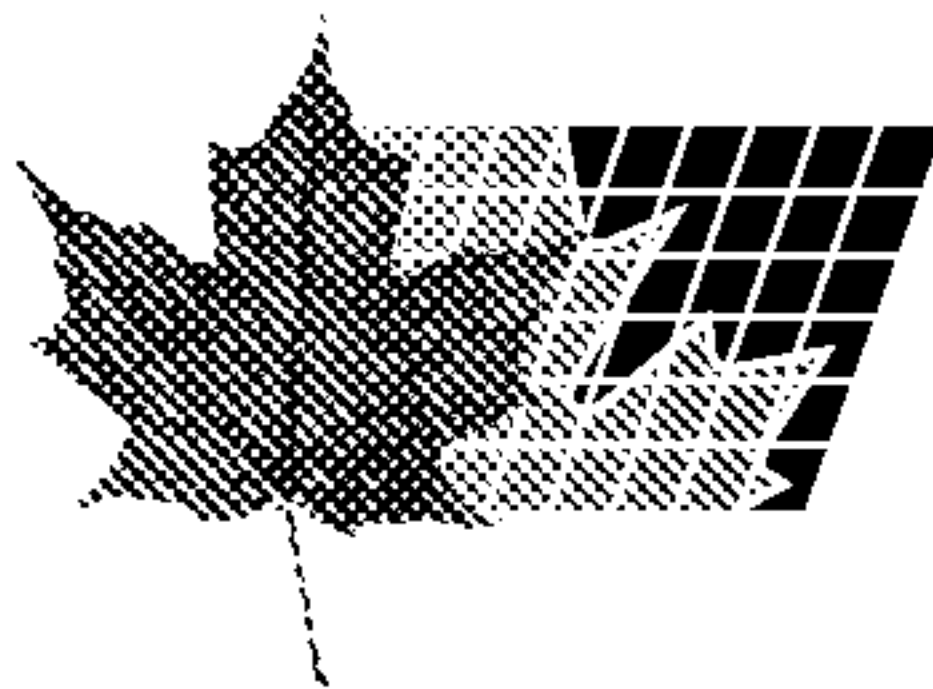




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(54) **PRODUCTION PAR RECOMBINAISON DE L'ANTIGENE SAG1
DE TOXOPLASME**

(54) **RECOMBINANT PRODUCTION OF TOXOPLASMA SAG1
ANTIGEN**

(57) L'invention concerne un procédé de production de l'antigène de toxoplasme SAG1 ou d'un fragment de celui-ci, qui comporte les étapes consistant à: (a) construire un plasmide comportant l'ADN codant pour SAG1 ou pour un fragment de celui-ci ; (b) transformer une cellule hôte de *P. pastoris* à l'aide dudit plasmide ; et cultiver l'hôte de façon à exprimer l'ADN codant pour SAG1 ou pour un fragment de celui-ci. L'invention a en outre trait à des protéines de SAG1 tronqué dans lesquelles la région d'ancrage est absente, notamment de SAG1 tronqué comportant les résidus d'acides aminés 48-307 de SAG1, et à des compositions de vaccin renfermant SAG1.

(57) The invention provides a method for the production of the toxoplasma antigen SAG1 or a fragment thereof, which comprises: (a) constructing a plasmid comprising DNA encoding SAG1 or a fragment thereof; (b) transforming a *P. pastoris* host cell with the said plasmid; and culturing the host such that the DNA encoding SAG1 or a fragment thereof is expressed. The invention further provides truncated SAG1 proteins in which the anchor region is absent, particularly truncated SAG1 comprising amino acid residues (48-307) of SAG1, and vaccine compositions comprising the SAG1.

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(21) International Application Number: PCT/EP99/03957 (22) International Filing Date: 8 June 1999 (08.06.99) (30) Priority Data: 9812773.1 12 June 1998 (12.06.98) GB 9908564.9 15 April 1999 (15.04.99) GB (71) Applicant (for all designated States except US): SMITHK- LINE BEECHAM BIOLOGICALS S.A. [BE/BE]; Rue de l'Institut 89, B-1330 Rixensart (BE). (72) Inventors; and (75) Inventors/Applicants (for US only): BIEMANS, Ralph [BE/BE]; SmithKline Beecham Biologicals s.a., Rue de l'Institut 89, B-1330 Rixensart (BE). BOLLEN, Alex [BE/BE]; (BE). HAUMONT, Michele [BE/BE]; Université Libre de Bruxelles, Rue de l'Industrie 24 B-1400 Nivelles (BE). (74) Agent: PRIVETT, Kathryn, Louise; Corporate Intellectual Property, SmithKline Beecham, Two New Horizons Court, Brentford, Middlesex TW8 9EP (GB).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
(54) Title: RECOMBINANT PRODUCTION OF TOXOPLASMA SAG1 ANTIGEN (57) Abstract The invention provides a method for the production of the toxoplasma antigen SAG1 or a fragment thereof, which comprises: (a) constructing a plasmid comprising DNA encoding SAG1 or a fragment thereof; (b) transforming a <i>P. pastoris</i> host cell with the said plasmid; and culturing the host such that the DNA encoding SAG1 or a fragment thereof is expressed. The invention further provides truncated SAG1 proteins in which the anchor region is absent, particularly truncated SAG1 comprising amino acid residues (48-307) of SAG1, and vaccine compositions comprising the SAG1.		

WO 99/66043

PCT/EP99/03957

RECOMBINANT PRODUCTION OF TOXOPLASMA SAG1 ANTIGEN

Toxoplasma gondii is an obligate intracellular protozoan parasite responsible for toxoplasmosis in warm-blooded animals, including man. Although it is generally clinically asymptomatic in healthy individuals, toxoplasmosis may cause severe complications in pregnant women and immunocompromised patients [1-4]. A live attenuated S48 *Toxoplasma* strain of the parasite is currently available for vaccination in sheep (Toxovax, Mycofarm) [5]. However, this vaccine cannot be administered to humans because of possible reversion to virulent forms. The development of subunit vaccines thus constitutes an alternative way to achieve effective protection of humans against congenital infection and to prevent infection of immunosuppressed individuals. In domestic animals like sheep and pigs, subunit vaccines could also prevent spontaneous abortion and reduce the reservoir of the parasite since tissue cysts in the muscles of these animals is a major cause of human toxoplasmosis.

Antigens which may be important in immunising against *toxoplasma gondii* are known, for example TG34 as described in WO 92/11366.

SAG1 (so called P30), the major surface antigen of *T. gondii*, is a putative candidate for a subunit vaccine. Indeed, SAG1 induces a strong immune response in human and experimental animal models [6-7]. Immunisation of mice with SAG1, purified from tachyzoites and adjuvanted with saponin Quil A or incorporated into liposomes leads to a nearly total protection after challenge [8-9]. This immunity appears to be primarily mediated by CD8⁺ cells specific for SAG1.

The gene encoding SAG1 has been cloned and sequenced. It is single copy and contains no introns [10]. Because native SAG1 is anchored to the plasma membrane via a glycosylphosphatidylinositol anchor (GPI) [11], its purification from tachyzoites is difficult and time consuming.

WO 99/66043

PCT/EP99/03957

Expression of *T. gondii* SAG1 antigen in *E. coli* or mammalian cells has generally been disappointing; indeed, the recombinant protein was either insoluble and misfolded or correctly folded but weakly produced [26-28].

- 5 It has been reported that SAG1 can be produced in *S. cerevisiae* (see WO 96/02654), in *S. pombe* and in insect cells. However the expression level is low and/or a heterogeneous product is obtained.

The present invention provides a method for the production of the toxoplasma
10 antigen SAG1 or a fragment thereof, which comprises:

- (a) constructing a plasmid comprising DNA encoding SAG1 or a fragment thereof;
- (b) transforming a *P. pastoris* host cell with the said plasmid; and
- (c) culturing the host such that the DNA encoding SAG1 or a fragment thereof
15 is expressed.

