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Immunogenicity of Genetically Engineered Glutathione *S*-Transferase Fusion Proteins Containing a T-Cell Epitope from Diphtheria Toxin

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Glutathione *S*-transferase (GST) has been shown to induce a marginal antibody response in experimental animals as well as partial protection against a number of parasitic worms, including *Schistosoma* and *Fasciola* species. The objective of our study was to increase the immunogenicity of GST by adding heterologous T-cell epitopes at the carboxy terminus of the protein. We generated recombinant GST proteins by attaching one or three tandem repeats of a T-cell epitope of CRM_{197}, a nontoxic variant of diphtheria toxin. This T-cell epitope encoding the region of amino acids 366 to 383 of CRM_{197}, when contained in a GST fusion protein and/or after purification as a recombinant peptide, retained the ability to induce a CRM_{197}-specific T-cell response. The fusion protein containing a single T-cell epitope induced a strong T-cell proliferative response to GST and also enhanced anti-GST antibody production in mice. The addition of three repeats of the epitope did not augment the responses when compared with the responses of GST itself. The results suggest that the addition of a single T-cell epitope to a larger protein like GST increases the immunogenicity of the protein.

Glutathione *S*-transferases (GST) play a pivotal role in the physiology of organisms. This family of isoenzymes has been reported to be responsible for the transport of anionic compounds and the metabolism of a wide range of xenobiotics (16, 20). Despite their role as physiological isoenzymes, the use of these proteins as potential vaccine candidates for a number of parasitic diseases has been appreciated in recent years (8–10, 22). GST has been shown to be present in three species of schistosomes (3) and in *Fasciola hepatica* (16). GST of 28 and 26 kDa have been shown to be protective antigens against schistosomiasis and fascioliasis in a number of animal models. However, the protection as measured by the reduction in worm burden is highly variable, ranging anywhere from 50 to 70% in rats (3, 16), 35 to 50% in mice (4, 23, 24), and 56 to 82% in cattle (9). But in a permissive host such as sheep, a 78% protection against fascioliasis has been observed following immunization with GST from *F. hepatica* (29, 30). In contrast, very poor anti-GST antibody titers have been observed in the sera of schistosome-infected humans (17). However, the presence of a small but significant amount of immunoglobulin A (IgA) antibody has been reported in humans infected with *Schistosoma mansoni* (13). This poor immunogenicity may be due to homologies between *Schistosoma* species and mammalian GST sequences, and thereby GST appears as a mammalian "self" protein. Smith and his colleagues (31) have provided support for this hypothesis by demonstrating that GST from *Schistosoma japonicum* shows 42 and 60% homology with rat and human GST, respectively.

The immunogenicity of a nonimmunogenic or weakly immunogenic antigen or hapten can be improved by coupling it to a strong carrier molecule (25) or to a synthetic T-cell-epitope peptide (6). However, in a recent study, fusion proteins of GST containing putative T-helper-cell epitopes from malarial and mycobacterial sequences have been shown to be less immunogenic than native GST protein in mice in terms of anti-GST antibody production (22). Similarly, cDNA coding for GST from *S. japonicum* has been cloned in *Escherichia coli* (32), and a recombinant GST–β-galactosidase fusion protein has also been shown to induce an inconsistent immune response in mice (23). A GST fusion protein containing *S. mansoni* sm20 has been shown to be immunogenic, whereas that fusion containing strain sm50 has been shown to down-regulate the anti-GST antibody response (34). It has been suggested that the poor immunogenicity of GST could be improved by conjugation of GST with a highly immunogenic carrier molecule (31). We have identified a T-cell epitope (CRM 366–383) from CRM_{197}, a nontoxic variant of diphtheria toxin, and have shown that a synthetic peptide corresponding to this epitope functions as a carrier for an oligosaccharide hapten derived from the capsule of *Haemophilus influenzae* type b (5). In current studies, we have constructed a genetic fusion protein by attaching one or three repeats of the T-cell epitope at the carboxyl-terminal end of the GST (Sj26-GST). The GST fusion protein containing a single T-cell epitope was more immunogenic in mice than the GST alone or GST with three T-cell epitopes.

