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Expression and Immunogenicity of an *Echinococcus granulosus* Fatty Acid-Binding Protein in Live Attenuated *Salmonella* Vaccine Strains

JOSÉ A. CHABALGOITY,[1]* JULIA A. HARRISON,[1] ADRIANA ESTEVES,[2] RAQUEL DEMARCO DE HORMAECHE,[1] RICARDO EHRLICH,[2] C. M. ANJAM KHAN,[1] AND CARLOS E. HORMAECHE[1]

Department of Microbiology, The Medical School, University of Newcastle, Newcastle Upon Tyne NE2 4HH, United Kingdom,[1] and Sección Bioquímica, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay[2]

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Fatty acid-binding proteins (FABPs) are candidate molecules for vaccines against several parasitic platyhelminths. A FABP from the cestode *Echinococcus granulosus* (EgDf1) was expressed in *Salmonella* vaccine strains as a C-terminal fusion to fragment C of tetanus toxin (TetC) by using expression vector pTECH. The fusion protein was equally expressed in several attenuated vaccine strains derived from bacteria with different genetic backgrounds and different attenuating mutations. Single-dose immunization experiments with the *aroA Salmonella typhimurium* strain SL3261 carrying the pTECH-EgDf1 construct were conducted with mice, using both the intravenous and the oral routes. Surprisingly, the antibody response to EgDf1 and the antigen-specific cytokine production in spleen cells were stronger in mice immunized orally. Furthermore, immune mouse sera strongly reacted with fixed sections of the worm's larval stage. Analysis of the isotype distribution of the specific anti-EgDf1 antibodies showed similar production of immunoglobulin G1 (IgG1) and IgG2a together with specific IgA antibodies. In addition, stimulation of spleen cells from mice immunized with the different constructs with either *Salmonella* lysate, TetC, or EgDf1 showed that, together with Th1-related cytokines (gamma interferon and interleukin 2 [IL-2]), significant levels of a Th2 cytokine (IL-5) were produced specifically, indicating a Th2 component to the response to the *Salmonella* carrier and to the recombinant antigens. Salmonellae expressing the TetC-rEgDf1 fusion are currently under evaluation as potential vaccines against *E. granulosus*.

The development of multivalent vaccines for human and veterinary use based on the expression of heterologous antigens in live attenuated salmonellae has received considerable attention in the last decade (15, 16, 33). Attenuated bacteria carrying precise deletions in their genome can be delivered orally to establish a self-limiting infection persisting in the tissues for a few weeks before being cleared. During that time, they can evoke a broad spectrum of immune responses against their own antigens as well as to recombinant heterologous antigens (3, 15, 16).

The successful construction of multivalent vaccines using the *Salmonella* delivery system requires that heterologous antigens be expressed at a level sufficient to trigger an immune response without being deleterious for the bacteria. Such a task has proven difficult to achieve, and several systems have been attempted. In many of these, only model antigens have been used, leaving open the question whether the systems will work with a broad range of antigens (15).

A new system for the expression of heterologous antigens in salmonellae as C-terminal fusions to tetanus toxin fragment C (TetC) driven by the anaerobically inducible promoter *nirB* has been described (18, 19). This system allows the expression of guest antigens either as a full-length protein (19) or as multiple tandem copies of a relevant peptide (18). Expression of the full-length *Schistosoma mansoni* glutathione *S*-transferase P28 as a fusion to TetC has led to an experimental vaccine capable of protecting mice against salmonellosis, tetanus, and schistosomiasis after a single dose (19). Furthermore, a single dose of a *Salmonella* vaccine strain expressing tandem copies of a peptide from glycoprotein D (gD) of herpes simplex virus as a fusion with TetC protected mice against virus challenge (4).

Hydatid infection caused by the cestode *Echinococcus granulosus* is one of the major world zoonoses, affecting humans as well as domestic and wild animals (37). The parasite requires two mammalian hosts for its complete life cycle. The adult worm develops in the intestine of the definitive host (commonly dogs), and when maturity is reached, worm eggs are shed into the environment in the host's feces. The eggs can infect a wide variety of herbivorous and omnivorous animals (and, accidentally, humans). In the intermediate host, the eggs develop into hydatid cysts which may produce thousands of larval worms (protoscolices), each of which if ingested by a dog can develop into an adult worm in the intestine of the dog.

Infection of the intermediate hosts constitutes a major public health problem and is a cause of important economic loss in areas of endemicity (31). Although control programs have been successfully implemented in a few countries, new foci of infection and regions of endemicity have recently been recognized (8). An effective vaccine for either the definitive host (dogs) or the intermediate host (sheep) should be an important way of breaking the cycle (13). A vaccine aimed at protecting dogs would directly stop the production of infecting eggs and, furthermore, would be the most cost-beneficial measure for cattle-rearing countries.

Several antigens in platyhelminths have been described as potential vaccine candidates (1). Parasitic platyhelminths cannot synthesize their own lipids de novo, so they depend on the

* Corresponding author. Present address: Cátedra de Inmunología, Facultad de Química, Casilla de Correo 1157, 11800 Montevideo, Uruguay. Phone: 598 2 47 43 34. Fax: 598 2 47 43 20. E-mail: crtn@bilbo.edu.uy.

uptake of lipids from the host (34), and fatty acid-binding proteins (FABPs) are believed to be involved in the transport of such molecules into the parasites (24). Homologous FABPs of *S. mansoni* and *Fasciola hepatica* confer a high degree of protection in experimental models (14, 36). Furthermore, the two molecules are cross-protective, and the *S. mansoni*-derived FABP has been proposed as a dual vaccine candidate for both helminth infections (36). A FABP from *E. granulosus* (EgDf1) that is differentially expressed in protoscolices has been isolated from a protoscolex cDNA library (10), and in this study, we describe the efficient delivery of this molecule to the mouse immune system by a live attenuated *Salmonella* vaccine strain.

MATERIALS AND METHODS

Plasmids, oligonucleotide, lambda clone, and bacterial strains. Plasmid pTECH2 (hereafter referred to as pTECH) has been described previously (18). The full-length gene coding for EgDf1 was amplified by PCR from a purified λgt11 recombinant clone (10) using primers Eg1 (forward primer: 5′-AATGG ATCCATGGAGGCATTCCTTGGTACC-3′) and Eg2 (reverse primer: 5′-TTA ACTAGTCGCCACCTTTGAGTAGGTTCG-3′), which allowed the directional cloning of EgDf1 into pTECH. PCR was performed by using the high-fidelity thermostable DNA polymerase from *Pyrococcus furiosus* (Stratagene, Cambridge, United Kingdom), which possesses an associated 3′,5′-exonuclease proofreading activity (21).

The bacterial strains used were *Escherichia coli* TG2 (*recA*) (29), *Salmonella typhimurium* SL5338 (*galE* $r^- m^+$) (2), and the *S. typhimurium* vaccine strains SL3261 (*aroA*), BRD726 (Δ*htrA*), C5046 (*htrA*::Tn*phoA*), and C5 *aroD htrA* (4).

Western blotting (immunoblotting) assays. Expression of the TetC fusion proteins in lysates of bacteria harboring the construct was tested by Western blotting with anti-TetC serum used as a probe as previously described (4).