The SAG1 protein, or fragment thereof, which is produced by the above process may be purified by conventional methods, for instance by a combination of anion exchange (for example Q-sepharose) and gel filtration (for example superdex 75HR)
20 chromatographies.

In one preferred aspect of the invention the DNA encoding the SAG1 protein or fragment thereof is positioned downstream from and in frame with a yeast secretion signal sequence, preferably the *S. cerevisiae* *prepro* α -mating factor secretion signal
25 sequence (MF α).

In a further preferred aspect of the invention the plasmid comprising DNA encoding the SAG1 protein or a fragment thereof is derived from a multicopy *P. pastoris* expression vector, preferably the vector pPIC9K.

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WO 99/66043

PCT/EP99/03957

In yet a further preferred aspect of the invention the DNA encoding SAG1 or a fragment thereof is expressed under the control of a methanol-inducible promoter, for example the AOX1 promoter.

- 5 One advantage of the present invention is that the secreted recombinant SAG1 level is at least ten times superior to that observed in *s. cerevisiae* (see WO 96/02654). Moreover, only two forms of the recombinant protein were secreted.

10 The *Pichia pastoris* expression system additionally leads to very high levels of secretion into an almost protein-free medium. As previously reported, the *Pichia pastoris* expression system is easy for fermentation to high cell density, is genetically stable and can be scaled-up without loss of yield [12-13].

15 The invention also provides a SAG1 protein or a fragment thereof when DNA encoding the said SAG1 protein or fragment thereof is expressed in the yeast *Pichia pastoris*.

20 In a preferred aspect of the invention the SAG1 protein or fragment thereof, in the form produced in *P. pastoris* according to the invention, is purified and when a fragment of the SAG1 protein is an immunological derivative of the SAG1 protein. The said fragment of SAG1 when produced in *P. pastoris* according to the invention is also preferably truncated, especially at the C-terminus. In a preferred aspect the said truncate is an anchor-less SAG1 protein, especially one lacking amino acids 308 to 336 of the SAG1 protein.

25

In a further aspect of the invention there is provided a truncated SAG1 protein comprising amino acids 48-307 of SAG1, and immunogenic derivatives thereof.

30 The term "immunogenic derivative" encompasses any molecule such as a truncated or other derivative of the protein which retains the ability to induce an immune response to the protein following internal administration to a human or to an animal or which retains the ability to react with antibodies present in the sera or other

WO 99/66043

PCT/EP99/03957

biological samples of *Toxoplasma gondii*-infected humans or animals. Such other derivatives can be prepared by the addition, deletion, substitution or rearrangement of amino acids or by chemical modifications thereof.

- 5 The recombinant truncated SAG1 protein appears correctly folded since it is recognised by antibodies specific for the native form of SAG1 and elicits proliferation of mononuclear cells from seropositive individuals. The recombinant truncated SAG1 protein is also capable of inducing a protective immune response against a toxoplasma challenge and in a congenital toxoplasmosis model. The
10 anchor-less SAG1 antigen is therefore useful in diagnosis of *T. gondii* infections and for development of a subunit vaccine.

Accordingly, the invention also provides a vaccine composition comprising the truncated SAG1 protein and a method of preventing toxoplasmosis infection which
15 comprises administering to a human subject in need thereof a vaccine composition according to the invention.

The present invention in a further aspect provides a vaccine formulation as herein described for use in medical therapy, particularly for use in the treatment or
20 prophylaxis of toxoplasmosis infections. The vaccine formulation will be useful in the prevention of both horizontal and vertical (congenital) transmission of toxoplasmosis.

The vaccine composition according to the invention will normally comprise a
25 protein according to the invention, as described hereinabove, admixed with a suitable adjuvant and/or carrier.

The vaccine composition according to the invention may comprise further components for the treatment or prophylaxis of infections other than toxoplasmosis
30 infections. In particular such further components may be one or more antigens from one or more other pathogens.

WO 99/66043

PCT/EP99/03957

The vaccine composition according to the invention may comprise one or more additional *T. gondii* antigens.

5 The vaccine of the present invention will contain an immunoprotective or immunotherapeutic quantity of the antigen and may be prepared by conventional techniques.

Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland,
10 U.S.A. 1978. Encapsulation within liposomes is described, for example, by Fullerton, U.S. Patent 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example, by Likhite, U.S. Patent 4,372,945 and by Armor et al., U.S. Patent 4,474,757.

15 The amount of protein in the vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed. Generally, it is expected that each dose will comprise 1-1000 mg of protein, preferably 2-100 mg, most preferably 4-40 mg. An optimal amount for a
20 particular vaccine can be ascertained by standard studies involving observation of antibody titres and other responses in subjects. Following an initial vaccination, subjects may receive a boost in about 4 weeks.

The proteins of the present invention are preferably adjuvanted in the vaccine
25 formulation of the invention. Adjuvants are described in general in Vaccine Design – the Subunit and Adjuvant Approach, edited by Powell and Newman, Plenum Press, New York, 1995.

Suitable adjuvants include an aluminium salt such as aluminium hydroxide gel
30 (alum) or aluminium phosphate, but may also be a salt of calcium, iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatised polysaccharides, or polyphosphazenes.

In the formulation of the invention it is preferred that the adjuvant composition induces a preferential Th1 response. However it will be understood that other responses, including other humoral responses, are not excluded.

5

Preferred Th1-type immunostimulants which may be formulated to form adjuvants suitable for use in the present invention include and are not restricted to the following.

- 10 Monophosphoryl lipid A, in particular 3-de-O-acylated monophosphoryl lipid A (3D-MPL), is a preferred Th1-type immunostimulant for use in the invention. 3D-MPL is a well known adjuvant manufactured by Ribi Immunochem, Montana. Chemically it is often supplied as a mixture of 3-de-O-acylated monophosphoryl lipid A with either 4, 5, or 6 acylated chains. It can be prepared by the methods
15 taught in GB 2122204 B. A preferred form of 3D-MPL is in the form of a particulate formulation having a small particle size less than 0.2 μ m in diameter, and its method of manufacture is disclosed in EP 0 689 454.

- Saponins are also preferred Th1 immunostimulants in accordance with the
20 invention. Saponins are well known adjuvants and are taught in: Lacaille-Dubois, M and Wagner H. (1996. A review of the biological and pharmacological activities of saponins. *Phytomedicine* vol 2 pp 363-386). For example, Quil A (derived from the bark of the South American tree *Quillaja Saponaria* Molina), and fractions thereof, are described in US 5,057,540 and "Saponins as vaccine adjuvants",
25 Kensil, C. R., *Crit Rev Ther Drug Carrier Syst*, 1996, 12 (1-2):1-55; and EP 0 362 279 B1. The haemolytic saponins QS21 and QS17 (HPLC purified fractions of Quil A) have been described as potent systemic adjuvants, and the method of their production is disclosed in US Patent No. 5,057,540 and EP 0 362 279 B1. Also described in these references is the use of QS7 (a non-haemolytic fraction of Quil-
30 A) which acts as a potent adjuvant for systemic vaccines. Use of QS21 is further described in Kensil *et al.* (1991. *J. Immunology* vol 146, 431-437). Combinations of QS21 and polysorbate or cyclodextrin are also known (WO 99/10008).

WO 99/66043

PCT/EP99/03957

Particulate adjuvant systems comprising fractions of QuilA, such as QS21 and QS7 are described in WO 96/33739 and WO 96/11711.

Another preferred immunostimulant is an immunostimulatory oligonucleotide
5 containing unmethylated CpG dinucleotides ("CpG"). CpG is an abbreviation for cytosine-guanosine dinucleotide motifs present in DNA. CpG is known in the art as being an adjuvant when administered by both systemic and mucosal routes (WO 96/02555, EP 468520, Davis *et al.*, *J.Immunol.*, 1998, 160(2):870-876; McCluskie and Davis, *J.Immunol.*, 1998, 161(9):4463-6). Historically, it was observed that
10 the DNA fraction of BCG could exert an anti-tumour effect. In further studies, synthetic oligonucleotides derived from BCG gene sequences were shown to be capable of inducing immunostimulatory effects (both in vitro and in vivo). The authors of these studies concluded that certain palindromic sequences, including a central CG motif, carried this activity. The central role of the CG motif in
15 immunostimulation was later elucidated in a publication by Krieg, *Nature* 374, p546 1995. Detailed analysis has shown that the CG motif has to be in a certain sequence context, and that such sequences are common in bacterial DNA but are rare in vertebrate DNA. The immunostimulatory sequence is often: Purine, Purine, C, G, pyrimidine, pyrimidine; wherein the CG motif is not methylated, but
20 other unmethylated CpG sequences are known to be immunostimulatory and may be used in the present invention.

