Oral *Salmonella*: Malaria Circumsporozoite Recombinants Induce Specific CD8+ Cytotoxic T Cells

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Summary

Oral immunization with an attenuated *Salmonella typhimurium* recombinant containing the full-length *Plasmodium berghei* circumsporozoite (CS) gene induces protective immunity against *P. berghei* sporozoite challenge in the absence of antibody. We found that this immunity was mediated through the induction of specific CD8+ T cells since in vivo elimination of CD8+ cells abrogated protection. In vitro studies revealed that this *Salmonella–P. berghei* CS recombinant induced class I–restricted CD8+ cytotoxic T cells that are directed against the *P. berghei* CS peptide epitope spanning amino acids 242–253. This is the same peptide that previously was identified as the target of cytotoxic T lymphocytes (CTL) induced by sporozoite immunization. *Salmonella–P. falciparum* CS recombinants were constructed that contained either the full-length CS gene or a repeatless gene consisting of CS flanking sequences. Both of these vaccines were able to induce CD8+ CTL directed against *P. falciparum* CS peptide 371–390, which is identical to the target of CTL induced by sporozoites and vaccinia CS recombinants. These results directly demonstrate the ability of an intracellular bacteria such as *Salmonella* to induce class I–restricted CD8+ CTL and illustrate the importance of CD8+ CTL in immunity to malaria.

Malaria occurs in hundreds of millions of people and kills one to two million children every year (reviewed in reference 1). A vaccine to prevent malaria is needed because control measures, including the use of antibiotics and insecticides, have been ineffective in most parts of the developing world. Under experimental conditions, humans have been protected from *Plasmodium falciparum* malaria by immunization with the bites of hundreds of infected mosquitoes that have been irradiated (2). Animals can also be immunized in this manner or by the direct injection of radiation-attenuated sporozoites (3). This protection is mediated in part by antibodies, some of which are directed against the repeat region of the circumsporozoite (CS)[1] protein, which covers the sporozoite surface (4–7). Early animal experiments, however, also demonstrated that μ-suppressed mice, which lack B cells and circulating Igs, could be immunized with radiation-attenuated sporozoites, indicating that T cells alone are sufficient for sporozoite-induced immunity in mice (8). This cellular immunity is more potent than antibody against the repeat region of the circumsporozoite protein in terms of the numbers of sporozoites that can be protected against (9).

Recent work has shown that in some mouse strains after immunization with radiation-attenuated sporozoites, CD8+ T cells (cytotoxic/suppressors) are induced that are involved in protection against malaria. Protection can be eliminated by in vivo depletion of CD8+ T cells (10, 11). CTL from *P. berghei*–immunized mice recognize the CS protein on the surface of infected hepatocytes and eliminate these cells from the culture in a genetically restricted manner (12). *P. falciparum* sporozoites have been shown to induce circumsporozoite protein–specific CD8+ CTL (13), and the epitope these CTL are directed against has been mapped to amino acid position 370–391. More recently, cloned cytotoxic T cell lines directed against *P. berghei* CS protein (position 249–260) have been shown to passively transfer protection against challenge (14).

We have previously reported that an orally administered live attenuated *Salmonella* recombinant vaccine, which expressed the full-length *P. berghei* circumsporozoite antigen, protected mice against malaria (15). Because the protection was achieved in the absence of antibody, we postulated that cellular immune mechanisms were involved. We proposed that intracellular targeting of the *Salmonella* recombinant or-

[1] *Abbreviations used in this paper:* CS, circumsporozoite; MF, microfluorometry; RLF, repeatless fragment.

ganisms led to expression of CS antigen on the cell surface in association with class I MHC molecules with the subsequent induction of specific CTL. To test this hypothesis, we constructed *Salmonella typhimurium–P. berghei* CS and *S. typhimurium–P. falciparum* CS recombinants, immunized mice, and tested for CS-specific CTL. We now report that orally administered *Salmonella*–CS recombinants induce CS-specific CTL. In vivo depletion experiments demonstrate that these CS-specific CTL are responsible for the protection against *P. berghei*.

Materials and Methods

Plasmids. pADE171 (16) was kindly provided by Dr. David Hone, University of Maryland (Baltimore, MD), and pMGB2 (15), plasmid AR13R-16 csp, and plasmid AR58 repeatless/NS (Gross, M., D.R. Sylvester, G. Sathe, and T. Theisen, manuscript submitted for publication) were from the Department of Molecular Genetics, Smith Kline Laboratories (Swedeland, PA).

