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Generation of a cytotoxic T-lymphocyte response using a *Salmonella* antigen-delivery system

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Summary

We have constructed a general-use vector for the cloning and stable expression of foreign genes in the chromosome of attenuated *Salmonella typhimurium*. Using this chromosomal expression vector (CEV), we expressed the circumsporozoite (CS) gene of the mouse malaria *Plasmodium yoelii* in an araA *S. typhimurium* strain. Mice immunized with CS-expressing *Salmonella* recombinants mount a CS-specific cytotoxic T-lymphocyte (CTL) response. This is the first demonstration that attenuated *Salmonella* can elicit a specific CTL response to a foreign protein in mice. The ability to easily and stably express foreign genes from the *Salmonella* chromosome and the generation of specific CTL greatly expands the potential of *Salmonella* as an antigen-delivery system.

Introduction

*Salmonella typhimurium* is a pathogen that causes a typhoid fever-like disease in mice. During infection, *Salmonella* elicits both humoral and cellular immune responses, both of which are necessary for protection against subsequent infection. Heat-killed *Salmonella* does not protect against a virulent challenge although strong antibody responses are generated. *Salmonella* strains with lesions in *galE* (Moser et al., 1980), *araA* (Hoiseth and Stocker, 1981), and *cyA* (Curtiss et al., 1987) genes, among others, have greatly increased lethal dose (50%) (LD₅₀) values for inbred mouse strains. Mice immunized with these attenuated strains are protected against a lethal challenge with virulent *Salmonella*. Therefore live strains appear to elicit the appropriate (B- and T-cell) immune responses for protection. Genes from a variety of pathogens have been expressed in attenuated *S. typhimurium* (Poirier et al., 1988; Newton et al., 1989; Sadoff et al., 1988) and immunization of mice with recombinant strains generated antibody (Poirier et al., 1988; Newton et al., 1989; Brown et al., 1987) or delayed-type hypersensitivity (DTH) (Sadoff et al., 1988) responses against the foreign antigen. For these reasons, there is a great deal of interest in attenuated *Salmonella* antigen-delivery systems as a means of examining the immune response to various antigens (Dougan et al., 1987).

Plasmids have been the primary method used to express foreign genes in *Salmonella*. The use of plasmids in antigen-delivery systems may have several drawbacks. The expression of many foreign antigens can result in plasmid instability, leading to loss of the plasmid (O’Callaghan et al., 1988); alternatively, the cloned sequences may undergo deletion or other rearrangements. High copy-number plasmids or a strong promoter can result in the overexpression of foreign proteins, which can be lethal to the bacterial cell (Shatzman et al., 1983). A plasmid can be integrated into the bacterial chromosome by a single recombinational event (Hone et al., 1988), but in the case of a protein which is deleterious to the cell, the plasmid could recircularize and be lost. Although a number of prokaryotic genes may be stably expressed and the proteins maintained in *Salmonella*, eukaryotic, including parasite, genes and gene products may be more difficult to stabilize. Antibiotic selection can maintain a reasonable level of an unstable plasmid *in vitro*, but it is not feasible to continue antibiotic selection in mice. One method for stabilizing foreign expressed DNA in *Salmonella* is to integrate the gene into the chromosome. There have been two reports of methods used to insert genes into the chromosome of *Salmonella* (Hone et al., 1988; Strugnell et al., 1990). We have also developed a general system for stably inserting foreign genes into the chromosome of *S. typhimurium*. This system uses a defective transposable element which stably inserts into the bacterial chromosome to express a gene from any organism of interest in *S. typhimurium*. We have termed this type of system...
'chromosomal expression vector' (CEV). In this report we describe lambdaBV, a CEV in which genes for foreign antigens are expressed in S. typhimurium.