In certain combinations of the six nucleotides a palindromic sequence is present. Several of these motifs, either as repeats of one motif or a combination of different
25 motifs, can be present in the same oligonucleotide. The presence of one or more of these immunostimulatory sequences containing oligonucleotides can activate various immune subsets, including natural killer cells (which produce interferon γ and have cytolytic activity) and macrophages (Wooldrige *et al* Vol 89 (no. 8), 1977). Other unmethylated CpG containing sequences not having this consensus sequence have
30 also now been shown to be immunomodulatory.

WO 99/66043

PCT/EP99/03957

CpG when formulated into vaccines, is generally administered in free solution together with free antigen (WO 96/02555; McCluskie and Davis, *supra*) or covalently conjugated to an antigen (WO 98/16247), or formulated with a carrier such as aluminium hydroxide ((Hepatitis surface antigen) Davis *et al. supra* ;

5 Brazolot-Millan *et al.*, *Proc.Natl.Acad.Sci.*, USA, 1998, 95(26), 15553-8).

Such immunostimulants as described above may be formulated together with carriers, such as for example liposomes, oil in water emulsions, and or metallic salts, including aluminium salts (such as aluminium hydroxide). For example, 3D-

10 MPL may be formulated with aluminium hydroxide (EP 0 689 454) or oil in water emulsions (WO 95/17210); QS21 may be advantageously formulated with cholesterol containing liposomes (WO 96/33739), oil in water emulsions (WO 95/17210) or alum (WO 98/15287); CpG may be formulated with alum (Davis *et al. supra* ; Brazolot-Millan *supra*) or with other cationic carriers.

15 Combinations of immunostimulants are also preferred, in particular a combination of a monophosphoryl lipid A and a saponin derivative (WO 94/00153; WO 95/17210; WO 96/33739; WO 98/56414; WO 99/12565; WO 99/11241), more particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153.

20 Alternatively, a combination of CpG plus a saponin such as QS21 also forms a potent adjuvant for use in the present invention.

Thus, suitable adjuvant systems include, for example, a combination of monophosphoryl lipid A, preferably 3D-MPL, together with an aluminium salt.

25 An enhanced system involves the combination of a monophosphoryl lipid A and a saponin derivative particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 is quenched in cholesterol containing liposomes (DQ) as disclosed in WO 96/33739.

30 A particularly potent adjuvant formulation involving QS21, 3D-MPL & tocopherol in an oil in water emulsion is described in WO 95/17210 and is another preferred formulation for use in the invention.

Another preferred formulation comprises an aluminium salt together with a CpG oligonucleotide.

- 5 In a further aspect of the present invention there is provided a method of manufacture of a vaccine formulation as herein described, wherein the method comprises mixing a protein according to the invention with a suitable adjuvant and, optionally, a carrier.
- 10 Particularly preferred adjuvant and/or carrier combinations for use in the formulations according to the invention are as follows:
- i) 3D-MPL + QS21 in DQ
 - ii) Alum + 3D-MPL
 - iii) Alum + QS21 in DQ + 3D-MPL
 - 15 iv) Alum + CpG
 - v) 3D-MPL + QS21 in DQ + oil in water emulsion

There is also provided a diagnostic kit for the diagnosis of toxoplasmosis infection in the blood of mammals which may be infected, which kit comprises an anchor-less
 20 SAG1 antigen or a fragment thereof.

The invention is illustrated in the accompanying examples and Figures in which the following are depicted:

- 25 Figure 1: Immunodetection of SAG1. Part a and b: respectively under reduced (lane 1 to 3, 5 and 7) and nonreduced (lane 4 and 6) conditions; lane 1, 4 and 5: total soluble *T. gondii* antigens (ARGENE); Lane 2, 6 and 7: recombinant SAG1; lane 3: molecular weight (Rainbow, Amersham). Part c: glycosylation of SAG1: Lane 1 and 2: respectively
 30 incubation without or with N-glycosidase F. Part d: Coomassie blue stain detection of purified SAG1. Lane 1: molecular weight (Rainbow, Amersham), lane 2 and 3: purified SAG1, respectively under reduced

and nonreduced conditions. SAG1 was detected with antibodies against residues 76-95 of SAG1 (part a and c) or with the monoclonal TG5.54 (part b).

5 Figure 2: Determination of human patients serology by ELISA. Plates were coated with soluble antigen extract from *Toxoplasma gondii* (A) or with purified recombinant SAG1 (B). To simplify the figure, only two negative and five positive sera (respectively D1, D6 and D2, D3, D4, D5 and D7) were represented.

10

Figure 3: Proliferative response of PBMC from immune (black box) and nonimmune (white box) individuals to soluble antigen extract from *T. gondii* (A) or to recombinant SAG1 (B). Proliferation was assessed by [³H]Thymidine incorporation. Results are expressed as the means $\pm$ standard deviation of 4 experiments.

15

Figure 4: Challenge and protection of mice immunized with recombinant SAG1. Groups of 5 mice received two injections of recombinant SAG1 before challenge with *T. gondii* C56 tachyzoites (see text for details). Results are plotted as number of surviving animals according to time (days) post challenge.

20

Figure 5: Construction of the plasmids for expression of the toxoplasma antigen SAG1 in *Saccharomyces cerevisiae* and in *Pichia pastoris*. See example 3 for details.

25

Figure 6: Schematic representation of the recombinant unglycosylated anchor-less SAG1 constructs. The details of the construction are described in Example 5.

30

Figure 7: Construction of unglycosylated anchor-less SAG1 expression vector for the methylotrophic yeast *P. pastoris*. HIS4, *P. pastoris* histidinol

WO 99/66043

PCT/EP99/03957

dehydrogenase gene to complement the defective *his4* genotype in *Pichia*
SMD1168 host strain. 5'AOX1, segment of about 1000 bp, including
the alcohol oxidase promoter. 3'AOX1 (TT), segment of about 260 bp
with the alcohol oxidase transcriptional terminating sequence. 3'AOX1,
5 segment of the alcohol oxidase focus which is necessary for gene
replacement.

The invention will now be illustrated by the following examples.

Examples

Example 1: Expression of SAG1 (P30) in *Pichia Pastoris*

5 *Bacterial and Yeast strains*

The DH5 α F'IQ *Escherichia coli* strain (Bethesda Research Laboratories) was used for bacterial transformation and recombinant plasmid propagation as described by Maniatis *et al* [14]. *P. pastoris* strain SMD1 168 (*his4*, *pep4*) was purchased from
10 Invitrogen.

Oligonucleotide synthesis

Oligonucleotides were synthesised by the solid-phase phosphoramidite method [15]
15 on an Applied Biosystems Synthesizer model 394.

Polymerase chain reaction (PCR)

PCR amplification was performed on a *T. gondii* tachyzoites RH strain λ gt11 cDNA
20 library [16]. The choice of the primers was based on the published sequence [10.]
Oligonucleotides 5'GGATCAAGCTTACCATGTTTCCGAAGGCAGTG3' and
5'TGATCGAATTCTCACGCGACACAAGCTGC3' were used to amplify the
sequence encoding amino acids 18 to 336 of SAG1. DNA was amplified in a 50 μ l
reaction mixture containing 10 mM Tris-HCl (pH 8.3), 2mM MgCl₂, 50 mM KCl,
25 0.01 % wt/vol gelatin, 200 μ M of each deoxynucleoside triphosphate, 20 pmol of
each primer, 1U of *Taq* polymerase (Perkin Elmer Cetus) and cDNA. Samples
were amplified for 30 cycles in a DNA thermal cycler (Perkins Elmer Cetus).
After an initial 10 min denaturation at 94°C, each cycle consisted of 1 min at 95° C,
2 min at 55°C and 3 min at 72°C. At the end of the 30 cycles of amplification, a
30 primer extension was continued for 10 min at 72°C. The PCR products were
analysed after electrophoresis on an 7.5% polyacrylamide gel.

