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(71) Applicant (*for all designated States except US*): **THE UNIVERSITY OF MELBOURNE** [AU/AU]; Royal Parade, Parkville, Victoria 3052 (AU).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): **REYNOLDS, Eric Charles** [AU/AU]; 14 Walsh Street, Balwyn, Victoria 3103 (AU). **O'BRIEN-SIMPSON, Neil Martin** [AU/AU]; 331 Brunswick Road, Brunswick, Victoria 3056 (AU). **PATHIRANA, Rishi Delan** [AU/AU]; 38 Cochrane Avenue, Keysborough, Victoria 3173 (AU).

(74) Agent: **BLAKE DAWSON WALDRON PATENT SERVICES**; Level 39, 101 Collins Street, Melbourne, Victoria 3000 (AU).

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(54) Title: ANTIGENIC COMPLEX FOR THE DIAGNOSIS AND TREATMENT OF *PORPHYROMONAS GINGIVALIS* INFECTION

(57) Abstract: The present invention provides a purified multimeric complex from *P. gingivalis*. The complex comprises at least one domain from each of RgpA, Kgp and HagA, and has a molecular weight greater than about 300 kDa.

ANTIGENIC COMPLEX FOR THE DIAGNOSIS AND TREATMENT OF *PORPHYROMONAS GINGIVALIS* INFECTION

FIELD OF INVENTION

This invention relates to a multimeric protein complex from *Porphyromonas gingivalis*. The
5 present invention also provides methods of obtaining the multimeric complex and to pharmaceutical compositions and associated agents based on the complex and components thereof for the detection, prevention and treatment of periodontal disease associated with *P. gingivalis*.

BACKGROUND OF THE INVENTION

10 Periodontal diseases are bacterial-associated inflammatory diseases of the supporting tissues of the teeth and range from the relatively mild form of gingivitis, the non-specific, reversible inflammation of gingival tissue to the more aggressive forms of periodontitis which are characterised by the destruction of the tooth's supporting structures. Periodontitis is associated with a subgingival infection of a consortium of specific Gram-negative bacteria that leads to the
15 destruction of the periodontium and is a major public health problem. One bacterium that has attracted considerable interest is *P. gingivalis* as the recovery of this microorganism from adult periodontitis lesions can be up to 50% of the subgingival anaerobically cultivable flora, whereas *P. gingivalis* is rarely recovered, and then in low numbers, from healthy sites. A proportional increase in the level of *P. gingivalis* in subgingival plaque has been associated with an increased
20 severity of periodontitis and eradication of the microorganism from the cultivable subgingival microbial population is accompanied by resolution of the disease. The progression of periodontitis lesions in non-human primates has been demonstrated with the subgingival implantation of *P. gingivalis*. These findings in both animals and humans suggest a major role for *P. gingivalis* in the development of adult periodontitis.

25 *P. gingivalis* is a black-pigmented, anaerobic, asaccharolytic, proteolytic Gram-negative rod that obtains energy from the metabolism of specific amino acids. The microorganism has an absolute growth requirement for iron, preferentially in the form of haeme or its Fe(III) oxidation product haemin and when grown under conditions of excess haemin is highly virulent in experimental animals. A number of virulence factors have been implicated in the pathogenicity of *P.*
30 *gingivalis* including the capsule, adhesins, cytotoxins and extracellular hydrolytic enzymes. In particular, proteases have received a great deal of attention for their ability to degrade a broad range of host proteins including structural proteins and others involved in defence. The proteins that have been shown to be substrates for *P. gingivalis* proteolytic activity include collagen types I and IV, fibronectin, fibrinogen, laminin, complement and plasma clotting cascade proteins, α_1-

antitrypsin, α_2-macroglobulin, antichymotrypsin, antithrombin III, antiplasmin, cystatin C, IgG and IgA. The major proteolytic activities associated with this organism have been defined by substrate specificity and are "trypsin-like", that is cleavage on the carboxyl side of arginyl and lysyl residues and collagenolytic although other minor activities have been reported.

5 *P. gingivalis* trypsin-like proteolytic activity has been shown to degrade complement, generating biologically active C5a, impair the phagocytic and other functions of neutrophils by modifying surface receptors, and abrogate the clotting potential of fibrinogen prolonging plasma clotting time. The trypsin-like proteolytic activity of *P. gingivalis* also generates Fc fragments from human IgG1 stimulating the release of pro-inflammatory cytokines from mononuclear cells and 10 is associated with vascular disruption and enhanced vascular permeation through the activation of the kallikrein-kinin cascade. *P. gingivalis* spontaneous mutants with reduced trypsin-like activity as well as wild-type cells treated with the trypsin-like protease inhibitor N-*p*-tosyl-L-lysine chloromethyl ketone are avirulent in animal models. Further, it has been shown that *P. gingivalis* grown under controlled, haemin-excess conditions expressed more trypsin-like and 15 less collagenolytic activity and were more virulent in mice relative to cells grown under haemin-limited but otherwise identical conditions.

There has been considerable endeavour to purify and characterise the trypsin-like proteases of *P. gingivalis* from cell-free culture fluids. Chen *et al*, (1992) [J Biol Chem 267:18896-18901] have purified and characterised a 50 kDa arginine-specific, thiol protease from the culture fluid of *P.* 20 *gingivalis* H66 designated Arg-gingipain. A similar arginine-specific thiol protease has been disclosed in JP 07135973 and the amino acid sequence disclosed in WO 9507286 and in Kirszbaum *et al*, 1995 [Biochem Biophys Res Comm 207:424-431]. Pike *et al* (1994) [J Biol Chem 269:406-411] have characterised a 60 kDa lysine-specific cysteine proteinase from the culture fluid of *P. gingivalis* H66 designated Lys-gingipain and the partial gene sequence for this 25 enzyme was disclosed in WO 9511298 and fully disclosed in WO 9617936. In addition, a cell surface protein complex of *P. gingivalis* comprising a 300 kDa complex of arginine-specific and lysine-specific proteases both containing adhesin domains is disclosed in US 6,511,666.

SUMMARY OF THE INVENTION

The present inventors have extracted from *P. gingivalis* a cell surface associated complex 30 comprising a multimeric complex of processed domains of RgpA, Kgp and HagA to form a high molecular weight (>300 kDa) proteinase-adhesin complex.

Accordingly in a first aspect the present invention consists in a purified multimeric complex from *P. gingivalis*, the complex comprising at least one domain from each of RgpA, Kgp and HagA, and having a molecular weight greater than about 300 kDa.

In a preferred embodiment the complex has a molecular weight greater than about 500 kDa, more preferably more than about 800 kDa.

In a second aspect of the present invention provides a method of obtaining a purified multimeric complex from *P. gingivalis*, the complex comprising at least one domain from each of RgpA,
5 Kgp and HagA, and having a molecular weight greater than about 300 kDa the method comprising detergent extraction of the complex from whole *Porphyromonas gingivalis* cells.

In a preferred embodiment the complex is subjected to further purification using ion exchange or ultrafiltration and diafiltration methods.

In a further preferred embodiment the detergent is Triton X114.

