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Expression of a *Schistosoma mansoni* 28-Kilodalton Glutathione *S*-Transferase in the Livers of Transgenic Mice and Its Effect on Parasite Infection

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Schistosomiasis is a debilitating tropical disease for which an effective vaccine is needed. A 28-kDa glutathione *S*-transferase from *Schistosoma mansoni* (Sm28GST) has been shown to induce protective immunity. Sm28GST possesses significant sequence identity to mammalian GST isoforms. In order to study self-reactivity in mice immunized with Sm28GST and the concomitant phenomena of immune tolerance and epitope suppression, as well as their consequences for the protective immunity induced by this vaccination, we developed transgenic (Tg) mice that express Sm28GST under the control of a part of the mouse transferrin gene promoter. A study of ^{P28}Tg mice showed that the expression of Sm28GST was strictly localized in pericentrolobular hepatocytes. No histological change, inflammatory infiltrates, or modification of seric L-aspartate: 2-oxoglutarate aminotransferase concentration was observed over an 18-month period, despite a cross-reactivity between Sm28GST and a mouse molecule of 30 kDa. The immunoglobulin G anti-Sm28GST response of ^{P28}Tg mice immunized with recombinant Sm28GST was lower ($P < 0.001$) than that observed in non-^{P28}Tg littermates and inversely proportional of Sm28GST liver expression. The response of non-^{P28}Tg mouse spleen cells to Sm28GST stimulation was greater ($P < 0.01$) than that observed with ^{P28}Tg mouse spleen cells. ^{P28}Tg mice infected with 40 *S. mansoni* furcocercariae harbored more worms ($P < 0.05$) than did non-^{P28}Tg control mice. The increase in the level of infection in ^{P28}Tg mice was reflected in concomitant increases in the numbers of adult worms and schistosome eggs found in livers and intestines after whole-body perfusion at 56 days postinfection, but no relative increase in the fertility of individual female worms was observed. The results obtained argue for the involvement of Sm28GST in reducing levels of infection and support the view that this enzyme has a central role in the maintenance of parasite viability, at least during its migration through host tissues.

Schistosomiasis is a debilitating disease that affects about 200 million people in 75 countries and is caused by three major species of parasitic trematode. Over the last few years, research into the development of an effective recombinant-subunit vaccine has led to the characterization of several candidate antigens (24). One problem associated with these antigens is that all of them are either enzymes or structural proteins that show significant sequence identities to their human counterparts, raising the possibility of autoimmune effects of vaccination.

The best defined of these vaccine candidates is a 28-kDa glutathione *S*-transferase from *Schistosoma mansoni* (Sm28GST) (3) that has been shown to induce protective immunity to challenge infection in both rodents and primates (7) in terms of both a reduction in worm burden and a diminution in female-worm fecundity and egg viability (34). Sm28GST possesses limited but significant sequence identity to mammalian GST isoforms (18). The target epitopes of the immune response have previously been defined (1, 2) and unsurprisingly are mainly outside areas of maximum sequence identity with human GSTs (29). Moreover, no evidence that indicates autoimmune consequences of vaccination with the complete recombinant protein or with derived peptides has so far been obtained.

However, the induction of autoimmune reactions is dependent on the adjuvant used, the route of administration, and the genetics of the recipient (11, 19); therefore, we cannot rule out such reactions to Sm28GST with new formulations and dose routes in outbred recipient populations.

In order to study further the problem of self-reactivity in animals immunized with Sm28GST and the concomitant phenomena of immune tolerance and epitope suppression, as well as their consequences for the protective immunity induced by this vaccination, we developed transgenic (Tg) mice that express this parasite antigen. Although most previous studies of tolerance have concerned Tg mice and expression in central lymphoid tissues, peripheral tolerance, including liver-specific tolerance in Tg mice, has begun to be examined (12, 14). We chose to express the protein specifically in the liver since this is the main site of schistosome egg deposition and thus of Sm28GST release during infection. We wished to determine

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how the immune system copes with a genetically engineered, self-reactive situation.

Here we describe ^{P28}Tg mice, which express Sm28GST in their livers under the control of the mouse transferrin gene promoter (mPTf). We show that these mice fail to respond to the antigen after immunization with recombinant Sm28GST (rSm28GST) and that they show increased parasitemia compared to that of non-^{P28}Tg controls.

MATERIALS AND METHODS

Construct used for microinjection. The Sm28GST open reading frame (ORF) was previously amplified by PCR with cloned cDNA and inserted into a *Bgl*II site 5′ to the human growth hormone (hGH) gene 3′ untranslated region present in the EV142 vector (kindly given by R. D. Palmiter, University of Washington). A 1.2-kb *Hin*dIII-*Bam*HI fragment containing 600 bp of mPTf directly 5′ to the ATG was subcloned from the original construct. mPTf was provided by M. Zakim (Institut Pasteur, Paris, France). An *Nco*I site was created at the ATG translation start site by oligonucleotide site-directed mutagenesis of the *Hin*dIII-*Bam*HI-mPTf construct. Finally, the 600-bp *Hin*dIII-*Nco*I fragment was ligated into the *Nco*I-*Kpn*I-digested Sm28GST ORF-hGH construct (see Fig. 1).

Creation and analysis of ^{P28}Tg mice. The DNA encoding mPTf linked to the Sm28GST coding sequence was freed from the vector by digestion with *Eco*RI (Boehringer Mannheim, Meylan, France). The linearized DNA was purified on a sucrose gradient. The purified fragment corresponding to the mPTf-Sm28GST ORF-hGH transgene was introduced into C57BL/6J × SJL mice by microinjection of one-cell embryos (17). Eggs were transferred into oviducts of OF_1 foster mothers. Animals that contained transgenes were identified by dot blot and Southern blot analyses of genomic DNAs from mouse tail tissues and hybridization with a ^{32}P-labelled Sm28GST cDNA fragment. The transgene copy number was determined by quantitative dot blot analysis with different copy numbers of the Sm28GST ORF fragment mixed with mouse genomic DNA. The DNA-positive founders were backcrossed with C57BL/6J mice. The DNA-negative littermates were used as non-^{P28}Tg control animals.