Bacteria. S. typhimurium LB 5010 r^-m^+, *S. typhimurium* WR4024 trp R^- were the generous gifts of Dr. Louis Barron, Walter Reed Army Institute of Research (Washington, DC).

Construction of Salmonella–P. berghei CS Recombinants. The full-length CS gene of *P. berghei* strain NK65 was cloned in the StuI restriction site of the P_L-based plasmid expression vector, pMG27 NSterm, as described elsewhere in detail (17, 18). In *Escherichia coli*, pMGB2 expresses full-length CS protein with six additional amino acids at its NH_2 terminus (Met, Asp, Pro, Trp, Arg, Lys). Plasmid pMGB2 was purified (19) and transformed into *Salmonella typhimurium* WR 4024 trp R^-. This strain is a derivative of WR4017, which was previously known as strain M206, an avirulent strain with impaired ability to multiply within macrophages (20, 21). Ampicillin-resistant colonies were examined for the expression of *P. berghei* CS antigen.

Construction of Salmonella–P. falciparum CS Recombinants. R16 CSP, containing 16 repeats of the CS repeat region fused to the full-length CS gene of *P. falciparum*, strain 7G8, and NS 1 81 repeatless fragment (RLF), containing the first 81 amino acids of influenza NS 1 protein fused to the CS gene containing no repeat sequences (RLF), were digested by BglII and SalI from plasmids AR 13R-16 csp and AR 58 repeatless/NS, respectively (Gross, M., D.R. Sylvester, G. Sathe, and T. Theisen, manuscript submitted for publication). The gel-purified inserts R16 CSP and NS 1 81 RLF were blunt-end ligated into the PstI site of plasmid pADE171 (16), which carries the *his OGD* region of *S. typhimurium*, resulting in plasmids containing the malaria gene flanked by *S. typhimurium his OGD* sequences (Fig. 1, Table 1). Plasmids pADE171 R16 and pADE171 RLF were purified and used to transform strain LB 5010, which is an r^-m^+ *S. typhimurium* strain. Spectinomycin-resistant colonies were examined for the expression of CS gene.

Plasmid pMGB2, pADE171 R16, and pADE171 RLF were transformed into *S. typhimurium* WR4024 trp R^- by a modification (22) of standard methods (23). Transformants WR4024/pMGB2 expressing *P. berghei* CS were identified by mAb 3.28 1 (24). *Salmonella* transformants WR4024/pADE171 R16 and WR4024/pADE171 RLF were examined for the expression of *P. falciparum* CS antigen using mAb 49.4D 9.1 and rabbit antirepeatless antibody 579.15, respectively. mAb 49.4D 9.1 (IgG) was made against the full-length CS gene by Dr. K. Esser, Walter Reed Army Institute of Research. Polyclonal rabbit sera raised against the CS repeatless molecule NS 1 81RLF 9 was a kind gift of Dr. D.M. Gordon (Walter Reed Army Institute of Research).

Immunization Protocol. BALB/c ($H2^d$) or B10.BR ($H2^k$) female mice, 5–6 wk old, were immunized with *Salmonella–P. berghei* or *Salmonella–P. falciparum* CS recombinants, respectively. Mice were immunized orally with three doses of 10^9 *Salmonella* CS recombinants on alternate days. *Salmonella* were cultured from liver homogenates for up to 3 wk.

Challenge. 4 wk after primary immunization with *Salmonella–P. berghei* CS recombinants, mice were injected intravenously with 1,000 NK65 *P. berghei* sporozoites. Thin blood films were made every day beginning 5 d after challenge, Giemsa stained, and scanned for parasites in 50 oil immersion fields. Mice were considered protected if no parasites were detected by day 21 after challenge. Serum was collected from individual mice, and anti-CS antibody was detected using an ELISA (15).

Depletion of T Lymphocytes. Groups of BALB/c mice immunized either with WR 4024/pMGB2 or WR4024, or unimmunized mice, were depleted of $CD8^+$ T cells or $CD4^+$ T cells by the method of Weiss et al. (10). Briefly, 4 wk after the immunization, mice were injected intraperitoneally with 300 μg of anti-CD8 antibody anti-Lyt-2.2 hybridoma clone 19/178 (mouse IgG 2a) (25), or anti-CD4 antibody, anti-L3T4 clone GK 1.5 (rat IgG 2b (26) for four successive days. These antibodies were purchased from Bioproducts for Science, Inc. (Madison, WI), and were kindly purified by Dr. Walter Weiss, Naval Medical Research Institute, Rockville, MD. 3 d after the last depletion dose, spleen cells from antibody-treated mice were stained with FITC-conjugated goat anti-mouse IgG2a or goat anti-rat IgG2b (Kierkegaard & Perry Laboratories, Inc., Gaithersburg, MD). Depletion was quantitated by microfluorometry (MF). Mice were challenged with 1,000 NK65 *P. berghei* sporozoites intravenously on the fourth day of depletion. These mice received additional injections of 300 μg of anti-CD8 or anti-CD4 antibody every third day for 21 d after challenge. A control group of mice received the same dose of a mouse IgG2a on the same schedule.