Animals and immunization protocols. Female BALB/c mice were purchased from Harlan Olac (Blackthorn, Bicester, United Kingdom) and used when at least 8 weeks old. For intravenous (i.v.) immunization, mice were injected once in a lateral tail vein with 10^6 CFU of the different constructs per mouse. For oral immunization, 4×10^9 CFU of each construct in 200 μl of phosphate-buffered saline (PBS) was given once intragastrically by gavage tube. The mice were bled at weeks 4 and 6 after immunization, and sera were stored individually at −20°C. Viable counts for organ homogenates were determined for mice immunized i.v. 7 days earlier as described elsewhere (19).

Antibody responses. Antibody responses were assessed by enzyme-linked immunosorbent assay (ELISA) as described previously (4). Briefly, 96-well microtiter plates (Nunc; GIBCO BRL, Life Technologies Ltd., Paisley, United Kingdom) were coated overnight at 4°C with either 0.1 μg of recombinant TetC (Boehringer Mannheim, Lewes, Sussex, United Kingdom) per well or 2 μg of a recombinant EgDf1 (rEgDf1) diluted in 0.1 M carbonate buffer (pH 9.6) per well. Individual sera were added at a dilution of 1/50 in PBS–1% bovine serum albumin, and the plates were incubated for 90 min at 37°C. Antibodies specific to each particular isotype (rabbit anti-mouse immunoglobulin A [IgA], IgG, IgG1, and IgG2a or a monoclonal rat anti-mouse IgE [Zymed Laboratories Inc., Cambridge BioScience, Cambridge, United Kingdom]) were added at a 1:500 dilution, and the plates were incubated for 1 h at 37°C. Swine anti-rabbit or rabbit anti-rat antibodies (peroxidase conjugated) (Dako Ltd., High Wycombe, Buckinghamshire, United Kingdom) were then added at 1:1,000, and the mixtures were incubated for another hour at 37°C. Color was developed for 15 min at 37°C with orthophenylene diamine (OPD) (Sigma, Poole, Dorset, United Kingdom) prepared according to the manufacturer's instructions, and the reaction was stopped with 2 M H_2SO_4 before readings were taken at 490 nm. Results for the anti-TetC ELISA are presented after substraction of the optical density (OD) obtained for the same microtiter plate for a pool of sera from mice immunized i.v. 6 weeks earlier with 10^6 CFU of strain SL3261 alone.

For ELISA assessment of anti-EgDf1 antibody responses, a recombinant protein (rEgDf1) was prepared as a glutathione *S*-transferase fusion protein by using vector pGEX-2T (Pharmacia Biotech, St. Albans, Herts, United Kingdom) and purified by affinity chromatography using glutathione-agarose beads (Sigma). An overnight culture of *E. coli* TG2 harboring the pGEX-2T construct was diluted 1:50 in fresh medium and grown for 2 h at 37°C with shaking. The culture was then induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and grown for another 4 h. Cells were collected by centrifugation, resuspended in 1/100 of the original volume of ice-cold PBS, and sonicated on ice. After sonication, Triton X-100 was added to a final concentration of 1%, cell debris were removed by centrifugation at 20,190 × *g* for 15 min, and supernatant was collected and filtered (0.45-μm-pore-size filter). The supernatant containing the fusion protein was incubated for 2 h at room temperature with a 2-ml volume of glutathione-agarose beads previously equilibrated with PBS. After the incubation, the mixture was transferred to a column. The sonicate was allowed to flow through, and the matrix was washed with 50 bed volumes of PBS. The bound fraction was then eluted by addition of elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0) in 1 bed volume and incubation for 10 min before collection. The elution was monitored by OD at 280 nm, and the fractions collected were checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Fractions containing the pure fusion protein were pooled, dialyzed against PBS, and stored at −20°C. Protein concentrations in samples were determined by using bicinchoninic acid (BCA) protein assay reagent (Pierce, Chester, United Kingdom).

Immunostaining of protoscolex sections. Immunoreactivity of the mouse sera against wax sections of *E. granulosus* protoscolices was determined by indirect peroxidase staining. Paraffin-embedded sections of *E. granulosus* protoscolices from Uruguayan sheep cysts were prepared by the Department of Pathology, University of Cambridge. The sections were dewaxed by two washings with xylene, and endogenous peroxidase activity was removed by incubation in methanol–1% hydrogen peroxide for 30 min. The sections were then washed twice for 5 min with distilled water and once with PBS. The washing buffer was drained off, and the excess was removed by carefully wiping around the sections. After blocking for 1 h with PBS–5% normal goat serum (Sigma), 1:100 dilutions of the mouse sera in PBS–1% normal goat serum were incubated with the sections for 2 h in a wet chamber. After four washes with PBS, the sections were incubated for 45 min with a 1/40 dilution of peroxidase-conjugated goat anti-mouse immunoglobulins (Dako) and washed again three times with PBS and finally once with acetate buffer (50 mM sodium acetate, pH 5.2) for 2 min. Freshly prepared substrate solution (10 mg of 3-amino 9-ethyl carbazole [AEC; Sigma] dissolved in 10 ml of dimethyl sulfoxide, mixed with 50 ml of acetate buffer, and filtered [0.2-μm filter] before addition of 4 μl of 30% hydrogen peroxide) was added to the sections, and color was allowed to develop for 10 to 15 min before the reaction was stopped by extensive rinsing with tap water. The sections were then counterstained with hematoxylin, rinsed, mounted with glycerol-gelatin (Sigma), and examined under a microscope.

Cytokine assays. Cytokine levels in spleen cell culture supernatants were measured after antigenic stimulation. Six weeks after immunization, three mice from each of the immunized groups plus three mice from a fifth, naive group were killed, and the spleens were aseptically removed and placed in RPMI 1640 (Sigma; HEPES modification). Single-cell suspensions were prepared by mashing the spleens through a sieve with a syringe plunger, washed in RPMI 1640, and harvested at 290 × *g* for 7 min at 20°C. The pellets were resuspended in 1 ml of complete RPMI (CRPMI) (RPMI 1640 with 10% heat-inactivated newborn calf serum [NBCS], 0.1% 0.05 M 2-mercaptoethanol, 2% 0.2 M glutamine, 10 mg of streptomycin per ml, and 100 U of penicillin per ml), and erythrocytes were lysed by addition of 5 ml of Gey's solution per spleen and incubated on ice for 4 min. The cells were then washed twice before being resuspended in CRPMI and counted. Cell cultures were set up in round-bottomed 96-well plates (Corning Glass Works, Corning, N.Y.) at 5×10^5 cells/culture in 200 μl and incubated at 37°C in 95% humidity–5% CO_2. Antigens were added in triplicate in volumes ranging between 5 and 20 μl. At intervals, supernatants were collected in flat-bottomed 96-well plates and frozen at −80°C until use in cytokine ELISAs. TetC and rEgDf1 were the same as those used for antibody determinations and were diluted in RPMI 1640 at concentrations of 5 and 10 μg per culture, respectively. For *Salmonella* antigens, a whole-cell extract designated C5 lysate was prepared as described previously (41). Briefly, a 50-ml overnight stationary culture of *S. typhimurium* C5 was pelleted by centrifugation, washed, resuspended in a small volume of PBS, and disrupted by sonication. The suspension was then clarified by centrifugation, filtered through a 0.22-μm-pore-size membrane, and stored at −20°C. The protein concentration of C5 lysate was determined by the BCA method.