MATERIALS AND METHODS

Genetic construction of GST-peptide fusion proteins. *E. coli* DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) was used for all cloning experiments and for the expression of fusion proteins. Stock cultures were stored at −70°C in 20% glycerol. Plasmid pGEX-2T (Pharmacia, Inc., Piscataway, N.J.), which carries the gene for GST with a multiple-cloning site at the carboxy terminus, was used to create a progressive series of recombinant fusions of GST and repeating sequences of amino acids 366 to 383 from the CRM_{197} protein. An initial construct, pPX 4000 (Table 1), was created by cloning one copy of a synthetic oligonucleotide encoding for amino acids 366 to 383 of diphtheria CRM_{197} (Fig. 1) into the *Bam*HI-*Eco*RI site of pGEX-2T as an in-frame fusion with GST. Additional stepwise *Sna*BI-*Eco*RI cloning was performed to create pPX 4002 and pPX 4003, which have two and three repeating sequences of amino acids 366 to 383, respectively (Table 1).

Production and purification of GST-peptide fusion proteins. *E. coli* DH5α-containing plasmids were cultured routinely in SOB (27) broth or SOB agar medium with ampicillin (50 μg/ml). Cells for the production of GST or GST fusion proteins were grown to the mid-log phase (150 Klett units) in SOB broth

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TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristics	Reference or source
pGEX-2T	GST gene fusion vector; the protein of interest is fused to the carboxyl terminus of GST from *S. japonicum*	Pharmacia
pPX 4000	Plasmid pGEX-2T with one copy of an oligonucleotide encoding for amino acids 366 to 383 of CRM_{197}; expresses a soluble GST fusion protein	This study
pPX 4002	Plasmid pGEX-2T with two copies of an oligonucleotide encoding for amino acids 366 to 383 of CRM_{197}; the fusion protein is degraded during expression	This study
pPX 4003	Plasmid pGEX-2T with three copies of an oligonucleotide encoding for amino acids 366 to 383 of CRM_{197}; the fusion protein is expressed as insoluble inclusion bodies	This study

with ampicillin (50 μg/ml), and protein production was induced for 3 h with 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

GST and the GST fusion protein with one copy of the peptide of amino acids 366 to 383 (peptide termed 366–383 peptide; fusion termed GST–366-1× fusion protein) were purified with a glutathione-Sepharose 4B affinity column (Pharmacia) as described in the manufacturer's instructions. The GST fusion protein with three copies of the 366–383 peptide (GST–366-3× fusion protein) was found to be produced in inclusion bodies. These materials were solubilized in 8 M urea and purified in a (DEAE) ion-exchange column with a 0.5 M NaCl salt gradient. The GST fusion protein with two copies of the 366–383 peptide (GST–366-2× fusion protein) was found to be degraded and, therefore, was not used in this study.

The recombinant T-cell 366–383 peptide (r-366) was generated by digesting GST–366-1× fusion protein with thrombin attached to Sepharose 4B. The fusion protein was incubated with the thrombin beads for 30 min at room temperature. Beads were removed by low-speed centrifugation, and the supernatant was passed through a glutathione-Sepharose affinity column to separate the free r-366 from the GST protein. The flowthrough was desalted on a Sephadex G-10 (Pharmacia) column and lyophilized. This material was tested routinely for the presence of any free GST by Western blotting (immunoblotting) a sodium dodecyl sulfate (SDS)–12% polyacrylamide gel (Bio-Rad, Hercules, Calif.) probed with rabbit anti-GST antibody and developed with a horseradish peroxidase-labeled goat anti-rabbit antibody (Boehringer Mannheim, Indianapolis, Ind.) and by Coomassie blue (R-250) staining.

Animals. Female SJL/j mice (Jackson Laboratories, Bar Harbor, Maine) or outbred Swiss Webster mice (Taconic, Germantown, N.Y.) were used at an age of 6 to 8 weeks. The SJL/j strain of mice has been shown in our previous studies (5) to respond to the synthetic T-cell epitope peptide derived from CRM_{197} protein (s-366).