CTL Assay for P. berghei Experiments. The CTL assay was performed as described by Townsend et al. (27). Spleen cells from WR4024/pMGB2, WR4024, or sporozoite-immunized BALB/c mice (haplotype $H\text{-}2^d$) were stimulated in vitro in the presence of various concentrations of the five different *P. berghei* CS peptides listed in Table 2. Cells were harvested after 6 d in culture, counted, and incubated for 6 h at E/T ratios from 2:1 to 50:1 with 5,000 ^{51}Cr-labeled P815 (haplotype $H\text{-}2^d$) or EL4 (haplotype $H\text{-}2^b$) target cells in the presence of each individual peptide. Controls included incubation with ^{51}Cr-labeled cells in the absence of peptide. After 6 h of incubation at 37°C, the plates were centrifuged, and 100 μl of supernatant was removed to measure chromium release. Percent lysis was determined as 100× (experimental cpm − medium control cpm)/(detergent-released cpm − medium control cpm).

CTL Assays for P. falciparum Experiments. The methods used for cytotoxicity assays and to identify peptide target epitope(s) in *P. falciparum* CS protein were essentially the same as described by Kumar et al. (13). Spleen cells (5×10^6) from B10.BR ($H\text{-}2^k$) mice immunized orally with WR4024/pADE171 R16, WR4024/pADE171 RLF, WR4024, or recombinant vaccinia (V-71) were incubated with 2×10^5 CS-transfected L cells or with 2×10^5 untransfected L cells. Cells were harvested after 6 d in culture, counted, and incubated for 6 h at various E/T ratios with 5,000 ^{51}Cr-labeled CS-transfected L cells or labeled untransfected L cells. Percent specific lysis was determined as described above.

To identify the CTL epitope, spleen cells from immunized mice were stimulated in vitro with CS-transfected L cells for 6 d. Putative CTL were then incubated with untransfected L cells at an E/T ratio of 40:1 for 6 h in the presence of various concentrations of

peptide 371–390 (DELDYENDIEKKICKMEKCSS). Peptide residue 291–310 was used as control. Killing was measured in 100 μl of supernatant as described above.

To demonstrate that CS-specific CTL were $CD8^+$ cells, CS-specific CTL were incubated with anti-CD8 mAb (clone 19/178) (25) followed by complement. Washed cells were then stained with FITC-conjugated goat anti-mouse IgG2a, and depletion was quantitated by microfluorimetry. Cells were incubated with 5,000 ^{51}Cr-labeled transfected L cells or untransfected L cells for 6 h at various E/T ratios. The percent lysis of target cells by complement-treated or CD8-depleted CTL was determined.

Results

For construction of *Salmonella–P. berghei* CS recombinants, expression plasmid pMGB2 containing the full-length *P. berghei* CS gene was transformed into *S. typhimurium* WR4024, an avirulent strain. Ampicillin-resistant transformants were examined for the expression of the CS protein. One of the transformants, WR4024/pMGB2, expressing *P. berghei* CS protein, was selected for further studies (Table 1).

For construction of *Salmonella–P. falciparum* CS recombinants, plasmid pADE171 R16 containing the full-length *P. falciparum* CS gene and plasmid pADE171 RLF containing the flanking regions without the repeats were constructed (Fig. 1). The plasmids were transformed into *S. Typhimurium* WR4024, and spectinomycin-resistant transformants were examined for expression of CS antigens. Two of these transformants were picked for further studies (Table 1).

Protection studies were performed by immunization of BALB/c mice with *Salmonella–P. berghei* CS recombinants. Mice were immunized orally with WR4024/pMGB2 or WR4024 and were intravenously challenged 5 wk later with 1,000 *P. berghei* (NK65) infectious sporozoites. This immunization provided protection in 55% of these animals, whereas all of the unimmunized and 94% of the mice receiving the WR4024 carrier strain were infected (Table 3). No antibody in the serum of individual mice could be demonstrated against recombinant CS protein using ELISA techniques (data not shown). Mice immunized intravenously with 50,000 irradiated sporozoites were fully protected.