Interleukin 2 (IL-2), gamma interferon (IFN-γ), IL-4, and IL-5 were routinely assayed by ELISA, using a pair of specific monoclonal antibodies (MAbs) (capture and detection) against each cytokine and dilutions of a recombinant cytokine for the construction of standard curves (all reagents supplied by Pharmingen). The ELISA plates were coated with 50 μl of 2-μg/ml capture MAb diluted in carbonate buffer (pH 8.2) per well and incubated for 2 h at 37°C and then overnight at 4°C. The plates were washed twice with PBS-Tween and blocked with 100 μl of 10% NBCS in RPMI per well for 1 h at 37°C. The plates were washed again twice, the samples and standards were added at 50 μl/well, and the plates were incubated for 2 h at 37°C. A standard curve was constructed for each plate by using twofold dilutions of the appropriate cytokine in duplicate. The ranges of concentrations used for the construction of the standard curves were 3 ng/ml to 15 pg/ml for IL-2 and IL-5, 6 ng/ml to 15 pg/ml for IL-4, and 20 ng/ml to 100 pg/ml for IFN-γ. Culture supernatants were added neat. After incubation, the plates were washed four times before 100 μl of the biotinylated detection MAb at 1 μg/ml in PBS–10% NBCS was added to each well and the plates were incubated for 1 h at 37°C. After a new round of washes, 100 μl of avidin peroxidase (Sigma) diluted to 2.5 μg/ml in PBS–10% NBCS was added to each well. The plates were incubated for 45 min at room temperature and washed five times, and color was developed by adding 50 μl of OPD substrate per well. The reactions were developed for 15 min and stopped with 15 μl of 2 M H_2SO_4 per well before readings were taken at 490 nm.

The reading for each sample less the mean of the three negative controls for each group of cells (cells without stimulation) was used to interpolate from the standard curve for quantification of the amount of cytokine present in each culture. Statistical significance of the differences in cytokine production was evaluated by Student's *t* test; differences were considered significant when P values of <0.05 were obtained.

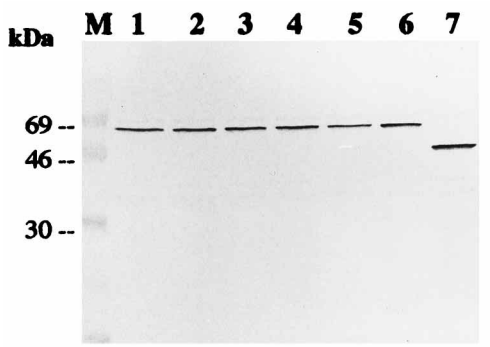

FIG. 1. Expression of the TetC-EgDf1 fusion in *E. coli* as determined by Western blotting and probing with a polyclonal rabbit anti-TetC serum. Lanes 1 to 6, whole-cell lysates from six different colonies of bacteria transformed with pTECH-EgDf1; lane 7, cells harboring the vector pTECH alone; lane M, rainbow molecular mass markers (Amersham, Amersham, United Kingdom).

RESULTS

Cloning and expression of TetC-EgDf1 fusion proteins in *E. coli* and in *Salmonella* vaccine strains. An EgDf1 expression cassette was produced by PCR using as a template a recombinant λgt11 clone comprising the full-length coding sequence of the gene. Oligonucleotide primers Eg1 and Eg2 were designed to amplify the full cDNA gene, beginning with the start codon and finishing at the stop codon. In addition, forward and reverse primers were tailored with *Bam*HI and *Spe*I to allow directional cloning into pTECH which had been previously digested with the same enzymes. The recombinant plasmid was first transformed into *E. coli* TG2, and expression on cells harboring the construct was evaluated by Western blotting and then probing with an anti-TetC serum. A single band of the expected size for the fusion protein was recognized in several different transformants (Fig. 1), and DNA restriction analysis of the plasmid showed the correspondent fragment sizes (data not shown). The construct was transferred to several *Salmonella* vaccine strains, and Western blot analysis showed that the fusion protein was similarly expressed in vaccine strains with different genetic backgrounds or carrying different attenuating mutations (Fig. 2).

Recovery of bacteria from mouse tissues 7 days after i.v. injection of SL3261 carrying pTECH or pTECH-EgDf1 showed no differences in the number of bacteria recovered from the liver or spleen (viable counts between 0.9×10^6 and 1.1×10^6 CFU/organ) or in the percentage of bacteria that retained the plasmid in both constructs (more than 99% in all cases).

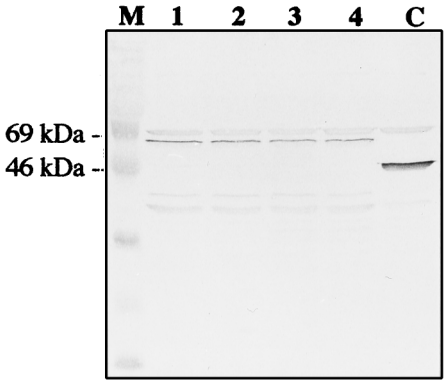

FIG. 2. Expression of the TetC-EgDf1 fusion in different *Salmonella* vaccine strains as determined by Western blotting and probing with rabbit anti-TetC serum. Vaccine strains: SL3261 (lane 1), BRD726 (lane 2), C5 *htrA* (lane 3), and C5046 (lane 4). Lane C, SL3261 harboring pTECH alone; lane M, rainbow molecular mass markers (Amersham).

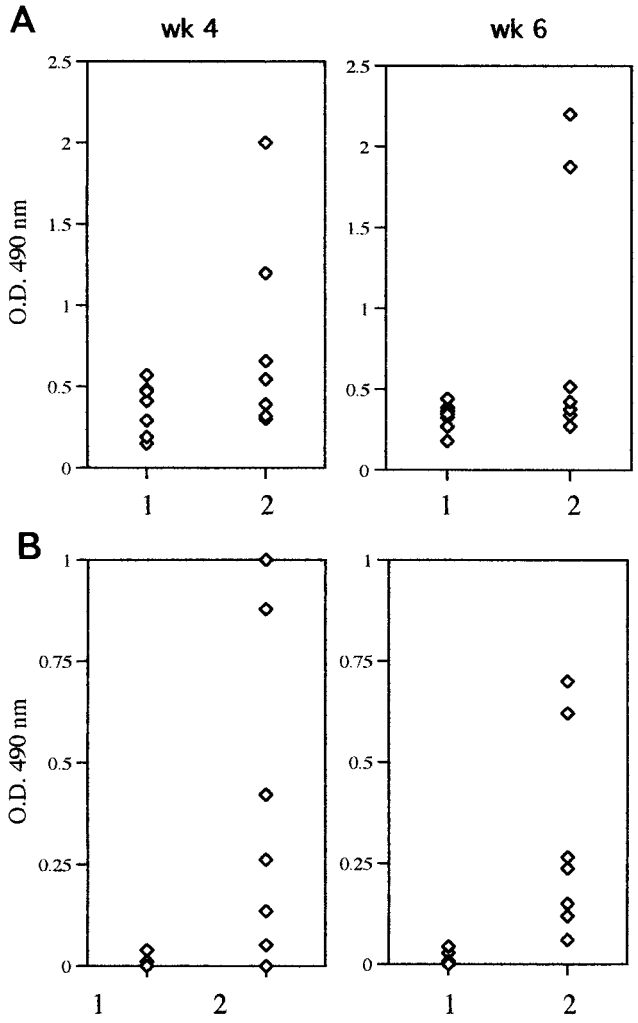

FIG. 3. Total IgG antibody response against rEgDf1 as detected by ELISA analysis of sera taken 4 and 6 weeks after immunization. Results are expressed as OD values for individual mice immunized i.v. (A) or orally (B) with SL3261 (pTECH) (1) or SL3261(pTECH-EgDf1) (2).