Immunization of animals. To evaluate in vitro proliferative responses, groups of three SJL/j mice were immunized subcutaneously (s.c.) at the base of the tail with 20 μg of GST or one of the GST fusion proteins, 50 μg of CRM_{197}, or 100 μg of s-366 in a 150 mM saline solution. Antigen preparations were emulsified in complete Freund's adjuvant (CFA; Difco Laboratories, Detroit, Mich.) before vaccination. Seven days postimmunization, periaortic and inguinal lymph node cells were used for an in vitro T-cell-proliferative assay.

Outbred Swiss Webster mice were used to evaluate antibody responses to GST and GST fusion proteins. Mice were preimmunized with 50 μg of diphtheria toxoid (Lederle-Praxis Biologicals, Pearl River, N.Y.) emulsified in CFA or absorbed onto 100 μg of aluminum phosphate ($AlPO_4$). One or 4 weeks after preimmunization, the mice were primed with 1.0 or 10.0 μg of GST, GST–366-1×, or GST–366-3× absorbed onto 100 μg of $AlPO_4$ and subsequently boosted at week 4. All antigens were administered s.c. in the flank. A minimum of five

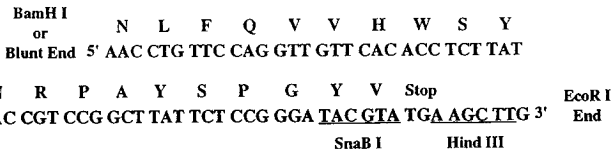

FIG. 1. Oligonucleotide sequence for amino acids 366 to 383 from diphtheria toxin CRM_{197}.

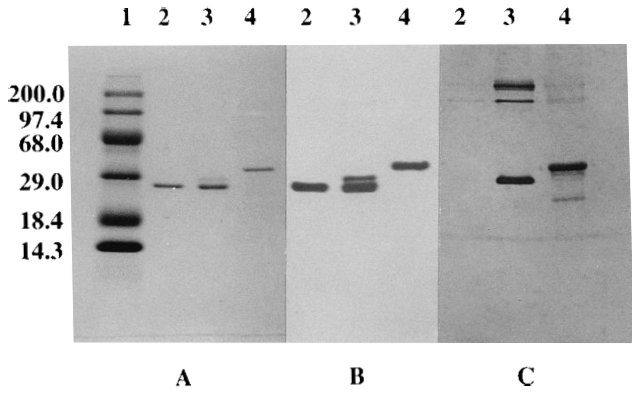

FIG. 2. Analysis of GST fusion proteins. Samples were run on an SDS–12% polyacrylamide gel and stained with Coomassie blue (A) or developed with rabbit anti-GST antibody (B) or rabbit-anti-366–383 peptide antibody (C) in a Western blot. Lanes: 1, low-molecular-weight standards; 2, GST protein; 3, GST–366-1×; 4, GST–366-3×. Numbers on the left indicate molecular masses in kilodaltons.

mice were used per group, and sera were collected before immunization and at 2-, 4-, and 6-week intervals.

In vitro T-cell proliferation assay. Periaortic and inguinal lymph node cells from SJL/j mice immunized 1 week earlier with the antigens indicated above were cultured at 3×10^5 cells per microtiter well in the presence of mitogens or various concentrations of CRM_{197}, diphtheria toxin (DT), GST, and GST fusion proteins. Cells were cultured in RPMI 1640 medium containing 2 mM L-glutamine, 100 U of penicillin and streptomycin, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; GIBCO-BRL, Grand Island, N.Y.), 10^{-5} M 2-β-mercaptoethanol (Sigma Chemical Co., St. Louis, Mo.), and 1.0% normal mouse serum for 3 days at 37°C in a 5.0% CO_2 incubator. Cultures were pulsed with 1.0 μCi of [^{3}H]thymidine per well for 16 h and harvested on a microcell harvester (Skatron, Lier, Norway). [^{3}H]thymidine incorporation was quantitated by liquid scintillation. The stimulation index was determined by dividing the experimental mean of triplicate cultures by the mean counts per minute from triplicate cultures stimulated with medium alone.