Plasmid construction

Amplified DNA fragment was digested by *HindIII* and *EcoRI* endonucleases
 5 before its insertion in the pUC19 (New England Biolabs) previously opened with
 the same enzymes, resulting in the plasmid pNIV3418. The resulting plasmid was
 then opened by *PstI* and *EcoRI* to permit the insertion of the annealed
 oligonucleotides 5'GGGTCATGATG3' and 5'AATTCATCATGACCCTGCA3'.
 The resulting plasmid contains the sequence encoding the amino acids 18 to 307 of
 10 SAG1. The sequence of the amplified DNA was confirmed by dideoxy sequencing.
 Digestion by *BamHI* and *EcoRI* generate a 781 bp DNA fragment which was
 introduced together with annealed oligonucleotides 5'TCGAGAAAAG
 AGAGGCTGAAGCTTCG3' and 5'GATCCGAAGCTTCAGCCTCTCTTTTC3'
 to provide the junction between the fragment obtained above and the *P. pastoris*
 15 secretion vector pPIC9 (Invitrogen) cut by *BamHI*, *XhoI* and *EcoRI*. The resulting
 plasmid, pNIV3464 was then cut by *BamHI*, *XhoI* and *EcoRI* to generate a 254 bp
BamHI - *XhoI* DNA fragment and a 807 bp *XhoI*-*EcoRI* DNA fragment which
 were introduced in the pPIC9K previously opened by *BamHI* and *EcoRI*. The
 resulting plasmid pNIV3488 contains the sequence encoding the amino acids 48 to
 20 307 of SAG1 downstream to, and in-frame with, the DNA sequence encoding the
 α -mating factor prepro secretion signal sequence of *Saccharomyces cerevisiae*.

Transformation of P. pastoris

25 The plasmid pNIV3488 was introduced into the *P. pastoris* strain SMD 1168 (*his4*,
pep4) by using the spheroplast transformation method (Invitrogen). Transformants
 were selected for histidinol dehydrogenase (His^+) prototrophy by plating on a
 dextrose-based medium without histidine supplementation. His^+ cells were then
 checked for methanol utilisation (Mut^+) by replica plating on both minimal
 30 methanol (MM) and minimal dextrose (MD). The screening of His^+ transformants
 for G418 resistance was realised by pooling and plating them on YPD agar
 containing increasing concentrations of G418 (0.25, 0.5, 1, 1/5 and 2 mg/ml)[17].

Culture conditions

All cell cultures were performed as described in the manufacturer's instruction
5 leaflet (Invitrogen). Briefly, expression assays were performed by inoculating 40
ml of minimal glycerol medium (MGY) or of buffered glycerol-complex medium
(BMGY) with transformants. Cells were grown at 30°C in a rotary shaker at 220
rpm to an A_{600} of ± 2 , then harvested by centrifugation at 3000 x g for 10 min at
room temperature and finally resuspended in 40 ml of minimal methanol medium
10 (MM) or buffered methanol-complex medium (BMMY) to induce expression.
Cultures were supplemented with 0.5% methanol every 24 hr.

SDS-PAGE and Western-blotting

15 Samples of harvested culture medium were analysed on a 15% sodium dodecyl
sulfate (SDS)-polyacrylamide gel [18]. Proteins were transferred onto nitrocellulose
sheets (Hybond C, Amersham) [19], which were successively incubated with an
anti-peptide 76-95 of SAG1, with an anti-rabbit 1gG alkaline phosphate conjugate,
and finally with 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitroblue
20 tetrazolium (NBT) (Promega). Protein blots were saturated and washed between
incubations with phosphate-buffered saline containing 0.05% Tween-20.

Peptides synthesis and purification

25 Two peptides derived from SAG1 were chosen according to predictive algorithms
for B-cell epitopes [20-22]: peptides NHFTLKCPKTACTEPPTLAY (aa 76-95) and
CNEKSFKDILPKLTEN (aa 238-253). They were synthesised by the Merrified
solid phase method on a fully automated peptide synthesiser (AB1 model 430 A,
Foster City, CA), according to the tertbutyloxycarbonyl/trifluoroacetic acid
30 (tBoc/TFA) strategy [23]. After synthesis, peptides were deprotected and cleaved
from the resin by hydrogen fluoride. The crude peptides were purified by gel-
filtration on TSK HW 40s (Merck, Rahway, NJ) and reverse phase HPLC on

WO 99/66043

PCT/EP99/03957

Nucleosil C₁₈ and thin-layer chromatography, and for identity by amino-acid analysis after total acid hydrolysis. Peptides were conjugated to the tetanus toxoid with coupling agents as carbodiimide for the first peptide and 6-maleimidocaproic acyl N-hydroxysuccinimide ester (MCS) for the second.

5

Immunisations with peptides

Rabbits were subcutaneously immunised at one month intervals using 500 µg of conjugated peptide emulsified in complete Freund's adjuvant for the first injection and Freund's incomplete adjuvant for the second one.

10

Purification of recombinant SAG-1

The *Pichia pastoris* culture supernatant (400 ml) from a high density fermentation was concentrated by ultrafiltration using YM 10 membrane (cut-off 10 kD) under a pressure of 3 bars. The concentrate was then dialysed against Tris-HCl 20mM, pH 8.5. The sample was then loaded onto a Q-sepharose fast flow (Pharmacia) column equilibrated in the same buffer. Recombinant SAG-1 was eluted with 100 mM NaCl in the same buffer. The sample was then concentrated and applied onto a superdex 75 HR column (Pharmacia LKB) equilibrated in 20 mM Tris-HCl pH 8.5, 150 mM NaCl. The protein content was determined by the method of Lowry with ovalbumin as standard [24].

15

20

N-glycanase treatment

25

Purified recombinant SAG1 was heat-denatured 10 min in presence of 0.05% SDS and 0.1% 2-mercaptoethanol in 50 mM sodium phosphate pH 8. The protein was then digested with 0.3 U N-glycanase F (Boehringer) for 6 hr at 37°C in presence of 0.7% Nonidet P-40. Samples were electrophoresed on a 15% SDS-polyacrylamide gel.

30

NH2 terminal amino acid sequencing

- 15 -

Automated Edman degradation of recombinant SAG1 or fragment thereof according to the invention was performed in an Applied Biosystems Model 477A sequencer equipped with a phenylthiohydantoin analyser. The samples were prepared after
5 electroblotting on polyvinylidene fluoride membrane.

Detection of human immune serum reactivity to recombinant SAG1

Heparinised blood from donors were centrifuged. Sera were collected and stored
10 at -20°C until used. The presence of specific anti-SAG1 antibodies in sera was detected using an ELISA assay. 96-Microwells plates were coated overnight at 4°C with 0.1 mg/well of purified recombinant SAG1 or with 8.3 mg/well of soluble *T. gondii* lysate (RH strain, ARGENE). Wells were washed four times with Tris-buffered saline (TBS)-0.1% Tween and saturated with TBS-Tween/1% BSA (bovine
15 serum albumine) (30 min. at 37°C). ELISA plates were then incubated with serial dilutions of sera (45 min. at 37°C). Immune complexes were detected with alkaline phosphatase-labeled goat anti-human IgG antibody (45 min. at 37°C) and N-p-nitrophenyl phosphate. The O.D. value at 415 nm were measured in a microplate reader (Biorad).