Dot blotting and Southern blotting. ^{P28}Tg mouse tail DNA (5 μg) was deposited on a nylon transfer membrane (Amersham) in an equal volume of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) solution and then fixed with 0.4 N NaOH. C57BL/6J mouse tail DNA was used as a negative control. The filter was hybridized with a Sm28GST probe that was ^{32}P labelled by random priming (Megaprime kit; Amersham). Mouse tail DNA was digested to completion by *Eco*RI and was subjected to electrophoresis on a 1% agarose gel. After denaturation and neutralization, DNA was transferred to a nylon transfer membrane (Amersham) by Southern blotting (27). The filter was hybridized with a ^{32}P-labelled Sm28GST probe.

RNA analysis. Tissues were removed quickly after the mice had been killed and were frozen immediately in liquid nitrogen. RNA was extracted from mechanically pulverized frozen tissue with RNAzol (1 ml/100 mg of tissue; Bioprobe, Montreuil, France) according to the manufacturer's specifications. The quality of the RNA was checked on a 1% agarose gel. RNA dot blots were prepared from 5 μg of total RNA as previously described (31) and hybridized with a ^{32}P-labelled Sm28GST probe. The presence of the 1.4-kb chimeric Sm28GST-hGH mRNA was checked by Northern blot analysis as previously described (13). Briefly, total RNAs of tissues were fractionated on a 1.2% formaldehyde–agarose gel and blotted onto nylon membranes, which were hybridized with the same DNA probe.

Western blot. Separated cells were harvested from mouse tissues by mechanical dissociation. Cell suspensions were boiled in sample buffer for 5 min and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5% concentration gel [pH 6.8] and 15% separation gel [pH 8.8]), and the protein was electrophoretically transferred to a nitrocellulose filter (0.45-μm pore size; Schleicher & Schuell, Dassel, Germany). Filters were incubated with anti-Sm28GST polyclonal rabbit serum (1/5,000) for 90 min at room temperature and then with anti-rabbit immunoglobulin G (IgG) peroxidase conjugate (1/10,000; Sigma, St. Louis, Mo.) for 45 min and stained with a Renaissance kit (Du Pont, Boston, Mass.).

Histological and immunohistochemical analyses. ^{P28}Tg mouse tissues were fixed in 0.1 M sodium phosphate–0.15 M NaCl (phosphate-buffered saline [PBS], pH 7.4)–4% formaldehyde and embedded in paraffin. Tissue sections (2 to 4 μm) were incubated with anti-Sm28GST polyclonal rabbit antiserum (1/2,000) for 2 to 3 h at room temperature. After three washes with 0.1 M Tris-HCl saline buffer (pH 7.4), tissue sections were incubated with anti-rabbit IgG alkaline phosphatase conjugate (1/200; Sigma) for 1 h. After three further washes, sections were stained with the new fuchsin substrate system (DAKO, Glostrup, Denmark) and counterstained with hematoxylin solution before examination. Sections for histological analysis were stained directly with hematoxylin-eosine saffron.

Parasite life cycle and reagents. The *S. mansoni* life cycle was maintained by using *Biomphalaria glabrata* snails as intermediate hosts and OF1 mice as definitive hosts. A full-length cDNA copy that encodes Sm28GST was expressed in *Escherichia coli* with a PL expression vector (4). rSm28GST was purified (95% purity) by passage through a glutathione-agarose column (Sigma). Anti-Sm28GST sera were prepared as previously described (4). New Zealand White rabbits were immunized subcutaneously with 250 μg (vol/vol) of rSm28GST in complete Freund's adjuvant (CFA; Difco, Detroit, Mich.). They were given subcutaneous booster injections of 250 μg of rSm28GST (vol/vol) in incomplete Freund's adjuvant (Difco) 3 weeks later and were bled 1 week after the last booster injection.

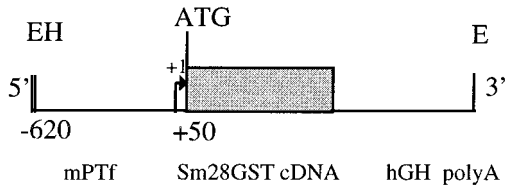

FIG. 1. Sm28GST transgene construct. The mPTf-Sm28GST-hGH fusion gene is represented. E, *Eco*RI; H, *Hin*dIII.

Soluble adult-worm preparation (SWAP). *S. mansoni* adult worms were collected by perfusion of the livers and mesenteric veins of golden hamsters at 40 days after infection (1,000 cercariae/animal). Adult worms were washed in PBS, homogenized with an Ultraturax (IKA Labortechnik, Strasbourg, France) for 1 min, disrupted by a 5-min sonication (B. Braun, Labsonic, Strasbourg, France), and centrifuged for 20 min at 10,000 × *g*. The supernatant was collected and stored in liquid nitrogen.

Assessment of liver function. Naive ^{P28}Tg and non-^{P28}Tg mice were bled every 3 months. After collection, plasma samples were separated at 4°C and 2,500 × *g* for 10 min and stored at −20°C before being tested. The seric aspartate aminotransferase or L-aspartate:2-oxoglutarate aminotransferase (GOT; EC 2.6.1.2.) activity was assayed at 37°C with a commercially available kit (AS 7148; Randox, Crumlin, United Kingdom) by the standardized procedure recommended by the International Federation of Clinical Chemistry (5). We measured enzymatic activity with a Hitachi 911 automatic analyzer (Boehringer, Mannheim, Germany).

