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Phage-Displayed T-Cell Epitope Grafted into Immunoglobulin Heavy-Chain Complementarity-Determining Regions: an Effective Vaccine Design Tested in Murine Cysticercosis

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A new type of immunogenic molecule was engineered by replacing all three complementarity-determining-region (CDR) loops of the human immunoglobulin (Ig) heavy-chain variable (V_H) domain with the *Taenia crassiceps* epitope PT1 (PPPVDYLYQT) and by displaying this construct on the surfaces of M13 bacteriophage. When BALB/c mice were immunized with such phage particles (PIgphage), a strong protection against challenge infection in very susceptible female hosts was obtained. When specifically stimulated, the in vivo-primed $CD4^+$ and $CD8^+$ T cells isolated from mice immunized with PT1, both as a free peptide and as the PIgphage construct, proliferated in vitro, indicating efficient epitope presentation by both major histocompatibility complex class II and class I molecules in the specifically antigen-pulsed macrophages used as antigen-presenting cells. These data demonstrate the immunogenic potential of recombinant phage particles displaying CDR epitope-grafted Ig V_H domains and establish an alternative approach to the design of an effective subunit vaccine for prevention of cysticercosis. The key advantage of this type of immunogen is that no adjuvant is required for its application. The proposed strategy for immunogen construction is potentially suitable for use in any host-pathogen interaction.

Over the last few years, M13 and other filamentous phages have been used as expression vectors in which foreign gene products are fused to the phage coat proteins and are displayed on the surfaces of the phage particles. Phage-displayed peptide (9, 25) and antibody (Ab) (1, 36) libraries have been widely used in numerous studies. One of the important properties of phage particles is their high immunogenicity in different animal systems, and the use of genetically engineered filamentous phages as antigens for Ab production has been reported (14, 23). There is, however, a single study in which a recombinant phage displaying a disease-specific protective B-cell epitope was used as a vaccine to confer protection against human respiratory syncytial virus infection in mice (2). Also, the phage particles displaying recombinant anti-idiotypic Ab ScFv (single-chain fragment-variable) fragments expressed on the phage were used in maternal immunization, protecting neonatal mice against streptococcal infection (18).

Recently, Abs carrying antigenic peptides grafted into their complementarity-determining-region (CDR) loops at the immunoglobulin (Ig) heavy-chain variable (V_H) region have been shown to be highly immunogenic and to serve as a very efficient vehicle to load the inserted epitopes onto major histocompatibility complex (MHC) molecules after processing by antigen-presenting cells (APC) (7, 37, 39, 41). Thus, it has been shown that a T-cell epitope of influenza virus nucleoprotein inserted into the CDR3 loop of the V_H region of Ig was able to prime the virus-specific T cells in vivo (38). When influenza virus T- and B-cell epitopes were introduced into the CDR2 and CDR3 loops of the Ig V_H domain, respectively, the DNA-immunized mice were protected against challenge with lethal doses of the virus (8).

So, taking advantage of the observations that Abs carrying T-cell epitopes inserted into CDR3 or CDR2 loops of the Ig V_H domain and phages displaying a B-cell epitope or anti-idiotypic Ab ScFv fragment are strong immunogens, we have developed a new concept for immunogen construction, designing a human Ig V_H domain grafted to a 10-amino-acid T-cell epitope, PT1, from the *Taenia crassiceps* antigen KETc7 (20) displayed on the M13 phage surface. The resulting PIgphage construct was used to immunize mice against experimental *T. crassiceps* cysticercosis, the simple disease model for testing candidate vaccine preparations against *Taenia solium* pig and human cysticercosis—a highly damaging and prevalent parasitic disease in the third world (20). To our knowledge, there is no report of the use of recombinant bacteriophages expressing any T-cell epitope alone or in the context of antigenized Abs or their fragments as immunogens. In our study, the mice immunized with the free synthetic T-cell epitope or with PIgphage developed a strong specific cellular immune response and resistance to challenge infection. The results point to this PT1 epitope as a promising vaccine candidate against cysticercosis and to the Ig V_H-phage construct as an effective and inexpensive strategy for large-scale production of vaccines against various diseases.

MATERIALS AND METHODS

Immunogen construction. A set of partially overlapping oligonucleotides collectively coding for the framework regions of the human Ig V_H domain DP47 (OL.1, -3, -5, -6, and -8) (34) and the *T. crassiceps* T-cell epitope PT1 (PPPVDYLYQT) (OL.2, -4, and -7) was synthesized at Operon Technologies, Inc., Alameda, Calif. The oligonucleotides used were as follows: OL.1, GAGGTGC AGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCC TGAGACTCTCCTGTGCA; OL.2 (PT1/H1), GCCTGGCGGACCCATGTCT