To determine the role of $CD8^+$ T cells in protection, mice were depleted of $CD8^+$ T cells by intraperitoneal injections of anti-CD8 mAb 4 wk after immunization. This procedure resulted in removal of >98% of the $CD8^+$ T cells from the spleens of these animals (data not shown). The depletion was confirmed by surface phenotyping of $CD8^+$ T cells from the spleen by single-color indirect fluorescent staining and monitored by microfluorometry. Elimination of $CD8^+$ T cells abrogated the protection after immunization with the *Salmonella* recombinant vaccine WR4024/pMGB2. Protection was reduced from 55% to 5% (Table 3). $CD8^+$ T cell–depleted control mice were all infected after challenge. Mice depleted of CD4 T cells did not lose their immunity (data not shown). These results indicate that $CD8^+$ T cells are the mediators of protection induced by *Salmonella* recombinant vaccines. However, in contrast to immunity induced by *Salmonella* recombinant vaccines, immunity achieved by immunization with radiation-attenuated sporozoites could not be eliminated even after complete depletion of $CD8^+$ T cells (Table 3).

To further characterize the $CD8^+$ T cells induced by *Salmonella–P. berghei* CS recombinant immunization, in vitro cytotoxic T cell assays were performed. For these assays, we used the methods of Townsend et al. (27), where target cells are made by incubation of histocompatible cells with short synthetic peptides that associate with class I molecules. We

Table 1. *Salmonella-CS Recombinant Vaccines*

Immunogen	Plasmid	Gene	Species
WR4024/pMGB2	pMGB2	Full-length CS	*P. berghei*
WR4024/pADE171 R16	pADE171	Full-length CS	*P. falciparum*
WR4024/pADE171 RLF	pADE171	Flanking regions	*P. falciparum*
WR4024	None	None	Carrier

Table 2. *Plasmodium berghei CS Peptides*

Sequence	Region	Residue
ADAPEGKKNEKKNEKIERNN	NH_2 terminus	69–78
(DPPPPNPN)3	Repeat	102–124
NDDSYIPSAEKI	COOH terminus	242–253
SYIPSAEKILEFVKQQIRDSITEEWSQ	COOH terminus	245–270
CFVKQIRDSITEEWSQCNVTCG	COOH terminus	256–276

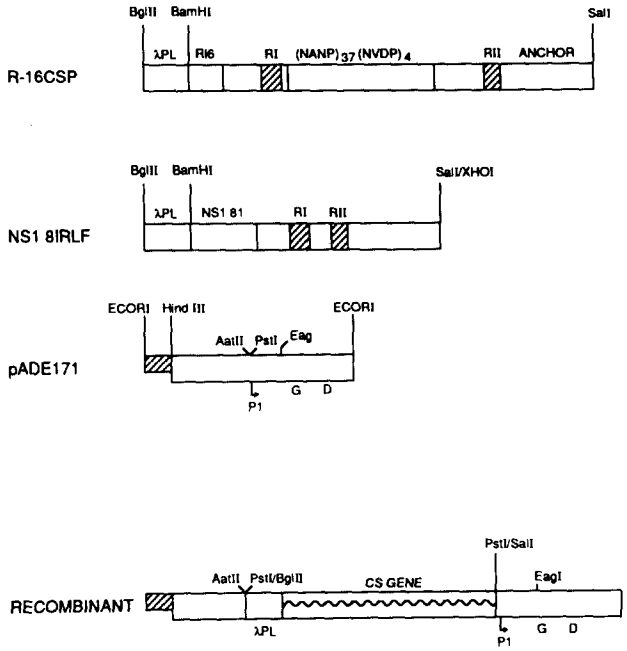

Figure 1. Construction of WR4024/pADE171 R16 and WR4024/pADE171 RLF. The plasmid containing R16 full-length or NS1 repeatless CS gene of *P. falciparum* was digested with BglII and SalI. The gel-purified insert was blunt-end ligated into the PstI site of plasmid pADE171, which carries *his* OGD region of *Salmonella*, resulting in the plasmid containing CS gene flanked by *Salmonella his* OGD.

designed peptides from the *P. berghei* CS protein based on the sequence identified previously by Romero et al. (14) as a CTL target. Five different peptides from NH_2 terminus, repeat region, and COOH terminus were used (Table 2). Spleen cells from mice immunized with either *Salmonella–P. berghei* CS recombinant WR4024/pMGB2, *S. typhimurium* carrier WR4024, or sporozoites were taken 4–10 wk after immunization. After in vitro stimulation with peptide for 6 d, the killing was measured by the ^{51}Cr release.