Immunization experiments. ^{P28}Tg mice and non-^{P28}Tg control mice were injected subcutaneously with 25 μg of either rSm28GST or keyhole limpet hemocyanin (KLH; Calbiochem-Behring, La Jolla, Calif.) in the presence of 1.25 mg of either alum (Intergen, Purchase, N.Y.) or CFA at the base of the tail. Mice were given booster injections under the same conditions 3 weeks later. Serum samples were collected every week and stored at −20°C.

Enzyme-linked immunosorbent assay. Plates (Nunc-Immuno Module; Inter Med) were incubated overnight at 4°C with 5 μg of rSm28GST per ml or 10 μg of peptides per ml in 100 ml of sodium carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$ [pH 9.6]). Plates were saturated for 1 h with PBS–5% gelatin. After three washes with PBS–0.1% Tween 20 (pH 7.2), 100 μl of serum from immunized or infected mice at various dilutions (1/5,000 for anti-Sm28GST IgG, 1/250,000 for anti-KLH IgG, and 1/200 for IgG1 and IgG2a) in PBS–0.1% Tween 20 was dispensed in each well and incubated overnight at 4°C. After five washes, peroxidase-labelled anti-mouse IgG-Fc (Jackson ImmunoResearch, West Grove, Pa.) was added at a dilution of 1/15,000 in the same buffer for 90 min at 37°C. After three washes, 1 mg of orthophenylenediamine (Sigma) per ml in 0.1 M sodium phosphate buffer (pH 5.5) containing H_2O_2 (1 ml/liter) was incubated for 30 min at 37°C. The reaction was stopped by the addition of 100 μl of 2 N HCl. The optical density at 492 nm was measured with a Titertek Multiskan MCC 1340 multichannel spectrophotometer. The results given are the means of duplicate wells after subtraction of the background.

TABLE 1. Characteristics of ^{P28}Tg mice

Mouse line	No. of transgene copies	Progeny[a]	Transgene expression[b]
1	5	−	ND[c]
3	5	−	ND
5	5	+	−
8	8	+	−
9	>50	+	+
21	20	+	−
23	50	−	ND
26	8	+	+
35	>10	+	−
37	>50	+	ND

[a] +, positive in hybridization assay; −, negative in hybridization assay.
[b] +, expression; −, no expression.
[c] ND, not determined.

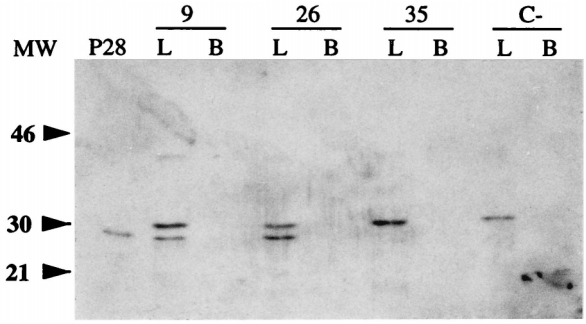

FIG. 2. Western blot analysis of Sm28GST protein in three lines (lines 9, 26, and 35) of ^{P28}Tg mice. MW, molecular mass (in kilodaltons); L, liver; B, brain; P28, rSm28GST (30 ng); C−, non-^{P28}Tg littermate.

Inhibition of GST activity. The GST-catalyzed reaction was performed with 1-chloro-2,4-dinitrobenzene (Sigma) as a substrate by the method of Habig et al. (16). rSm28GST (0.11 μg) was incubated with 50 μl of immunized-^{P28}Tg-mouse serum for 1 h at 37°C and then for 4 h at 4°C. The enzymatic reaction was carried out in 200 μl of potassium phosphate buffer (50 mM; pH 6.5) with 5 mM glutathione and 5 mM 1-chloro-2,4-dinitrobenzene. The absorbance at 340 nm was measured with a spectrophotometer (DU-64; Beckman Instruments, Inc., Fullerton, Calif.) every 30 s for up to 3 min at room temperature. Similar enzyme inhibition tests were performed in parallel with appropriate controls and sera of immunized non-^{P28}Tg mice and nonimmunized control mice.

Lymphocyte proliferation assay. Spleen cells were isolated from immunized and infected mice. For in vitro assays, 5×10^5 lymphocytes were maintained at 37°C in a 5% CO_2 atmosphere in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (JRH BioSciences, Lenexa, Kans.), 50 μg of gentamicin (Schering-Plough, Le-Vallois-Perret, France) per ml, 2 mM L-glutamine (Seromed, Berlin, Germany), 1% nonessential amino acids (Seromed), 1 mM sodium pyruvate (Sigma), and 5×10^{-5} M β-mercaptoethanol (Merck, Darmstadt, Germany) and incubated alone or in the presence of rSm28GST (20 μg/ml), SWAP (100 μg/ml), or concanavalin A 2.5 mg/ml; Sigma) in a total volume of 0.2 ml on 96-well flat-bottom microtiter tissue culture plates (Nunc, Roskilde, Denmark). Cells were exposed to 1 μCi of [^{3}H]thymidine (37 GBq/mmol; CEA, Gif-sur-Yvette, France) per well for the last 18 h of a 5-day culture period. Finally, cells were harvested by filtration on fiberglass discs with a multiharvester (Skatron, Lierbyen, Norway) and the amount of incorporated [^{3}H]thymidine was measured with a liquid scintillation counter (LKB; Wallac, Turku, Finland). Data are expressed as the arithmetic means of the change in counts per minute (Δcpm) ± standard deviations for triplicate cultures, as follows: $\Delta cpm = (cpm_x - cpm_{medium})/cpm_{medium}$, where cpm_x is the splenocyte responses (in counts per minute) for individual mice stimulated with various antigens and cpm_{medium} is the splenocyte responses for individual mice without stimulation.