Antibody responses. Figure 3 shows the individual total IgG antibody responses against rEgDf1 in mice immunized i.v. (Fig. 3A) or orally (Fig. 3B). Only mice immunized with SL3261 (pTECH-EgDf1) elicited antibodies to EgDf1, although the background response against rEgDf1 in mice immunized with SL3261(pTECH) was much higher in mice immunized i.v. than in orally immunized mice. However, whereas most mice developed an anti-EgDf1 response in the group immunized orally with SL3261(pTECH-EgDf1), only two of the seven mice immunized i.v. developed a detectable response in week 4 or 6. On the other hand, all mice developed anti-TetC antibodies (Fig. 4). However, mice immunized i.v. with the pTECH-EgDf1 construct developed a weaker anti-TetC response than those immunized with bacteria carrying pTECH alone (Fig. 4A). The anti-TetC responses elicited by the two constructs were similar when the recombinant bacteria were delivered

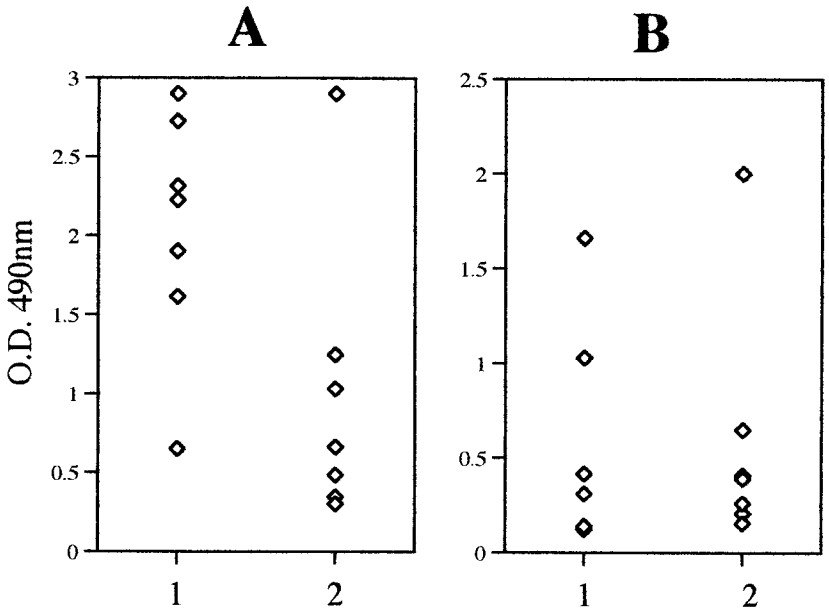

FIG. 4. Total IgG antibody response against TetC as detected by ELISA analysis of sera taken 4 weeks after immunization. Results are expressed as OD values for individual mice immunized i.v. (A) or orally (B) with SL3261(pTECH) (1) or SL3261(pTECH-EgDf1) (2).

orally (Fig. 4B), although they were slightly weaker than the response elicited by SL3261(pTECH) in i.v. immunizations.

Analysis of the isotype distribution of the anti-EgDf1 antibody response in mice immunized orally 6 weeks earlier showed that most of the mice developed specific IgG1 and IgG2a antibodies (Fig. 5). In addition, specific IgA antibodies were detected in all the mice that had previously shown an IgG anti-rEgDf1 response (Fig. 5). An IgE response to rEgDf1 was detectable only in one mouse (result not shown).

Immunohistochemical staining of protoscolices with mouse sera. Sera from mice immunized orally with SL3261(pTECH-EgDf1) were assessed for their capacity to react with larval worms, with the sera of mice immunized with SL3261(pTECH) used as a control.

Indirect immunoperoxidase staining of sections of protoscolices with sera from mice immunized once with SL3261 (pTECH-EgDf1) showed a strong positive response (Fig. 6A) compared with that of sections stained with the control sera (Fig. 6B). The pattern of staining (Fig. 6C) correlates with that obtained previously by using a monospecific anti-EgDf1 serum (10), namely, at the subtegumental level of the protoscolex.

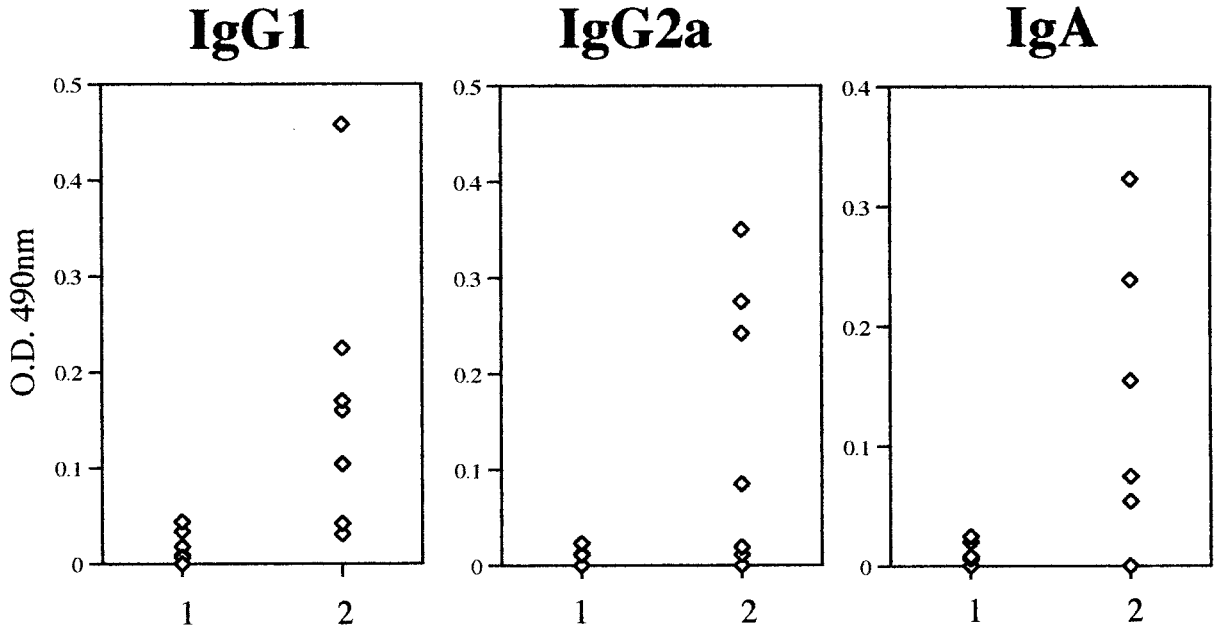

FIG. 5. Isotype distribution of the antibody response against rEgDf1. Results are expressed as OD values for individual mice immunized orally with SL3261 (pTECH) (1) or SL3261(pTECH-EgDf1) (2).