20

Cell Proliferation assay

PBMC were isolated from heparinised blood as previously described [25]. One hundred thousand peripheral-blood mononuclear cells (PBMC) were incubated in
25 the presence of different concentrations of recombinant SAG1 or *T. gondii* lysate (RH strain, ARGENE). After 4 days at 37°C in a humidified atmosphere containing 5% CO_2 , cells were pulsed with 0.5 μCi of [^3H]thymidine (Amersham). The results were expressed as mean counts per minute (c.p.m) $\pm$ standard deviation of 4 experiments.

30

Plasmid construction and expression experiments

WO 99/66043

PCT/EP99/03957

In a first step, the sequence coding for SAG1 (336 amino acids residues) was recovered by PCR amplification from a lambda gt11 tachyzoite cDNA library, as described above. This sequence, verified by automatic dideoxysequencing, carries a 3' terminal region coding for a stretch of hydrophobic amino acids (residues 308 to 336) which serves as acceptor of the so-called GPI group, i.e. a phosphatidylinositol glycolipid. Native SAG1 is in fact anchored in toxoplasma membranes via this GPI group. The SAG1 coding sequence was engineered to remove the region specifying amino acids 308 to 336, then inserted, downstream to and in frame with the *S. cerevisiae* prepro α -mating factor secretion signal sequence (MF α), into the multicopy *P. pastoris* expression vector pPIC9K. The resulting plasmid, pNIV3488, thus carries, under the control of the methanol-inducible AOX1 promoter, the fused sequences of MF α and anchor-less SAG1, together with a kanamycin resistance gene cassette necessary for subsequent selection of multicopy integrants by the antibiotic G418. Plasmid pNIV3488 was linearised with *Bgl*II to orient integration events at the *AOX1* locus *P. pastoris* recipient cells, strain SMD1168 (*his4*, *pep4*) were transformed with linearised plasmid by the spheroplast method.

Altogether 52 clones, obtained after selection of transformants on histidine-deficient medium, were tested for methanol utilisation and then for growth in the presence of G418 (0.25 to 2 mg/ml) in order to identify those carrying multiple copies of the resistance gene and of the gene of interest.

Four transformants resisting to 2 mg/ml G418 were tested for expression; supernatants of methanol-induced cultures were analysed, in reducing conditions, on SDS-polyacrylamide gels and proteins were detected by immunoblotting using anti-peptide antibodies targetting SAG1 residues 76 to 95 and 230 to 253 respectively.

As seen in Figure 1a lane 2, in all cases, two secreted immunoreactive products were detected, having molecular masses of 34.5 and 31.5 kDa respectively. Since

SAG1 is a highly conformational antigen, it was of interest to analyse the recombinant products under non-denaturing conditions using in this case for detection the monoclonal antibody TG5.54 which is specific for native SAG1 [29] (gift of Prof. Capron, Lille). Two observations arose from this experiment. First, as expected and already reported, the non-reduced native SAG1 antigen migrated with a higher mobility than its denatured equivalent (FIG. 1a, lane 1 and 1b: lane 4, 30 kDa versus 33 kDa). This phenomenon results from the preservation of correct disulfide pairing in the SAG1 molecule under non-reducing conditions. Likewise, the two recombinant products secreted by *P. pastoris* transformants were not only detected by the conformation-specific antibody but migrated also with a higher mobility, banding at apparent molecular masses of 27.5 and 31.5 kDa respectively (FIG. 1b, lane 6 and 1d; lane 3). These results indicate that recombinant SAG1 antigens were secreted under an appropriate conformation for recognition by the monoclonal antibody TG5.54 and suggest that correct disulfide pairing was achieved. The SAG1 antigen molecule presents a potential N-Glycosylation site which, in the parasite, is not effectively glycosylated [30]. However, in experiments where SAG1 was produced in mammalian cells, glycosylation was observed [28]. We thus checked this possibility for yeast-derived SAG1 antigen by treating samples with N-glycanase F. As seen in FIG 1c; lane 2, a single immunoreactive band of 31.5 kDa was detected. In another experiment, it was found that the 34.5 kDa form of recombinant SAG1 was recognised by the GNA lectin (*Galanthus nivalis* agglutinin) which identifies mannose residues (data not shown). It appears therefore that *P. pastoris* achieved N-glycosylation, at least in part, of the SAG1 anchor-less antigen and also that this modification had no significant effect on the conformation of the recombinant product since, as said above, it was clearly recognised by the specific monoclonal antibody TG5.54.

Recombinant anchor-less SAG1 was then purified to near homogeneity starting from spent culture medium of the highest secreting yeast transformant. The combination of anion exchange (Q-sepharose) and gel filtration (superdex 75HR) chromatographies yielded about 12 mg of $\geq 95\%$ pure product per litre of culture (FIG. 1d). Both purified 34.5 and 31.5 kDa forms of the recombinant SAG1 were

WO 99/66043

PCT/EP99/03957

submitted to N-terminal amino acid analysis which indicated the occurrence of two additional amino acid residues. Glu-Ala, on each N-terminal end. This result revealed the incomplete processing of the prepro MF α signal peptide by the dipeptidyl aminopeptidase STE13, a phenomenon already reported in other cases [31-32]. The presence of these excendatary amino acids had obviously no effect on the conformation of the molecule since, as shown above, recognition by the conformation-specific monoclonal antibody TG5.54 was demonstrated.

In order to evaluate the seroreactivity of recombinant SAG1, sera from *T. gondii*-positive and negative individuals were tested in an ELISA format where either total soluble *T. gondii* antigens or purified recombinant SAG1 were coated onto microtiter plates. All sera has been characterised beforehand by commercial immunoassays (anti-Toxoplasma immunoglobulin M (IgM) and IgG detection kit from Behring) for the presence of IgG and IgM. All 20 sera tested were IgM negative, 8 were clearly IgG-positive, 4 probably IgG-positive and 8 IgG-negative. The absence of IgM and the presence of IgG indicates that the individuals were chronically infected by *T. gondii*. As seen in FIG.2, all positive samples reacted with recombinant SAG1 indicating recognition of the antigen by IgG antibodies. Having no IgM-containing sera, it is impossible at this stage to evaluate the seroreactivity of recombinant SAG1 in instances of acute infection.

Purified recombinant SAG1 was further characterised in terms of cellular proliferative capability. To this end, polymorphonuclear cells, derived from four *T. gondii*-seropositive individuals, were isolated then stimulated *in vitro* either with total soluble antigens of *T. gondii* or with purified recombinant SAG1. As seen in FIG.3, a significant proliferative response (Stimulation Index=4) was observed with the recombinant protein. This result strengthens the interest of yeast-derived SAG1 as a putative antigen for the preparation of a toxoplasmosis vaccine.

30

Example 2: protection against a Toxoplasma challenge

Mouse immunization and parasite challenge

Female BALB/c (8-week-old) were subcutaneously immunized twice at two weeks intervals with 10 µg of purified recombinant SAG1 combined either with the
5 SBAS1c adjuvant (10 µg of 3D-MPL, 10 µg of DQ) or with 100 µg of aluminium hydroxide; 1 dose = 100 µl. Two weeks after the last injection, all mice were injected interperitoneally with 10⁴ *T. gondii* C56 strain tachyzoites.

Parasites

10

The *T. gondii* C56 strain (kindly donated by Darde, Centre Hospitalier Régional et Universitaire de Limoges, France) was maintained by serial passage in the peritoneal cavities of BALB/c mice. Tachyzoites were collected from the peritoneal cavity of infected mice as previously described (Saavedra et al, 1991 b).