Human malaria, caused by the parasite Plasmodium falciparum, is endemic in many developing countries and is responsible for 2.5 million deaths per year (Sturchler, 1988). Plasmodium species have a narrow host range and the species infective for mice include Plasmodium berghei and Plasmodium yoelii. Irradiated P. yoelii and P. berghei sporozoites can induce immunity in mice against a sporozoite challenge (Nussenzweig et al., 1969). Antibodies to the CS protein that covers the sporozoite are generated in the host and may play a role in sporozoite-induced immunity. However, mice that have been α-suppressed, and therefore lack B-cells and circulating immunoglobulins, can be protected against challenge by immunization with irradiated sporozoites (Chen et al., 1977). Recent studies in mice indicate that cellular immune responses induced by immunization with sporozoites are essential for immunity. In particular, CD8+ T-cell (cytotoxic/α-suppressor) responses are necessary for protection against a P. yoelii or P. berghei sporozoite challenge (Weiss et al., 1988; Scholfield et al., 1987).

It was previously shown that BALB/c mice immunized orally with attenuated S. typhimurium expressing the P. berghei CS protein from a plasmid vector were partially protected against a P. berghei sporozoite challenge in the absence of an anti-CS antibody response (Sadow et al., 1988). We have tested our CEV system by expressing the CS gene of P. yoelii in S. typhimurium and immunizing mice with the recombinant strain to examine the immune response to the recombinant CS protein. Using lambdaBV, the gene encoding the malarial circumsporozoite protein of P. yoelii was expressed from the chromosome of an araA mutant of S. typhimurium. Immunization of BALB/c mice with this recombinant strain resulted in a specific cytotoxic T-lymphocyte (CTL) response against a peptide of the CS protein. This is the first demonstration of a specific, genetically restricted CTL response to a foreign antigen expressed from the chromosome in Salmonella.

Results

Description and use of lambdaBV

High-frequency insertion of foreign genes into the chromosome of S. typhimurium was effected by using a modified version of the mini-transposon, mini-Tn10-Kan (Way et al., 1984) carried on bacteriophage lambda (see the Experimental procedures and Fig. 1A). This CEV, termed lambdaBV, carries a transposase gene separated from the mini-Tn10 that encodes kanamycin resistance and a lacUV5 promoter with an α-lacZ fragment. The transposase gene is outside the transposable portion of the transposon and is under the control of a strong promoter (p lac). The α-lacZ portion has a unique NotI site useful for constructing transcriptional fusions of foreign DNA with the lacUV5 promoter or translational fusions with the α-lac peptide. The NotI site permits cloning of DNA without a methylation step, as there are few genes with internal NotI sites.

The lacUV5 promoter is susceptible to catabolite repression and so will not be regulated by sugar levels once the Salmonella enters the macrophages of the mouse. Transposition of the mini-Tn does not occur in Escherichia coli infected with lambdaBV since the expression of transposase and the cloned gene is under the control of lac repressor. Salmonella has no lac repressor so transposition occurs at a high frequency and the cloned foreign gene is expressed. Lambda does not infect Salmonella as it can neither adsorb to the cell surface nor replicate in the cell. However, lambda DNA can be introduced into Salmonella if its receptor, LamB, is expressed on the cell surface. Therefore lambdaBV will infect S. typhimurium harbouring a lamB clone and act as a 'suicide' vehicle for the mini-transposon. Once the phage DNA enters Salmonella the transposase is expressed at a high level (in the absence of lac repressor) and the mini-Tn element carrying a foreign gene transposes randomly into the chromosome. The lambda phage DNA is unable to replicate and is lost. The mini-Tn insertions are stable since the transposase is lost along with the phage genome. Using this CEV, transposition into the Salmonella chromosome occurs at a frequency of 5 × 10^-3. These transposition events are easily selected by plating the Salmonella on kanamycin following adsorption of lambdaBV.