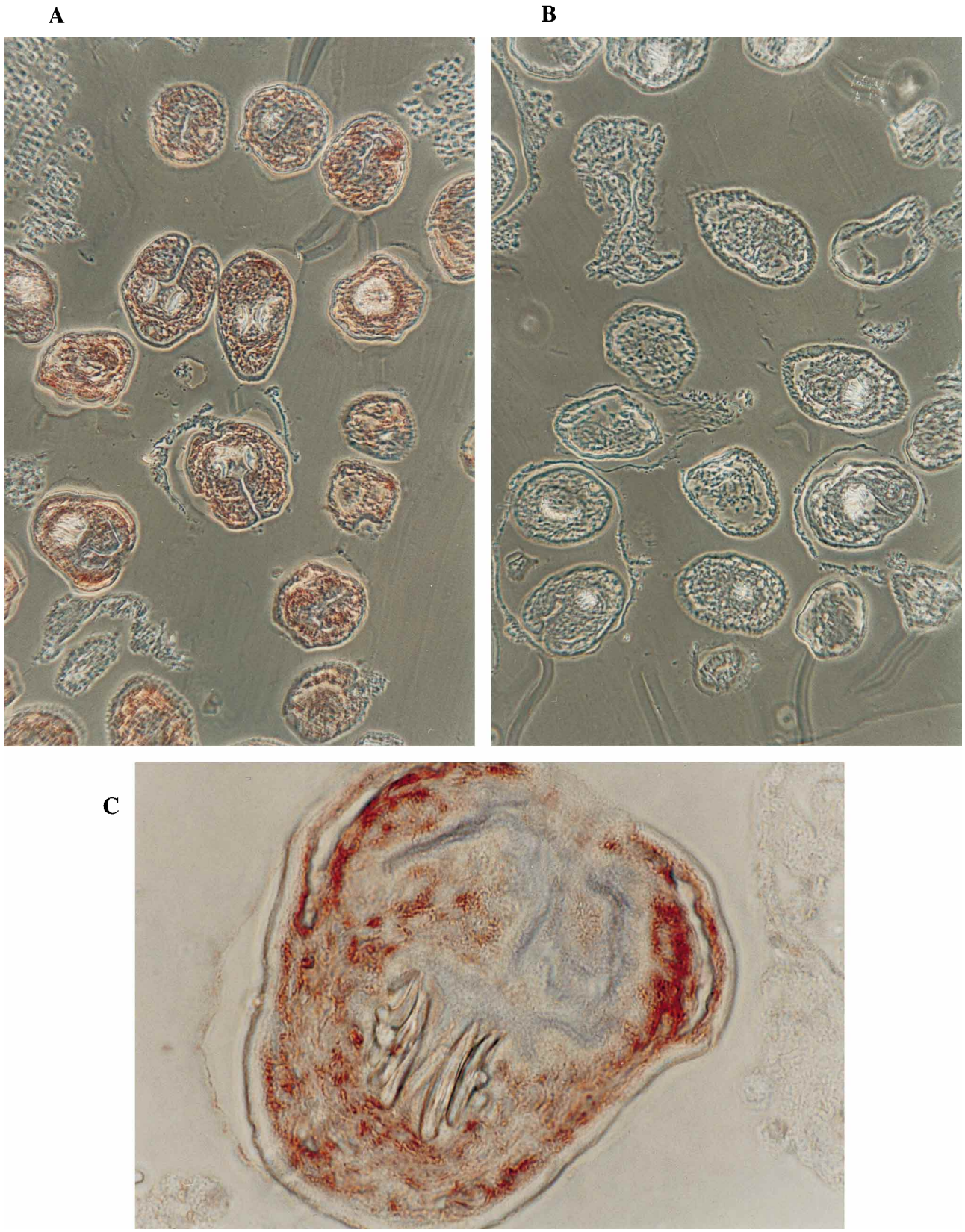

FIG. 6. Light micrograph illustrating the immunoperoxidase staining of sections of protoscolices with pooled sera (1/100) from mice immunized orally with SL3261 (pTECH) (B) or SL3261(pTECH-EgDf1) (A and C). The sections were counterstained with hematoxylin.

In vitro production of IFN-γ and IL-2. Figure 7 shows the IFN-γ response upon stimulation with the different antigens. Stimulation with C5 lysate induced similar responses in all immunized groups (Fig. 7A). A response seen at 24 h in naive mice was no longer present in supernatants collected on the following days.

On the other hand, the IFN-γ response upon stimulation with TetC varied markedly with both the characteristics of the construct and the route of immunization (Fig. 7B). The responses in cells from mice immunized with bacteria expressing TetC or the fusion with EgDf1 were very similar when the mutants were delivered orally. However, for i.v. immunization, a marked reduction of cytokine production was obtained for mice immunized with the pTECH-EgDf1 construct compared with that of the cells from mice that received the construct expressing TetC alone. Furthermore, the response obtained with the latter was greater than those obtained with any of the cultures from mice immunized orally (Fig. 7B).

Stimulation with rEgDf1 elicited production of IFN-γ in cells from all mice, particularly in supernatants collected at day 4. However, a significantly greater response was obtained for cells from mice immunized orally with the bacteria expressing TetC-EgDf1, in supernatants collected at days 1 and 2 after stimulation (Fig. 7C). It is noteworthy that the amount of IFN-γ produced at day 2, when the response seemed to peak, was similar in magnitude to the response to C5 lysate and greater than the values obtained upon stimulation with TetC. Cells from mice immunized i.v. with bacteria carrying the pTECH-EgDf1 construct failed to generate a specific response above the background.

IL-2 production upon stimulation with C5 lysate was detectable only on the first 2 days of stimulation (Fig. 8A). The cytokine response to TetC was again stronger in cells from mice immunized i.v. with SL3261 carrying pTECH alone; in the other immunized groups, it was detectable mainly on day 2 (Fig. 8B). A small but significant production of IL-2 upon stimulation with rEgDf1 was detected on day 1 after stimulation in cells from mice immunized orally with *Salmonella* carrying the pTECH-EgDf1 construct (Fig. 8C).

In vitro production of IL-4 and IL-5. There was no detectable IL-4 production upon stimulation with any of the antigens, although mitogenic stimulation with concanavalin A induced IL-4 production in all cultures, with values ranging from 400 to 700 pg/ml (data not shown).

However, upon stimulation with the different antigens, IL-5 responses were detectable (Fig. 9). Although the responses against either C5 lysate or rTetC showed appreciable scatter between individual cultures, as seen from the high standard deviations obtained, clear specific responses could be detected for supernatants collected 6 days after stimulation (Fig. 9A and B). Moreover, there was a small specific response on days 2 and 4 upon stimulation with rEgDf1 in cells from mice immunized orally with bacteria expressing the fusion protein TetC-EgDf1 (Fig. 9C).