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GG TACAGATAATCAAC TGGCGG TGG TGCACAGGAGAG TC T; OL.3, TGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCA; OL.4 (PT1/H2), GCCCTTCACGGAGTCTGTCTGGTACAGATAATCAACTGGC GGTGGTGGTGAGACCCACTCCA; OL.5, GACTCCGTGAAGGGCCGGT TCACCATCTCCAGAGACAAT TCCAAGAACACGC TG TATC TGCAAAT GAAC; OL.6, ACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGG ACACGGCCGTATATTACTGTGCG; OL.7 (PT1/H3), GCCGTATATTACT GTGCGCCACCGCCAGTTGATTATCTGTACCAGACATGGGCCAGGGA ACCCTGGTC; OL.8, TGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA; 5′VR, GATGAATTCTGAGGTGCAGCTGTTGGAGTCTGG; and 3′VS, CTCG TCGACACGGTGACCAGGGTTCCCTGGCCC. Oligonucleotides 1 to 8 listed above (4 pmol each; the overlaps between the complementary oligonucleotides are 14 to 21 nucleotides) were combined and assembled in PCR (27) with *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) by cycling the reaction mixture (50 μl) 30 times (95°C for 2 min; 56°C for 2 min; 72°C for 1 min). An aliquot from this reaction (5 μl), containing a 350-bp DNA fragment coding for the Ig V_H domain with the CDRs replaced by PT1 sequence, was amplified by PCR (50 μl) by cycling 30 times (94°C for 1 min; 65°C for 1 min; 72°C for 1 min) with the 5′VR and 3′VS primers (30 pmol each), which introduce *Eco*RI and *Sal*I restriction sites at the 5′ and 3′ ends of the synthesized Ig V_H domain, respectively. The assembly and amplification of PCR products were checked by agarose gel electrophoresis, and the DNA of the engineered V_H domains, after purification from the gel with a Master Kit (Bio-Rad Laboratories, Hercules, Calif.), was cut with *Eco*RI and *Sal*I (Stratagene) and purified again. Then, 1 μg of this DNA was ligated with 10 U of T4 DNA ligase (Amersham-Life Science, Cleveland, Ohio) to approximately 1 μg of *Eco*RI- and *Sal*I-digested DNA of the pFACIB3 phagemid vector (kindly provided by J. Gavilondo) to fuse Ig V_H in frame with M13 minor coat protein III (cpIII). The competent XL-1 Blue cells (Stratagene) were transformed with the ligation mixture, and the PIgphage phagemid clone obtained was rescued and amplified with helper phage VCS-M13 (Stratagene) as described previously (24). The correct PCR assembly and cloning were verified by dideoxy sequencing with [α-^{35}S]dATP (Amersham) and the T7 Sequenase Quick-Denature plasmid sequencing kit (Amersham). Also, the same Ig V_H domain gene with the CDRs replaced by PT1 sequence was cloned under the cytomegalovirus promoter into the eukaryotic expression vector pcDNA3 (Invitrogen) at *Eco*RI/*Sal*I restriction sites, resulting in the pcDVH18 clone.

A nonrelated phage (NF) was previously isolated from a phage display heptapeptide library; it contains an autoimmune thrombocytopenic purpura-related epitope (ATSAIHG) displayed on the M13 phage surface (11).

Synthetic peptide. To predict T-cell epitopes in *T. crassiceps* KETc7 protein (20), the method described by Margalit et al. (22) was used and the sequence PPPVDYLYQT with the highest amphipathic score was selected. The peptide PT1 (AAPPPVDYLYQTA) was prepared by stepwise solid-phase synthesis by the N^{α}-tert-BOC strategy, essentially as described previously (12). Purification was carried out by reverse-phase high-performance liquid chromatography on a Waters (Milford, Mass.) Delta Pak C_{18} column (7.8 by 150 mm) with a linear gradient (water-acetonitrile in 0.1% trifluoroacetic acid). The correct amino acid sequence was confirmed by protein sequencing on a pulsed liquid-phase protein sequencer (Applied Biosystems) at the Instituto Nacional de Cardiología by F. Masso. The molecular weight of the peptide was determined by fast-atom bombardment mass spectrometry on a JEOL JMS-SX102A mass spectrometer at the Instituto de Quimica, Universidad Nacional Autonoma de Mexico (Mexico D.F., Mexico) by L. Velasco.

Immunization and protection assays. Four- to 6-week-old female BALB/c mice, originally purchased from Jackson Laboratories (Bar Harbor, Maine) and maintained at our animal facilities, were used. On days 0, 14, and 28, groups of 7 or 10 mice were immunized by intradermal (i.d.) inoculation and then boosted two times intraperitoneally (i.p.) with PIgphage (2×10^{10} phage), NF (2×10^{10} phage), Tris-buffered saline (TBS), or PT1 peptide (50 μg) in a 200-μl volume. Groups of six mice were immunized by single i.d. inoculations with PIgphage and NF. PT1 was administered in complete Freund's adjuvant with two boosts in incomplete Freund's adjuvant, and the phages were applied in TBS. Fourteen days after the last inoculation, the mice were challenged with *T. crassiceps* cysticerci, and 7 weeks later the individual parasite load in the peritoneal cavity of each mouse was counted as described previously (20). The data presented are representative of two experiments performed.