Infection. ^{P28}Tg and non-^{P28}Tg mice were exposed percutaneously to 40 *S. mansoni* (Guadeloupe strain) furcocercariae by the method first described by Smithers and Terry (26). Parasite burdens were evaluated by whole-body perfusion at 56 days postinfection. Livers and intestines were digested by 4% potassium hydroxide solution by the method of Sturrock et al. (28). Tissue egg counts were expressed as the numbers of eggs per gram of tissue analyzed and per gram of tissue per female worm. The organs of each mouse were treated and analyzed individually. The data presented are means ± standard errors.

Statistics. The significance of the result was calculated by Student's *t* test and F test with StatView software.

RESULTS

Generation of Sm28GST ^{P28}Tg Mice. The microinjected transgene consisted of Sm28GST cDNA under the regulatory control of a 600-bp fragment containing mPTf and an hGH poly(A) signal downstream of the cDNA (Fig. 1). The DNA recovered after tail biopsies of weaning animals was screened for the presence of Sm28GST cDNA by DNA dot blot analysis with a parasite cDNA probe. Examinations of 53 mice identified 10 mice that contained at least one copy of the transgene (Table 1). Breeding colonies for founder animals containing the Sm28GST gene were established by mating them to C57BL/6J mice, and the second-generation mice that were positive in the hybridization assay were tested for Sm28GST expression.

All founders that harbored transgenes yielded ^{P28}Tg progeny, except for the three designated 1, 3, and 23 (Table 1). The diploid copy number of each ^{P28}Tg line was estimated by semiquantitative dot blot analysis and ranged from 5 to 50 copies of

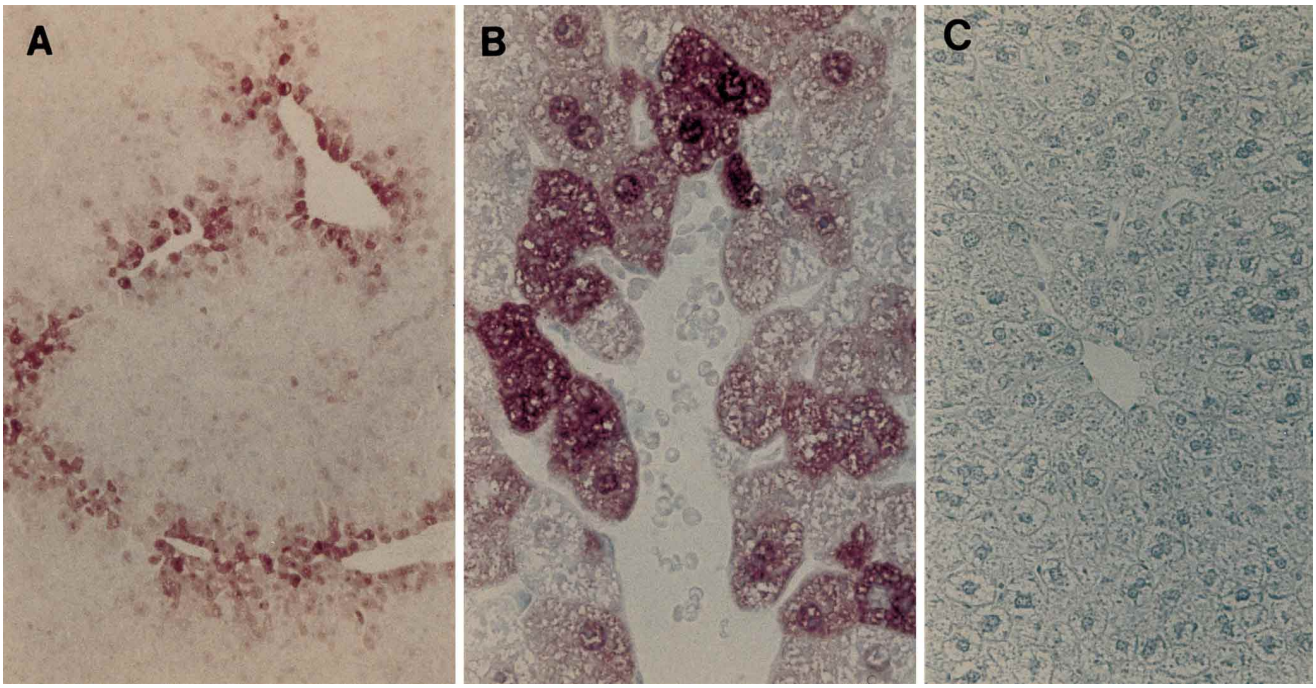

FIG. 3. Immunohistochemical analysis of Sm28GST protein in the livers of ^{P28}Tg mice. Immunoperoxidase staining revealed the protein in center-lobular hepatocytes (cytoplasm and/or nucleus) (A and B), whereas no labelling was observed in the livers of non-^{P28}Tg control mice (C). Hepatitis or abnormal liver histology was not observed. Magnification: ×50 (A); ×400 (B); ×200 (C).