10 In a preferred embodiment the *Porphyromonas gingivalis* is a virulent strains. It is also preferred that the *P. gingivalis* has high arginine and/or lysine proteolytic activity.

In a third aspect the present invention consists in a composition for use in eliciting an immune response directed against *Porphyromonas gingivalis*, the composition comprising an effective amount of the complex of the first aspect of the present invention and a suitable adjuvant and /or
15 acceptable carrier.

In a fourth aspect the present invention consists in an antibody preparation comprising antibodies specifically directed against the complex of the first aspect of the present invention. The antibodies may be polyclonal antibodies or monoclonal antibodies.

In a fifth aspect the present invention consists in a method of treating a subject suffering from
20 *Porphyromonas gingivalis* infection, the method comprising administering to the subject an amount of the antibody preparation of the fourth aspect of the present invention.

As will be recognised by those skilled in the art the antibody preparation may be administered by any of a number of well known routes, however, it is presently preferred that the preparation is administered orally.

25 In a sixth aspect the present invention consists in a method of reducing the prospect of *Porphyromonas gingivalis* infection in an individual and/or severity of disease, the method comprising administering to the individual an amount of the composition of the third aspect of the present invention effective to induce an immune response in the individual directed against *Porphyromonas gingivalis*.

30 In use the antibodies of the fourth aspect of the present invention may be blended into oral compositions such as toothpaste, mouthwash, toothpowders and liquid dentrifices, mouthwashes,

trouches, chewing gums, dental pastes, gingival massage creams, gargle tablets, dairy products and other food stuffs.

In another aspect the invention provides a method of diagnosis for the presence of *Porphyromonas gingivalis* characterised by the use of the complex of the first aspect of the present invention or antibody of the fourth aspect of the present invention. These methods will involve known techniques including for example, enzyme linked immunosorbent assay.

The invention also provides diagnostic kits comprising the complex of the first aspect of the present invention or antibody of the fourth aspect of the present invention.

The invention also provides a method of treatment of a patient human and/or animal either suffering from *Porphyromonas gingivalis* infection comprising active vaccination of said patient with a composition according to the third aspect and/or passive vaccination of said patient with an antibody of the fourth aspect of the present invention.

BRIEF DESCRIPTION OF FIGURES

Figure 1. Arg-Sepharose affinity chromatography of *P. gingivalis* cell Triton X-114 extract. *P. gingivalis* extracts were added to an Arg-Sepharose column and unbound proteins (peak A) were eluted at a flow rate of 1 mL/min. Non-specifically bound proteins (peak B) were eluted with a linear gradient of 0-40% TC 500 buffer (500 mM NaCl, 50 mM Tris/HCl, 5 mM $CaCl_2$, pH 7.4) at a flow rate of 1.0 mL/min. The complex (peak C) was eluted with TC 50-Arg buffer (500 mM arginine, 50 mM NaCl, 50 mM Tris/HCl, 5 mM $CaCl_2$, pH 7.4) at a flow rate of 1 ml/min. The arrows indicate the start of each step gradient.

Figure 2. SDS-PAGE of Arg-affinity purified *P. gingivalis* Triton X114 extracted complex. Lane 1, Invitrogen molecular weight standards (kDa); lane 2, Triton X114 extracted complex. Gels stained with Coomassie blue. Protein bands (1 to 9) were excised or transferred onto PVDF membrane and identified by peptide mass finger printing analysis or N-terminal sequence analysis, respectively, as described.

Figure 3. Diagrammatic representation of RgpA, Kgp and HagA showing the processed proteinase catalytic and adhesin domains and the N-terminal sequences of each domain. Shaded areas represent the mature, processed domains.

Figure 4: Size exclusion chromatography of the Triton X114 extracted complex. Arginine-affinity purified Triton X114 extracted complex was applied to a size exclusion column (macrosphere 300Å, 7µm, 250 x 4.6 mm, Alltech, Australia). V_0 indicates the void volume of the column (Dextran Blue >2 million Da, was used to determine the void volume). The elution

volumes of the standard proteins A = thyroglobulin (667 kDa), B = ferritin (440 kDa) and C = catalase (232 kDa) are marked.

Figure 5. Murine lesion model of *P. gingivalis* infection; average lesion size of mice immunized with antigenic complex extracted by sonication or by Triton X-114 methodologies. BALB/C mice (10 mice per group) were immunized subcutaneously (s.c.) with complex extracted by Triton X114 and sonication for the primary and secondary immunisations and challenged s.c. 12 days after the second immunisation with *P. gingivalis* ATCC 33277 (1×10^9 viable cells). Animals were monitored over a period of 14 days for the development and size of lesions. Lesion sizes were statistically analyzed using Kruskal-Wallis test and Mann-Whitney U-Wilcoxon rank sum test with a Bonferroni correction for type 1 error. *, ** group significantly different ($p<0.05$, $p<0.01$, respectively) from the control (IFA/PBS) group.

Figure 6. Murine periodontitis model of P. gingivalis-induced periodontal bone loss. Periodontal bone loss of mice immunised with the Triton X114 extracted complex, non-specific immunogenic protein (diphtheria toxoid) and adjuvant alone (Control, IFA/PBS) or unimmunised orally infected (control, infected) mice. Measurement of bone loss is the mean area measured in mm2 from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) of the buccal side of each maxillary molar of both the left and right maxillae. Data was normally distributed as measured by Levene's homogeneity of variance and are presented as mean ± SD (n = 10) and were analyzed using the One-Way analysis of variance and Dunnett's T3 test and Cohen's Effect size. * group significantly different ($p<0.001$) from the orally infected control group and the orally infected control groups immunised with IFA/PBS or the non-specific immunogenic protein, diphtheria toxoid.

Figure 7. Serum antibody subclass responses of immunised mice with the complex extracted using the Triton X114 and sonication methodologies. Sera from mice immunised with the Triton X114 (black bars) and sonication (white bars) extracted complex were used in the ELISA with the complex as the absorbed antigen. Antibody responses are expressed as the ELISA titre OD415 determined as the reciprocal of the dilution at which absorbance was double the background level, with each titre representing the mean ± standard deviation of three values.

Figure 8. Western blot analysis of the antigenic complex (Triton X114 extracted) and the RgpA-Kgp complex (sonication extracted) probed with antigenic complex or RgpA-Kgp complex antisera, respectively. The antigenic complex (Triton X114 extracted, lane 2) and RgpA-Kgp complex (sonication extracted, lane 1) were separated by SDS-PAGE, transferred onto PVDF membrane and probed with anti-complex antisera (1:50 TN buffer, lane 2), and anti-RgpA-Kgp complex antisera (1:50 TN buffer, lane 1). Molecular weight markers are shown in kilodaltons.

DETAILED DESCRIPTION OF THE INVENTION

The intra-oral bacterium *Porphyromonas gingivalis* possesses on its cell surface major trypsin-like proteinases as a >300 kDa multimeric protein complex of Arg-specific and Lys-specific thiol endopeptidases with hemagglutinins (adhesins) herein designated the RgpA-Kgp-HagA complex or antigenic complex. The antigenic complex can be purified from *P. gingivalis* cells by detergent extraction or ultrasonication followed by ultrafiltration/diafiltration or anion exchange and Lys-sepharose or Arg-sepharose chromatography. The extracted and purified complex is a >300 kDa multimeric protein aggregate.

