Epitopes Recognized by Human T Lymphocytes in the ROP2 Protein Antigen of *Toxoplasma gondii*

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The ROP2 protein of *Toxoplasma gondii* possesses immunological and biological properties which have led to its proposal as a vaccine candidate. To identify epitopes recognized by human T cells in the ROP2 antigen, we submitted the sequence of this protein to three reported T-cell epitope prediction algorithms. Three sequences that were predicted by all three methods were selected (sequences 197 to 216, 393 to 410, and 501 to 524), and the corresponding peptides were synthesized. The peptides were first tested in a proliferation assay with a DPw4-restricted, ROP2-specific human T-cell clone, and the peptide corresponding to residues 197 to 216 was shown to stimulate the T-cell clone. The three peptides were further tested in proliferation assays with peripheral blood mononuclear cells from a panel of *T. gondii*-seropositive and -seronegative individuals. We found that cells from a high proportion of the seropositive donors (64%) recognized at least one of the three peptides. The most frequently recognized ones were peptides 197 to 216 (45%) and 501 to 524 (36%). None of the seronegative donors responded to any peptide. These results show that the ROP2 antigen of *T. gondii* contains T-cell epitopes recognized by a high percentage of the immune population and further strengthen its potential as a vaccine candidate.

Toxoplasma gondii is an intracellular protozoan parasite and the etiological agent of toxoplasmosis. This infection has a worldwide distribution, and it has been estimated that one-third of the human population is infected (13). Although toxoplasmosis is generally asymptomatic in immunocompetent individuals, it may cause severe complications in immunocompromised persons and pregnant women. In immunodeficient or immunosuppressed patients, toxoplasmosis is one of the major opportunistic infections and is caused by the reactivation of cysts that persist in the tissues of the host after a primary infection; release of the tachyzoites can cause severe disseminated toxoplasmosis and/or encephalitis, which is often fatal (17). If toxoplasmosis is first acquired during pregnancy, transplacental transmission can occur, leading to abortion or neonatal malformations (19, 31).

A vaccine against this parasite would be highly valuable. Since protective immunity against *T. gondii* is cell mediated (8, 9, 18, 26, 27), parasite antigens that could be used as vaccine candidates must be identified at the T-cell level. In this context, we have reported the identification and molecular cloning of a potentially protective 54-kDa protein antigen of *T. gondii* (22). This antigen was recognized by a human T-cell clone (TCC32) isolated from an immune donor, and it is specific for the parasite and produces high levels of gamma interferon, the major mediator of resistance against toxoplasmosis (23). We found that the TCC32-defined epitope was conserved among different strains of *T. gondii* (virulent and cyst forming) and was recognized by TCC32 in association with major histocompatibility complex (MHC) class II molecule DPw4, the most

frequent allele in the Caucasian population (22). This antigen was recently shown to be a component of the rhoptries, more precisely the ROP2 protein (12). It is expressed in the three stages of the *T. gondii* life cycle (tachyzoites, bradyzoites, and sporozoites) (24) and is also found inserted into the parasitophorous vacuole membrane surrounding the intracellular parasite (1). Moreover, 89% of *T. gondii*-seropositive individuals possess antibodies against a recombinant truncated form of this antigen (28). ROP2 has also been shown to be a target of mucosal immune mechanisms (3).

The purpose of the present study was to localize epitopes of the ROP2 antigen recognized by human T cells and particularly by DPw4-restricted TCC32. We used the methods developed by Margalit et al. (16), Rothbard and Taylor (20), and Claverie et al. (4) to predict ROP2 sequences containing potential T-cell epitopes. The corresponding peptides were synthesized and tested for their abilities to induce proliferation of TCC32 and peripheral blood mononuclear cells (PBMC) from a panel of naturally infected donors. The identification of T-cell epitopes of the ROP2 antigen of *T. gondii* could be valuable for the design of a synthetic vaccine against toxoplasmosis.

