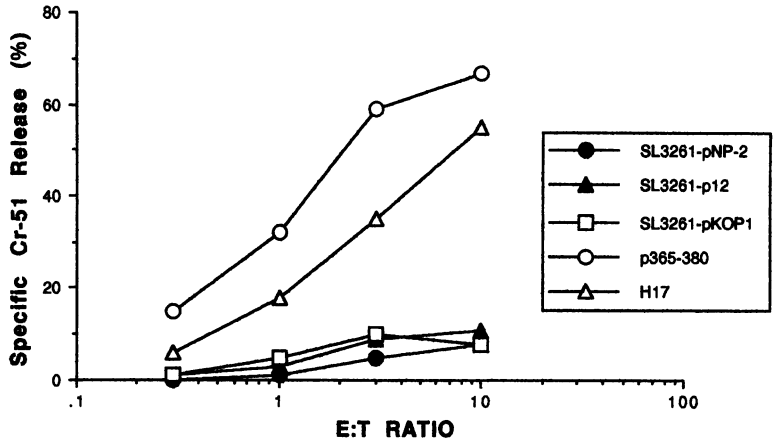

FIG. 7. SL3261-NP constructs did not sensitize CHO/D^b for F5 recognition. CHO/D^b cells were infected with either SL3261-p12, SL3261-pKOP1, or SL3261-pNP-2, as indicated. The infected cells were labelled with ^{51}Cr and used as target cells for the F5 CTL killing assay. Peptide p365-380-pulsed and influenza A virus H17-infected CHO/D^b cells were also included. The results were expressed as the percentage of ^{51}Cr release. The spontaneous release was less than 20% of the maximum release. E:T ratio, effector cell to target cell ratio.

with live SL3261-p12 and SL3261-pKOP1. Since none of the SL3261 constructs described here sensitized target cells for CTL recognition in vitro, it was important to determine if any of these was capable of inducing NP-specific CTL responses in vivo. All four constructs were used to infect C57BL/6 (H-2^b) mice. Spleens were collected from the infected mice 3 to 5 weeks after the challenge, and the spleen cells were stimulated in vitro with peptide p365-380-pulsed syngeneic spleen cells for 6 days. They were then tested on ^{51}Cr-labelled, p365-380-pulsed CHO/D^b cells. As shown in Fig. 8A, spleen cells from both SL3261-p12- and SL3261-pKOP1-infected mice induced significant lysis of the target cells, whereas the spleen cells from mice infected with SL3261-pNP-2 and SL3261 were unable to do so. The induced cytotoxicity was H-2 D^b-restricted, since only p365-380-pulsed CHO/D^b, but not CHO or CHO/B27, cells were recognized by these cells (Fig. 8B). Thus, the *Salmonella* constructs that expressed fragments of NP in the periplasm, rather than in inclusion body, were able to prime CTL responses in vivo.

DISCUSSION

Strains of *S. typhimurium* have been rendered avirulent by nonreverting deletion mutations in the *aroA* gene (14). Although it has been suggested that the avirulent mutants of *Salmonella* species are not capable of surviving within macrophages (8, 31), our results demonstrate that the *aroA* mutants of *S. typhimurium* can invade and replicate within nonphagocytic CHO cells. However, our attempts to sensitize HLA-B27 and H-2 D^b transfectant CHO cells for NP-specific CTL recognition in vitro by infecting them with various recombinant *S. typhimurium* constructs expressing the NP molecule were not successful. The bacteria did replicate (Fig. 1 and 2A) and they synthesized NP within the