The >300 kDa RgpA-Kgp-HagA proteinase-adhesin complex is referred to herein as the "antigenic complex". It is believed that the antigenic complex contains unique epitopes not displayed on the individual domains or processed proteins. The previously disclosed arginine-specific and lysine-specific thiol proteases discussed above do not exhibit a number of the features of the "antigenic complex" and have proven of limited application to date. However, in experiments conducted to date the antigenic complex has shown characteristics required for development of diagnostic and immunoprophylactic products. The cell surface extracted antigenic complex is accordingly of particular interest for diagnostics and neutralisation by passive immunity through oral compositions containing neutralising antibodies and by vaccine development.

Accordingly in a first aspect the present invention consists in a purified multimeric complex from *P. gingivalis*, the complex comprising at least one domain from each of RgpA, Kgp and HagA, and having a molecular weight greater than about 300 kDa.

In a preferred embodiment the complex has a molecular weight greater than about 500 kDa, more preferably more than about 800 kDa.

RgpA comprises the domains $RgpA_{cat}$, $RgpA_{A1}$, $RgpA_{A2}$ and $RgpA_{A3}$; Kgp comprises the domains Kgp_{cat}, Kgp_{A1} and Kgp_{A2} and HagA comprises the domains $HagA_{A1*}$, $HagA_{A1**}$, $HagA_{A2}$ and $HagA_{A3}$. The sequence of these polyproteins and the locations of the domains in a type strain of *P. gingivalis* is as follows:

RgpA polyprotein from *Porphyromonas gingivalis.*

Accession number; AAC18876.

RgpA protein domains.

RgpA domain	Residues (numbered from the initial methionine)
$RgpA_{cat}$	228 - 688
$RgpA_{A1}$	720 -1081
$RgpA_{A2}$	1139 - 1257
$RgpA_{A3}$	1274 - 1404
$RgpA_{A4}$	1432 - 1706

RgpA protein sequence:

5

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   1 MKNLNKFVSI ALCSSLLGGM AFAQQTELGR NPNVRLLEST QQSVTKVQFR MDNLKFTEVQ
  61 TPKGIGQVPT YTEGVNLSEK GMPTLPILSR SLAVSDTREM KVEVVSSKFI EKKNVLIAPS
 121 KGMIMRNEDP KKIPYVYGKT YSQNKFFPGE IATLDDPFIL RDVRGQVVNF APLQYNPVTK
 181 TLRIYTEITV AVSETSEQGK NILNKKGTFA GFEDTYKRMF MNYEPGRYTP VEEKQNGRMI
 241 VIVAKKYEGD IKDFVDWKNQ RGLRTEVKVA EDIASPVTAN AIQQFVKQEY EKEGNDLTYV
 301 LLIGDHKDIP AKITPGIKSD QVYGQIVGND HYNEVFIGRF SCESKEDLKT QIDRTIHYER
 361 NITTEDKWLG QALCIASAEG GPSADNGESD IQHENVIANL LTQYGYTKII KCYDPGVTPK
 421 NIIDAFNGGI SLANYTGHGS ETAWGTSHFG TTHVKQLTNS NQLPFIFDVA CVNGDFLFSM
 481 PCFAEALMRA QKDGKPTGTV AIIASTINQS WASPMRGQDE MNEILCEKHP NNIKRTFGGV
 541 TMNGMFAMVE KYKKDGEKML DTWTVFGDPS LLVRTLVPTK MQVTAPAQIN LTDASVNVSC
 601 DYNGAIATIS ANGKMFGSAV VENGTATINL TGLTNESTLT LTVVGYNKET VIKTINTNGE
 661 PNPYQPVSNL TATTQGQKVT LKWDAPSTKT NATTNTARSV DGIRELVLLS VSDAPELLRS
 721 GQAEIVLEAH DVWNDGSGYQ ILLDADHDQY GQVIPSDTHT LWPNCSVPAN LFAPFEYTVP
 781 ENADPSCSPT NMIMDGTASV NIPAGTYDFA IAAPQANAKI WIAGQGPTKE DDYVFEAGKK
 841 YHFLMKKMGS GDGTELTISE GGGSDYTYTV YRDGTKIKEG LTATTFEEDG VATGNHEYCV
 901 EVKYTAGVSP KVCKDVTVEG SNEFAPVQNL TGSAVGQKVT LKWDAPNGTP NPNPNPNPNP
 961 NPGTTTLSES FENGIPASWK TIDADGDGHG WKPGNAPGIA GYNSNGCVYS ESFGLGGIGV
1021 LTPDNYLITP ALDLPNGGKL TFWVCAQDAN YASEHYAVYA SSTGNDASNF TNALLEETIT
1081 AKGVRSPEAM RGRIQGTWRQ KTVDLPAGTK YVAFRHFQST DMFYIDLDEV EIKANGKRAD
1141 FTETFESSTH GEAPAEWTTI DADGDGQGWL CLSSGQLDWL TAHGGTNVVS SFSWNGMALN
1201 PDNYLISKDV TGATKVKYYY AVNDGFPGDH YAVMISKTGT NAGDFTVVFE ETPNGINKGG
1261 ARFGLSTEAD GAKPQSVWIE RTVDLPAGTK YVAFRHYNCS DLNYILLDDI QFTMGGSPTP
1321 TDYTYTVYRD GTKIKEGLTE TTFEEDGVAT GNHEYCVEVK YTAGVSPKKC VNVTVNSTQF
1381 NPVKNLKAQP DGGDVVLKWE APSAKKTEGS REVKRIGDGL FVTIEPANDV RANEAKVVLA
1441 ADNVWGDNTG YQFLLDADHN TFGSVIPATG PLFTGTASSD LYSANFESLI PANADPVVTT
1501 QNIIVTGQGE VVIPGGVYDY CITNPEPASG KMWIAGDGGN QPARYDDFTF EAGKKYTFTM
1561 RRAGMGDGTD MEVEDDSPAS YTYTVYRDGT KIKEGLTETT YRDAGMSAQS HEYCVEVKYT
1621 AGVSPKVCVD YIPDGVADVT AQKPYTLTVV GKTITVTCQG EAMIYDMNGR RLAAGRNTVV
1681 YTAQGGYYAV MVVVDGKSYV EKLAIK
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10 15 20 25 30

35 (SEQ ID No:1)

<u>Kgp polyprotein from *Porphyromonas gingivalis*.</u>

Accession number; AAB60809.