MATERIALS AND METHODS

Parasites and preparation of *T. gondii*-soluble antigen (TSA2). The Wiktor strain (7) of *T. gondii* was maintained by serial passage in the peritoneal cavities of CD1 mice. For the preparation of parasite antigen, tachyzoites were obtained by in vitro culture in Vero cells as previously described (22). One billion tachyzoites were suspended in 5 ml of Dulbecco's phosphate-buffered saline (PBS), sonicated on ice for 5 min at 40 W with a 4710 series ultrasonic homogenizer (Cole-Parmer, Chicago, Ill.), and centrifuged at 10,000 × g at 4°C for 30 min. The supernatant was recovered, sterile filtered (0.22-μm-pore-size filter), and stored in aliquots at -20°C. The protein concentration was determined by the Bio-Rad protein assay (Bio-Rad, Richmond, Calif.), with serum albumin being used as the standard.

T-cell epitope prediction. The algorithms of Margalit et al. (16), Rothbard and Taylor (20), and Claverie et al. (4), programmed in Fortran 77, were used to predict T-cell epitopes in the ROP2 protein sequence. Predictions derived by each method were compared, and only those sequences identified by all three methods were selected. With respect to the algorithm of Margalit et al. (16), amphipathic scores (AS) were calculated for stretches of 11 residues. So far as

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TABLE 1. Amino acid sequences of the ROP2-derived peptides

Peptide	Sequence ^a	AS(s)
197 to 216	TDPGDVIEELFNRI [*] PETSV	28.9
393 to 410	LQLIRLAASLQHYGLVHA [*]	8.6 and 4.3
501 to 524	IEWIYRRCKNIPQPV [*] RALLEGFLR [*]	54.7

^a The Rothbard motifs are underlined. The asterisks indicate null occurrences of the tetrapeptides in the human proteins data bank and are located below the first amino acids of the tetrapeptides.

the method of Claverie et al. (4) is concerned, tetrapeptides were searched for in human protein sequences in the SWISSPROT data bank.

Peptide synthesis. Peptides were synthesized by the solid-phase method according to the *tert*-butyloxycarbonyl-trifluoroacetic acid strategy (14) on a fully automated peptide synthesizer (ABI model 430A; Applied Biosystems, Foster City, Calif.). After low-high hydrogen fluoride cleavage, the crude peptides were purified by gel filtration on a TSK HW 40S column (Merck, Rahway, N.J.) and by reverse-phase high-pressure liquid chromatography (HPLC) on a Nucleosil C₁₈ column. The peptides were checked for homogeneity by reverse-phase HPLC on a Vydac C₁₈ column and by thin-layer chromatography and for identity by amino acid analysis after total acid hydrolysis.

ELISA. The enzyme-linked immunosorbent assay (ELISA) procedure was adapted from reference 30. *T. gondii* tachyzoites obtained by in vitro culture in Vero cells were washed three times with PBS and resuspended at 10⁷ parasites per ml in a fresh solution of 2% *p*-formaldehyde in PBS. After fixation for 15 min at room temperature (RT), the suspension was diluted with PBS to 2 × 10⁶ tachyzoites per ml, and 100 µl of this suspension was distributed in each well of a 96-well polystyrene microtiter plate (E.I.A./R.I.A. plate; Costar, Cambridge, Mass.). The plates were allowed to dry at RT and were then kept at -20°C until use. At the moment of their use, the wells were washed three times with PBS containing 0.05% Tween 20 (PBS-T20) and blocked with 0.5% casein in PBS containing 0.25 M glycine for 2 h at RT. After four cycles of washing with PBS-T20, human serum samples diluted 1:50 (vol/vol) in 10 mM sodium phosphate (pH 7.4)-0.35 M NaCl-0.5% casein-0.2% Triton X-705 were incubated in the wells for 2 h at RT (100 µl per well). After washing of the wells, the bound antibodies were revealed by incubation with horseradish peroxidase-labeled rabbit antibodies to human immunoglobulin G (DAKO, Glostrup, Denmark) and the chromogenic substrate as previously described (28). The serum samples were tested in duplicate. Fifteen reference negative serum samples (as determined by the Sabin-Feldman test) were included in each ELISA, and their absorbance values were used to determine the cutoff value. A serum was considered positive when its absorbance value was greater than the mean plus 3 standard deviations of the absorbance values of the 15 reference negative serum samples. A serum was considered negative when its absorbance value was less than the mean plus 1 standard deviation of the absorbance values of the 15 reference negative serum samples.