Separately, groups of five mice were immunized by single i.d. inoculations with PIgphage, NF, or PT1 peptide and with 100 μg of total antigen extract of *T. crassiceps* (TAg) (20) and used in lymphoproliferation assays. Also, sera from *T. crassiceps*-infected and noninfected mice were obtained and used in enzyme-linked immunosorbent assays (ELISAs).

Lymphoproliferation and cytokine assays. Peritoneal macrophages (Mϕ) were used as APC in all cell cultures, as previously reported (21). Briefly, Mϕ were elicited in healthy female BALB/c mice by i.p. injection with 3 ml of 3% thioglycolate medium. Four days later, the mice were killed and Mϕ were isolated by washing the peritoneal cavity with cold phosphate-buffered saline (PBS), following the procedure described elsewhere (19). The Mϕ were scraped, adjusted to 10^6/ml, and seeded in the presence of PT1 (50 μg/ml), TAg (50 μg/ml), phage particles (2×10^9), pcDVH18 plasmid DNA (10 μg), or pcDNA3 plasmid DNA (10 μg). The plasmid DNAs were isolated with a Plasmid Midi kit (Qiagen Inc., Chatsworth, Calif.). After 3 h of incubation at 37°C in 5% CO_2, the Mϕ were scraped again and adjusted to 10^6/ml, and 100 μl of this suspension was seeded in flat-bottomed 96-well plates (Falcon, Oxnard, Calif.). The Mϕ were cocultured with pools of $CD4^+$ or $CD8^+$ splenic cells (5×10^4) isolated from the mice (five mice per group) 10 days after the single inoculations with corresponding antigens, as described above. These cells were magnetically isolated with anti-CD4 and anti-CD8 monoclonal antibodies (PharMingen, San Diego, Calif.) bound to ferritin on a magnetic column (Miltenui Biotec, Bergisch Gladbach, Germany), as recommended by the manufacturer. Typically, the cell preparations were >90% pure as determined by staining and flow cytometric analysis. Plates were cultured in triplicate for 5 days, and 18 h before harvesting, 0.5 μCi of tritiated thymidine ([*methyl*-^{3}H]TdR; specific activity, 247.9 GBq/mmol; NEN, Boston, Mass.) was added to the wells. The radioactivity in pelleted cells was measured with a Betaplate scintillation counter (Wallac). The results were expressed as total counts per minute. The levels of cytokines (gamma interferon [IFN-γ], interleukin-2 [IL-2], and IL-4) produced by T cells after 72 h of incubation with the corresponding antigen-pulsed Mϕ were measured by sandwich ELISA with cytokine-specific monoclonal antibodies (PharMingen) according to the manufacturer's instructions. The tests were done in duplicate. The data presented are representative of two experiments performed.

ELISA. The levels of *T. crassiceps*- and phage-specific Abs in immune sera pooled from mice within each group were measured by ELISA essentially as described previously (11, 12). Briefly, flat-bottomed microtitration plates (96 well; Nunc, Roskilde, Denmark) were coated (in duplicate) with *T. crassiceps* TAg or PT1 peptide diluted in 0.2 M carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. Separate plates were coated with PIgphage or NF (10^9 phage/well) and incubated overnight at 4°C. After being washed with PBS, the plates were incubated with test sera diluted in PBS (1:20) containing 1% bovine serum albumin at 37°C for 1 h. Washing with PBS was repeated, and alkaline phosphatase-conjugated anti-mouse IgG (whole molecule) (Sigma, St. Louis, Mo.) was added. The subsequent reaction with *p*-nitrophenyl phosphate substrate (Sigma) in diethanolamine buffer (pH 9.8) was stopped by addition of 2 N NaOH. The absorbance was read at 405 nm with an automated ELISA reader. Another ELISA was performed with plates coated with murine sera diluted in carbonate-bicarbonate buffer. After overnight incubation at 4°C, the plates were washed with PBS and PIgphage or NF (10^9 phage/well) was added. The plates were incubated overnight at 4°C and washed with PBS, and rabbit anti-M13 serum (Stratagene) diluted in PBS (1:5,000) was added. After incubation at 37°C and washing with PBS, alkaline phosphatase-conjugated anti-rabbit IgG (whole molecule) was added, followed by reaction with *p*-nitrophenyl phosphate. The absorbance was read as described above.

Statistical analysis. Parasite load data were analyzed for homoscedasticity by Levene's test. Analysis of variance was then applied, considering all of the groups tested. Pairwise comparisons were made with Tukey's post hoc test.