The gene of interest is cloned into the NotI site of the lambda vector, fusing the coding sequence to the lacZ transcription and translation signals. The ligation is packaged in vitro with lambda phage extracts and the lysate used to infect E. coli strain Y1090 (supF). This strain carries a plasmid that expresses high levels of the lac repressor gene, so neither the transposase gene nor the cloned foreign gene are transcribed. Positive clones can be detected by DNA hybridization or antibody screening of plaques induced in the presence of 10 mM isopropyl-β-D-thiogalactoside (IPTG). IPTG induction of the lacUV5 promoter should only be performed with replica plaques, as IPTG also induces the transposase which could cause transposition of the mini-transposon in E. coli. A liquid lysate of the positive clone is used to infect S. typhimurium strain LB5000 carrying the lamB plasmid pTROY11 (de Vries et al., 1984) at a multiplicity of infection (m.o.i.) of 5–10, and kanamycin-resistant colonies selected (Fig. 1B). Strain LB500 is deficient in DNA restriction systems, and therefore does not degrade the incoming DNA from E. coli-grown phage lambda. However, LB500, a laboratory strain, is not a useful attenuated strain for inducing
immune responses in mice. For this reason, after generating insertions in LB5000, a Salmonella transducing phage P22 lysate is made on a pool of these kanamycin-resistant LB5000 colonies. This lysate is used to transduce the chromosomal insertions to an attenuated S. typhimurium strain, selecting for kanamycin resistance. A pool of insertions in the chromosome is used to diminish the effect of a single insertion in a Salmonella gene important for survival in the mouse.

**Expression of P. yoelii CS protein in Salmonella**

To test the ability of the CEV constructed here to stably maintain and express a parasite gene in *S. typhimurium* upon introduction into mice, we cloned the CS gene from *P. yoelii* into lambdaBV (Fig. 2A). An Alul DNA fragment of *P. yoelii* encoding all but the first 36 amino acids of CS protein (Lal et al., 1987) was subcloned into lambdaBV. In *E. coli* strain Y1090, plaques were first screened by DNA hybridization for those containing the CS insert. Positive plaques were replaques in the presence of 10mM IPTG, transferred to nitrocellulose, and screened with anti-CS monoclonal antibody (NYS1) for production of CS protein.

A lambda lysate from a positive clone was used to transfect S. typhimurium strain LB5000(pTROY11). Phage P22 was used to transduce the CS gene from LB5000 to the chromosome of the attenuated (aroA) S. typhimurium strains SL3261 and SL3235. The recombinant S. typhimurium stably expressed the truncated CS protein, as demonstrated by immunoblots with NYS1 monoclonal antibody (Fig. 2).

**Specific CTL response in mice immunosed with Salmonella producing CS**

The usefulness of a *Salmonella* antigen-delivery system for examining the complete repertoire of immune responses to a variety of foreign antigens depends, in part, on the ability of the bacteria to induce a specific CTL response to the foreign protein in mice. We tested this with our CS-producing recombinant *S. typhimurium*. An essential role for CD8+ T-cells in protection against a *Plasmodium* sporozoite challenge has been demonstrated. *P. yoelii* sporozoite-immunized mice depleted of CD8+ T-cells were no longer resistant to a *P. yoelii* sporozoite challenge. In contrast, depletion of CD4+
T-cells had no effect on the sporozoite-induced immunity (Weiss et al., 1988). Experiments using \textit{P. berghei} sporozoites also demonstrated an essential role for CD8$^+$ T-cells (Schofield et al., 1987). Mice immunized with \textit{Plasmodium falciparum} sporozoites or recombinant vac- cinia virus expressing the \textit{P. falciparum} CS protein were shown to contain CTL specific for a single epitope on the CS protein (Kumar et al., 1988). Similarly, only a single CTL epitope has been identified recently on the CS proteins of \textit{P. berghei} (Romero et al., 1989) and \textit{P. yoelii} (Weiss et al., 1990). The 16-amino-acid peptide PYCTL1 (residues 281–300) from the \textit{P. yoelii} CS protein was specifically recognized by CTL in sporozoite-immunized BALB/c mice. This peptide was shown to stimulate CTL from immunized animals \textit{in vitro}, and to label target cell lines for lysis by these cells (Weiss et al., 1990).