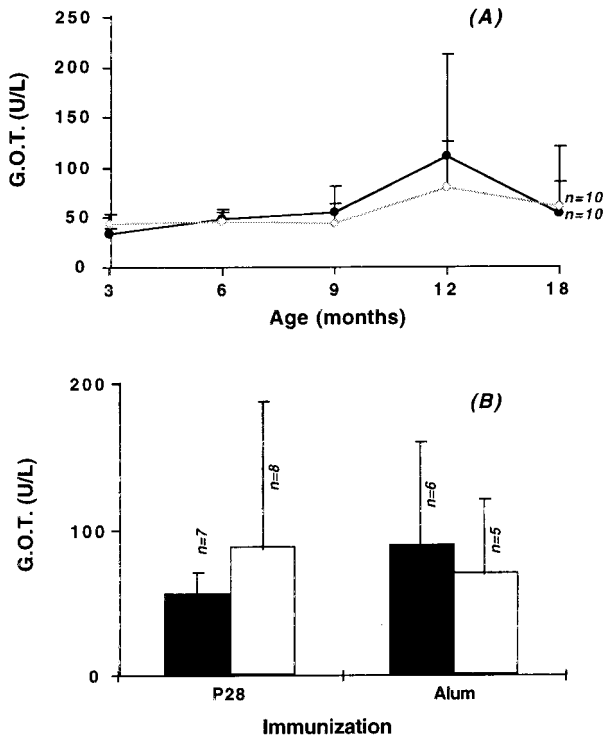

FIG. 4. Measurement of liver function in ^{P28}Tg mice. Liver function was monitored by measurements of the GOT concentration (in units per liter [U/L]) in serum. (A) GOT concentrations in sera of naive (○) and ^{P28}Tg (●) mice. (B) GOT concentrations in sera of ^{P28}Tg mice (■) and non-^{P28}Tg mice (□) immunized with rSm28GST and alum (P28) or alum alone (Alum).

the transgene. Southern blot analysis of genomic DNAs obtained from ^{P28}Tg mouse lines revealed the 1.9-kb *Eco*RI fragment characteristic of the Sm28GST gene (data not shown). Northern blot analysis demonstrated the presence of Sm28GST mRNA in the livers and brains of ^{P28}Tg animals from lines 9 and 26, but not from lines 5, 8, 21, and 35 (data not shown).

In the next generation, the transgenes segregated, as assessed by dot blot analysis, in an approximate 1:1 ratio of male and females, with no evidence of transmission ratio distortion, except for line 9. An analysis of the progeny of line 9 showed transmission of the transgene in the X chromosome.

Expression of Sm28GST in the livers of ^{P28}Tg mice. Both mouse lines 9 and 26 expressed the Sm28GST protein in their livers, as detected on immunoblots probed with a monospecific polyclonal antibody (Fig. 2). The cross-reactive band from the livers of non-^{P28}Tg mice (approximately 30 kDa) is thought to represent a host GST. No specific Sm28GST expression was observed in the livers of lines 5, 8, 21, and 35 (Fig. 2). A control non-^{P28}Tg littermate did not show specific reactivity.

To assess Sm28GST expression in other tissues, brains, skin, spleens, lungs, kidneys, and muscles were collected from lines 9 and 26. Western blot analysis failed to detect Sm28GST reactivity in any tissue other than liver. A blot of brain homogenate is shown in Fig. 2 as a representative control experiment. Due to the low fecundity of line 9, we thereafter used line 26.

The distribution of Sm28GST was examined by immunohistological analysis. Stained hepatocytes were localized mainly at the pericentrolobular areas. The staining was heterogeneous, and approximately 10% of total hepatocytes were stained. The expression was cytoplasmic and nuclear. No histological changes or inflammatory infiltrates were observed (Fig. 3).

In order to determine whether Sm28GST expression is toxic for the liver, we measured the serum GOT concentrations of ^{P28}Tg mice. Figure 4A shows that the levels of this enzyme remained similar to those of control animals over an 18-month period. The slight peak at 12 months is not statistically significant and may have been due to the presence of hemoglobin in some sera.

Analysis of the humoral immune responses in ^{P28}Tg mice immunized with rSm28GST. The antibody responses to Sm28GST in ^{P28}Tg mice and non-^{P28}Tg littermates were measured after immunization with rSm28GST or with KLH used as a control. Figure 5A shows the IgG anti-Sm28GST responses throughout the experiment. Non-^{P28}Tg mice displayed a consistent response, which peaked 2 weeks after the second injection and remained stable until the end of the experiment. In ^{P28}Tg mice, the antibody levels peaked at the same time (2 weeks after the second injection) but were significantly lower ($P < 0.01$) and tended to decline. Furthermore, immunizations of ^{P28}Tg and non-^{P28}Tg mice with rSm28GST failed to affect the serum GOT concentration (Fig. 4B). The antibody responses against individual peptide epitopes of Sm28GST (24 to 43, 87 to 111, 103 to 153, 115 to 131, and 190 to 221) were measured at day 56 after infection, which corresponded to the day of sacrifice, because insufficient amounts of serum were available on other sampling days. No significant responses to peptides were measured by enzyme-linked immunosorbent assay (34) (data not shown).