RESULTS

Engineering phage particles carrying PT1 grafted into the Ig V_H domain. First, the T-cell epitope PT1 (PPPVDYLYQT) was predicted (with GeneWorks from IntelliGenetics, Campbell, Calif.) by analyzing the amino acid sequence of the *T. crassiceps* proline-rich protective antigen KETc7 isolated from a cDNA expression library (20). The algorithm of Margalit et al. (22) was used, and the calculated amphipathic score was highest for the PT1 sequence.

The general strategy of immunogen construction is summarized in Fig. 1. A set of synthetic overlapping oligonucleotides based on the DNA sequences of the framework regions of the human Ig germ line DP47 segment were used in PCR assembly to construct an entirely synthetic Ig V_H domain carrying a 30-bp DNA fragment which codes for a PT1 epitope inserted in all three V_H CDR loops. After PCR reamplification with 5′ and 3′ flanking primers carrying restriction sites, the antigenized V_H segment was cloned into the *Eco*RI and *Sal*I sites of the phagemid vector pFACIB3, resulting in the PIgphage clone, in which the V_H domain was fused in frame to phage cpIII. The correct PCR assembly and cloning were verified by DNA sequencing. The nucleotide sequence of the CDR-grafted PT1 epitope and a diagram of the V_H-PT1 chimeric polypeptide are shown in Fig. 1. Using the rescue procedure, the PIgphage clone was amplified and the phage particles were directly used in immunization trials. In parallel, the same Ig V_H domain containing CDRs replaced by the PT1 epitope was cloned into the eukaryotic expression vector pcDNA3 (Invitrogen) by using *Eco*RI/*Sal*I sites to obtain pcDVH18, which was used to pulse Mϕ in a T-cell proliferation assay.

To evaluate the immunogenicity of the *T. crassiceps* epitope

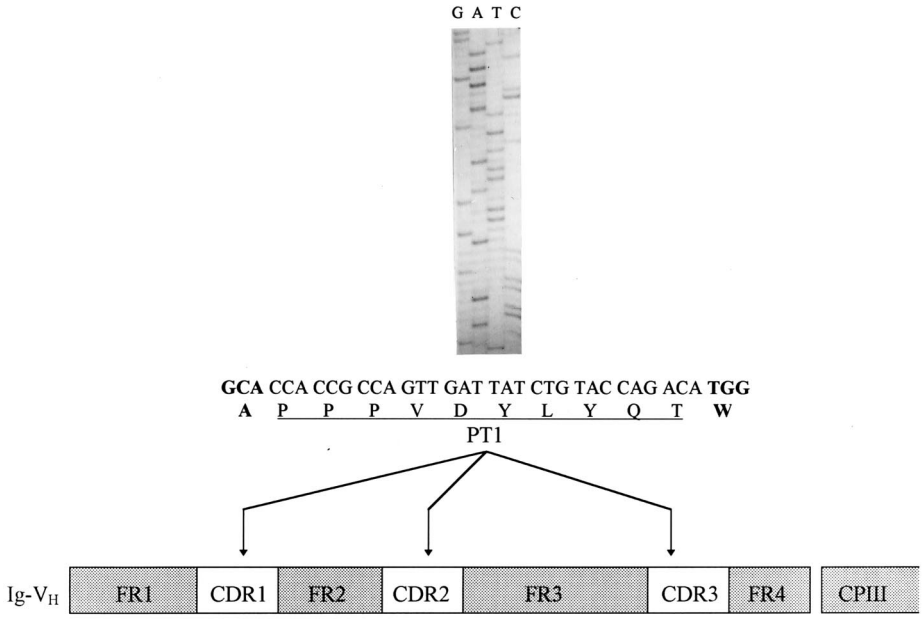

FIG. 1. Schematic presentation of the engineered PIgphage immunogen. Oligonucleotides coding for the PT1 epitope (underlined) were inserted by PCR assembly into the CDRs of the Ig V_H domain and cloned into the phagemid vector as a fusion with cpIII (see Materials and Methods). The correct PCR assembly was verified by DNA sequencing, and the sequence of the PT1 epitope, inserted into the CDR1 loop, is shown. Indicated in boldface letters are the alanine and tryptophan residues from the FR1 and FR2 regions, respectively. FR, immunoglobulin heavy-chain variable-domain framework region.

as a free peptide, the PT1 peptide was produced by solid-phase synthesis as reported previously (12).

Immunization and protection assays. In order to analyze the protective potential of the PT1 epitope integrated in a PIgphage construct and as the free peptide, female BALB/c mice were immunized and tested in challenge experiments. Ten mice were immunized i.d. with 2×10^{10} PIgphage particles without adjuvant and boosted two times with the same immunogen by the i.p. route at 2-week intervals. The free peptide was injected i.d. in complete Freund's adjuvant, and the animals were boosted two times with PT1 in incomplete Freund's adjuvant. Mice from control groups were injected in the same way with NF and TBS alone. Two weeks after the last injection, the mice were challenged i.p. with *T. crassiceps* cysticerci, and 7 weeks later the level of protection was estimated by counting the individual parasite load in the peritoneal cavity of each mouse as described in detail elsewhere (20). Separately, mice were immunized with TAg, and T cells from these mice were used later in lymphoproliferation assays. These mice were not challenged with the parasite.