In summary, a cellular (cytokine) response against heterologous antigens expressed in a *Salmonella* vaccine strain was detectable after a single dose of vaccine given orally or i.v. The response was measured as in vitro production of IFN-γ, IL-2, and IL-5 upon stimulation with *Salmonella* antigens, TetC, and rEgDf1. Cells from mice immunized orally with the pTECH-EgDf1 construct produced significantly larger amounts of IFN-γ, IL-2, and IL-5 upon stimulation with rEgDf1 than cells from all other groups. Cells from mice immunized i.v. with the pTECH-EgDf1 construct had a reduced response against rTetC compared with that of cells from mice immunized i.v. with the pTECH construct and failed to generate a stronger response upon stimulation with rEgDf1. However, the cytokine responses to *Salmonella* antigens were similar among all the groups immunized with any of the mutants.

DISCUSSION

The results presented in this study reinforce previous observations (4, 5, 18, 19) on the feasibility of developing multivalent vaccines by constructing C-terminal fusions of recombinant bacterial, viral, or parasitic antigens with TetC by using the vector pTECH and live attenuated *Salmonella* strains as a delivery system. We extend those results here, showing that a single oral dose of recombinant bacteria expressing a FABP of the helminth *E. granulosus* is able to trigger a strong antibody response against the parasite, as detected by the reactivity of the mouse sera with sections of protoscolices, and that cellular responses against the bacteria and the heterologous antigens can be detected as in vitro cytokine production. Furthermore, these results demonstrate that the immune responses against heterologous antigens delivered by *Salmonella* vaccine carriers, as well as against *Salmonella* antigens, comprise antibody isotypes and cytokines currently associated with activation of T cells from both Th1 and Th2 subsets.

A particularly surprising finding was that the antibody response against EgDf1 was greater in mice immunized by the oral route than in mice immunized i.v. (Fig. 3), whereas the reverse was true for the responses to TetC (Fig. 4). However, it is noteworthy that similar results showing stronger immune responses when the oral route is used have been found also by other authors using the *Salmonella* delivery system. The antibody response to a *Chlamydia trachomatis* major outer membrane protein was stronger in orally than in i.v.-immunized mice when the antigen was delivered by SL3261 as a fusion with the *E. coli* LamB protein (12). Also, a recombinant SL3261 strain expressing the cholera toxin B subunit (CT-B) elicited a strong systemic and secretory antibody response against CT-B when the mice were immunized orally, whereas the intraperitoneal route failed to induce a response (38). In addition, it has been shown that protection against *Bacillus anthracis* in mice could be afforded by a protective antigen expressed in SL3261 when the mice were immunized orally but not when they were immunized i.v. (7). Since antigens delivered i.v. or orally are processed by different lymphoid tissues, it is possible that differences in the lymphocyte repertoire could be affecting the efficiency of processing of the antigens and thus promoting differences in the stimulation of the immune response. Moreover, it has been suggested that, as in the case of CT-B, it may be the characteristics of the heterologous antigen itself that determine the immunization route by which it will be more immunogenic (38).

Clear cytokine responses were obtained. Stimulation with either C5 lysate or TetC produced significant amounts of IFN-γ, IL-2, and IL-5 in cells from all immunized groups. In addition, cells from mice immunized orally with the pTECH-EgDf1 construct also produced these cytokines when stimulated with rEgDf1 (Fig. 7 to 9, panels C). Of particular interest was the induction of cells to produce IL-5 upon stimulation with the corresponding antigens (Fig. 9), indicating a Th2 component to the mainly Th1 response observed. However, no IL-4 response could be detected. We have also observed an IL-5 response in the absence of IL-4 in mice immunized with a C5 *htrA* vaccine, alone or carrying the vector pTECH or pTECH fused to tandem copies of a herpes simplex virus gD-derived peptide, in response to C5 lysate and TetC (reference 4 and our unpublished observations). The failure of others to detect IL-5 (2, 40) may be due to the fact that cultures