To determine if attenuated \textit{S. typhimurium} expressing the CS protein could generate a similar specific CTL response in mice, BALB/c mice were immunized orally (1–5 × 10$^6$ bacteria every third day for a total of three inoculations) with \textit{S. typhimurium} strain SL3235 expressing the cloned CS protein (CS11) or with SL3235 carrying the mini-Tn10 without an insert (BV). As a positive control, mice were immunized intravenously (i.v.) with irradiated sporozoites. Six weeks after the last immunization, spleens were removed and the cells were used as effector cells in a $^{51}$Cr-release CTL assay using mouse tumour cell line P815 (H-2$^b$), labelled with CS peptide PYCTL1, as target cells. Specific lysis of the target cells was observed with effector cells from mice immunized either with sporozoites (54%) or with \textit{S. typhimurium} CS11 (37%) (Fig. 3A). T-cells from mice immunized with \textit{S. typhimurium} BV gave only 8% lysis of peptide labelled target cells. The specific lysis seen is due to CD8$^+$ CTL since treatment of the effector cell cultures with anti-CD8 monoclonal antibody and complement resulted in loss of lytic activity against the peptide-labelled target cells, whereas treatment with a control antibody plus comple- ment had no effect (Fig. 3B). When EL4 (H-2$^b$) cells labelled with PYCTL1 were used as target cells in an identical CTL assay, CTLs from mice immunized with \textit{S. typhimurium} expressing CS gave low levels of lysis (Fig. 4). Similar low levels of PYCTL1-labelled EL4 target cell lysis were observed with CTLs from mice immunized with \textit{P. yoelii} sporozoites (data not shown). These results demonstrate that \textit{Salmonella} can induce a CTL response in mice and that the CTL response to the recombinant CS protein is MHC-(H-2)-restricted.
CTL response generated by Salmonella antigen-delivery system

Response does not seem to be related to loss of CS expression since *S. typhimurium* expressing the CS protein could be recovered from spleens three weeks after the first immunization. In one out of ten isolates recovered from mice, a small deletion in the cloned CS gene was detected. This is not unexpected since there are repeats within the gene which could be subject to recombination. However, we did not observe deletions after repeated subculturing of the CS-expressing strains *in vitro*. These observations indicate that the chromosomal copy of this gene is generally quite stable.

**Challenge of immunized mice with sporozoites**

Since a CTL response has been suggested to be important in sporozoite immunity, we investigated whether the mice immunized with *S. typhimurium* producing the CS protein would be protected against a sporozoite challenge. Mice immunized with SL3235 BV or CS11 (orally) or irradiated sporozoites (i.v.) were challenged eight weeks post-immunization with 200 *P. yoelii* sporozoites (i.v. in the tail vein). Only mice immunized with irradiated sporozoites were protected against challenge. The recombinant-Salmonella-immunized mice developed parasitaemia at the same time as untreated mice.

**Discussion**

We have constructed a general-use system for the stable expression of foreign genes from the *S. typhimurium* chromosome. Insertion of a foreign gene into the chromosome increases the likelihood of stable maintenance and expression of the antigen in mice. Recombinant attenuated Salmonella can generate both humoral and cellular responses to a foreign protein. These features make
Salmonella antigen-delivery systems very useful for examining the immune response to foreign antigens, both peptide epitopes and whole proteins. Previously, antibody and delayed-type hypersensitivity (DTH) responses to a foreign protein in Salmonella have been reported (Poirier et al., 1988; Newton et al., 1989; Sadoff et al., 1988). We demonstrate here that cytotoxic T-cells can also be elicited against a specific foreign antigen expressed in Salmonella.