Assays for antibody responses. Pooled sera from immunized Swiss-Webster mice were assayed for the presence of anti-GST-specific IgG antibodies by an enzyme-linked immunosorbent assay (ELISA). Briefly, microtiter plates were coated with 1.0 μg of GST per ml in phosphate-buffered saline. Specific antibody was detected with horseradish peroxidase-conjugated goat anti-mouse IgG (Zymed Laboratories, South San Francisco, Calif.). The reaction was developed with 2,2′-azino-di(3-ethyl-benzthiazoline sulfonate) (ABTS; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) for 20 min at room temperature. Optical densities were read at 410 nm (referenced against readings at 630 nm) on an MR5000 microplate reader (Dynatech, Chantilly, Va.). Each assay was repeated at least twice. Twofold serial dilutions of serum were tested, and serum antibody titers were expressed as endpoint dilutions (average of duplicate wells minus background) at an optical density of 0.3.

In a competition ELISA, the binding of polyclonal rabbit anti-GST antibody to s-366 was subjected to competition with either s-366, r-366, or a nonspecific synthetic peptide from amino acids 306 to 334 of CRM_{197} (CRM 306–334). Briefly, microtiter plates were coated with s-366 and reacted with rabbit anti-GST containing various concentrations of the antigens. The reaction was developed as outlined above.

RESULTS

Characterization of GST fusion proteins. Fusion proteins purified by glutathione affinity column chromatography or ion-exchange columns were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting with anti-peptide and anti-GST antibodies. The data are presented in Fig. 2. *E. coli* cells expressing GST–366-1× fusion protein showed two bands after induction with IPTG for 3 h; a distinct 28-kDa band representing GST–366-1× fusion protein and a less-intense 26-kDa breakdown product of the fusion protein (Fig. 2A). Both the low- and high-molecular-weight bands reacted to rabbit anti-GST antibodies in a Western blot (Fig. 2B), whereas only the high-molecular-weight band reacted with anti-366–383 peptide antibody (Fig. 2C). The digestion of

TABLE 2. Comparison of antigenicities of r-366, s-366, and CRM 306–334[a]

Antigen concn (μg/ml)	% Inhibition		
	s-366	r-366	CRM 306–334[b]
100	93.3	92.2	ND[c]
50	88.8	87.8	7.9
10	63.6	70.4	0
5	52.8	56.4	0

[a] ELISA plates were coated with s-366 and reacted with rabbit antibody to s-366. The reactivity was inhibited by various concentrations of antigens as indicated.
[b] CRM 306–334, a nonspecific peptide, was included in the assay.
[c] ND, not done.

this material with thrombin abrogated the anti-peptide reactivity in the Western blot. These data suggest that the high-molecular-weight protein band contains GST–366-1× fusion protein. We estimate that 30 to 40% of the material remains as intact fusion protein.

The GST–366-3× fusion protein purified by ion-exchange chromatography was also analyzed by SDS-PAGE. A gradual increase in molecular size of GST-peptide fusions (GST–366-1× [Fig. 2A, lane 3, the upper band] and GST–366-3× [Fig. 2A, lane 4]) was observed. The increase in molecular weight corresponded to the number of peptides added to the GST molecule. Rabbit anti-GST antibody reacted with GST, GST–366-1×, and GST–366-3× in a Western blot (Fig. 2B), whereas rabbit anti-366–383 peptide antibody reacted with fusion proteins only (Fig. 2C, lanes 3 and 4).

Antigenicity and immunogenicity of the recombinant T-cell-epitope peptide. r-366 purified from GST–366-1× fusion protein was compared with s-366 for in vitro antigenicity and immunogenicity. Antigenicity was measured in a competition ELISA. Both s-366 and r-366 competed, in a dose-dependent manner, for the binding of rabbit polyclonal antibody to the synthetic peptide in the solid phase (Table 2). A nonspecific peptide (CRM 306–334) showed no reactivity in this assay. Thus, the data suggest that r-366 retained antigenicity comparable to that of s-366.

The immunogenicities of r-366 and the GST–366-1× fusion protein were studied in an in vitro T-cell proliferation assay. Draining lymph node cells from mice immunized with CRM_{197} protein were stimulated in vitro with various concentrations of synthetic and recombinant peptides or the fusion protein. CRM_{197} protein induced a secondary antigen-specific T-cell-proliferative response, while the tetanus toxoid did not induce any T-cell proliferation (Table 3). The purified recombinant peptide (r-366) induced a lower response than that of synthetic peptide (s-366). The GST fusion protein induced a T-cell response which is comparable to that induced by s-366. Thus, the data from these experiments suggest that the 366–383 peptide in a fusion protein is still immunogenic and can induce appropriate T helper cells.