As shown in Fig. 2A, very similar protection effects were observed with the same epitope in both forms of presentation, as the free peptide and as PIgphage. Five of 10 and 4 of 7 mice immunized with PT1 peptide and the PIgphage construct, respectively, were completely protected against challenge, while in the rest of the immunized mice, with a single exception in both cases, a dramatic reduction in parasite load was obtained (80%) (Fig. 2A). Although few animals were tested, a single i.d. inoculation with PIgphage also induced strong protection against parasite challenge (Fig. 2B). In the mice immunized with the nonrelated phage NF, only a slight reduction in parasite load was observed compared to that in the TBS-immunized mice.

Immune response to vaccination. To study the type of protective immune response induced by the PT1 epitope in both forms of presentation and to find out whether immunization elicited a T-cell-proliferative response to this epitope, spleen lymphocytes and sera were obtained from the immunized mice (five mice in each group). $CD4^+$ and $CD8^+$ T cells were separated and tested in in vitro-proliferation assays to determine whether these in vivo-primed lymphocytes could be stimulated with the antigens. In general, proliferation was observed when $CD4^+$ and $CD8^+$ T cells from mice immunized with PT1 peptide, PIgphage, and TAg were stimulated in vitro with the same immunogens, with Mϕ as the APC (Fig. 3). In contrast,

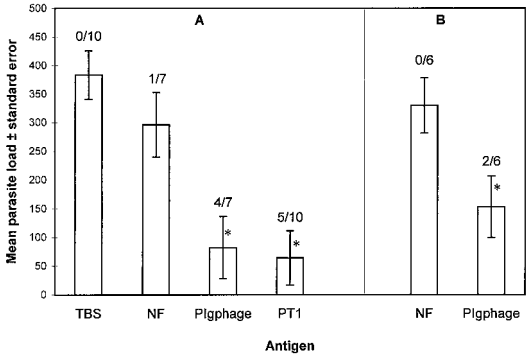

FIG. 2. Protection against cysticercosis in immunized mice. The individual parasite loads were counted in mice immunized with corresponding antigen 7 weeks after challenge with *T. crassiceps* cysticerci. The number of completely protected mice of the total number of mice tested in each group is shown above each bar. (A) On days 0, 14, and 28, groups of mice were immunized by i.d. inoculation and boosted two times i.p. with PIgphage (2×10^{10} phage), nonrelated phage NF (2×10^{10} phage), and buffer solution (TBS) or free peptide (PT1) (50 μg) in a 200-μl volume. Fourteen days after the last inoculation, the mice were challenged with *T. crassiceps* cysticerci. (B) Mice were immunized by a single i.d. inoculation with PIgphage (2×10^{10} phage) and NF (2×10^{10} phage). Fourteen days later, the mice were challenged with *T. crassiceps* cysticerci. *, contrast with the negative controls (immunized with buffer and nonrelated phage) is statistically significant ($P < 0.05$).