It has become clear that a CTL response is an essential component of the protective immune response to a number of pathogens. Immunization of mice with soluble proteins can give antibody and T-helper cell responses, but cannot generally induce CD8* CTL responses. To examine the potential of Salmonella in eliciting a CTL response to heterologous antigens we chose to study the CS protein of P. yoelii. A CTL epitope on the CS protein of P. yoelii has been identified (Weiss et al., 1988). We were able to generate a CTL response to this CS epitope in BALB/c mice immunized with our S. typhimurium strain producing the CS protein, but protection against a P. yoelii challenge was not observed. To date, it has not been proven that the CS protein of P. yoelii is a protective antigen. Our data and other recent evidence (W. R. Weiss, unpublished) suggest that a strong CTL response to this particular epitope is not sufficient for protection against a P. yoelii sporozoite challenge. Interestingly, adoptive transfer of cloned CTL lines specific for a corresponding P. berghei CS epitope (differing from the P. yoelii epitope by two amino acids) to BALB/c mice confers protection against a subsequent P. berghei sporozoite challenge (Romero et al., 1989). It is possible that in BALB/c mice the P. yoelii CS peptide (PYCTL1) is not a protective CTL epitope. It may be that in BALB/c mice, epitopes in addition to those of the CS protein are necessary for protective immunity. These results emphasize the need for research into additional malarial antigens as potential vaccine candidates, and suggest that immunologically important antigens may differ among species of malaria parasites.

Although we cannot easily explain the lack of antibody response to the CS protein in mice immunized with the recombinant Salmonella, the data are consistent with the previous observation that Salmonella carrying the CS gene from P. berghei on a plasmid did not induce a strong anti-CS antibody response in mice (Sadoff et al., 1988). In contrast, other genes expressed from plasmids in Salmonella, for example the streptococcal M protein (Poirier et al., 1988) and E. coli β-galactosidase (Brown et al., 1987), elicited antibody responses in mice. Using the lambdaBV CEV system, Salmonella expressing the Trypanosoma cruzi neuraminidase gene elicited a strong antibody response to the T. cruzi neuraminidase protein (J. L. Flynn, unpublished).

Recently it has been shown that some intracellular bacteria, most notably Listeria monocytogenes, can induce a CTL response in mice (Kaufmann, 1988). Live Salmonella appear to be required to induce the immune response (both B- and T-cell) necessary for protection against a virulent Salmonella challenge. Salmonella reside in the macrophages and other cells of the host. Although it has not been extensively studied, it is thought that S. typhimurium replicates inside the phagolysosome. Certain S. typhimurium strains that are too attenuated to survive for any length of time inside the host are not as effective in the induction of protective immunity (O’Callaghan et al., 1988; Curtiss et al., 1987). Thus it seems that persistence in the host cell is necessary to induce cellular immunity. The araA attenuated strains we used in these studies persist for four to six weeks and protect against a virulent Salmonella challenge (Hoiseth and Stocker, 1981).

Unlike L. monocytogenes, Salmonella probably replicate in the phagolysosome; the attenuated strains are killed and the proteins are degraded. The generation of a CTL response to an intracellular antigen (the recombinant CS protein) implies that the antigens from Salmonella can associate with Class I major histocompatibility complex (MHC) molecules in the cell and are presented to CD8+ T-cells. It is unclear where these antigens associate with the Class I molecules and it may be possible to address this question using a Salmonella antigen-delivery system.

The ability of recombinant attenuated Salmonella strains to generate a foreign antigen-specific CTL response in mice indicates that our chromosomal expression system could be useful for studying viruses and other parasites whose clearance requires CTL responses. This CEV system has also been used to express the T. cruzi neuraminidase gene in S. typhimurium and immunization of mice with this recombinant strain resulted in both antibody production against T. cruzi neuraminidase and a specific T-helper response (J. L. Flynn, G. Harth, and M. So, unpublished). Generation of a CTL response to a foreign antigen, in conjunction with the induction of antibody and T-cell responses, makes antigen delivery by S. typhimurium a very attractive system for examining the immunological potential of various proteins, while obviating the need for purifying large quantities of the protein or using a foreign adjuvant. This novel CEV system will be valuable for assessing the protection capacity of antigens from a wide variety of pathogens that have an appropriate animal model, and may lead to the development of new vaccine strategies for infectious diseases.