The immunogenicities of the fusion proteins containing one repeat (GST–366-1×) and three repeats (GST–366-3×) of the T-cell epitope were compared in an in vitro T-cell proliferation assay. The addition of three repeats of a single T-cell epitope in tandem at the carboxyl terminus of the GST did not show any beneficial effect over the addition of a single epitope (data not shown). Although in some experiments GST–366-3× induced some response, the antigen-specific T-cell proliferation observed was very inconsistent. These data indicate that a single epitope is sufficient to induce T-helper-cell responses.

Induction of GST-specific T-cell response. To further explore the immunogenicity of GST or GST fusion protein, lymph node cells from mice immunized with GST or GST fusion protein were cultured in vitro and stimulated with different concentrations of the antigens shown in Table 4. For simplicity, the peak response observed is presented as a stimulation index. GST-fusion protein-primed animals showed a twofold increase in T-cell proliferation when compared with GST-primed mice. The response observed was specific in that GST-primed cells did not show any response when stimulated with DT or CRM_{197} proteins. As expected, GST fusion protein-primed animals showed a measurable T-cell-proliferative response to DT and CRM proteins. Thus, these in vitro studies show that the addition of an immunogenic T-cell epitope to a larger protein like GST may have some influence on the immunogenicity of the protein.

TABLE 3. Comparison of immunogenicities of CRM_{197}, tetanus toxoid, r-366, s-366, and the GST–366-1× fusion protein[a]

Antigen concn (μg/ml)	Stimulation index (mean ± SD)				
	CRM_{197}	TT[b]	s-366	r-366	GST–366-1×
100	102.0 ± 4.1	0.2 ± 0.01	12.3 ± 2.0	10.7 ± 0.3	ND[c]
50	103.0 ± 1.9	ND	10.9 ± 1.3	14.7 ± 1.3	ND
10	55.3 ± 4.0	0.7 ± 0.04	39.9 ± 2.9	12.1 ± 1.2	ND
5	37.2 ± 6.5	ND	37.5 ± 1.3	11.5 ± 0.4	23.7 ± 4.9
1	37.4 ± 7.4	0.5 ± 0.01	35.2 ± 2.1	5.3 ± 0.3	14.2 ± 1.0

[a] SJL/j mice were immunized with 50 μg of CRM_{197} in CFA s.c. at the base of the tail. Seven days postimmunization, lymph node cells were stimulated with the antigen concentrations indicated and proliferation was monitored by [^{3}H]thymidine incorporation. Positive controls were lipopolysaccharide and concanavalin A (stimulation indexes, 47.6 and 60.9, respectively).
[b] TT, tetanus toxoid (negative control).
[c] ND, not done.

Antibody response to GST or GST fusion proteins. After characterizing in vitro immunogenicity, the ability of these proteins to induce an antibody response was evaluated in outbred Swiss Webster mice. To maximize T-helper-cell responses to the 366–383 epitope, mice were preprimed with 50 μg of DT in CFA s.c. at the base of the tail. Control groups received an equal concentration of CFA without DT. One week postvaccination, animals were immunized s.c. with $AlPO_4$-adsorbed GST or GST fusion proteins at 1.0- or 10-μg concentrations. All mice were boosted 4 weeks after the primary immunization. Mice immunized with 1 μg of GST on aluminum phos-

TABLE 4. Induction of GST-specific T-cell proliferative responses in mice[a]

In vitro stimulation by:	Stimulation index (maximum ± SD)	
	GST[b]	GST–366-1×[b]
GST	74.6 ± 8.3	156.5 ± 13.9
s-366	1.9 ± 0.04	20.1 ± 2.9
GST–366-1×	140.1 ± 17.3	359.9 ± 20.4
DT	1.2 ± 0.03	6.8 ± 0.3
CRM_{197}	1.7 ± 0.01	7.1 ± 0.1
ConA[c]	227.7 ± 1.3	206.9 ± 4.4
LPS[d]	8.6 ± 0.2	29.5 ± 2.5

[a] Two groups of three SJL/j mice were immunized with 10 μg of GST or GST–366-1× in CFA per mouse at the base of the tail. Seven days postimmunization, lymph node cells were cultured with various concentrations of the proteins listed in the table. Cultures were then monitored for proliferation by [^{3}H]thymidine incorporation.
[b] Priming protein.
[c] ConA, Concanavalin A.
[d] LPS, lipopolysaccharide.