The spleen cells from mice immunized with WR4024/pMGB2 caused 24% specific lysis of P815 (H-2^d) target cells incubated with peptide 242–253 at a concentration of 50 μg/ml and an E/T ratio of 40:1 (Fig. 2). Spleen cells from WR4024/pMGB2-immunized mice had no cytolytic activity without in vitro peptide stimulation. In vitro stimulated cells did not show activity if the target cells were not incubated with peptide 242–253. Spleen cells from unimmunized mice or mice immunized with the carrier *Salmonella* WR4024 and stimulated with the peptide 242–253 had <4% cytolytic activity, indicating that the CTL arose after immunization and were not due to in vitro peptide stimulation. Specific cytolytic activity of 34% was measured with peptide 242–253-stimulated spleen cells from mice immunized with radiation-attenuated sporozoites.

None of the other four peptides stimulated spleen cells from mice immunized either with *Salmonella* recombinants or radiation-attenuated sporozoites. These peptides also failed to generate P815 cell targets. There was no specific lysis of P815 cells after incubation with any of the five peptides at the concentration used, demonstrating that they were not directly toxic to cells. The CTL lytic activity generated by WR4024/pMGB2 immunization was genetically restricted. After stimulation of spleen cells with peptide 242–253, no specific lytic activity was observed when EL4(H-2^b) cells incubated with the same peptide were used as targets.

We asked whether *Salmonella* recombinant vaccines could also induce CTL responses to the human malaria parasite *P. falciparum*. For this purpose, spleen cells from B10. BR(H-2^k) mice immunized with WR4024/pADE171 R16, WR4024/pADE171 RLF, WR4024, or recombinant vaccinia (V-71) containing full-length *P. falciparum* CS gene were stimulated in vitro for 6 d with CS-transfected L cells (H-2^k). These cells were then assessed for lytic activity against transfected or untransfected L cells. At an E/T ratio of 40:1, in three different sets of experiments, we observed 37 ± 2.2% specific killing of CS gene–transfected cells by CTL induced by WR4024/pADE171 R16 compared with 20 ± 2.5%

Table 3. *Protective Immunity Induced by S. typhimurium (WR4024) Transformed with a Plasmid Expressing Full-Length P. berghei CS Gene (WR4024/pMGB2)*

Immunogen	Anti-CD8 antibody	No. infected/ no. challenged	Percent protection
WR40	–	29/31	6
WR4024	+	8/8	0
WR4024/pMGB2	–	10/22	55
WR4024/pMGB2	+	18/19	5
None	–	30/30	0
None	+	8/8	0
Irradiated Spz	–	0/10	100
Irradiated Spz	+	0/10	100

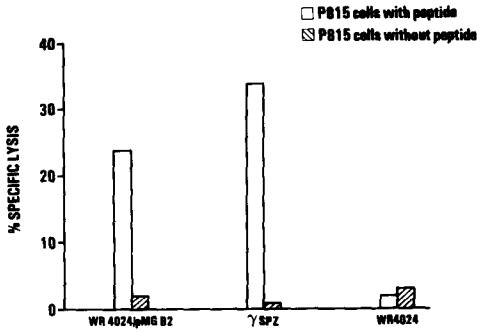

Figure 2. Results of cytotoxic assays performed with P815 target cells and CTL in the presence of synthetic peptides. Spleen cells from mice immunized with *Salmonella–P. berghei* CS recombinant vaccine were stimulated in vitro for 6 d with 50 μg of peptide, and 6 d after in vitro stimulation cells were incubated with ^{51}Cr-labeled P815 cells in the presence of different peptides, and ^{51}Cr release was measured. This experiment is representative of four independent experiments.