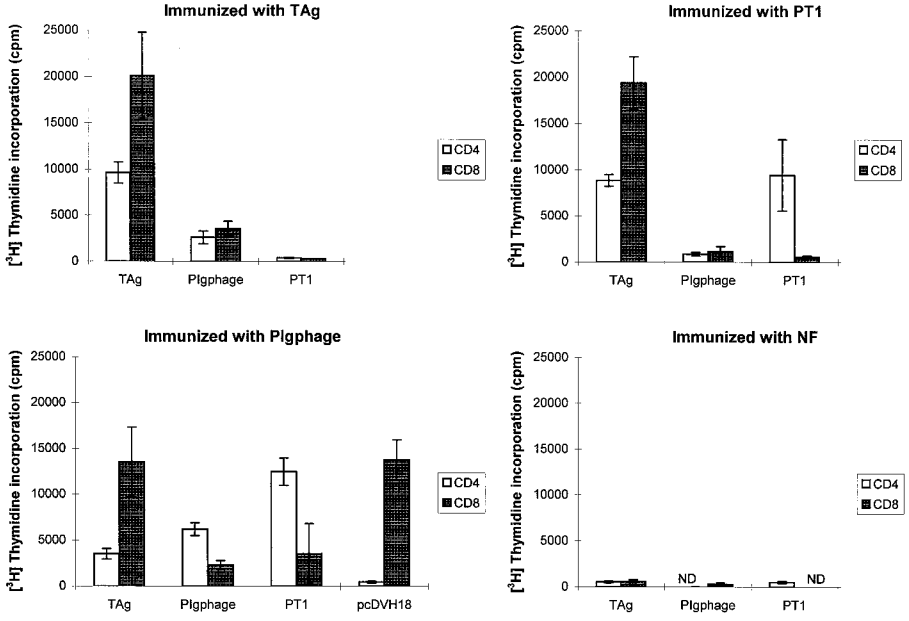

FIG. 3. T-cell proliferation assay. The pools of $CD4^+$ and $CD8^+$ T cells (5×10^4) were isolated from the spleens of mice immunized with various antigens (five mice in each group) and were cultured for 5 days with intact Mϕ pulsed in vitro with *T. crassiceps* TAg (50 μg/ml), recombinant PIgphage (2×10^9), synthetic PT1 peptide (50 μg/ml), and pcDVH18 plasmid DNA (10 μg). T cells not cultured with Mϕ have shown a basal level of [^{3}H]thymidine incorporation of <500 cpm. Each point represents the mean of determinations from triplicate wells ± standard deviation. ND, not determined.

no proliferation was observed in T cells isolated from mice immunized with NF phage (Fig. 3) or when T cells were cultured with NF-pulsed Mϕ (data not shown). The levels of efficiency of the epitope presentation, however, differed for the three antigens. In almost all cases, the most efficient antigen was TAg, although for T cells from PIgphage-immunized mice, PIgphage and PT1 peptide were better stimulators for $CD4^+$ cells, and $CD8^+$ cells proliferated equally upon stimulation by TAg and pcDVH18 DNA. The nonresponsiveness of T cells from TAg-immunized mice to PT1 peptide stimulation possibly indicates the relative insignificance of the PT1-specific immune response when a complex antigen mixture is used as the immunogen. In contrast, the same cells respond positively to stimulation with the same epitope on a PIgphage carrier, indicating that the PT1 epitope as part of a PIgphage particle is more efficiently processed and presented to T cells by Mϕ than as a free peptide. Similarly, PIgphage and PT1 peptide were stimulatory for the T cells from PIgphage-immunized mice but not for the T cells from PT1 peptide-primed mice stimulated with the same antigens, except for $CD4^+$ cells stimulated with the peptide. Mϕ were also pulsed with pcDVH18 plasmid DNA carrying the V_H domain with CDRs grafted by the PT1 epitope to test the presentation of endogenously synthesized epitope. As expected, in this case the PT1 epitope was effectively presented by MHC class I molecules to $CD8^+$ T cells but not to $CD4^+$ T cells from PIgphage-immunized mice, as demonstrated in the T-cell proliferation assay (Fig. 3). The same T cells from PIgphage-immunized mice were cocultured with Mϕ pulsed by pcDNA3 vector plasmid DNA as a control and did not proliferate (data not shown).

Cytokine production was measured in supernatants of cultured T cells with different immunization protocols. In most cases, $CD4^+$ and $CD8^+$ T cells produced high levels of IFN-γ after stimulation by Mϕ pulsed with TAg, PIgphage, and PT1 peptide (Table 1). Although the PIgphage- and PT1 peptide-stimulated $CD8^+$ cells from PIgphage-immunized mice showed less proliferation than the $CD4^+$ cells (Fig. 3), they produced more IFN-γ. The highest levels of IFN-γ were produced by PIgphage-stimulated $CD4^+$ cells from TAg- and PT1-immunized mice and by PIgphage-stimulated $CD8^+$ cells from TAg-immunized mice, while the lowest levels were detected in supernatants of NF-stimulated $CD4^+$ cells. Surprisingly, low levels of IFN-γ were produced by PIgphage- and TAg-stimulated $CD4^+$ cells from mice immunized with the

TABLE 1. IFN-γ production by antigen-stimulated T cells[a]

Mice immunized with:	IFN-γ production (pg/ml) by:							
	$CD4^+$ cells stimulated with:				$CD8^+$ cells stimulated with:			
	PIgphage	TAg	PT1	NF	PIgphage	TAg	PT1	NF
PIgphage	411	2,504	1,130	474	1,362	ND	2,081	1,003
TAg	4,051	138	491	103	2,588	2,546	1,700	<50
PT1	3,011	2,948	72	116	85	<50	<50	138
NF	<50	<50	<50	<50	ND	ND	ND	ND

[a] The pools of $CD4^+$ and $CD8^+$ cells (5×10^4) from mice (five mice in each group) immunized with corresponding antigen were cultured in the presence of Mϕ stimulated with different antigens (see the legend to Fig. 2). Supernatants from duplicate cultures were harvested after 72 h and assayed for IFN-γ production. ND, not determined.