40 Kgp protein domains.

Kgp domain	Residues (numbered from the initial methionine)
Kgp_{cat}	229 – 710
Kgp_{A1}	738 – 1099
Kgp_{A2}	1157 -1275
Kgp_{A3}	1292 – 1424
Kgp_{A4}	1427 – 1546
Kgp_{A5}	1548 - 1732

Kgp protein seq20
uence:

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   1 MRKLLLLIAA SLLGVGLYAQ SAKIKLDAPT TRTTCTNNSF KQFDASFSFN EVELTKVETK
  61 GGTFASVSIP GAFPTGEVGS PEVPAVRKLI AVPVGATPVV RVKSFTEQVY SLNQYGSEKL
 121 MPHQPSMSKS DDPEKVPFVY NAAAYARKGF VGQELTQVEM LGTMRGVRIA ALTINPVQYD
 181 VVANQLKVRN NIEIEVSFQG ADEVATQRLY DASFSPYFET AYKQLFNRDV YTDHGDLYNT
 241 PVRMLVVAGA KFKEALKPWL TWKAQKGFYL DVHYTDEAEV GTTNASIKAF IHKKYNDGLA
 301 ASAAPVFLAL VGDTDVISGE KGKKTKKVTD LYYSAVDGDY FPEMYTFRMS ASSPEELTNI
 361 IDKVLMYEKA TMPDKSYLEK VLLIAGADYS WNSQVGQPTI KYGMQYYYNQ EHGYTDVYNY
 421 LKAPYTGCYS HLNTGVSFAN YTAHGSETAW ADPLLTTSQL KALTNKDKYF LAIGNCCITA
 481 QFDYVQPCFG EVITRVKEKG AYAYIGSSPN SYWGEDYYWS VGANAVFGVQ PTFEGTSMGS
 541 YDATFLEDSY NTVNSIMWAG NLAATHAGNI GNITHIGAHY YWEAYHVLGD GSVMPYRAMP
 601 KTNTYTLPAS LPQNQASYSI QASAGSYVAI SKDGVLYGTG VANASGVATV SMTKQITENG
 661 NYDVVITRSN YLPVIKQIQV GEPSPYQPVS NLTATTQGQK VTLKWEAPSA KKAEGSREVK
 721 RIGDGLFVTI EPANDVRANE AKVVLAADNV WGDNTGYQFL LDADHNTFGS VIPATGPLFT
 781 GTASSNLYSA NFEYLIPANA DPVVTTQNII VTGQGEVVIP GGVYDYCITN PEPASGKMWI
 841 AGDGGNQPAR YDDFTFEAGK KYTFTMRRAG MGDGTDMEVE DDSPASYTYT VYRDGTKIKE
 901 GLTATTFEED GVAAGNHEYC VEVKYTAGVS PKVCKDVTVE GSNEFAPVQN LTGSSVGQKV
 961 TLKWDAPNGT PNPNPNPNPN PGTTLSESFE NGIPASWKTI DADGDGHGWK PGNAPGIAGY
1021 NSNGCVYSES FGLGGIGVLT PDNYLITPAL DLPNGGKLTF WVCAQDANYA SEHYAVYASS
1081 TGNDASNFTN ALLEETITAK GVRSPKAIRG RIQGTWRQKT VDLPAGTKYV AFRHFQSTDM
1141 FYIDLDEVEI KANGKRADFT ETFESSTHGE APAEWTTIDA DGDGQGWLCL SSGQLDWLTA
1201 HGGSNVVSSF SWNGMALNPD NYLISKDVTG ATKVKYYYAV NDGFPGDHYA VMISKTGTNA
1261 GDFTVVFEET PNGINKGGAR FGLSTEANGA KPQSVWIERT VDLPAGTKYV AFRHYNCSDL
1321 NYILLDDIQF TMGGSPTPTD YTYTVYRDGT KIKEGLTETT FEEDGVATGN HEYCVEVKYT
1381 AGVSPKKCVN VTVNSTQFNP VQNLTAEQAP NSMDAILKWN APASKRAEVL NEDFENGIPA
1441 SWKTIDADGD GNNWTTTPPP GGSSFAGHNS AICVSSASYI NFEGPQNPDN YLVTPELSLP
1501 GGGTLTFWVC AQDANYASEH YAVYASSTGN DASNFANALL EEVLTAKTVV TAPEAIRGTR
1561 AQGTWYQKTV QLPAGTKYVA FRHFGCTDFF WINLDDVVIT SGNAPSYTYT IYRNNTQIAS
1621 GVTETTYRDP DLATGFYTYG VKVVYPNGES AIETATLNIT SLADVTAQKP YTLTVVGKTI
1681 TVTCQGEAMI YDMNGRRLAA GRNTVVYTAQ GGHYAVMVVV DGKSYVEKLA VK
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(SEQ ID No:2)

HagA polyprotein from *Porphyromonas gingivalis*.

Accession number; P59915.
HagA protein domains.

HagA domain	Residues (numbered from the initial methionine)
$HagA_{A1}$	26 - 351
$HagA_{A1*}$	366 – 625
$HagA_{A1**}$	820 – 1077 and 1272 - 1529
$HagA_{A2}$	685 – 803 and 1137 – 1255 and 1589 - 1707
$HagA_{A3}$	1724 - 1856
$HagA_{A4}$	1859 – 1978
$HagA_{A5}$	1980 - 2164

HagA protein sequence:

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   1 MRKLNSLFSL AVLLSLLCWG QTAAAQGGPK TAPSVTHQAV QKGIRTSKAK DLRDPIPAGM
  61 ARIILEAHDV WEDGTGYQML WDADHNQYGA SIPEESFWFA NGTIPAGLYD PFEYKVPVNA
 121 DASFSPTNFV LDGTASADIP AGTYDYVIIN PNPGIIYIVG EGVSKGNDYV VEAGKTYHFT
 181 VQRQGPGDAA SVVVTGEGGN EFAPVQNLQW SVSGQTVTLT WQAPASDKRT YVLNESFDTQ
 241 TLPNGWTMID ADGDGHNWLS TINVYNTATH TGDGAMFSKS WTASSGAKID LSPDNYLVTP
 301 KFTVPENGKL SYWVSSQEPW TNEHYGVFLS TTGNEAANFT IKLLEETLGS GKPAPMNLVK
 361 SEGVKAPAPY QERTIDLSAY AGQQVYLAFR HFGCTGIFRL YLDDVAVSGE GSSNDYTYTV
 421 YRDNVVIAQN LTATTFNQEN VAPGQYNYCV EVKYTAGVSP KVCKDVTVEG SNEFAPVQNL
 481 TGSAVGQKVT LKWDAPNGTP NPNPGTTTLS ESFENGIPAS WKTIDADGDG NNWTTTPPPG
 541 GSSFAGHNSA ICVSSASYIN FEGPQNPDNY LVTPELSLPN GGTLTFWVCA QDANYASEHY
 601 AVYASSTGND ASNFANALLE EVLTAKTVVT APEAIRGTRV QGTWYQKTVQ LPAGTKYVAF
 661 RHFGCTDFFW INLDDVEIKA NGKRADFTET FESSTHGEAP AEWTTIDADG DGQGWLCLSS
 721 GQLGWLTAHG GTNVVASFSW NGMALNPDNY LISKDVTGAT KVKYYYAVND GFPGDHYAVM
 781 ISKTGTNAGD FTVVFEETPN GINKGGARFG LSTEANGAKP QSVWIERTVD LPAGTKYVAF
 841 RHYNCSDLNY ILLDDIQFTM GGSPTPTDYT YTVYRDGTKI KEGLTETTFE EDGVATGNHE
 901 YCVEVKYTAG VSPKECVNVT VDPVQFNPVQ NLTGSAVGQK VTLKWDAPNG TPNPNPGTTT
 961 LSESFENGIP ASWKTIDADG DGNNWTTTPP PGGTSFAGHN SAICVSSASY INFEGPQNPD
1021 NYLVTPELSL PNGGTLTFWV CAQDANYASE HYAVYASSTG NDASNFANAL LEEVLTAKTV
1081 VTAPEAIRGT RVQGTWYQKT VQLPAGTKYV AFRHFGCTDF FWINLDDVEI KANGKRADFT
1141 ETFESSTHGE APAEWTTIDA DGDGQGWLCL SSGQLDWLTA HGGTNVVASF SWNGMALNPD
1201 NYLISKDVTG ATKVKYYYAV NDGFPGDHYA VMISKTGTNA GDFTVVFEET PNGINKGGAR
1261 FGLSTEANGA KPQSVWIERT VDLPAGTKYV AFRHYNCSDL NYILLDDIQF TMGGSPTPTD
1321 YTYTVYRDGT KIKEGLTETT FEEDGVATGN HEYCVEVKYT AGVSPKECVN VTVDPVQFNP
1381 VQNLTGSAVG QKVTLKWDAP NGTPNPNPGT TTLSESFENG IPASWKTIDA DGDGNNWTTT
1441 PPPGGTSFAG HNSAICVSSA SYINFEGPQN PDNYLVTPEL SLPNGGTLTF WVCAQDANYA
1501 SEHYAVYASS TGNDASNFAN ALLEEVLTAK TVVTAPEAIR GTRVQGTWYQ KTVQLPAGTK
1561 YVAFRHFGCT DFFWINLDDV EIKANGKRAD FTETFESSTH GEAPAEWTTI DADGDGQGWL
1621 CLSSGQLGWL TAHGGTNVVA SFSWNGMALN PDNYLISKDV TGATKVKYYY AVNDGFPGDH
1681 YAVMISKTGT NAGDFTVVFE ETPNGINKGG ARFGLSTEAN GAKPQSVWIE RTVDLPAGTK
1741 YVAFRHYNCS DLNYILLDDI QFTMGGSPTP TDYTYTVYRD GTKIKEGLTE TTFEEDGVAT
1801 GNHEYCVEVK YTAGVSPKEC VNVTINPTQF NPVQNLTAEQ APNSMDAILK WNAPASKRAE
1861 VLNEDFENGI PASWKTIDAD GDGNNWTTTP PPGGSSFAGH NSAICVSSAS YINFEGPQNP
1921 DNYLVTPELS LPGGGTLTFW VCAQDANYAS EHYAVYASST GNDASNFANA LLEEVLTAKT
1981 VVTAPEAIRG TRVQGTWYQK TVQLPAGTKY VAFRHFGCTD FFWINLDDVV ITSGNAPSYT
2041 YTIYRNNTQI ASGVTETTYR DPDLATGFYT YGVKVVYPNG ESAIETATLN ITSLADVTAQ
2101 KPYTLTVVGK TITVTCQGEA MIYDMNGRRL AAGRNTVVYT AQGGHYAVMV VVDGKSYVEK
2161 LAVK
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(SEQ ID No:3)

In a preferred embodiment at least seven proteins are present in the complex. In a preferred embodiment these proteins are selected from the group consisting of Kgp_{cat}, $RgpA_{cat}$, $RgpA_{A1}$, Kgp_{A1}, $RgpA_{A3}$, $KgpA_3$, $HagA_{A3}$, $HagA_{A1**}$, $RgpA_{A2}$, Kgp_{A2}, $HagA_{A2}$ and $HagA_{A1}$.

As the purified antigenic complex normally has enzymatic activity it is preferred in a number of uses the thiol proteinases are rendered inactive. This may be achieved in a number of ways, for example by oxidation, mutation or by small molecular weight inhibitors. It is presently preferred that inactivation is by oxidation.

As used herein the term "purified" means that the antigenic complex has been removed from its natural surrounds in that the antigenic complex is substantially free of *P. gingivalis* cells.

As will be understood by those skilled in this field in order for the antigenic complex to have the preferred molecular weight the antigenic complex is made up of multiple copies of various

domains from RgpA, KgpA and HagA. It is believed that the antigenic complex has a core molecular weight of about 223 to about 294 kDa which forms large aggregates >300 kDa.

The antigenic complex can be used to generate antibodies using standard techniques. The animals used for antibody generation can be rabbits, goats, chickens, sheep, horses, cows etc. When a high antibody titre against the complex is detected by immunoassay the animals are bled or eggs or milk are collected and the serum prepared and/or antibody purified using standard techniques or monoclonal antibodies produced by fusing spleen cells with myeloma cells using standard techniques. The antibody (immunoglobulin fraction) may be separated from the culture or ascites fluid, serum, milk or egg by salting out, gel filtration, ion exchange and/or affinity chromatography, and the like, with salting out being preferred. In the salting out method the antiserum or the milk is saturated with ammonium sulphate to produce a precipitate, followed by dialyzing the precipitate against physiological saline to obtain the purified immunoglobulin fraction with the specific anti-complex antibodies. The preferred antibody is obtained from the equine antiserum and the bovine antiserum and milk. In this invention the antibody contained in the antiserum and milk obtained by immunising the animal with the inactivated complex is blended into the oral composition. In this case the antiserum and milk as well as the antibody separated and purified from the antiserum and milk may be used. Each of these materials may be used alone or in combination of two or more. Antibodies against the complex can be used in oral compositions such as toothpaste and mouthwash to neutralise the complex and thus prevent disease. The anti-complex antibodies can also be used for the early detection of *P. gingivalis* in subgingival plaque samples by a chair-side Enzyme Linked Immunosorbent Assay (ELISA).

For oral compositions it is preferred that the amount of the above antibodies administered is 0.0001 - 50 g/kg/day and that the content of the above antibodies is 0.0002 - 10% by weight preferably 0.002 - 5% by weight of the composition. The oral composition of this invention which contains the above-mentioned serum or milk antibody may be prepared and used in various forms applicable to the mouth such as dentifrice including toothpastes, toothpowders and liquid dentifrices, mouthwashes, troches, periodontal pocket irrigating devices, chewing gums, dental pastes, gingival massage creams, gargle tablets, dairy products and other foodstuffs. The oral composition according to this invention may further include additional well known ingredients depending on the type and form of a particular oral composition.

In certain highly preferred forms of the invention the oral composition may be substantially liquid in character, such as a mouthwash or rinse. In such a preparation the vehicle is typically a water-alcohol mixture desirably including a humectant as described below. Generally, the weight ratio of water to alcohol is in the range of from about 1:1 to about 20:1. The total amount of water-alcohol mixture in this type of preparation is typically in the range of from about 70 to

about 99.9% by weight of the preparation. The alcohol is typically ethanol or isopropanol. Ethanol is preferred.