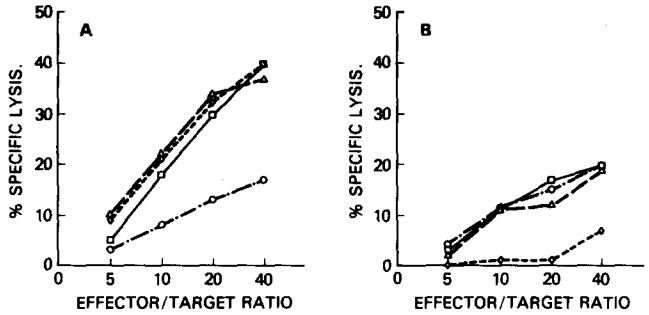

Figure 3. Malaria *Salmonella* CS recombinants stimulate CS-specific CTL. In vitro stimulated spleen cells from mice immunized with *Salmonella–P. falciparum* CS recombinants were incubated with ^{51}Cr-labeled CS-transfected (*A*) or untransfected L cells (*B*). Percent specific lysis was determined. (◇) Vaccinia CS recombinant; (△) R16; (□) RLF; (○) vector. This experiment is representative of five independent experiments.

killing of untransfected L cells ($p < 0.05$). Likewise, at this E/T ratio, spleen cells from animals immunized with WR4024/pADE171 RLF killed 36 ± 3.1% of CS gene–transfected L cells compared with 20 ± 4% killing of untransfected L cells ($p < 0.05$). These CTL responses were comparable with those obtained after immunization with the recombinant vaccinia (Fig. 3). CTLs from mice immunized with the *Salmonella* carrier WR4024 gave 20 ± 4.5% ^{51}Cr release.

To determine if the cytotoxic activity in the spleen cell cultures was due to CD8$^+$ T cells, in vitro depletions were performed. Removal of CD8$^+$ T cells from the cultures on the day of the CTL assay was confirmed by MF (data not shown). WR4024/pADE171 R16–induced CS-specific CTL, when treated with anti-CD8 mAb and complement, yielded only 5 ± 2.8% specific lysis of CS-specific target cells at an E/T ratio of 40:1 compared with specific lysis of 37 ± 2.2% ($p < 0.05$), when the cells were treated with complement alone (Fig. 4). WR4024/pADE171 RLF induced CTL with similar activity (12 ± 3.5 vs. 36 ± 3.1; $p < 0.05$). This demonstrated that the CS-specific cytotoxicity was mediated by CD8$^+$ cells. Reductions in specific cytotoxicity by depletion of CD8$^+$ T cells were similar for spleen cells from animals immunized with each of the three vaccines (Fig. 4).

Immunization of B10.BR mice with *Salmonella* also induced CTL that caused 20 ± 4.5% lysis of untransfected cells. This nonspecific effect was induced by both recombinants and the control *Salmonella* vaccine, and contrasts to the 7 ± 1.5% lysis seen with recombinant vaccinia-immunized mice (Fig. 3). After CD8 depletion, the lysis of untransfected cells by WR4024/pADE R16–induced CTL was reduced from 18 ± 2.8 to 4 ± 2% ($p < 0.05$), and killing by WR4024/pADE171 RLF–induced CTL was reduced from 16 ± 3.5 to 7 ± 1.5% ($p < 0.05$). These findings suggest that the majority of induced nonspecific cytotoxic T cells were CD8$^+$ (Fig. 4). Immunization of BALB/c mice with *Salmonella* vaccines followed by in vitro stimulation of spleen cells with peptides did not yield CTL reactive with non–peptide-pulsed targets. This may be attributed to differences between in vitro stimulation with transfected cells vs. in vitro stimulation with peptides.

To determine the epitope specificity of CTL induced by oral immunization with *Salmonella–P. falciparum* CS constructs in B10.BR mice, spleen cells from immunized mice were stimulated in vitro with CS-transfected L cells. After 6 d, untransfected L cells were incubated with 100 μg/ml of peptide 371–390, single letter code DELDYENDIEKKICKMEKCSS, or peptide 291–310 from the *P. falciparum* CS gene (12), and lysis was determined at an E/T of 40:1. Peptide with amino acid sequence 371–390 was recognized by CTL from mice immunized with the two recombinant *Salmonella* vaccines or the recombinant vaccinia (Fig. 5). This same peptide had previously been identified as the target of CTL induced by sporozoite or recombinant vaccinia-CS immunization (12). L cells incubated with peptide 291–310 were not lysed by immune CTL above the levels seen with target cells alone (data not shown).

Discussion

We have demonstrated that oral immunization of mice with attenuated *S. typhimurium* recombinants expressing full-length *P. berghei* CS gene or full-length or repeatless *P. falciparum* CS gene induce specific CD8$^+$ CTL. We also provide evidence that these CS-specific CTL are responsible for the protection induced against *P. berghei* sporozoites after immunization of BALB/c mice with *Salmonella* recombinant vaccines.