Donors. Blood donors were 21- to 62-year-old individuals with no apparent symptoms of toxoplasmosis at the time of the study. They were classified as seropositive or seronegative for *T. gondii* according to the results of the ELISA described above.

T-cell clone 32 (TCC32). Human TCC32, specific for the ROP2 protein antigen of *T. gondii*, was isolated and characterized as previously described (22, 23). It was maintained in culture by stimulation every 2 to 3 weeks in the presence of allogeneic irradiated PBMC, phytohemagglutinin, and human recombinant interleukin-2 as previously described (22).

Cell proliferation assay. The culture medium consisted of RPMI 1640 (GIBCO, Grand Island, N.Y.) supplemented with sodium pyruvate (1 mM), nonessential amino acids (0.1 mM), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; 25 mM), 2-mercaptoethanol (5 × 10⁻⁵ M), and 10% (vol/vol) pooled human serum obtained from *T. gondii*-seronegative donors. PBMC were isolated from heparinized blood with Ficoll-Paque (Pharmacia, Uppsala, Sweden) as previously described (23). One hundred thousand PBMC were incubated in the presence of different concentrations of peptides, TSA2, phytohemagglutinin (the M form in a 1:300 [vol/vol] dilution of stock solution provided by the manufacturer [GIBCO]), or medium alone in a total volume of 200 µl in quadruplicate wells of 96-well, flat-bottom plates (Costar) for 6 days (for peptides and TSA2) or 3 days (for phytohemagglutinin) at 37°C in a humidified atmosphere containing 5% CO₂ in air. The cells were pulsed with 0.5 µCi of [³H]thymidine (45 Ci/mmol; Amersham, Buckinghamshire, England) for the last 18 h and were harvested onto glass fiber filters with an automatic cell harvester (Skatron, Sterling, Va.). Radioactivity uptake was measured by liquid scintillation spectroscopy. The results were expressed in terms of stimulation indices (SI) (the SI is the ratio between the mean of the counts per minute in wells with antigen and the mean of the counts per minute in wells without antigen).

RESULTS

Prediction of T-cell epitopes. In order to localize T-cell epitopes in the ROP2 antigen of *T. gondii*, the protein sequence (22) was submitted to a T-cell epitope prediction analysis using the methods developed by Margalit et al. (16), Rothbard and Taylor (20), and Claverie et al. (4). Three sequences predicted by all three methods were selected and are shown in Table 1. (i) Sequence 197 to 216 contains an amphipathic alpha helix with an AS of 28.9, two contiguous Rothbard motifs, and one tetrapeptide of null occurrence in the human data bank. (ii) Sequence 393 to 410 contains two overlapping amphipathic alpha helices with ASs of 8.6 and 4.3, two noncontiguous Rothbard motifs, and three consecutive tetrapeptides with null occurrence in the human data bank. (iii) Sequence 501 to 524 contains an amphipathic alpha helix with an AS of 54.7, three Rothbard motifs (two of which are contiguous), and tetrapeptides in the amino-terminal part with low occurrence values in the human data bank. The peptides corresponding to these sequences were synthesized, purified by gel filtration and reverse-phase HPLC, and checked by amino acid analysis.

Identification of the T-cell epitope recognized by ROP2-specific human T-cell clone TCC32. The three peptides were first tested for their abilities to induce proliferation of ROP2-specific human T-cell clone TCC32. As can be seen in Fig. 1, a high level of proliferation was induced by peptide 197 to 216; maximum response was obtained with concentrations ranging between 1 × 10⁻² and 3 × 10⁻² µg/ml. No response was observed with the two other peptides at any of the concentrations tested. These results clearly show that amino acid sequence 197 to 216 of the ROP2 antigen contains the epitope recognized by DPw4-restricted TCC32.