The pH of such liquid and other preparations of the invention is generally in the range of from about 4.5 to about 9 and typically from about 5.5 to 8. The pH is preferably in the range of from 5 about 6 to about 8.0, preferably 7.4. The pH can be controlled with acid (e.g. citric acid or benzoic acid) or base (e.g. sodium hydroxide) or buffered (as with sodium citrate, benzoate, carbonate, or bicarbonate, disodium hydrogen phosphate, sodium dihydrogen phosphate, etc).

Other desirable forms of this invention, the oral composition may be substantially solid or pasty in character, such as toothpowder, a dental tablet or a dentifrice, that is a toothpaste (dental 10 cream) or gel dentifrice. The vehicle of such solid or pasty oral preparations generally contains dentally acceptable polishing material. Examples of polishing materials are water-insoluble sodium metaphosphate, potassium metaphosphate, tricalcium phosphate, dihydrated calcium phosphate, anhydrous dicalcium phosphate, calcium pyrophosphate, magnesium orthophosphate, trimagnesium phosphate, calcium carbonate, hydrated alumina, calcined alumina, aluminium 15 silicate, zirconium silicate, silica, bentonite, and mixtures thereof. Other suitable polishing material include the particulate thermosetting resins such as melamine-, phenolic, and urea-formaldehydes, and cross-linked polyepoxides and polyesters. Preferred polishing materials include crystalline silica having particle sized of up to about 5 microns, a mean particle size of up to about 1.1 microns, and a surface area of up to about 50,000 cm^2/gm., silica gel or colloidal 20 silica, and complex amorphous alkali metal aluminosilicate.

When visually clear gels are employed, a polishing agent of colloidal silica, such as those sold under the trademark SYLOID as Syloid 72 and Syloid 74 or under the trademark SANTOCEL as Santocel 100, alkali metal alumino-silicate complexes are particularly useful since they have refractive indices close to the refractive indices of gelling agent-liquid (including water and/or 25 humectant) systems commonly used in dentifrices.

Many of the so-called "water insoluble" polishing materials are anionic in character and also include small amounts of soluble material. Thus, insoluble sodium metaphosphate may be formed in any suitable manner as illustrated by Thorpe's Dictionary of Applied Chemistry, Volume 9, 4th Edition, pp. 510-511. The forms of insoluble sodium metaphosphate known as 30 Madrell's salt and Kurrol's salt are further examples of suitable materials. These metaphosphate salts exhibit only a minute solubility in water, and therefore are commonly referred to as insoluble metaphosphates (IMP). There is present therein a minor amount of soluble phosphate material as impurities, usually a few percent such as up to 4% by weight. The amount of soluble phosphate material, which is believed to include a soluble sodium trimetaphosphate in the case of 35 insoluble metaphosphate, may be reduced or eliminated by washing with water if desired. The

insoluble alkali metal metaphosphate is typically employed in powder form of a particle size such that no more than 1% of the material is larger than 37 microns.

The polishing material is generally present in the solid or pasty compositions in weight concentrations of about 10% to about 99%. Preferably, it is present in amounts from about 10% 5 to about 75% in toothpaste, and from about 70% to about 99% in toothpowder. In toothpastes, when the polishing material is silicious in nature, it is generally present in amount of about 10-30% by weight. Other polishing materials are typically present in amount of about 30-75% by weight.

In a toothpaste, the liquid vehicle may comprise water and humectant typically in an amount 10 ranging from about 10% to about 80% by weight of the preparation. Glycerine, propylene glycol, sorbitol and polypropylene glycol exemplify suitable humectants/carriers. Also advantageous are liquid mixtures of water, glycerine and sorbitol. In clear gels where the refractive index is an important consideration, about 2.5 - 30% w/w of water, 0 to about 70% w/w of glycerine and about 20-80% w/w of sorbitol are preferably employed.

15 Toothpaste, creams and gels typically contain a natural or synthetic thickener or gelling agent in proportions of about 0.1 to about 10, preferably about 0.5 to about 5% w/w. A suitable thickener is synthetic hectorite, a synthetic colloidal magnesium alkali metal silicate complex clay available for example as Laponite (e.g. CP, SP 2002, D) marketed by Laporte Industries Limited. Laponite D is, approximately by weight 58.00% SiO_2, 25.40% MgO, 3.05% Na_2O, 0.98% Li_2O, 20 and some water and trace metals. Its true specific gravity is 2.53 and it has an apparent bulk density of 1.0 g/ml at 8% moisture.

Other suitable thickeners include Irish moss, iota carrageenan, gum tragacanth, starch, polyvinylpyrrolidone, hydroxyethylpropylcellulose, hydroxybutyl methyl cellulose, hydroxypropyl methyl cellulose, hydroxyethyl cellulose (e.g. available as Natrosol), sodium 25 carboxymethyl cellulose, and colloidal silica such as finely ground Syloid (e.g. 244). Solubilizing agents may also be included such as humectant polyols such propylene glycol, dipropylene glycol and hexylene glycol, cellosolves such as methyl cellosolve and ethyl cellosolve, vegetable oils and waxes containing at least about 12 carbons in a straight chain such as olive oil, castor oil and petrolatum and esters such as amyl acetate, ethyl acetate and benzyl 30 benzoate.

It will be understood that, as is conventional, the oral preparations are to be sold or otherwise distributed in suitable labelled packages. Thus, a jar of mouthrinse will have a label describing it, in substance, as a mouthrinse or mouthwash and having directions for its use; and a toothpaste, cream or gel will usually be in a collapsible tube, typically aluminium, lined lead or plastic, or

other squeeze, pump or pressurized dispenser for metering out the contents, having a label describing it, in substance, as a toothpaste, gel or dental cream.

Organic surface-active agents are used in the compositions of the present invention to achieve increased prophylactic action, assist in achieving thorough and complete dispersion of the active agent throughout the oral cavity, and render the instant compositions more cosmetically acceptable. The organic surface-active material is preferably anionic, nonionic or ampholytic in nature which does not denature the antibody of the invention, and it is preferred to employ as the surface-active agent a detersive material which imparts to the composition detersive and foaming properties while not denaturing the antibody. Suitable examples of anionic surfactants are water-soluble salts of higher fatty acid monoglyceride monosulfates, such as the sodium salt of the monosulfated monoglyceride of hydrogenated coconut oil fatty acids, higher alkyl sulfates such as sodium lauryl sulfate, alkyl aryl sulfonates such as sodium dodecyl benzene sulfonate, higher alkylsulfo-acetates, higher fatty acid esters of 1,2-dihydroxy propane sulfonate, and the substantially saturated higher aliphatic acyl amides of lower aliphatic amino carboxylic acid compounds, such as those having 12 to 16 carbons in the fatty acid, alkyl or acyl radicals, and the like. Examples of the last mentioned amides are N-lauroyl sarcosine, and the sodium, potassium, and ethanolamine salts of N-lauroyl, N-myristoyl, or N-palmitoyl sarcosine which should be substantially free from soap or similar higher fatty acid material. The use of these sarconite compounds in the oral compositions of the present invention is particularly advantageous since these materials exhibit a prolonged marked effect in the inhibition of acid formation in the oral cavity due to carbohydrates breakdown in addition to exerting some reduction in the solubility of tooth enamel in acid solutions. Examples of water-soluble nonionic surfactants suitable for use with antibodies are condensation products of ethylene oxide with various reactive hydrogen-containing compounds reactive therewith having long hydrophobic chains (e.g. aliphatic chains of about 12 to 20 carbon atoms), which condensation products ("ethoxamers") contain hydrophilic polyoxyethylene moieties, such as condensation products of poly (ethylene oxide) with fatty acids, fatty alcohols, fatty amides, polyhydric alcohols (e.g. sorbitan monostearate) and polypropyleneoxide (e.g. Pluronic materials).