Antibody-dependent cellular cytotoxicity has been shown after immunization of humans with *S. typhi* mutant strain

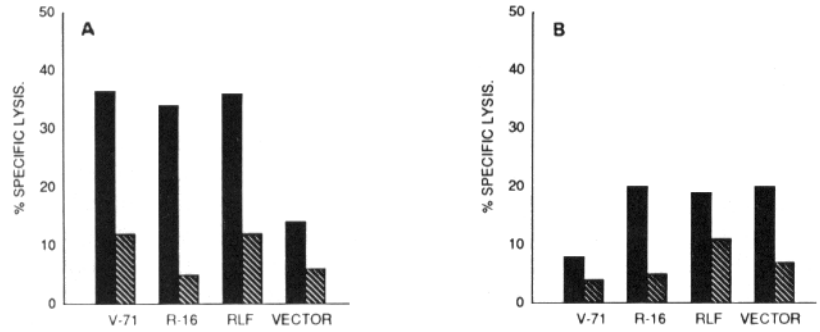

Figure 4. Effect of CD8 depletion on CS-specific CTL. In vitro stimulated cells from the mice immunized with *Salmonella–P. falciparum* CS recombinants were incubated with anti-CD8 mAb (19/178) followed by complement. Washed cells were then incubated with 5,000 ^{51}Cr-labeled transfected (*A*) or untransfected (*B*) L cells at various E/T ratios. The percent specific lysis of target cells by complement-treated (■) or CD8-depleted (▨) CTL was determined. A representative experiment at the 40:1 ratio is presented.

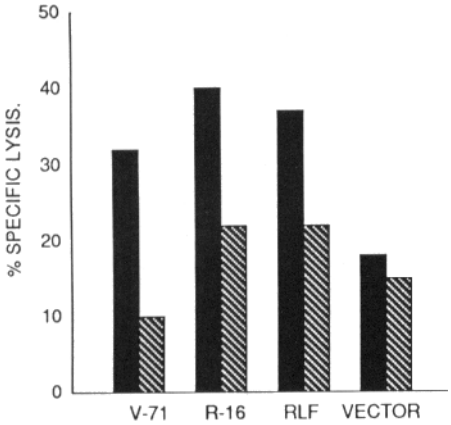

Figure 5. Identification of the CTL epitope on the *P. falciparum* CS protein. Spleen cells from *Salmonella–P. falciparum* recombinant-immunized mice were stimulated in vitro with CS-transfected L cells. ^{51}Cr-labeled target cells were incubated with peptide and the in vitro stimulated spleen cells at an E/T ratio of 40:1 (■); or without peptide (▨), and ^{51}Cr release was measured. This experiment is representative of three independent experiments.

Ty21a (28) or auxotrophic *S. typhi* 541Ty(Vi$^+$) or 543Ty(Vi$^-$) (29). The effector cell has been identified as a nonadherent T3$^+$, T8$^-$, T4$^+$ lymphocyte (28). After immunization, delayed-type hypersensitivity responses to *Salmonella* carbohydrate antigens (30) and recombinant expressed proteins have been demonstrated (15, 31). Macrophage activation directly or through T cell–mediated mechanisms has also been seen after immunization or infection (32, 33). To our knowledge, induction of protective class I–restricted antigen-specific CD8$^+$ CTL by immunization or infection with *Salmonella* has not, however, been previously reported.

After oral ingestion, attenuated *Salmonella* vaccines are soon translocated from the intestinal lumen to an intracellular location, primarily inside macrophages (30). Attenuated *Salmonella* carrier strains, such as *S. typhimurium* WR4024 used in this study and the aro A mutant of *S. dublin* SL1438 used by us in other studies (34), have limited ability to multiply within macrophages but do survive for variable periods of time. The intracellular expression of recombinant malaria CS antigens is probably responsible for processing and presentation in association with class I antigen with the subsequent induction of CTL. Bacterial enzymes may play some role in degradation of the recombinant expressed protein before processing by intracellular enzymes, although we have no direct evidence for this possibility. The presence of *Salmonella* inside macrophages may also activate these macrophages and induce the release of cytokines thought to be important as differentiation factors for induction of CTL (35).