15

Results

The protective potential of recombinant SAG1 was evaluated in a lethal toxoplasmosis mouse model. To this end, groups of five BALB/c mice were
20 subcutaneously immunized twice at two weeks intervals with 10 µg of recombinant SAG1 combined either with the SBAS1c adjuvant (proprietary composition of SmithKline Biologicals, Rixensart, Belgium), which induces a Th1-type response or with aluminium hydroxide known to induce a Th2-type response. Control groups of mice received adjuvants alone. 15 days after the second injection, all mice were
25 challenged with 10⁴ tachyzoites of the *T. gondii* C56 strain administered intraperitoneally. As seen in Figure 4, and as expected, all mice from control groups died within 8 days post challenge. In addition, all mice immunized with SAG1 adjuvanted with aluminium hydroxide also died in this time span, indicating that a Th2-type response is inappropriate to confer protection. In contrast, an
30 overall protection of 60% was observed in the group of mice immunized with recombinant SAG1 combined to the Th1-type inducing adjuvant. Although two

WO 99/66043

PCT/EP99/03957

mice in this group died, their survival time was nevertheless significantly extended with respect to that of control mice (13 and 18 days respectively versus 8 days). These results thus indicate that immunization with recombinant SAG1, properly adjuvanted to induce a Th1-type immune response can protect mice against a lethal
5 challenge. The level of protection obtained here is of the same magnitude (60% versus 67%) as observed before (Khan et al, 1991) in bred A/J mice immunized with purified parasite-derived SAG1 adjuvanted with Quil A.

In conclusion, the set of data presented here strengthens the interest of yeast-derived
10 SAG1 as a potential vaccine antigen.

Example 3: Expression of the toxoplasma antigen SAG1 in *Saccharomyces cerevisiae* and in *Pichia pastoris*. Comparison between the two systems.

15

The DNA sequence coding for SAG1 with its native sequence signal or with the signal sequence of the yeast pheromone MF α -1 was introduced in the *S. cerevisiae* expression plasmid TCM97 (pRIT13145). The resulting plasmids respectively pNIV3433 and pNIV3435, contain, under the control of the ARG3 promoter, the
20 sequence encoding the residues 18 to 336 of SAG1 for the first one and the sequence encoding the 19 amino acids of the signal sequence of MF α -1 followed by residues 48 to 336 of SAG1 for the second one (FIG. 5). In order to obtain secretion of SAG1 by yeast cells, the sequence encoding the SAG1 hydrophobic carboxy-terminal (residues 308 to 336) was deleted to prevent addition of the GPI
25 group. The resulting plasmids, pNIV3448 and pNIV3441, contain respectively the sequence encoding the residues 18 to 307 of SAG1 and the sequence encoding the 19 amino acids of the signal sequence of MF α -1 followed by residues 48 to 307 of SAG1 (FIG. 5). The sequence coding for the residues 48 to 307 of SAG1 was also introduced downstream of the α factor prepro peptide in the *P. pastoris* expression
30 vector, pIC9K, to give pNIV3488 (FIG. 5).

WO 99/66043

PCT/EP99/03957

In *S. cerevisiae*, SAG1 will be constitutively expressed under the control of the ARG3 promotor placed on a 2- μ -based high copy plasmid TCM97 with *dLEU2* selection maker. Complementation of the leucine auxotrophy requires a higher copy number since the expression level of the *dLEU2* gene is low due to its deleted promoter.

The transformed *S. cerevisiae* strains were grown for 72 hours in 40 ml YNB at 30°C and 200 rpm shaking. Cells were harvested by centrifugation, lysed and the soluble protein extracts and culture medium (20 μ l) were analysed for the presence of SAG1 by proteins separation on SDS-PAGE and transfer onto nitrocellulose membrane. However, immunodetection of SAG1 was only observed after TCA (trichloroacetic acid) precipitation of proteins from 40 ml of culture medium. Indeed, two proteins of about 33 and 36kDa were detected in immunoblot but not visualised by silver-staining detection, confirming the very low secretion of SAG1. Curiously, these bands were principally observed with the yeast cells producing the GPI-anchored SAG1 (pNIV3433 plasmid). Soluble forms of a variety of GPI-anchored proteins occurring extracellularly were recently reported (Wang et al, Biochemistry, 36: 14583-14592 (1997)). However, the molecular mechanisms governing their release are not entirely clear. An intracellular form of SAG1 was also observed but only in a 8 M urea extract of the insoluble fraction.

20

In conclusion in *S. cerevisiae*, we observed a very low production of SAG1. It is possible that overproduction conditions represent a stress situation for the yeast cells leading to a growth advantage for cells with reduced expression and thus reduced copy number.

25

The highly inducible and stringently regulated methanol oxidase gene (AOX1) promoter was used for the production of SAG1 in *P. pastoris*. Recombinant SAG1 was easily detected after proteins separation from 20 μ l of the culture medium (40 ml) on SDS-PAGE followed by coomassie or silver staining. Two proteins of about 31.5 and 34.5 were immunodetected using antipeptides targetting SAG1 residues 76 to 95 and 230 to 253 respectively. The combination of anion exchange

30

WO 99/66043

PCT/EP99/03957

(Q-sepharose) and gel filtration (superdex 75HR) chromatographies yielded about 12 mg of $\downarrow$ 95% pure product per litre of culture.

It has been reported that SAG1 has been produced in *S. cerevisiae* (see WO 96/02654). The DNA sequence encoding the amino acids 48 to 316 of SAG1 was also placed downstream to, and in-frame with, the DNA sequence encoding the α -mating factor prepro secretion signal sequence. SAG1 was expressed under the control of the α -mating factor promoter. The *dURA3* gene was used as selection marker and the KEX2 gene used in order to circumvent an eventual problem of incomplete processing of the prepro region of MF α . The recombinant SAG1 was secreted under a heterogeneous form suggesting an incomplete processing by KEX2 and/or heterogeneous glycosylation of the protein (SAG1 and the pro region of MF α possess respectively one and three potential site of N-glycosylation). Expression level of SAG1 seems to be low: secreted SAG1 production obtained in WO 96/02654 in *Schizosaccharomyces pombe*, *S. cerevisiae* or in insect cells is between 0.1 mg/l to 0.3 mg/l. In *Schizosaccharomyces pombe*, only a major protein of about 35 kDa (28 kDa if the N-glycosylation site is mutated) was observed in the culture medium.

In conclusion, the *P. pastoris* expression system is more efficient for the production of a recombinant SAG1. Indeed, the secreted recombinant SAG1 level is at least ten times superior to the one observed in WO 96/02654 in *S. cerevisiae*, in *S. pombe* and in insect cells. Moreover, only two forms of the recombinant protein were secreted in *P. pastoris* in contrast to a heterogeneous product in *S. cerevisiae*. The preferred mode of expression in *P. pastoris* is by chromosomal integration using one of the integrative plasmids. There are several advantages for using integrative transformation which include expression cassette stability, generation of multicopy strains, control of site of integration and ability to engineer different modes of integration using appropriately cleaved DNA.

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WO 99/66043

PCT/EP99/03957

Both *S. cerevisiae* and *P. pastoris* have a majority of N-linked glycosylation of the high-mannose type; however, the length of the oligosaccharide chains added post-translationally to proteins in *Pichia* (average 8-14 mannose residues per side chain) is much shorter than those in *S. cerevisiae* (50-100 mannose residues).

5

Example 4: Protective effect of vaccination with recombinant SAG1 against congenital toxoplasmosis in Guinea Pig

Materials and methods

10 **T.gondii**

The C56 medium-virulent strain of *Toxoplasma gondii* (Supplied by ML Darde, CHU Limoges), maintained by passage of infective brain homogenate in the peritoneum of BalbC mice, was used for experimental infections in Durkin-Hartley
15 guinea pigs.

Vaccination procedure

Groups of 20 guinea pigs were immunized three times at 3 weeks intervals using
20 either 10µg of recombinant SAG1 formulated with SBAS1c adjuvant (10µg of 3D-MPL, 10µg of DQ) or SBAS1c alone (SC route). SAG1 was produced in *Pichia pastoris* and purified according to the procedure described in Example 1 above.

Measurement of antibody response

25

Two weeks after the last immunization, animals were bled and sera were checked for the presence of anti-SAG1 IgG antibodies in an enzyme-linked immunosorbent assay (ELISA) using recombinant SAG1 as coating reagent (100ng/well). Serial dilutions of each serum were tested and titer was expressed as the reciprocal of the dilution giving
30 one-half the maximal optical density.