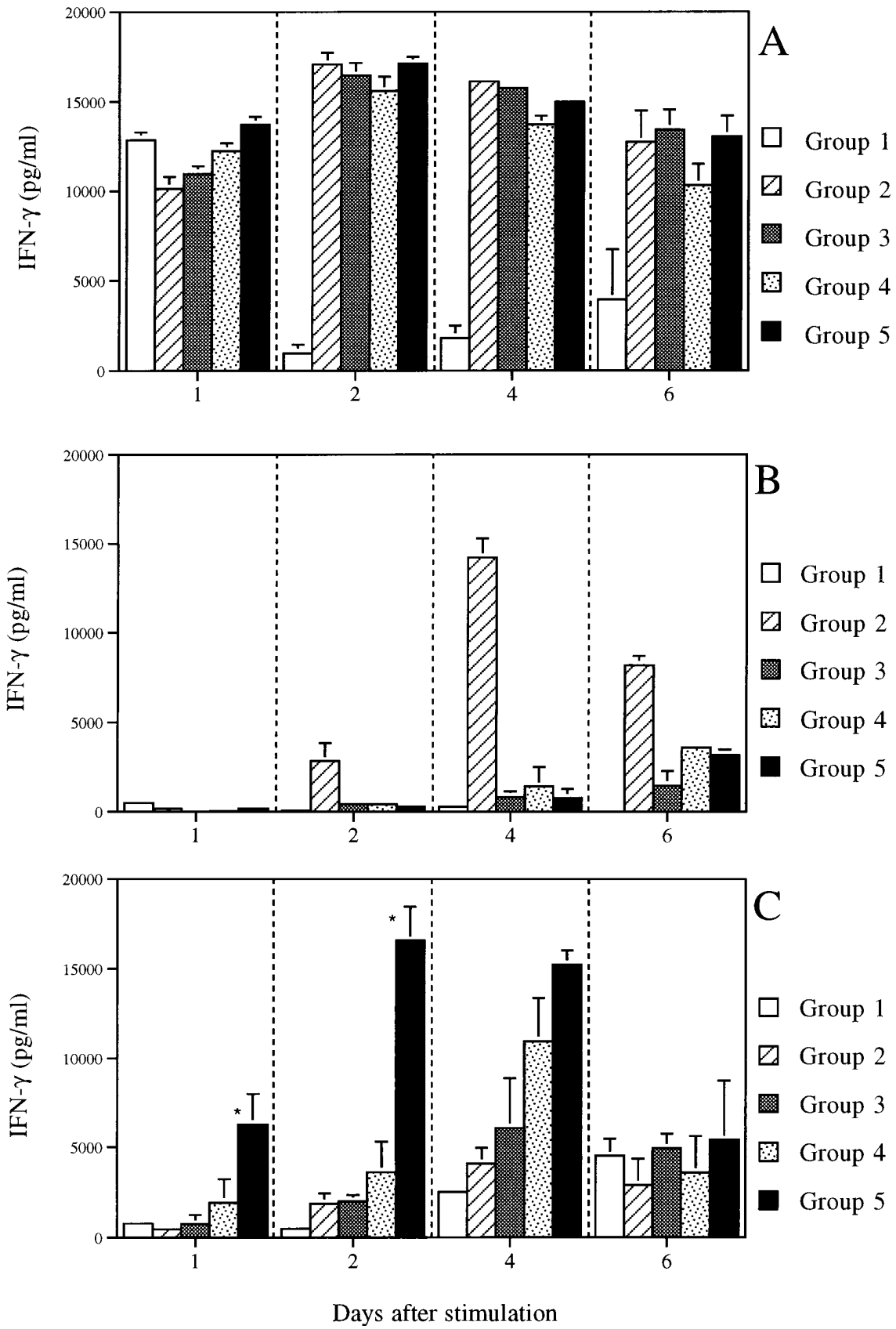

FIG. 7. IFN-γ production by whole spleen cells after stimulation with C5 lysate (A), rTetC (B), or rEgDf1 (C). Results are expressed as the concentration of cytokine in supernatants of cell cultures collected on different days after stimulation, as detected by ELISA. Each value is the average for three different cultures plus the standard deviation. *, $P < 0.05$ compared with group 4. Groups: 1, naive mice; 2 and 3, mice immunized i.v. with SL3261(pTECH) or SL3261(pTECH-EgDf1), respectively; and 4 and 5, mice immunized orally with SL3261(pTECH) or SL3261(pTECH-EgDf1), respectively.

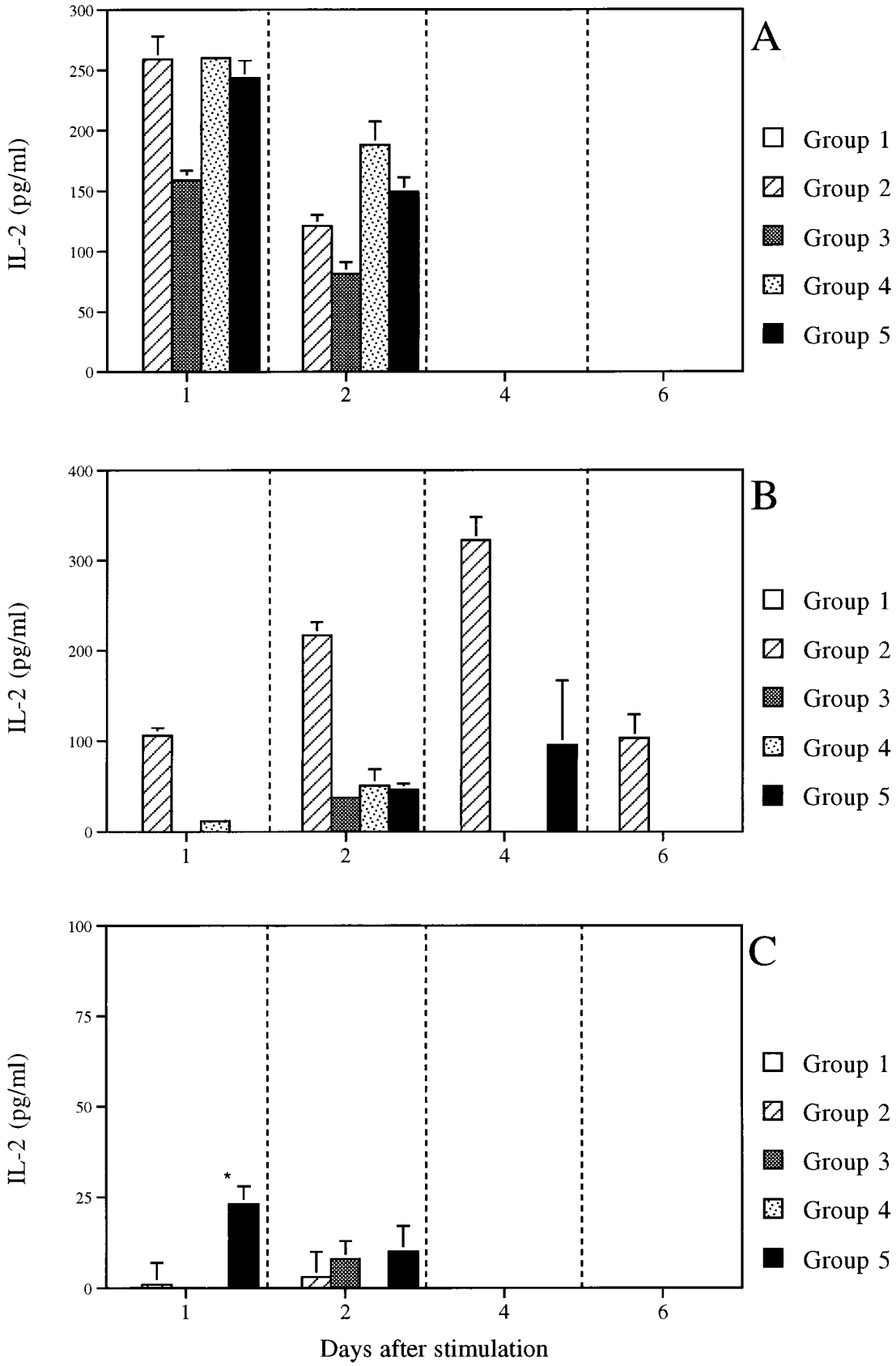

FIG. 8. IL-2 production by whole spleen cells after stimulation with C5 lysate (A), rTetC (B), or rEgDf1 (C). Results are expressed as the concentration of cytokine in supernatants of cell cultures collected on different days after stimulation, as detected by ELISA. Each value is the average for three different cultures plus the standard deviation. *, $P < 0.05$ compared with any other group. Groups are as for Fig. 7.