TABLE 2. Antibody response in immunized mice[a]

Plate coated with	Response when incubated with:					
	Normal serum	Positive serum	Anti-PIgphage	Anti-NF	PIgphage	NF
PT1	0.184	0.248	0.135	0.168		
TAg	0.554	2.258	0.469	0.547		
PIgphage	0.484	0.366	1.856	1.720		
Normal serum					0.289	0.525
Anti-PIgphage					1.624	1.627
Anti-TAg					0.247	0.533
Anti-NF					1.929	1.855

[a] The presence of specific antibodies was measured by ELISA as described in Materials and Methods. Sera from *T. crassiceps*-infected and noninfected mice were used as positive and negative controls, respectively. Phage particles (PIgphage and NF) were used at 2×10^9. Sera used were diluted 1:20. Anti-PIgphage, anti-TAg, and anti-NF are pooled sera from mice immunized with the corresponding antigen (five mice in each group).

respective antigens (Table 1). In the same supernatants, IL-4 was not detected and IL-2 was found only when $CD8^+$ cells from PIgphage-immunized mice and $CD4^+$ cells from PT1-immunized mice were cultured with PIgphage-pulsed Mϕ (data not shown).

The levels of *T. crassiceps*-specific Abs in pooled sera from the immunized mice was measured by ELISA (12). As shown in Table 2, no positive signal was detected after incubation of the antigens containing the PT1 epitope (PT1, TAg, and PIgphage) with sera from PIgphage-immunized mice. Similar results were obtained with ELISA plates coated with the respective antisera and incubated in the presence of PIgphage (Table 2). In contrast, high levels of phage-specific antibodies were detected in sera of PIgphage and NF-immunized mice (Table 2), confirming the high immunogenicity of the phage itself. Interestingly, the same high levels of phage-specific Abs were detected in two different ELISA protocols: when plates were coated with PIgphage particles and incubated with the sera from PIgphage- and NF-immunized mice and when the plates were coated with the same sera and incubated in the presence of phages (Table 2).

DISCUSSION

The principal goal of the present research was to explore the immunogenic capacity of the Ig V_H domain with CDRs replaced by a pathogen-specific epitope and expressed as a fusion product coupled to cpIII on the M13 phage surface. To achieve this aim, an entirely synthetic Ig V_H segment was engineered by PCR assembly by simultaneously inserting a T-cell epitope, PT1 (PPPVDYLYQT), from *T. crassiceps* recombinant antigen KETc7 previously isolated from a cDNA expression library (20) into all of the CDRs of the V_H domain (Fig. 1). In KETc7 proline-rich antigen (29% proline), prolines are tandemly repeated and are possibly involved in multiple epitopes. Generally, the proline-containing peptides are involved in many immunologically important phenomena and are commonly present in proteins at solvent-exposed sites, such as loops and turns (29), and V_H loops seemed to be suitable sites for the introduction of peptide epitopes. According to our experimental design, we were expressing only the Ig V_H domain, so altering the CDR structure by peptide insertion was not problematic, as it could be when expressing Fab fragments or the whole Ig molecule, where changes in CDRs can cause misfolding of Ig chains (37). As an example of a successful application of phages for vaccination purposes, the construction of recombinant anti-idiotypic Ab ScFv fragments expressed on the phage was reported and the phage particles displaying this Ab ScFv fragment were used in maternal immunization, leading to the protection of neonatal mice against streptococcal infection (18). In a recent elegant work, CDR-like loops in the Ig constant-region domains were replaced with antigenic peptides, and the ability of the mutant Ig to stimulate $CD4^+$ T cells, by both the endogenous and the exogenous routes for class II presentation, was demonstrated (17). Those authors have identified another attractive site for the insertion of epitopes: the segments between β-strands of Ig C-region domains, although only in vitro efficiency of the epitope presentation by APC to peptide-specific T-helper 1 (Th1) cells has been shown.

Experimental murine *T. crassiceps* cysticercosis is a well-characterized model to study immunological (31), genetic (10), and gender-associated (30) factors of resistance and susceptibility to this parasite. This experimental system was also widely used for the evaluation of different vaccine preparations, such as recombinant antigens (20), synthetic peptides (33), and naked DNA (21). In order to test the immunogenic properties of the PT1 epitope, female BALB/c mice extremely susceptible to *T. crassiceps* infection were used in vaccination trials, and more than 50% of the mice immunized with PIgphage and free PT1 peptide were completely protected against pathogen challenge while in the rest of the immunized mice, the parasite load was dramatically reduced (Fig. 2A). Moreover, we have demonstrated that a single inoculation with PIgphage is able to confer protection against challenge (Fig. 2B). The results clearly indicate the importance of the PT1 epitope in immune protection against cysticercosis.

The detected immune response reflects an additional interesting phenomenon. We have shown that $CD4^+$ and $CD8^+$ T cells from PT1-, PIgphage-, and TAg-immunized mice proliferated in vitro upon stimulation by Mϕ pulsed with TAg, PT1, and PIgphage (Fig. 3). These data indicate that the exogenously applied T-cell epitope in different molecular contexts (PT1 peptide and PIgphage) was effectively processed and presented in vivo to $CD8^+$ and $CD4^+$ T cells in the context of both MHC class I and class II molecules, respectively, and the same epitope was presented on Mϕ to stimulate the T cells in vitro. Importantly, the T cells from TAg-immunized mice proliferated in the presence of PIgphage and TAg was stimulatory for T cells from PIgphage-immunized mice, suggesting that the PT1 epitope displayed on PIgphage contributes to the development of protective immunity to this pathogen. The positive response of T cells from peptide-immunized mice to TAg stimulation is an additional support for this suggestion.