WO 99/66043

PCT/EP99/03957

Congenital infection model

Before antigen injection, all guinea pigs were monitored for absence of seroreactivity against toxoplasma. Females were mated with males for breeding
 5 three weeks after the last immunization ; challenge infection using 5×10^5 tachyzoites (ID route) followed 7 weeks later.

Infectious status of pups delivered from guinea pigs was evaluated in a mouse assay :
 pups were sacrificed within 48 hours following delivery, each brain was homogenized
 10 in 1ml of PBS and intra peritoneally injected into two female BalbC mice (0.5 ml each). Mice that did not survive from 21 days onwards after brain homogenate injection were considered infected and their mortality indicated the infection status of the pups ; it was assessed that a pup was infected once one of the two injected mice died.

15

Results**IgG response of guinea pigs immunized with rSAG1**

20 The geometrical mean was 63065 with values between 24226 and 248217.
 The titers in the mock-immunized group were below the detectable level.

Congenital transmission

25 After challenge, 18 SAG1- and 17 mock-immunized guinea pigs produced respectively 69 and 59 pups ; of those 19 and 17 respectively were excluded from further analysis because, in a precedent experiment, we observed that stillborn pups or pups retrieved from dead mothers were always negative in the mouse assay even when they originated from the mock-immunized group, probably due to parasite
 30 inactivation.

After exclusion of the 36 pups, 50 and 42 pups, originated from 17 and 15 litters

WO 99/66043

PCT/EP99/03957

respectively, were analysed.

Protection against vertical transmission was observed and summarized in Table 1.

5

Table 1

Read out	SAG1 immunized	mock-immunized
Number of litters	17	15
Overall mortality in mice injected with brain's pup	11%	77%
Number of litters without infected pups	11	0
Number of litters partially infected	5	7
Number of litters fully infected	1	8
Number of pups	50	42
Proportion of infected pups	7/50 (14%)	36/42 (86%)

Similar results were obtained in a further experiment.

10

Example 5: Expression of unglycosylated SAG1 protein in *P. pastoris*

The SAG1 gene encodes a consensus N-linked glycosylation site (Asn-X-Ser/Thr) which is not used by the parasite (Odenthal-Schnittler et al, 1993, Biochem. J. 291: 713-721). Elimination of the consensus N-glycosylation site can prevent glycosylation of SAG1 by the yeast. To this end, the asparagine at the potential N-linked glycosylation site (amino acid 259) was mutated to glutamine.

20 The sequence encoding the unglycosylated anchor-less SAG1 was obtained as follows:

to change the Asn in position 250 to glutamine the following mutagenic oligonucleotide was synthesized 5'AGCGTGGCACCCCTTATCACTCGAAGCTTGA CCCTG3' and used as antisense primer with the sense oligonucleotide

25 5'AGACAACAATCAGTACTGTTCCGGGAC3' to amplify a 129 bp DNA fragment. The DNA sequence (pNIV3418) encoding SAG1 was used as template.

WO 99/66043

PCT/EP99/03957

The amplified DNA fragment was then digested by *ScaI* and *BanI* endonucleases. A 642 bp *HindIII-ScaI* and a 149 bp *BanI-EcoRI* DNA fragments were recovered from plasmid pNIV3418 and inserted with the digested PCR fragment in the pUC19 previously opened with *HindIII* and *EcoRI* to give pNIV4710 (FIG. 6).

5

A 781 bp *BamHI-EcoRI* DNA fragment was recovered from plasmid pNIV4710 and introduced together with annealed oligonucleotides

5'TCGAGAAAAGAGAGGCTGAAGCTTCG3' and

5'GATCCGAAGCTTCAGCCT

10 CTCTTTTC3' to provide the junction between the fragment obtained above and the *P. pastoris* secretion vector pPIC9 (Invitrogen) cut by *XhoI* and *EcoRI* (FIG. 7).

The resulting plasmid, pNIV4729 contains as the plasmid pNIV3488 the sequence encoding the amino acids 48 to 307 of SAG1 except that the Asn in position 259 was mutated to glutamine. The sequence encoding the unglycosylated SAG1 was

15 also introduced in the *P. pastoris* vector pPIC9K to give pNIV4732.

Plasmid pNIV4729 or pNIV4732 was introduced into the *P. pastoris* strain SMD1168 (*his4, pep4*) by using the spheroplast transformation method. Cell culture were performed as described for the N-glycosylated anchor-less SAG1.

20

Supernatants were collected every 24 hr and analyzed for protein expression by silver-stained SDS-PAGE and western blot. Elimination of the N-glycosylation site did not affect the secretion of SAG1. Indeed, a protein of an apparent molecular mass of 31.5 kDa was detected with antibodies targeting SAG1 residues 76-95 and 230-253 respectively. Weaker proteins with slower mobility at apparent molecular masses of 36 and 40 kDa were also detected suggesting an incomplete processing of the prepro MFa signal peptide. Under non-reducing conditions, only a single protein at apparent molecular mass of 27.5 kDa was detected with conformation-specific antibodies as the monoclonal antibodies TG5.54 (gift of Prof. Capron,

30 Lille) or GII.9 (ARGENE), suggesting a correct disulfide pairing.

Unglycosylated recombinant SAG-1 purification

The *P.pastoris* culture supernatant from a high density fermentation was concentrated by ultrafiltration using YM 10 membrane (cut-off, 10 kDa, Amicon) under a pressure of 3 bar. The concentrate was desalted onto a sephadex G-25 column (2.6 x 35 cm, Pharmacia) equilibrated in 20 mM citrate bufer, pH 3.3. The sample was then directly applied onto a macroprep S column (2.6 x 10 cm, Bio-rad) conditioned in the citrate buffer. Unglycosylated recombinant SAG-1 was eluted with 400-500 mM NaCl in the same buffer. Enriched SAG-1 fractions were pooled, concentrated by ultrafiltration and loaded onto a superdex 75 HR column (1 x 30 cm, Pharmacia) equilibrated in phosphate-buffered saline (PBS) pH 7.3. Unglycosylated recombinant SAG-1 migrated on SDS-PAGE as a molecule of approximately 30 kDa; the protein was recognized as a single band on western blot by antipeptide antibodies directed to SAG-1 residues 230-253. The final yield was about 16mg of purified unglycosylated recombinant SAG-1 per liter of yeast culture. The product was estimated more than 95% pure.

20 Example 6: Recognition of Recombinant SAG1 by Sera and PBMC

Human seroreactivity to SAG-1

Heparinized blood from donors was centrifuged; the presence of specific anti-SAG1 antibodies was detected in the supernatant using an enzyme-linked immunosorbent assay (ELISA). 96 Microwell plates were coated overnight at 4°C with either 100 ng per well of purified recombinant SAG1 produced as described in example 1 or with unglycosylated SAG1 produced as described in example 5. Wells were washed five times with Tris-buffered saline (TBS)-0.1% Tween 80 and saturated in the same buffer supplemented with 1% bovine serum albumin (BSA) for 1 hour at 37°C. Plates were then incubated with serial dilutions of sera (1 hour at 37°C). Plates were washed as described above and alkaline phosphatase-labelled goat anti-

WO 99/66043

PCT/EP99/03957

human IgG antibody diluted 1 :7500 was used as the secondary antibody (1 h at 37°C). After washing, immune complexes were developed with p-nitrophenyl phosphate as chromogenic substrate. Absorbance at 415 nm was measured in a microplate reader (Biorad).

- 5 ELISA titer was calculated as the reciprocal dilution giving 50% of the maximal O.D. signal.