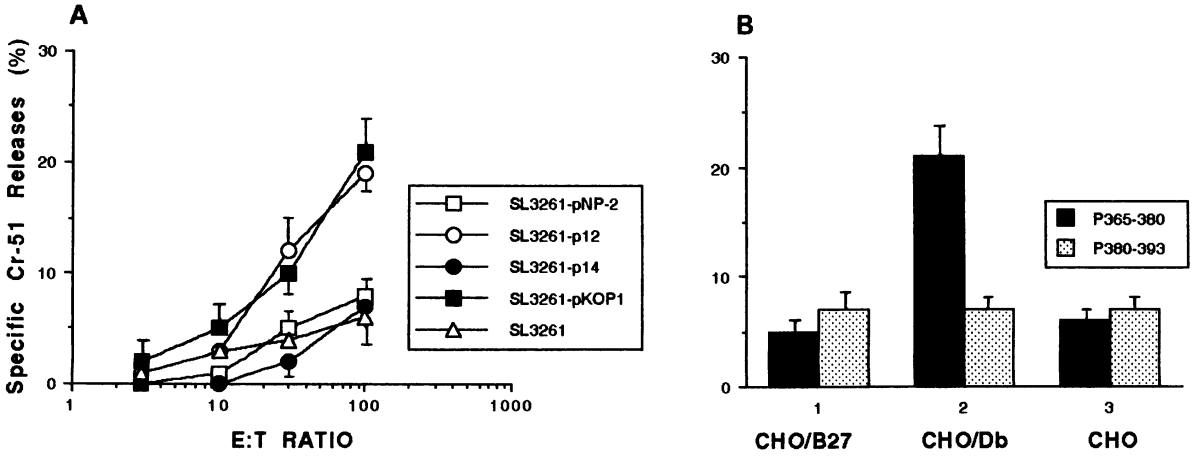

FIG. 8. NP-specific cytotoxicity of spleen cells from mice infected with recombinant *S. typhimurium*. (A) Spleens were collected from mice infected with either SL3261-p12, SL3261-pKOP1, SL3261, or SL3261-pNP-2 3 to 5 weeks previously, and spleen cells were stimulated in vitro with p365-380-pulsed syngeneic spleen cells for 6 days. These cells were titrated against p365-380- or p380-393-pulsed CHO/D^b cells. Supernatants were harvested 6 hours later, and the results were expressed as the percentage of specific ^{51}Cr release. (B) Results of similar experiments using spleen cells from mice primed with SL3261-pKOP1 as effector cells and peptide-pulsed CHO/B27 (1), CHO/D^b (2), and CHO (3) cells as target cells.

host CHO cells (Fig. 2B and 4), but the infected cells were not recognized by NP-specific CTL (Fig. 3 and 7; Table 2).

Unlike macrophages, CHO cells are not professional antigen-presenting cells. Thus, their inability to process intracellular bacteria and then present digested antigens to CTL may resemble that of the nonphagocytic, parenchymal cells, such as hepatocytes and epithelial cells in the gut. When liver parenchymal cells from C57BL/6 mice were infected in vitro with various recombinant SL3261 and used as target cells for CTL clone F5 (H-2 D^b restricted), the cells were not lysed (data not shown). However, transfected CHO cells were able to present virus-derived antigens to CTL, indicating that if an antigen reaches the cytosol, it can be processed and presented through MHC class I molecules. Similarly, *Salmonella*-infected CHO transfectants presented viral antigen as well as added peptide to CTL, demonstrating that the antigen presentation system was not impaired by the bacterial infection.

SL3261-pNP-2 expressed the full-length NP as a fusion protein in an inclusion body (34) (Fig. 2B and C). We demonstrated that this NP failed to reach a detectable level in the cytosol of the host cells (Fig. 2 and 4). On the other hand, constructs SL3261-p14, SL3261-p12, and SL3261-pKOP1 expressed fragments of NP in the periplasm, where expressed products were secreted. However, the level of periplasmic expression of foreign proteins in bacteria is often very low. Although the NP fragments were clearly demonstrated in the periplasmic extracts of *Salmonella* SL3261 harboring p14, p12 (Fig. 6), and pKOP1 (not shown), attempts to identify the expression products in CHO cells infected with these constructs by Western blot, immunoprecipitation, and immunoelectron microscopy were not successful (data not shown). It should be emphasized, however, that previous results with influenza A virus NP-transfected L cells indicated that CTL could readily recognize target cells in which NP was unidentifiable by immunostaining or immunoprecipitation (35).

Intracellularly surviving *S. typhimurium* are located within membrane-bound vacuoles derived from the host cells (10, 15, 20) (Fig. 2A). In nonphagocytic cells, this may form a protective barrier for the bacteria. Thus, proteins made by the surviving bacteria may not be able to cross this membrane and gain access into the cytosol of the host cells. This would make presentation of intracellular *Salmonella* antigens through the class I pathway in nonphagocytic cells, such as CHO and liver parenchymal cells, almost impossible. Attempts were also made to infect two macrophage lines (THP-1 and U936) with recombinant salmonellae and use them as positive antigen-presenting cells for CTL recognition; but we found, as previously reported (8, 31), that the *aroA* mutants of *S. typhimurium* do not grow efficiently in macrophages, and it was not possible to test these cells as targets for CTL lysis.