Immunizations of ^{P28}Tg mice and non-^{P28}Tg mice with KLH

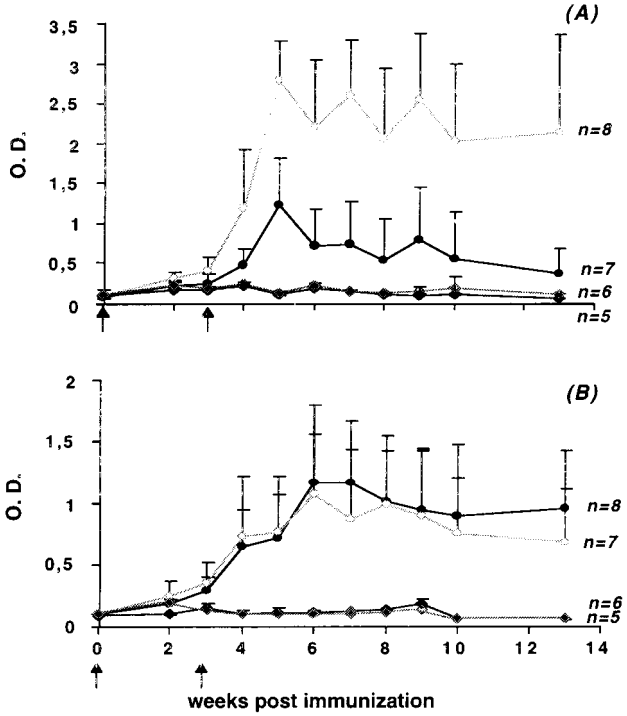

FIG. 5. Kinetics of IgG responses of ^{P28}Tg mice immunized with rSm28GST, alum, and KLH. Shown are the levels of anti-Sm28GST antibodies (serum dilution, 1/5,000) (A) and anti-KLH antibodies (serum dilution, 1/25,000) (B) of ^{P28}Tg mice immunized with rSm28GST and alum (●), non-^{P28}Tg mice immunized with rSm28GST and alum (○), ^{P28}Tg mice immunized with alum (◆), and non-^{P28}Tg mice immunized with alum (◇). Arrows indicate the times of primary immunizations and booster injections. O.D., optical density.

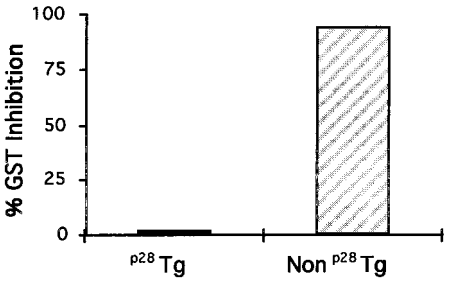

FIG. 6. Inhibition of Sm28GST enzyme activity by pooled anti-rSm28GST antisera of ^{P28}Tg ($n = 6$) and non-^{P28}Tg ($n = 7$) mice. Sera of mice immunized with alum alone were used as negative controls to calculate the percentage of inhibition.

were performed as a control. As shown in Fig. 5B, no differences in the anti-KLH responses of ^{P28}Tg and non-^{P28}Tg mice were observed.

In light of the previously reported positive association between the anti-Sm28GST antibody response and the inhibition of GST catalytic activity (34), the immune sera of ^{P28}Tg and non-^{P28}Tg mice were tested for the ability to inhibit Sm28GST enzyme activity. As shown in Fig. 6, 93% of GST catalytic activity was ablated by sera from immunized non-^{P28}Tg mice, whereas sera from immunized ^{P28}Tg mice failed to inhibit GST enzyme activity.

Individual variation in Sm28GST expression. In order to determine whether variations in the level of antibody response in individual ^{P28}Tg mice can be related to the levels of Sm28GST expression in their livers, we estimated Sm28GST expression by immunohistological analysis.

Four ^{P28}Tg mice and one non-^{P28}Tg mouse from the same litter, housed together, were immunized with rSm28GST. The various degrees of Sm28GST expression in these mice were estimated as follows: +, ++, +++, or −. Figure 7 shows the time courses of individual antibody responses to Sm28GST. The level of the anti-Sm28GST response was approximately inversely proportional to the level of Sm28GST expression in the liver.

Lymphocyte proliferative responses of ^{P28}Tg mice immunized with rSm28GST. The proliferative responses of lymphocytes from rSm28GST-immunized mice were determined by

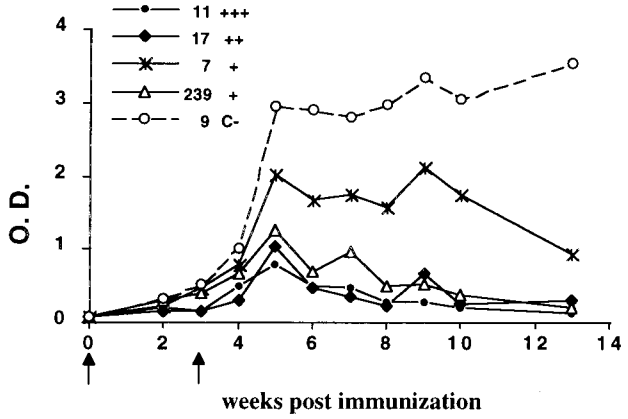

FIG. 7. Individual kinetic antibody responses of rSm28GST-immunized ^{P28}Tg mice with high (+++), medium (++), or low (+) Sm28GST expression in their livers. C−, non-^{P28}Tg littermate (negative control). Arrows indicate the times of primary immunizations and booster injections. O.D., optical density.

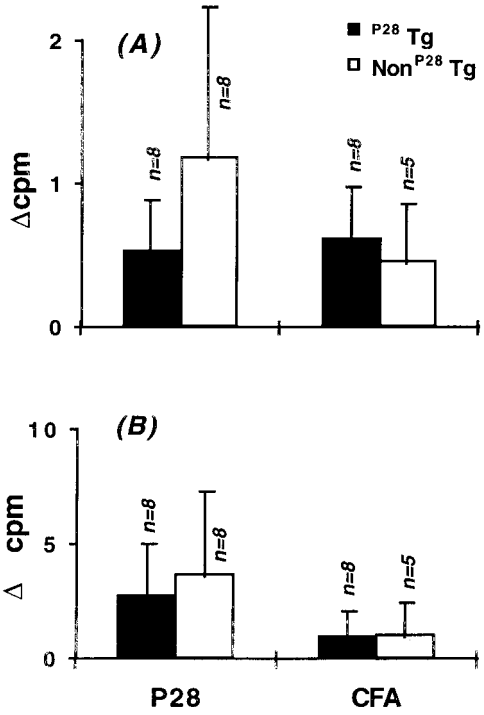

FIG. 8. Proliferation assay of spleen lymphocytes from rSm28GST-immunized mice. Lymphocytes were stimulated with rSm28GST (A) and SWAP (B).

incubating spleen cells of ^{P28}Tg mice and non-^{P28}Tg mice with rSm28GST and with SWAP as a control. rSm28GST stimulation of non-^{P28}Tg mouse spleen cells was significantly greater ($P < 0.01$) than that observed with ^{P28}Tg mouse spleen cells (Fig. 8A). No differences were observed between cells isolated from ^{P28}Tg and non-^{P28}Tg mice immunized with CFA. As a control, SWAP stimulations of spleen cells from rSm28GST-immunized and infected ^{P28}Tg and non-^{P28}Tg mice were performed (Fig. 8B). Strong reactivities were observed in both ^{P28}Tg and non-^{P28}Tg mice, with no differences between the responses of the two groups ($P < 0.05$).