Experimental procedures

Bacterial strains, plasmids, phage and parasites

E. coli strain Y1090 (Young and Davis, 1984) was used as a host for lambdaBV. Plasmid pNK862 (AmpR, KanR) which carries
Construction of the lambdaBV vector

A 200 bp EcoRI–HindIII fragment containing the lacUV5 promoter region from pRZ5320 was isolated. This promoter fragment was ligated to a 166 bp Haell–HindIII gel-isolated fragment from pUC9–NotI containing the alpha-lac peptide of $\beta$-galactosidase with an in-frame NotI adaptor linker replacing the EcoRI site in the multiple cloning site. XbaI linkers were added to this lacUV5/\alpha-lac fragment and it was cloned into the XbaI site of the mini-Tn10 on plasmid pNK862. A 6 kb EcoRI fragment containing the transposase and mini-Tn10 Kan (with the lac cartridge) was cloned into the EcoRI site of Lambda long-c.

Cloning the CS gene from pPY2053 into lambdabV

NotI linkers were added to the 1.4 kb Alul fragment of the CS gene from plasmid pPY2053. This fragment encodes all but the first 36 amino acids of CS protein and was cloned into the NotI site of the lambdabV in-frame expression from the lacUV5 promoter in Salmonella. This construct does not contain the signal sequence for the CS protein, since this sequence may be partially responsible for poor expression of CS protein in E. coli (J. L. Flynn, unpublished). The ligated was packaged in vitro (Gigapack, Stratagene), and plaqued on E. coli Y1090. 

Immunoblots

S. typhimurium whole-cell lysates were prepared by boiling late-log cultures in sodium dodecyl sulphate (SDS) sample buffer with 2-mercaptoethanol. Following centrifugation (13,000 × g, 10 min) to remove insoluble debris, the samples were run on 10% SDS–polyacrylamide gels and transferred to nitrocellulose. The filters were blocked with 2% bovine serum albumin (BSA) and incubated with monoclonal antibody NYS1 (1:128 dilution) (Charoenwit et al., 1987) for 2 h at room temperature. The secondary antibody was anti-mouse alkaline phosphatase conjugate (Promega). Mouse sera were used at 1:50 dilution against nitrocellulose blots containing SDS-solubilized sporozoites dissected from mosquitoes.

Mice

Female BALB/cByJ mice (6–10-week-old) from Jackson Laboratories or the breeding facility of Scripps Clinic were used for all mouse studies. Mice were immunized i.p. with S. typhimurium (1–2 × 10^9) or orally (1–5 × 10^9, three times within 10 d). Mice were immunized i.v. with irradiated (12,000 rad of gamma radiation from a Cs-137 source) sporozoites.

CTL assays

These assays were performed as previously described (Weiss et al., 1990). Spleen cells (5 × 10^6) were aliquoted into 10% fetal calf serum, 5 × 10^-3 2ME). Peptide antigen was added to a final concentration of 5 μM, and the cells were incubated at 37°C, 5% CO2. After 2 d, 0.2 ml of rat concanavalin A culture supernatant (Collaborative Research, Inc.) was added to each well, cultures were incubated for an additional 5 d, and viable cells were harvested for effector cells. The target cell line used was P815 (H-2k) (American Type Culture Collection). Target cells (1 × 10^5) were labelled by 18-h culture in 2 ml RPMI, 10% fetal calf serum and 0.1 μCi of 51 Chromium as a sterile sodium chromate solution (Dupont-New England Nuclear, Inc.) with peptide antigen at 5 μM. Following incubation, cells were washed extensively and plated at 500 cells/well in a 96-well U-bottom plate. Effector cells at the desired concentration were added as well as peptide antigen at a final concentration of 5 μM. Experimental wells were reproduced in triplicate. After 6 h at 37°C, 5% CO2, supernatants were harvested and released 51 Chromium was detected by scintillation counting. Lysis of CD8+ cells was accomplished by incubating 1 × 10^6 effector cells with 100 μg of monoclonal antibody 2.43 (anti-CD8) for one hour at 4°C, followed by incubation with rabbit low-toxicity complement (Accurate Scientific) for a further hour at 37°C. Control cells were incubated with rat serum immunoglobulin and complement at the same concentration. Maximum 51 Chromium release was determined by lysing cells with 10% SDS. Spontaneous 51 Chromium release was determined to be 20% of maximum release. Calculation of % specific lysis = (experimental release – spontaneous release)/(maximum release – spontaneous release) × 100.

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