Surface active agent is typically present in amount of about 0.1-5% by weight. It is noteworthy, that the surface active agent may assist in the dissolving of the antibody of the invention and thereby diminish the amount of solubilizing humectant needed.

Various other materials may be incorporated in the oral preparations of this invention such as whitening agents, preservatives, silicones, chlorophyll compounds and/or ammoniated material such as urea, diammonium phosphate, and mixtures thereof. These adjuvants, where present, are incorporated in the preparations in amounts which do not substantially adversely affect the properties and characteristics desired.

Any suitable flavouring or sweetening material may also be employed. Examples of suitable flavouring constituents are flavouring oils, e.g. oil of spearmint, peppermint, wintergreen, sassafras, clove, sage, eucalyptus, marjoram, cinnamon, lemon, and orange, and methyl salicylate. Suitable sweetening agents include sucrose, lactose, maltose, sorbitol, xylitol, sodium cyclamate, perillartine, AMP (aspartyl phenyl alanine, methyl ester), saccharine, and the like. Suitably, flavour and sweetening agents may each or together comprise from about 0.1% to 5% more of the preparation.

In the preferred practice of this invention an oral composition according to this invention such as mouthwash or dentifrice containing the composition of the present invention is preferably applied regularly to the gums and teeth, such as every day or every second or third day or preferably from 1 to 3 times daily, at a pH of about 4.5 to about 9, generally about 5.5 to about 8, preferably about 6 to 8, for at least 2 weeks up to 8 weeks or more up to a lifetime.

The compositions of this invention can be incorporated in lozenges, or in chewing gum or other products, e.g. by stirring into a warm gum base or coating the outer surface of a gum base, illustrative of which may be mentioned jelutong, rubber latex, vinylite resins, etc., desirably with conventional plasticizers or softeners, sugar or other sweeteners or such as glucose, sorbitol and the like.

The composition of this invention also includes targeted delivery vehicles such as periodontal pocket irrigation devices, collagen, elastin, or synthetic sponges, membranes or fibres placed in the periodontal pocket or used as a barrier membrane or applied directly to the tooth root.

Another important form of the invention is a vaccine based on the inactivated complex and suitable adjuvant delivered by nasal spray, orally or by injection to produce a specific immune response against the complex thereby reducing colonisation of *P. gingivalis* and neutralising the complex thereby preventing disease. A vaccine can also be based upon a recombinant component of the complex incorporated into an appropriate vector and expressed in a suitable transformed host (eg. *E. coli, Bacillus subtilis, Saccharomyces cerevisiae*, COS cells, CHO cells and HeLa cells) containing the vector. Unlike whole *P. gingivalis* cells or other previously prepared antigens based on fimbriae or the capsule the complex is a safe and effective antigens for the preparation of a composition for use in the prevention of *P. gingivalis*-associated periodontal disease. The complex can be produced using recombinant DNA methods as illustrated herein, or can be synthesized chemically from the amino acid sequence disclosed in the present invention. Additionally, according to the present invention, the complex may be used to generate *P. gingivalis* antisera useful for passive immunization against periodontal disease and infections caused by *P. gingivalis*.

Various adjuvants are used in conjunction with vaccine formulations. The adjuvants aid by modulating the immune response and in attaining a more durable and higher level of immunity using smaller amounts of vaccine antigen or fewer doses than if the vaccine antigen were administered alone. Examples of adjuvants include incomplete Freunds adjuvant (IFA), Adjuvant 65 (containing peanut oil, mannide monooleate and aluminium monostrearate), oil emulsions, Ribi adjuvant, the pluronic polyols, polyamines, Avridine, Quil A, saponin, MPL, QS-21, and mineral gels such as aluminium salts. Other examples include oil in water emulsions such as SAF-1, SAF-0, MF59, Seppic ISA720, and other particulate adjuvants such as ISCOMs and ISCOM matrix. An extensive but exhaustive list of other examples of adjuvants are listed in Cox and Coulter 1992 [In: Wong WK (ed.) *Animals parasite control utilising technology.* Bocca Raton; CRC press, 1992; 49-112]. In addition to the adjuvant the vaccine may include conventional pharmaceutically acceptable carriers, excipients, fillers, buffers or diluents as appropriate. One or more doses of the vaccine containing adjuvant may be administered prophylactically to prevent periodontitis or therapeutically to treat already present periodontitis.

In another preferred composition the preparation is combined with a mucosal adjuvant and administered via the oral or nasal route. Examples of mucosal adjuvants are cholera toxin and heat labile *E. coli* toxin, the non-toxic B sub-units of these toxins, genetic mutants of these toxins which have reduced toxicity. Other methods which may be utilised to deliver the complex orally or nasally include incorporation of the complex into particles of biodegradable polymers (such as acrylates or polyesters) by micro-encapsulation to aid uptake of the microspheres from the gastrointestinal tract or nasal cavity and to protect degradation of the proteins. Liposomes, ISCOMs, hydrogels are examples of other potential methods which may be further enhanced by the incorporation of targeting molecules such as LTB, CTB or lectins (mannan, chitin, and chitosan) for delivery of the complex to the mucosal immune system. In addition to the vaccine and the mucosal adjuvant or delivery system the vaccine may include conventional pharmaceutically acceptable carriers, excipients, fillers, coatings, dispersion media, antibacterial and antifungal agents, buffers or diluents as appropriate.

Another mode of this embodiment provides for either, a live recombinant viral vaccine, recombinant bacterial vaccine, recombinant attenuated bacterial vaccine, or an inactivated recombinant viral vaccine which is used to protect against infections caused by *P. gingivalis.* Vaccinia virus is the best known example, in the art, of an infectious virus that is engineered to express vaccine antigens derived from other organisms. The recombinant live vaccinia virus, which is attenuated or otherwise treated so that it does not caused disease by itself, is used to immunise the host. Subsequent replication of the recombinant virus within the host provides a continual stimulation of the immune system with the vaccine antigens such as the antigenic complex, thereby providing long lasting immunity.