Effects of Sm28GST expression in ^{P28}Tg mice on *S. mansoni* infection. To determine the effects of Sm28GST expression by hepatocytes on *S. mansoni* infection in mice, we compared ^{P28}Tg and non-^{P28}Tg control mice infected with 40 *S. mansoni* furcocercariae. Eggs and adult worms were collected from the livers and intestines of ^{P28}Tg mice and controls at 56 days postinfection. The representative results of three challenge infection experiments are shown in Table 2.

A significant increase in worm burden was observed in ^{P28}Tg mice, compared to that in non-^{P28}Tg mice (27%; $P < 0.05$). The number of eggs per gram of tissue was significantly increased in the intestines of ^{P28}Tg mice, compared to that of non-^{P28}Tg mice (74%; $P < 0.05$), and there was a similar tendency in livers. However, when individual female-worm fecundity was considered, there were no significant differences in the total number of eggs produced per gram of tissue and per female worm between ^{P28}Tg and non-^{P28}Tg mice.

In a separate experiment, anatomopathological analysis was performed with mouse liver sections sampled at different times (8, 12, 14, 16, and 21 weeks) after infection. No differences in the granuloma surface area and level of inflammation between ^{P28}Tg and non-^{P28}Tg mice were noticeable.

TABLE 2. Effects of *S. mansoni* infection on ^{P28}Tg mice

Mouse group (*n*)	Worm burden (no. of worms/mouse)[a]	No. of eggs/g of tissue[a]		No. of eggs/g of tissue/couple[b]	
		Liver	Intestines	Liver	Intestines
^{P28}Tg (9)	25 ± 7 (39; <0.05)	4,164 ± 1,085 (23; >0.05)	6,417 ± 1,659 (74; <0.05)	900 ± 345 (>0.05)	1,354 ± 367 (>0.05)
Non-^{P28}Tg (6)	18 ± 8	3,388 ± 1,909	3,685 ± 1,493	1,246 ± 592	1,378 ± 515

[a] Parenthetical data are the percent increases in the means for ^{P28}Tg mice compared with those for non-^{P28}Tg mice and the *P* values determined by Student's *t* test.
[b] Parenthetical data are the *P* values determined by Student's *t* test.

DISCUSSION

Here we describe the construction of Tg mice that express a heterologous protein, the 28-kDa GST of the trematode parasite *S. mansoni*. This molecule is one of the best-defined vaccine candidates for this debilitating disease. One of the objectives of this work was to produce a mouse tolerant for this molecule. In this case, it would be possible to show, by the absence of an immune response toward a crucial parasite molecule, how important this is to parasite survival and host resistance. We show here that the expression of this nonself antigen by liver cells did indeed induce a functional tolerance but that this tolerance was not absolute, since an antibody response was detectable after immunization with the corresponding recombinant antigen. This may have been due to localized expression of the transgene in the liver. However, despite this partial tolerance, we observed that ^{P28}Tg mice were more susceptible to *S. mansoni* infection, indicating that immune reactions toward this molecule may be an important component of resistance toward infection.

We deliberately chose to express this antigen in the liver by making use of a liver-specific promoter since the pathological effects of schistosome infection implicate this organ in particular. This pathology is due to the entrapment of schistosome eggs in the portal vein radicles which stimulate an inflammatory response, in turn leading to extensive fibrosis. Sm28GST is a major component of egg secretions (25) and may be a target of the inflammatory response.

A fused gene containing 600 bp of upstream mPTf genomic sequence linked to the Sm28GST ORF and the hGH poly(A) signal was used to produce Tg mice. Two (of 10) lines that incorporated 8 and 60 complete copies of the transgene expressed substantial levels of Sm28GST mRNA in the liver and lower levels in the brain. However, Western blot analysis showed that Sm28GST protein was present only in the liver, not in the brain. This result is not in accordance with the work of Lu et al. (21), who showed that Tg mice carrying 0.67 kb of the 5′ flanking region of the human Tf gene fused to the bacterial chloramphenicol acetyltransferase gene expressed the transgene in the liver and the brain at both transcriptional and translational levels. The absence of Sm28GST protein in the brains of our two lines of Tg mice may indicate that Sm28GST protein is not stable in the mouse central nervous system. Expression of the transgene could thus be regulated at a posttranscriptional level. Immunohistological analysis showed that Sm28GST was localized at the pericentrolobular area of the liver. The locations of other transgenes under the control of mPTf (0.6 kb) in Tg mice have never been studied precisely at the cellular level, and therefore it is not possible to infer a particular cellular specificity for the portion of the promoter that we utilized.

A significant observation in this study was the absence of any change in the concentration of GOT in serum or of any histological alterations over an 18-month period. Despite the relatively low level of Sm28GST expression (no more than 10% of total hepatocytes were immunostained), these data show that the expression of Sm28GST is not toxic in the Tg-mouse model. This evidence can be added to unpublished data accumulated for different protocols of immunization in ruminants (8), baboons (7), *Erythrocebus patas* monkeys (9), mice (7), and rats (15), in which no evidence of toxicity after immunization with the recombinant protein was observed (in some cases over very long periods).