Proliferative response of PBMC from *T. gondii*-immune individuals to the ROP2 peptides. The three peptides were further tested in proliferation assays with PBMC from a panel of 11 *T. gondii*-seropositive and 9 seronegative donors, as classified by the ELISA described above. As can be seen from Fig. 2, a heterogeneous response to the peptides was obtained. Peptide 197 to 216 was recognized by 45% of the seropositive donors tested (5 of 11); the highest response was observed with a peptide concentration of 5.0 µg/ml, except for donor 4,

FIG. 1. Identification of the epitope recognized by TCC32. T cells (10⁴ per well) and cryopreserved irradiated autologous B lymphoblastoid cells (5 × 10⁴ per well) were incubated in the presence of different peptide concentrations or in medium alone. Proliferation was assessed 3 days later by [³H]thymidine incorporation. Results are expressed as mean counts per minute of triplicate cultures ± standard deviations (error bars). ●, peptide 197 to 216; ○, peptide 393 to 410; ■, peptide 501 to 524.

FIG. 2. Proliferative response of PBMC from immune and nonimmune individuals to ROP2-derived peptides. One hundred thousand PBMC from seropositive (bars 1 to 11) or seronegative (bars 12 to 20) donors were cultured in medium alone or in the presence of 0.5 or 5 µg of peptides 197 to 216 (a), 393 to 410 (b), and 501 to 524 (c) per ml and in the presence of 0.3 or 1 µg of TSA2 per ml (d) for 6 days, and the proliferation was determined by [³H]thymidine incorporation. The results are expressed as mean SIs from quadruplicate cultures. For each antigen concentration tested, we set the cutoff at the mean plus 4 standard deviations of the SIs of the nine seronegative donors. Proliferative responses with SIs above the cutoff are marked with asterisks. The count-per-minute ranges were as follows: no antigen, 111 to 733 cpm (seropositives) and 116 to 1,553 cpm (seronegatives); peptide 197 to 216, 133 to 8,407 cpm (seropositives) and 112 to 1,355 cpm (seronegatives); peptide 393 to 410, 123 to 2,061 cpm (seropositives) and 131 to 945 cpm (seronegatives); peptide 501 to 524, 113 to 10,738 cpm (seropositives) and 131 to 1,118 cpm (seronegatives); TSA2, 457 to 215,035 cpm (seropositives) and 253 to 19,641 cpm (seronegatives).

whose maximum response was obtained at 0.5 µg/ml. Peptide 393 to 410 was recognized by only one individual (9% of the donors), and the response observed was low (an SI of <8). Peptide 501 to 524 was recognized by 4 of the 11 donors (36%); maximal SIs were obtained with concentrations between 0.5 and 5 µg/ml. None of the seronegative individuals showed proliferation in response to any of the peptides. We also tested the proliferative response of the PBMC to total soluble antigen of *T. gondii* (TSA2); 9 of the 11 (82%) seropositive individuals responded to TSA2. The two individuals who did not respond to TSA2 did not respond to any peptide. All donors responded to phytohemagglutinin with SIs of >130 (data not shown). In addition, it can be observed in Fig. 2 that 7 of the 11 seropositive individuals tested (64%) responded to at least one peptide. One donor (donor 9) responded to the three peptides tested, one donor (donor 1) responded to two peptides (197 to 216 and 501 to 524), and five donors responded to only one peptide (donors 3 and 5 responded to peptide 501 to 524; donors 4, 8, and 10 responded to peptide 197 to 216). There was no apparent relationship between the level of response to TSA2 and the response to any of the peptides.

DISCUSSION

In this report, we show that the epitope recognized by *T. gondii*-specific and DPw4-restricted TCC32 is localized in sequence 197 to 216 of the ROP2 protein, one of the three sequences identified by all three methods of T-cell epitope prediction used in this study. We compared the 197-to-216

sequence with the recently described DPw4-binding motif deduced by the pool-sequencing technique (6). As can be seen from Fig. 3, the sequence partially aligns with the reported motif. The sixth amino acid of the peptide (Val) could correspond to the relative position 1 of the motif; although Val was not considered part of the motif, it was frequently found in peptides eluted by the pool-sequencing technique (6). A Phe is found in relative position 7 and an Ile is found in relative position 10, which are parts of the motif. It was also reported that Pro is frequently found on both extremities of the epitopes (6); the 197-to-216 peptide presents a Pro in relative position 11, which aligns well with the motif.