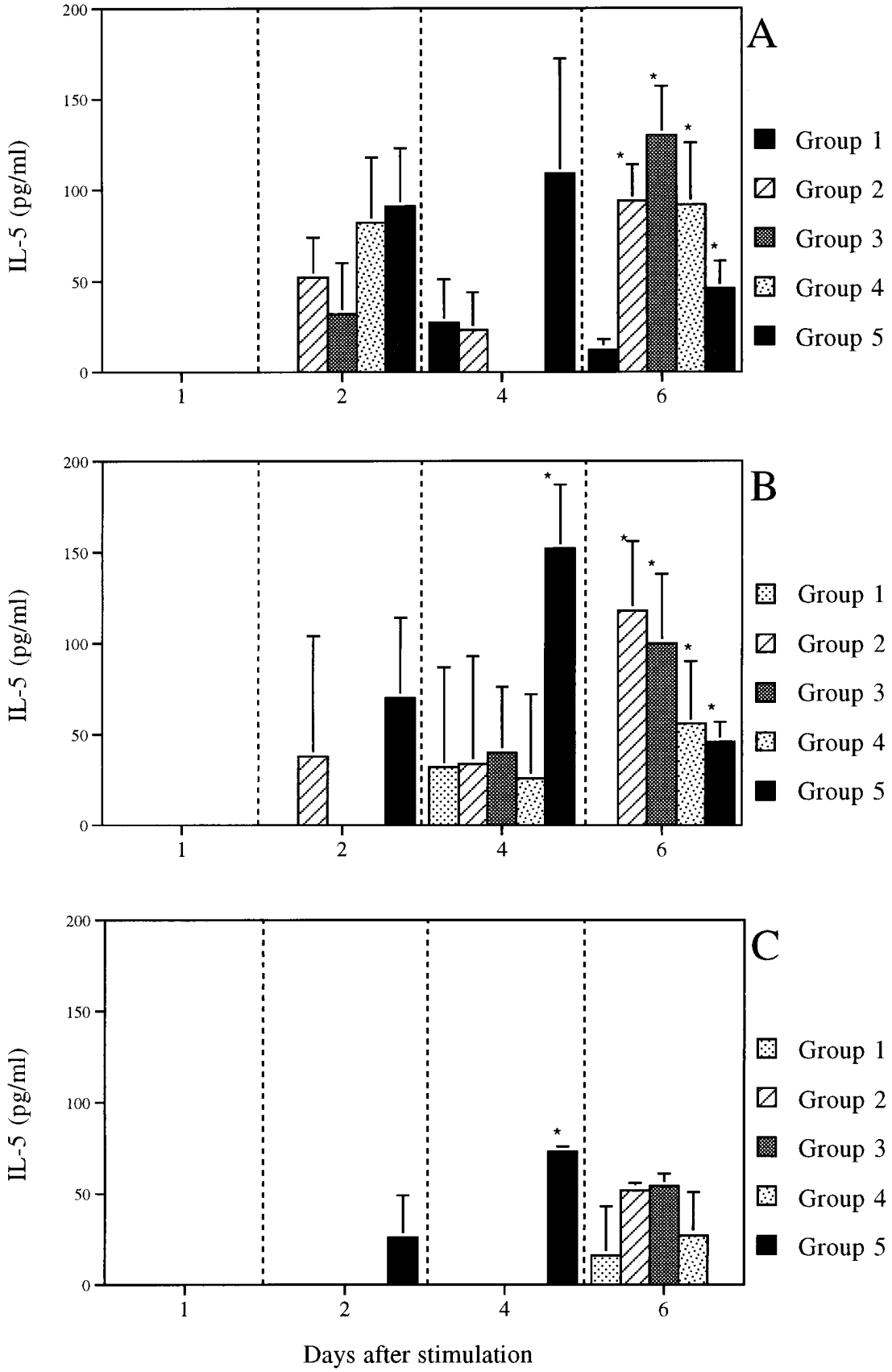

FIG. 9. IL-5 production by whole spleen cells after stimulation with C5 lysate (A), rTetC (B), or rEgDf1 (C). Results are expressed as the concentration of cytokine in supernatants of cell cultures collected on different days after stimulation, as detected by ELISA. Each value is the average for three different cultures plus the standard deviation. *, $P < 0.05$ compared with group 1. Groups are as for Fig. 7.

were not monitored for up to 6 days, as was the case in this study.

Analysis of subclasses of antibody to rEgDf1 in orally immunized mice showed IgG1, IgG2a, and IgA antibodies. This is consistent with the cytokine pattern we observed, IFN-γ being associated with a Th1 response and IgG2a production whereas IL-5 corresponds to a Th2 response and IgG1 and IgA production (6, 11, 20, 25–27, 39). There are few reports on the profile of the IgG subclasses and the T-cell-derived cytokines elicited by heterologous antigens in *Salmonella* vaccine strains. Studies of the immune response against gp63 of *Leishmania major* expressed in an *S. typhimurium* vaccine strain have shown a restricted profile in the B- and T-cell responses elicited in BALB/c mice (42, 43). Anti-gp63 antibodies of the IgG2a but not the IgG1 subclass could be detected, and upon in vitro stimulation of spleen and lymph node cells with freeze-thawed parasites, only IFN-γ and IL-2 were detected (42, 43). Furthermore, the antibody response against the hepatitis B nucleocapsid antigen (HBc) delivered by *Salmonella* comprised significantly higher levels of IgG2a than IgG1 antibodies (30). Other authors, studying the immune response to the influenza virus nucleoprotein (NP) expressed in a *Salmonella* carrier, have found high levels of both IgG1 and IgG2a anti-NP antibodies (2). However, they could not detect IL-5 production in lymph node cells from mice immunized with the construct, although high levels of IFN-γ were produced upon stimulation with a recombinant NP (2). Interestingly, in a more recent work using an *aroA aroD Salmonella* strain expressing TetC from the parental plasmid of pTECH (pTET*nir*15), the anti-TetC response showed significant lower IgG1 than IgG2a antibody levels, and IL-5 production could not be detected in Peyer's patches or spleen cells (40).

In contrast, the results obtained by us using the pTECH system have consistently shown that high levels of IgG1 antibodies and IL-5 production can be elicited against several recombinant antigens, delivered by different *Salmonella* vaccine strains and by using both oral and i.v. routes for the immunizations (references 4 and 5 and our unpublished observations). Furthermore, it has been shown that increasing levels of specific IgG1 antibodies can explain the increase in the total IgG response against a peptide when the number of tandem copies fused to TetC by this system is increased (4).

The reason for these differences in isotype and cytokine responses are not clear, but, as mentioned above, in most cases we could clearly detect IL-5 only after 6 days or more of culture. In any case, the differences found in the kinetics of production of cytokines for each particular antigen suggest that the collection of supernatants at several time points may be advisable when the *Salmonella* delivery system is used.

Although it is not possible to draw conclusions on the association of cytokine production seen in vitro and the production of particular antibody isotypes found in serum, it is noteworthy that in our results the production of Th1- and Th2-derived cytokines correlates with the induction of antibody responses comprising a nonrestricted profile of antibody isotypes and that the presence of IL-5-producing cells is consistent with the generation of specific IgA responses. However, alternative pathways for induction of IgA and IgG1 isotypes in the absence of Th2 cells producing IL-4 and IL-5 have been proposed (9, 40). The induction of IgG1 synthesis dependent on IL-2 has been demonstrated in vitro using Th1 clones stimulated with IL-2 (9). Furthermore, it has been suggested that antigen-specific IgA responses to heterologous antigens expressed in salmonellae may be the result of an alternative pathway in which macrophages producing IL-6 and Th2 cells producing IL-10 provide the signals for the development of mucosal IgA (40). However, it could also be that in vivo other signals are operating and even that IL-4 which could not be detected in vitro could have been produced in vivo.

Finally, the ability of rEgDf1, either as a killed preparation or delivered by a *Salmonella* vaccine strain, to evoke a protective response against *E. granulosus* remains to be tested. However, the ability of FABPs to confer protection against other parasitic helminths is clearly established, suggesting that EgDf1 should be investigated as a possible candidate component of a vaccine for *E. granulosus*. The mechanisms of immunity to *E. granulosus* remain unclear, but expulsion of intestinal parasites appears to involve an immunologically mediated local inflammatory response (reviewed in reference 22). Salmonellae are able to invade through the intestinal epithelium, colonizing Peyer's patches (17), and can evoke local immunity as demonstrated by the production of secretory antibodies (23, 32, 35) and IFN-γ production by gut-associated lymphoid tissues (28), making this an attractive delivery system for relevant antigens of an intestinal parasite. We have shown that a recombinant salmonella expressing EgDf1 elicits humoral and cellular responses to EgDf1. The ability of these constructs to confer protection against *E. granulosus* is currently under evaluation.

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