Cell proliferation assay

- 10 Peripheral-blood mononuclear cells (PBMC) were isolated from heparinized blood. Two hundred thousand PBMC were resuspended in RPMI 1640 medium supplemented with 10% human serum AB (PAA laboratories), 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2 ethanesulfonic acid), 2mM L-glutamine, 1 mM sodium pyruvate, $5 \cdot 10^{-5}$ mM β -mercaptoethanol and 50 IU/ml penicilline-
- 15 streptomycine. Cells were incubated in the presence of different concentrations of glycosylated and unglycosylated recombinant SAG1 (50, 5, 0.5 and 0.05 μ g/ml) . After 4 days at 37°C, cells were pulsed with 1 μ Ci per well of (^{3} H)-thymidine. 16 hours later, they were harvested and incorporated radioactivity was determined
- 20 expressed as the stimulation index (SI), calculated as the ratio of mean cpm in SAG1 stimulated wells to control wells, in the presence of 5 μ g/ml of SAG1. A positive response was defined as an SI > 2

WO 99/66043

PCT/EP99/03957

Serum	Anti-Toxoplasma titer (e)	Anti-SAG1 ELISA titer		Stimulation Index	
		<i>SAG1 ungly</i> (a)	<i>SAG1</i> (b)	<i>SAG1 ungly</i> (c)	<i>SAG1</i> (d).
1	> 300	3300	3274	5,2	3,2
2	196	869	3635	3,7	5,4
3	186	645	2703	4,6	4,4
4	112	150	452	2,1	2,9
5	85	638	574	2,2	2,4
6	81	572	481	1,3	1,2
7	44	969	1425	3,8	3,9
8	44	540	2738	1,5	1,3
9	32	243	1920	1,4	1,5
10	30	223	330	1,5	1,3
11	24	267	1130	1,5	1,2
12	21	80	420	1,2	1,3
13	13	208	415	1,5	0,8
14	10	50	382	2,2	1,3
15	4	105	475	1,1	1,0
16	0	10	274	1,2	1,2
17	0	0	549	1,6	1,3

(a) coating antigen, prepared as described in example 5

5 (b) coating antigen, prepared as described in example 1

(c) stimulating antigen, prepared as described in example 5

(d) stimulating antigen, prepared as described in example 1

(e) IgG anti-Toxoplasma ELISA was performed by Dr Bigaignon (UCL) and expressed in IU (ELISA VIDAS, Bio-Mérieux), serum was considered as

10 *Toxoplasma* seropositive for value ≥ 8 UI.

WO 99/66043

PCT/EP99/03957

Like SAG1 described in example 1, the unglycosylated form of SAG1 was able to induce proliferative response of T lymphocytes from *Toxoplasma* seropositive donors and was also recognized by antibodies from the same donors.

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WO 99/66043

PCT/EP99/03957

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WO 99/66043

PCT/EP99/03957

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WO 99/66043

PCT/EP99/03957

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WO 99/66043

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Claims

1. A method for the production of the toxoplasma antigen SAG1 or a fragment thereof, which comprises:
 - 5 (a) constructing a plasmid comprising DNA encoding SAG1 or a fragment thereof;
 - (b) transforming a *P. pastoris* host cell with the said plasmid; and culturing the host such that the DNA encoding SAG1 or a fragment thereof is expressed.
- 10 2. A method as claimed in claim 1 which further comprises the step of purifying the SAG1 protein or fragment thereof from the culture medium.
- 15 3. A method as claimed in claim 1 or claim 2 in which the DNA encoding the SAG 1 protein or a fragment thereof encodes a truncated SAG1 protein lacking the anchor domain (lacking amino acids 308 to 336 of the SAG1 protein).
- 20 4. A method as claimed in claim 3 in which the DNA encoding the truncated SAG1 protein encodes amino acids 48-307 inclusive of the SAG1 protein.
- 25 5. A method as claimed in any one of claims 1 to 4 in which the DNA encoding the SAG1 protein or fragment thereof is positioned downstream from and in frame with a yeast secretion signal sequence.
6. A method as claimed in any one of claims 1 to 5 in which the said plasmid is derived from a multicopy *P. pastoris* expression vector.
- 30 7. A method as claimed in any one of claims 1 to 6 in which the DNA encoding SAG1 or a fragment thereof is expressed under the control of a methanol-inducible promoter.

WO 99/66043

PCT/EP99/03957

8. Plasmid pNIV3488.
9. A SAG1 protein or fragment thereof when expressed in *P. pastoris*.
- 5 10. A SAG1 protein or fragment as claimed in claim 9, which is a truncated SAG1.
- 10 11. A truncated SAG1 protein as claimed in claim 10 in which the anchor region of SAG1 is absent.
12. A truncated SAG1 protein as claimed in claim 10 or claim 11 which comprises amino acid residues 48-307 of SAG1, and immunogenic derivatives thereof.
- 15 13. A vaccine composition comprising the protein of any one of claims 9 to 12 in combination with a suitable adjuvant and/or carrier.
14. A vaccine composition as claimed in claim 13 wherein the adjuvant is a Th1-
20 type inducing adjuvant.
15. A vaccine composition as claimed in claim 14 wherein the adjuvant comprises at least one immunostimulant chosen from the group consisting of 3D-MPL, QS21 and CpG.
- 25 16. A vaccine composition as claimed in claim 14 or claim 15, formulated with at least one carrier chosen from the group consisting of an oil in water emulsion, an aluminium salt and cholesterol-containing liposomes.
- 30 17. A vaccine composition as claimed in claim 15 or claim 16, wherein the adjuvant comprises 3D-MPL.

WO 99/66043

PCT/EP99/03957

18. A vaccine composition as claimed in any one of claims 15 to 17, wherein the adjuvant comprises a saponin such as QS21.
19. A vaccine composition as claimed in claim 18 wherein the adjuvant is 3D-MPL, in combination with QS21 in a cholesterol-containing liposome.
20. A vaccine composition as claimed in any one of claims 17 to 19, further comprising alum.
21. A vaccine composition as claimed in claim 18 wherein the adjuvant is 3D-MPL and QS21 in an oil in water emulsion.
22. A vaccine composition as claimed in claim 15 or claim 16 wherein the adjuvant comprises CpG and alum.
23. A truncated SAG1 protein in which the anchor region of SAG1 is absent.
24. A truncated SAG1 protein as claimed in claim 23 which comprises amino acid residues 48-307 of SAG1, and immunogenic derivatives thereof.
25. A vaccine composition comprising the protein of claim 23 or claim 24 in combination with a suitable adjuvant and/or carrier.
26. A vaccine composition as claimed in claim 25 wherein the adjuvant is a Th1-type inducing adjuvant.
27. A vaccine composition as claimed in claim 26 wherein the adjuvant comprises at least one immunostimulant chosen from the group consisting of 3D-MPL, QS21 and CpG.

WO 99/66043

PCT/EP99/03957

28. A vaccine composition as claimed in claim 26 or claim 27, formulated with at least one carrier chosen from the group consisting of an oil in water emulsion, an aluminium salt and cholesterol-containing liposomes.
- 5 29. A vaccine composition as claimed in claim 27 or claim 28, wherein the adjuvant comprises 3D-MPL.
30. A vaccine composition as claimed in any one of claims 27 to 29, wherein the adjuvant comprises a saponin such as QS21.
- 10 31. A vaccine composition as claimed in claim 30 wherein the adjuvant is 3D-MPL, in combination with QS21 in a cholesterol-containing liposome.
- 15 32. A vaccine composition as claimed in any one of claims 29 to 31 further comprising alum.
33. A vaccine composition as claimed in claim 30 wherein the adjuvant is 3D-MPL and QS21 in an oil in water emulsion.
- 20 34. A vaccine composition as claimed in claim 27 or claim 28, wherein the adjuvant comprises CpG and alum.
35. A vaccine composition as claimed in any one of claims 25 to 34 which additionally comprises another *Toxoplasma gondii* antigen.
- 25 36. A vaccine composition as claimed in any one of claims 25 to 35 wherein the truncated SAG1 is expressed in yeast, such as *P. pastoris*.
- 30 37. Use of a protein according to any of claims 9 to 12, 23 and 24 in the manufacture of a medicament for the prevention or treatment of toxoplasmosis infections in mammals.

WO 99/66043

PCT/EP99/03957

38. The use according to claim 37, for the prevention of congenital toxoplasmosis infections.
- 5 39. A diagnostic kit for the diagnosis of toxoplasmosis infection in the blood of mammals which may be infected, which kit comprises an anchor-less SAG1 antigen or a fragment thereof.