TABLE 5. Antibody responses of mice preimmunized at week −1 and immunized with GST or a GST fusion protein[a]

Vaccine	Dose (μg/mouse)	Pre-imm	Anti-GST IgG endpoint titer at week:			
			0	2	4	6
GST	1	CFA	<50	<50	769	23,128
	10	CFA	<50	258	306	1,812
GST–366-1×	1	CFA	<50	329	372	32,552
	10	CFA	<50	3,766	7,877	495,995
GST–366-3×	1	CFA	<50	<50	135	150
	10	CFA	<50	1,370	676	36,656
GST	1	DT-CFA	<50	<50	739	9,168
	10	DT-CFA	92	2,225	2,477	7,764
GST–366-1×	1	DT-CFA	<50	370	1,292	5,621
	10	DT-CFA	<50	492	1,398	24,962
GST–366-3×	1	DT-CFA	<50	227	3,153	4,100
	10	DT-CFA	82	1,805	9,373	88,085

[a] Swiss Webster mice ($n = 5$) were preimmunized (Pre-imm) at −7 days with DT in CFA (DT-CFA) or saline in CFA (CFA) and vaccinated subsequently at weeks 0 and 4 with GST or a GST fusion protein adsorbed on $AlPO_4$. Mice were immunized s.c. in the flank. Pooled sera were analyzed for anti-GST antibody response by an ELISA.

TABLE 6. Antibody responses of mice preimmunized at week −4 and immunized with GST or a GST fusion protein[a]

Vaccine	Dose (μg/mouse)	Pre-imm	Anti-GST IgG endpoint titer at week:			
			0	2	4	6
GST	1	Saline	118	<50	255	319
	10	Saline	<50	137	95	192
GST–366-1×	1	Saline	119	315	556	6,744
	10	Saline	104	758	816	51,855
GST–366-3×	1	Saline	103	194	164	132
	10	Saline	<50	193	267	3,364
GST	1	DT	202	243	80	369
	10	DT	<50	<50	72	1,072
GST–366-1×	1	DT	67	89	79	197
	10	DT	<50	298	506	11,786
GST–366-3×	1	DT	<50	67	83	83
	10	DT	86	106	124	1,246

[a] Swiss Webster mice ($n = 5$) were preimmunized (Pre-imm) at −4 weeks with DT (10 μg per mouse) or saline and subsequently vaccinated with GST or a GST fusion protein at weeks 0 and 4. All vaccines were adsorbed to $AlPO_4$. Pooled sera were analyzed for antibody by an ELISA.

phate showed a low primary anti-GST antibody response at week 4 (endpoint titer of 1:750) and exhibited a threefold increase in anti-GST titer 2 weeks after a secondary immunization. When the antigen dose was increased to 10 μg per animal, the animals showed no increase in antibody titers. In fact, the antibody titers were lower than that observed with the 1-μg-dose group (Table 5).

In contrast, mice immunized with 1 μg of GST–366-1× fusion protein elicited an anti-GST response after primary vaccination with a 10-fold increase in response 2 weeks after the secondary immunization. Increasing the dose of the antigen from 1 to 10 μg per animal correspondingly increased the antibody titers to a maximum of 500,000 after two vaccinations. The primary antibody response was also 10- to 20-fold higher than that observed in animals immunized with GST. Mice immunized with GST–366-3× produced very low primary and secondary antibody responses but showed an increased response when the immunization dose was increased from 1 to 10 μg per animal (Table 5). The data suggest that the inclusion of a single CRM-specific T-cell epitope with GST as a fusion protein enhanced the anti-GST antibody response in mice, and the addition of multiple repeats of the same epitope has no beneficial effect. In contrast to this observation, animals preprimed with DT showed a lower response to GST and GST–366-1× fusion protein than animals which received CFA alone (Table 5). However, when given 10 μg of antigen, these mice showed a slightly better response to GST–366-3× fusion protein. A clear difference between GST–366-1× and GST–366-3× immunizations was not observed in this set of experiments.