Two variants of the DPw4 allele (DPw4.1 and DPw4.2) have been reported (2). The motif described above was deduced by sequencing peptides eluted from a cell line which expresses the DPw4.1 allele (6); lymphoblastoid cell line JAH, used in our

DPw4 motif	1	2	3	4	5	6	7	8	9	10									
	F						F		V										
	L						L		Y										
	Y						Y		I										
	M						M		A										
									L										
Peptide 197-216	T	D	P	G	D	V	V	I	E	E	L	F	N	R	I	P	E	T	S

FIG. 3. Alignment of the DPw4-restricted epitope of the *T. gondii* ROP2 antigen with the DPw4-binding motif described by Falk et al. (6).

previous work to identify the restriction element of TCC32 (22), is also known to express the DPw4.1 allele (2). These observations suggest that the motif could correspond to the DPw4.1 variant. In fact, Hammond et al. (11) previously reported a DPw4.2-specific motif which is different from that shown in Fig. 3 (6).

The peptides corresponding to the three predicted epitopes were also tested with PBMC from a panel of *T. gondii*-seropositive and -seronegative donors. A high proportion of the seropositive individuals (45%) responded to DPw4-restricted peptide 197 to 216, 36% of seropositive individuals responded to peptide 501 to 524, and only 9% of the individuals responded to peptide 393 to 410. These results clearly show that sequences 197 to 216 and 501 to 524 contain T-cell epitopes recognized by a high percentage of the immune human population. This observation could be related to the high frequency of the particular MHC allele(s) able to bind these peptides in the population or to a promiscuous binding of the peptides to different MHC molecules. For peptide 197 to 216, we demonstrated that it can bind to DPw4, the most frequent allele in the Caucasian population (25), which may explain in part the high frequency of response to this peptide. MHC class II typing of the seropositive donors could allow us to confirm the correlation between the response to peptide 197 to 216 and expression of DPw4. However, we cannot rule out the possibility that both peptides can bind to several MHC alleles or contain more than one T-cell epitope. In fact, promiscuous binding seems to be a common characteristic of MHC class II epitopes (6, 21). Promiscuous epitopes are of great interest for the development of synthetic vaccines, since they could be immunogenic in a large proportion of the population.

Although the proportions of responders to peptides 197 to 216 and 501 to 524 were similar, the SIs obtained with peptide 501 to 524 were higher, even at low concentrations (0.5 µg/ml). This observation could be due to a higher frequency of T cells recognizing peptide 501 to 524.

As can be seen in Fig. 2, the extent of T-cell proliferation induced by TSA2 (SIs of ≥ 100) exceeds the peptide-induced proliferation (SIs of ≤ 58). These results suggest the presence in TSA2 of other T-cell epitopes either derived from ROP2 or derived from other antigens of the parasite. A previously published study indicates that major tachyzoite surface antigen P30 or SAG1 of *T. gondii* is also able to induce proliferation of PBMC from seropositive individuals and production of gamma interferon by these cells (15). However, the epitopes of this antigen recognized by the human T cells were not localized. Although there are reports on the localization of T-cell epitopes in the SAG1 antigen (10, 29) and in dense granule antigen GRA1 or P24 (5), these studies were carried out in the rat or mouse model of toxoplasmosis. So, to our knowledge, this paper contains the first report of *T. gondii* epitopes recognized by T lymphocytes from immune individuals.

The main finding of this study is that a high proportion of the *T. gondii*-seropositive subjects (64%) responds at least to one of the three ROP2-derived peptides tested. Two of the seropositive donors did not respond to TSA2 in the proliferation assay and could be false seropositives; this suspicion was in fact shown to be the case for one of them. If we consider only the subjects who responded to TSA2 in the proliferation assay, the percentage of responders to the peptides rises to 78%. Thus, the ROP2 antigen elicits a T-cell response in a large majority of the *T. gondii*-immune human population. This feature could be related to the fact that this antigen is conserved among different strains (22) and among the three stages of *T. gondii* (24) and to its immunodominance at the T-cell level. These observations further strengthen its potential as a vaccine can-

didate and suggest that these epitopes should be included in a future vaccine.

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