Until recently, it was generally thought that viruses were the only pathogens capable of presenting endogenous antigens on the surface of infected cells in association with class I molecules with the subsequent induction of CD8$^+$ CTL. This view was challenged by the evidence for a protective role of CD8$^+$ CTL induced by sporozoites in murine malaria (10, 11), induction of CTL in mice against *P. falciparum* CS by sporozoites (13), and the protective role of CD8$^+$ T cells in immunity to *Toxoplasma gondii* (36). There is also evidence that protection against intracellular pathogens such as *L. monocytogenes*, *M. tuberculosis*, *M. leprae*, and others may in part be mediated through CD8$^+$ T cells (37). Our findings directly prove that an intracellular bacteria such as *S. typhimurium* can induce specific CD8$^+$ CTL and suggest that the antigen processing and presentation is probably not very different from that which occurs with vaccinia or sporozoites, since the recognized peptide and quantitative data are very similar.

Our finding that *Salmonella–P. berghei* CS recombinant–induced protection is mediated through specific CD8$^+$ cytotoxic T cells further demonstrates the importance of CTL in immunity against malaria. This is consistent with the recent finding that CD8$^+$ CTL clones directed against *P. berghei* CS by themselves are able to provide complete protection (14). These CTL clones were induced by immunization with radiation-attenuated sporozoites. The CTL we induced with *Salmonella* CS recombinants are directed against the same peptide in the CS sequence as the CTL clones defined by Romero et al. (14). Not all of the CTL clones found by Romero were able to protect. Our study demonstrates that at least some of the CTL induced by *Salmonella–P. berghei* CS recombinants are protective. To increase the number of protective CTL induced by oral immunization with *Salmonella* CS recombinants, we are inserting malarial genes into the bacterial chromosome in an attempt to stabilize and increase the expression of malaria gene products. We are also utilizing *Salmonella* auxotrophs as carrier strains, which are safe but more invasive than the attenuated *S. typhimurium* WR4024 we utilized in this study.

We were able to achieve CTL that gave similar levels of killing by immunization with *Salmonella–P. falciparum* CS recombinant vaccines that expressed the entire CS gene or a gene from which the central repeat region was deleted. Both of these recombinant constructs produced the malaria CS antigens as fusion proteins. The complete CS molecule is therefore not required for the in vivo induction of CD8$^+$ CTL by *Salmonella*, and modification of the original gene by addition also does not interfere. Presentation of antigens in association with class I molecules is generally dependent on intracellular enzymatic cleavage to produce a peptide fragment that can bind to the class I molecule. Our findings suggest a possible method for determining the minimal structure from any given molecule that is necessary for such a process to occur. This could be done by cloning deletion fragments as fusion proteins into *Salmonella* and looking for the ability of these *Salmonella* recombinants to induce CTL.

We were able to determine the *Salmonella–P. berghei* CS recombinant–induced protection by depletion of CD8$^+$ T cells. This contrasts with our inability to eliminate protection by this technique in BALB/c mice immunized with radiation-attenuated sporozoites. This result may be due to involvement of antibody, effector CD4$^+$ T cells, other immune cell types or other sporozoite or preerythrocytic antigens in BALB/c immunity to *P. berghei*. Schofield et al. (11)

were able to eliminate sporozoite-induced protection in A/J mice by depletion of $CD8^+$ T cells. This shows that the mechanism of radiation in attenuated sporozoite-induced immunity is dependent on the mouse haplotype. Although we were not able to eliminate sporozoite-induced protection in BALB/c mice, Weiss et al. (10) were able to accomplish this, but the parasite was *P. yoelii*.

A vaccine for malaria that overcomes the sequence variation in the CS CTL site as well as differences due to genetic restriction (13) will likely contain several different versions of the CS CTL site recognized by humans as well as other sporozoite and liver stage antigens important for CTL recognition. Such multivalent vaccines can be constructed in *Salmonella* because of the size of the bacterial genome and the flexibility of its manipulation. Because new *Salmonella* vaccine strains designed for human use are auxotrophs (38), which can only replicate in the body to a limited extent, they have been safe in studies of immunocompromised hosts (39, 40). This inherent safety plus their sensitivity to antibiotics is important for immunization of human populations that may have been exposed to HIV.

Our findings that recombinant *Salmonella* constructs provide some protection and induce CTL effector mechanisms similar to those induced by irradiated and live sporozoites provide a basis for further work on the development of oral vaccines against malaria. The CTL epitope, which has been identified in the CS protein of the human malaria parasite, *P. falciparum*, has only been shown to be recognized by primed mouse lymphocytes. Whether this peptide sequence is also recognized by human CTL is currently unknown. Immunization of humans with irradiation-attenuated sporozoites or possibly *Salmonella* recombinants may help answer this question. These findings suggest that live attenuated *Salmonella* recombinants may also be useful in the study of other diseases where CTL mediated immunity may be important.

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