Effect of induction of memory T helper cells on the immunogenicity of GST or GST fusion proteins. Prepriming animals with DT in CFA to induce carrier-specific T helper cells before vaccination with GST or GST fusion proteins did not induce an anti-GST response any greater than that observed with CFA-pretreated animals (Table 5). In this experiment, animals were preprimed 1 week before vaccination with the fusion proteins, and this may not be an ideal situation to induce carrier-specific T helper cells. Animals were then preprimed with DT on aluminum phosphate 1 month before vaccination with GST or GST fusion proteins (Table 6). Preimmunization with DT elicited DT-specific T-cell-proliferative response in these mice (data not shown). Depending on the dose of GST antigen used, mice which were not preimmunized with DT showed a 4- to 30-fold increase in anti-GST antibody response over that of animals which were preimmunized with DT. Thus, the data suggest that GST fusion proteins function effectively as immunogens even in the absence of preexisting T helper cells to the carrier peptide (Table 6).

DISCUSSION

The results presented in this paper provide evidence that a T-cell epitope or multiple repeats of an epitope from CRM_{197}, a nontoxic variant of diphtheria toxin, can be successfully inserted at the carboxyl-terminal end of the GST protein from *S. japonicum* by use of recombinant genetic engineering technology. The biological activities of the recombinant T-cell epitope (i.e., r-366) are comparable to those of the synthetic T-cell epitope (i.e., s-366) in terms of antigenicity (Table 2) and immunogenicity (Table 3). Furthermore, the recombinant GST–366-1× protein is more immunogenic than GST or the GST–366-3× protein in eliciting GST-specific T-cell responses and inducing an anti-GST antibody response in mice (Table 6). It should be noted that the amount of GST–366-1× fusion protein injected may be less than that reported (Tables 5 and 6); only a certain percentage of the material injected is actual fusion protein (Fig. 2). Therefore, the immunogenicity of this fusion protein could be greater if the concentration of the protein were increased to an appropriate level.

The generation of antibody response to protein antigens in general and GST in particular requires the induction of T helper cells that recognize certain regions of the protein called T-cell determinant or epitope. The T helper cells support or help B cells that react to another region of the protein molecule, resulting in the generation of a protein-specific antibody response. Therefore, the presence of T- and B-cell epitopes on the GST protein are very critical for the induction of anti-GST antibody response. In fact, Auriault et al. (2) have shown, by use of synthetic peptides, the presence of T- and B-cell epitopes on the GST protein in a rat model. These T helper cells have been shown to play an important role in protection against *S. mansoni* in rat (1) and mouse (26, 35) model systems. Our data suggest that the inclusion of a T-cell epitope from DT at the carboxyl terminus of GST greatly enhances the immu-

nogenicity of the protein. Therefore, it is apparent that the foreign T-cell epitope in concert with the native epitope on GST supports a B-cell response to the GST protein. In fact, a similar conclusion was derived in a foot-and-mouth disease virus system in which a weakly immunogenic VP1 peptide containing both T- and B-cell epitopes was made more immunogenic by the addition of a known T-cell-epitope peptide from ovalbumin or sperm-whale myoglobin (11). However, such positive results were not always observed. GST fusion proteins containing putative T-cell epitopes from malarial and mycobacterial sequences were not any more immunogenic than the native GST protein (22). Although the reason for the difference between our studies and this observation is not clear at this point, it is probable that the T-cell-stimulating ability of the T-helper-cell epitopes used in this study makes the difference. It is interesting to note that in the current study, the T-cell-epitope peptide retains its immunogenicity even after it has been coupled to GST (Tables 3 and 4).