Our results raise the possibility that, for some nonphagocytic cell types, intracellular *S. typhimurium* may be safe from lysis by CTL. Such cells would shield the bacteria from attack by both major arms, humoral and cellular, of the immune response. It should be emphasized, however, that the data on this point are preliminary, and further proof will be required. Similarly, it should not be assumed that the existence of CTL, induced by *Salmonella* infection, indicates a protective role.

In contrast to the results in vitro, it was possible to prime an H-2 D^b-restricted, NP-specific CTL response in C57BL/6 mice by infection with SL3261-pKOP1 and SL3261-p12. Although the level of CTL response induced by SL3261-p12 and SL3261-pKOP1 was not great, it was definitely positive compared with the lack of response to SL3261-pNP-2 and SL3261 (Fig. 8). Our data are in agreement with those of other investigators (2, 11) who have demonstrated that recombinant *Salmonella* infection can generate CTL responses in vivo to the inserted antigen. In addition, we show that the site of antigen expression can be important; this will be relevant in determining which *Salmonella* products are recognized by CTL. Although the role of CTL in immunity to viral infection is well established, their function in bacterial infection is less secure. Further study of CTL in protection against *Salmonella* infection is merited.

Priming of CTL in vivo also argues that the inability of SL3261-p12 and SL3261-pKOP1 to sensitize CHO/D^b for CTL (F5) recognition in vitro is unlikely to be simply due to the low level of expression of the NP fragments in the periplasm of the bacteria. It is not known which cells present *Salmonella* antigens to CTL precursors in vivo. *Salmonella* species can certainly invade and survive within hepatocytes and epithelial cells in the gut, but as these cells are not classical antigen-presenting cells, it is possible that, like CHO cells in vitro, they fail to present bacterial antigens to $CD8^+$ T cells through MHC class I products. Macrophages and dendritic cells are obvious candidates, although the ability of the avirulent mutants of *Salmonella* species to survive within phagocytic cells is limited (8, 31).

The possibility that bacteria, killed by opsonization, release proteins or peptides that prime is less likely because SL3261-pNP-2, which produces the full-length NP, failed to prime CTL. This inability of SL3261-pNP-2 to induce CTL in C57BL/6 mice confirms a previous report by Tite et al. (33) that when BALB/c mice were infected with this construct, NP-specific CTL were not detectable, although strong NP-specific helper T cells and antibody responses were elicited. Comparison of SL3261-pKOP1 and SL3261-p12, both of which expressed NP fragments in the periplasm, with SL3261-pNP-2, which expressed greater quantity of NP in an inclusion body, for their ability to induce CTL responses in vivo suggests that the compartment of the bacteria in which antigen was expressed might determine the ability to induce CTL responses in vivo. It is also possible, however, that the solubility of the expressed protein plays a role or that instability of NP fragments as a result of fusion to LTB or the Omp-A leader sequence may be critical for CTL induction.

This and previous studies indicate that *Salmonella* infection can prime CTL responses to recombinant gene products. It is reasonable, therefore, to expect that CTL specific for *Salmonella* proteins will also be generated, although the antigenicity may be restricted by their location in the bacteria. However, if *Salmonella*-infected nonphagocytic cells are resistant to killing by bacterial antigen-specific CTL, the role played by CTL in the acquired immunity against *Salmonella* infection could be limited. It is possible that macrophages and other mononuclear phagocytes are susceptible to CTL lysis, but as these cells form a major defense against *Salmonella* infection in vivo, it is difficult to imagine how the destruction of these cells by CTL could benefit the host. However, it cannot be ruled out at present that the inability of the nonphagocytic cells to present recombinant NP to specific CTL in vitro is due to unknown deficiencies of this experimental system. Further experimental analysis will require development of a target cell that can, after *Salmonella* infection, be recognized by specific CTL. In addition, identification of the in vivo target cells for these CTL would

allow us to answer the question whether these CTL cells play any protective role in a *Salmonella* infection.

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