The presentation of the PT1 epitope to $CD8^+$ cells in the context of PIgphage is rather surprising, because peptides from exogenous sources are usually effectively presented on MHC class II but not class I molecules (39). Probably, the PT1 epitope attached to the phage particle accesses a classical or alternative MHC class I processing pathway to be delivered to $CD8^+$ cells (4), acting like some bacterial toxins capable of delivering catalytic protein moieties to the cytosol of eukaryotic cells (13). Interestingly, PT1 peptide was presented by Mϕ to both $CD4^+$ and $CD8^+$ cells from PIgphage-immunized mice in lymphoproliferation assays, indicating association of this 13-amino-acid peptide with both MHC class I and class II molecules, although the loading of class II molecules was much more efficient than that of class I molecules. In general, naturally occurring peptides eluted from MHC class I molecules are composed of 8 to 10 amino acid residues, and short peptide fragments (8 to 15 amino acids) derived from antigen are bound to both classes of MHC molecules (3). With respect to MHC class II-mediated peptide presentation to $CD4^+$ cells,

the efficient loading of identical viral peptides onto MHC class II molecules by antigenized Ig and influenza virus applied exogenously has already been demonstrated (7). As expected, in our experiment, when Mϕ were pulsed with plasmid DNA (pcDVH18) carrying antigenized Ig V_H domain to generate PT1 epitopes endogenously, only MHC class I molecules appeared to be charged with the PT1 epitope, resulting in proliferation of $CD8^+$ but not $CD4^+$ T cells isolated from the PIgphage-immunized mice (Fig. 3). Although the mechanism by which synthetic peptides induce cell-mediated immunity or stimulate T cells is not clear, possibly they can bind directly to MHC class II molecules (6) or to class I molecules by direct penetration into the cytoplasm (15).

Our data indicate that we have preferentially induced a type 1 (T1) immune response with production of IFN-γ by both $CD4^+$ and $CD8^+$ T cells and the absence of IL-4. The data presented in this study support the previous observations concerning the role of the immune response and cytokines in murine cysticercosis. Thus, it was shown that neonatal thymectomy of mice greatly increases susceptibility to *T. crassiceps* infection and that T-cell replacement restores it to normal levels (5), whereas the bulk of antiparasite Abs were not clearly related to protection and might even enhance parasite growth (16).

Importantly, Th1-type response has been shown to be protective in cysticercosis, while Th2-type response is permissive in this parasitic disease, and a progressive shift from Th1- to Th2-type response was observed during the experimental infection in mice (31, 35). By immunization with the PIgphage novel immunogen, we were able to strengthen this balance in favor of a Th1-type response. Moreover, in a recent work we have shown that treatment of mice with monoclonal anti-IFN-γ Ab resulted in a dramatic increase in susceptibility to pathogen challenge (32), while the mice receiving recombinant IFN-γ and IL-2 showed a low parasite load. In contrast, IL-10 induced a significant increase in parasite load (32). In the present study, no correlation was observed between the proliferative response and IFN-γ production. Similar results were reported in human (28) and murine (26) cells. No parasite- or PT1-specific antibodies were detected in the sera of PIgphage- and PT1-immunized mice, indicating that in our case the humoral immune response is not participating in immunoprotection against murine cysticercosis. Based on the presented data, we can conclude that cell-mediated T1 immune response is probably involved in the induction of resistance to the *T. crassiceps* challenge. At present, the exact mechanism and the role of components of immunity responsible for the protection obtained in this study are not clear. Obviously, more experiments are necessary to clarify the issues mentioned above, as well as to determine the MHC restriction element (*H-2* haplotype) and the duration of the immune response, which were beyond the scope of this study.

In conclusion, in this study we demonstrated the engineering and use of a new kind of immunogen: a phage-displayed epitope grafted into Ig heavy-chain CDRs that seems to favor a T1 immune response. We showed that a CDR-grafted epitope is effectively processed and presented in vitro by APC to T cells and confers protection against pathogen challenge. Probably, using any other T-cell epitope, such an immunogen could serve as a universal vehicle to target specific cellular immune responses. Furthermore, the proposed approach for vaccine development has clear advantages over other systems described, since it is highly cost-effective and simple to manage. Importantly, no adjuvant is required for this type of immunogen application, and although there are no available data concerning the safety of the bacteriophage for use in humans, we hope that their use in veterinary medicine as a vaccine platform will be practical. Finally, although the proposed vaccine development strategy was successfully tested in a murine cysticercosis model, its application in other disease models, especially when the induction of cellular immune response is desirable, seems promising, considering that the engineering of Ig molecules grafted with biologically relevant peptides is already a well-established technique.

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