The use of a defined T-cell-epitope region rather than the whole carrier protein has several advantages. For the fusion protein generated in this study, it may be easier to genetically fuse a smaller T-cell peptide than to fuse a larger carrier protein. Furthermore, the addition of a larger protein may induce tertiary conformational changes in the B-cell epitope regions of the protein, reducing the immunogenicity. A study using a neutralizing B-cell epitope from rotavirus VP6 protein supports this concept. A conjugate of the VP6 peptide and a T-cell peptide from influenza hemagglutinin was more immunogenic than the corresponding VP6 peptide-bovine serum albumin conjugate (7). The use of T-helper-cell peptide as carrier for B-cell epitopes has been shown to have unique advantages in making conjugate vaccines against infectious agents such as malaria (12), hepatitis B (21), and human immunodeficiency virus (14).

In developing vaccines for humans against parasitic diseases, the selection of an appropriate T-cell epitope may be very critical. T-cell epitopes from diphtheria toxin may be viewed to be appropriate since diphtheria toxoid has been used in childhood immunization practices and most humans have been primed to T-cell determinants of diphtheria toxin. We have shown that a conjugate vaccine made by coupling oligosaccharide from the capsule of *H. influenzae* to s-366 induces an antibody response to the capsule of the bacteria (5) without producing any humoral response to the T-cell peptide. Triebel et al. have shown that B-cell determinants of diphtheria toxin are present in both the A and B fragments of the toxin, whereas T-cell determinants are present in the B fragment only (33). Therefore, the GST fusion protein could stimulate a strong T-cell-dependent response to GST in humans without inducing an antibody response to the carrier protein. However, our results do not provide evidence to support this concept. Mice preprimed with DT 1 or 4 weeks prior to vaccination with a GST fusion protein do not show an increase in antibody response when compared with that of the nonpreprimed animals (Tables 5 and 6). It is possible that the reduced response in our experiments is due to epitopic suppression; preexisting carrier immunity may suppress the response to the hapten coupled to the same carrier (15). However, the existence of epitopic suppression is described for some conjugates (28) but not for others (18). Further experiments are necessary to explain the reduction of antibody response obtained in carrier-primed animals in our experiments.

The addition of three repeats of a single T-cell epitope in tandem to GST does not provide any advantage over the addition a single repeat of the T-cell epitope (Tables 5 and 6) either for T-cell responses or antibody production. These results are in contrast to the observation made by Lowenadler et al. (19), who have shown that anti-STa antibody responses are higher to fusion proteins containing more than one or two ovalbumin-specific T-cell epitopes than to those with only one or two. However, it is interesting to note that these authors have not shown any difference in T-cell response between the fusion proteins containing different numbers of ovalbumin-specific T-cell epitopes (19). Our results show that three repeats of a single T-cell epitope has no advantage over a single repeat of the same T-cell epitope in a GST fusion protein. It is not clear at this point whether this effect is due to the addition of multiple copies of a T-cell epitope or the denaturation of B-cell epitopes of GST during the purification procedure using 8 M urea. We have observed a low level of binding of GST–366-3× fusion protein to a glutathione column when compared with the binding of GST–366-1× fusion protein. This observation suggests that a slight change in the secondary structure of GST might have occurred during the solubilization of the fusion protein with urea and that this may account for the reduction in the immune response with GST–366-3×.

Although we have demonstrated a strong antibody response to GST with the GST–366-1× fusion protein in a murine model, it does not necessarily mean that the antibodies generated are protective. Francis and colleagues (11) have shown that a fusion protein containing B-cell epitopes of rotavirus capsid protein fused to an ovalbumin T-cell epitope induces neutralizing anti-rotavirus antibodies, whereas the same B-cell epitope fused to a myoglobin T-cell epitope induces only non-neutralizing anti-rotavirus antibodies. Thus, different T-cell epitopes may differentially control antibody production of specific B-cell clones. This B-cell regulation by T cells may be due to the characteristics of the T-cell epitope itself and the position of the T-cell epitope in relation to the B-cell epitope (11). Thus, in our system, it is very important to delineate the regulation of anti-GST B-cell response by DT-specific T-cell epitopes in a murine model system. Furthermore, if neutralizing antibodies are produced against GST by the GST–366-1× fusion protein, it is important to evaluate whether this fusion protein would bypass the genetic restriction phenomenon and induce protective antibodies in outbred human and animal species.

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