Another point to come out of this study was the clear evidence for cross-reactivity between Sm28GST and a mouse molecule of 30 kDa (Fig. 2) in the liver. GSTs are abundant in mammals and are multifunctional but are particularly implicated in detoxification and the transport of lipophilic molecules (22). This superfamily can be divided into five groups, and Sm28GST has significant sequence homology to the human α isoform (28%) and slightly lower homology to the π isoform (18). The fact that a polyclonal rabbit antiserum to the recombinant schistosome protein can recognize a mouse GST isoform is therefore not surprising. This cross-reactivity is not limited to liver tissue, since the 30-kDa protein was also detected on Western blots with brain and muscle extracts.

In terms of vaccination, this may in part explain the relatively feeble immunogenicity of Sm28GST that we observed (Fig. 4A). Indeed, an immunization protocol which led to a strong immune response might also induce an autoimmune process, although this is more dependent on the type of response produced. Viral models of autoimmune disease underline the role of $CD8^+$ cytotoxic T lymphocytes in the process (30). A protocol aimed at inducing this type of response will be tested in order to determine the conditions for the rupture of tolerance in this model. However, immunizations of non-^{P28}Tg mice with rSm28GST led to protection of 27% (data not shown), with humoral (Fig. 4A) and cellular (Fig. 8) immune responses and without any evidence of histological lesions of the liver. In addition, no autoimmune hepatitis has been observed during the various experimental protocols performed so far.

Sm28GST is one of the components of adult-worm excretions and secretions, and indeed its absence from the worm gut mucosa implies that it is secreted by or released from the tegument (25). In fact, the N-terminal peptide sequence possesses the characteristics of a eukaryotic signal sequence (23a), but the mature schistosome protein lacks only the first Met residue (6). However, it was tempting to speculate that this sequence is functional in a mammalian cell. Thus, it was interesting to compare the behavior of the protein expressed in a mammalian system with its expression in the helminth. Sm28GST was found in both the cytoplasm and nuclei of hepatocytes; this distribution is similar to that recently reported for parasite cells (20). This raises the as-yet-unanswered question of the role of this enzyme in the nucleus.

Both B- and T-cell mediated immune responses toward Sm28GST were analyzed. ^{P28}Tg mice showed low levels of antibody and cellular responsiveness, but clearly these were

not totally abolished. This unresponsiveness was specific since ^{P28}Tg mice produced normal responses to both KLH and SWAP. The basic mechanisms of nonresponsiveness are being investigated. It has been previously reported for neo-self-reactive Tg products, such as virally encoded proteins, that expression of the transgene led to functional unresponsiveness (32). In almost all models, this in vivo state was reversible by various manipulations in vivo and in vitro, suggesting that peripheral tolerance was due to the inability of nonlymphoid cells in peripheral tissues to present the transgene products (32).

When Tg mice were infected by *S. mansoni* cercariae, they harbored significantly more worms than did non-Tg littermates. We observed similar results after immunization with rSm28GST. Tg mice preimmunized with rSm28GST and challenged with *S. mansoni* cercariae 3 weeks after the second immunization showed significantly increased worm burdens compared to those of immunized non-Tg mice (35). The increase in the level of infection of ^{P28}Tg mice (Table 2) was reflected in a concomitant increase in the numbers of adult worms and schistosome eggs found in various tissues, but no relative increase in the fertility of individual female worms was observed. This may, however, be due to a crowding effect in which increased numbers of worms do not lead to increases in egg numbers (10, 23). This may be due to a relative reduction in nutrient availability, and it should be remembered that the infection of a mouse by 30 worms is the equivalent of a human infection with 15,000 worms, a very heavy infection indeed. In addition, the absence of measurable antibody responses toward defined peptide epitopes, although a significant antibody response toward recombinant Sm28GST was observed, may indicate that the antibody responses of ^{P28}Tg mice were principally derived against conformational epitopes and may explain the absence of an effect on fertility, since this has been shown to be linked to recognition of the C-terminal epitope (34).

We noted that the infection levels in individual ^{P28}Tg mice were variable, and we attempted to correlate this variation to the different levels of Sm28GST expression in their livers. The mice studied were heterozygote for the Sm28GST gene and were obtained by backcrossing to C57BL/6J mice. The results presented here are for fifth- or sixth-generation mice, but the Tg mice used were obviously not congenic. Host strain-dependent differences among various mouse strains infected with *S. mansoni* have been well documented in regard to the worm burden during primary infection and thus may explain some of the variability in the results obtained even with Tg mice from the same litter. In addition, the levels of Sm28GST expression in the liver estimated by immunohistochemistry showed that there was significant variability in the individual levels of expression of the transgene. We did not attempt to measure this quantitatively due to the cross-reactivity previously mentioned. Figure 7 shows the relationship between the kinetics of the appearance of anti-Sm28GST antibodies in the sera of ^{P28}Tg mice immunized with rSm28GST and the levels of Sm28GST expressed in their livers. The results show that the lower the expression level, the stronger the response. We have not attempted to establish a relationship of Sm28GST expression to protection levels obtained after immunization, since it would be difficult to estimate the level of transgene expression in a parasitized liver (schistosome eggs secrete large amounts of this protein).

While the variability in the levels of expression may explain a large part of the individual differences observed, the nonconsanguinity of mice may also be involved. Five backcrosses are insufficient to establish total homogeneity in the major histocompatibility complex, and the anti-Sm28GST response has been shown to be haplotype dependent (33). It should be noted that it required 2 years and the screening of 600 mice to establish the Tg-mouse line over five generations.

The results we have obtained argue that Sm28GST plays a role in reducing levels of infection and in turn support the view that this enzyme has a central role in the maintenance of parasite viability, at least during its migration through host tissues. The line we have established is being used to dissect T-cell responses to specific epitopes of Sm28GST. Future work will aim at testing the effects of various immunization protocols on the induced tolerance of ^{P28}Tg mice.

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