Other live vaccine vectors include: adenovirus, cytomegalovirus, and preferably the poxviruses such as vaccinia (Paoletti and Panicali, U.S. Patent No. 4,603,112) and attenuated *salmonella* strains (Stocker *et al.*, U.S. Patent Nos. 5,210.035; 4,837,151; and 4,735,801; and Curtis *et al.*, 1988, Vaccine 6: 155-160). Live vaccines are particularly advantageous because they continually
5 stimulate the immune system which can confer substantially long-lasting immunity. When the immune response is protective against subsequent *P. gingivalis* infection, the live vaccine itself may be used in a protective vaccine against *P. gingivalis*. In particular, the live vaccine can be based on a bacterium that is a commensal inhabitant of the oral cavity. This bacterium can be transformed with a vector carrying a recombinant inactivated complex and then used to colonise
10 the oral cavity, in particular the oral mucosa. Once colonised the oral mucosa, the expression of the recombinant protein will stimulate the mucosal associated lymphoid tissue to produce neutralising antibodies. For example, using molecular biological techniques the genes encoding the complex may be inserted into the vaccinia virus genomic DNA at a site which allows for expression of epitopes but does not negatively affect the growth or replication of the vaccinia
15 virus vector. The resultant recombinant virus can be used as the immunogen in a vaccine formulation. The same methods can be used to construct an inactivated recombinant viral vaccine formulation except the recombinant virus is inactivated, such as by chemical means known in the art, prior to use as an immunogen and without substantially affecting the immunogenicity of the expressed immunogen.

20 As an alternative to active immunisation, immunisation may be passive, i.e. immunisation comprising administration of purified immunoglobulin containing antibody against the complex.

In the context of this disclosure, the terms "adhesin" and "hemagglutinin" may be considered to be synonymous.

Throughout this specification the word "comprise", or variations such as "comprises" or
25 "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

All publications mentioned in this specification are herein incorporated by reference. Any discussion of documents, acts, materials, devices, articles or the like which has been included in
30 the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia or elsewhere before the priority date of each claim of this application.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications
35 may be made to the invention as shown in the specific embodiments without departing from the

spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

In order that the nature of the present invention may be more clearly understood preferred forms thereof will now be described with reference to the following Examples.

5 **EXAMPLE 1**

(1) Preparation of Antigenic complex.

A. Triton X-114 extraction and affinity chromatography.

Porphyromonas gingivalis was grown in an anaerobic chamber (MK3 anaerobic workstation; Don Whitley Scientific Ltd., Shipley, England) at 37°C on horse blood agar plates supplemented
10 with 10% (v/v) lysed horse blood. Bacterial colonies were used to inoculate brain heart infusion media containing 5 μg/ml of hemin and 0.5 μg/ml of cysteine for batch culture growth. Batch culture growth was monitored at 650 nm using a spectrophotometer (Perkin-Elmer model 295E). Culture purity was routinely checked by Gram stain, microscopic examination and using a variety of biochemical tests. Stocks were maintained as lyophilised cultures. *P. gingivalis* cells
15 (2L) were grown to late exponential phase and harvested by centrifugation (7500 g, 30 min, 4°C) and washed twice with PG buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, and 5 mM cysteine-HCl, pH 8.0) in the anaerobic workstation. Cells were resuspended in PG buffer, total volume 60 mL, containing 0.5% v/v Triton X114 and gently mixed at either (a) room temperature for 45 min or (b) 4°C overnight. For comparison cells were resuspended in PG
20 buffer, total volume 60 mL and subjected to mild sonication using a Branson sonifier 250 with an output control of 3 and a 50% duty cycle. The cell extract was centrifuged (7500 g, 30 min, 4°C) and the collected supernatant centrifuged (40,000g, 30 min, 4°C). The supernatant was then filtered (0.2μm) and the complex purified by arginine affinity chromatography. Fast protein liquid chromatography (FPLC) was performed at room temperature at a flow rate of 1.0 mL/min.
25 *P. gingivalis* cell supernatant was applied to an Arg-Sepharose column (Hiload XK 16/10 Q, Pharmacia), installed in a Pharmacia GP-250 FPLC system, in TC 50 buffer (buffer A) (50 mM Tris/HCl, 50 mM NaCl, 5 mM $CaCl_2$, pH 7.4) at a flow rate of 1 mL/min. Non-specifically bound proteins were eluted with a linear gradient of 0-40% TC 50 buffer containing, 500 mM NaCl, 50 mM Tris/HCl, 5 mM $CaCl_2$, pH 7.4 (buffer B) at a flow rate of 1.0 mL/min. The
30 column was re-equilibrated with buffer A and bound proteins eluted with TC 50 buffer containing 500 mM arginine, pH 7.4 at a flow rate of 1 mL/min. The eluent was monitored at 280 nm. All fractions were collected at 4°C and stored at -70°C before further processing. A typical affinity chromatogram of the complex is shown in Figure 1. Arginine eluted FPLC fractions were concentrated using Vivaspin 20 concentrator (10,000 MWCO) (Sartorius, NSW,
35 Australia) by centrifugation at 3000 × *g* for 15 min periods at 4°C until the eluant was reduced to

a volume of approximately 1 mL. The filter membrane of the Vivaspin 20-concentrator was then rinsed with 1 mL of TC 50 buffer. This procedure purifies and inactivates by oxidation the complex which is then stored frozen (-70°C) and used as an immunogen.

Benzoyl-L-Arg-*p*-nitroanilide (Bz-L-Arg-*p*NA) Sigma, NSW, Australia) and benzyloxycarbonyl-
5 L-Lys-*p*-nitroanilide (z-L-Lys-*p*-NA) (Novabiochem, NSW, Australia) were used to assay FPLC fractions for Arg- and Lys proteolytic activity, respectively. Samples of each chromatographic fraction were diluted in TC 150 buffer (total volume of 360 μL) and incubated for 10 minutes at 37°C with 40 μL of 100 mM cysteine, pH 8. After incubation, 400 μL of either Bz-L-Arg-*p*NA or z-L-Lys-p-NA substrate [2 mM Bz-L-Arg-*p*NA or 2 mM z-L-Lys-*p*-NA dissolved in 3 mL
10 isopropan-2-ol and mixed with 7 ml of enzyme buffer (400 mM Tris-HCl, 100 mM NaCl and 20 mM cysteine), pH 8] was added and the proteolytic activity determined by measuring the absorbance at 410 nm using a diode Array spectrophotometer (model 8452A, Hewlett Packard, Germany) over 3 minutes. The proteolytic activity is expressed in U, where U - μmol substrate converted min^{-1} at 37°C. The protein concentration of FPLC fractions and purified samples was
15 determined using the Bradford protein assay (BioRad) with BSA as a standard. The protein concentration and proteolytic activity of the complex extracted via the Triton X114 method or sonication method and purified by affinity chromatography is shown in Table 1. The Triton X114 extraction method produced the antigenic complex in a higher yield and higher purity compared to the traditional sonication method, Table 1.

20 **Table 1.** Purification of the antigenic complex using Triton X114 and sonication methodologies.

	Protein (mg)	**Arg Proteolytic activity (U)***	**Lys Proteolytic activity (U)#**	**Arg Proteolytic activity (U mg^{-1})**	**Lys Proteolytic activity (U mg^{-1})**	**Purification (-fold)**	
						Arg	**Lys**
Crude cell sonicate^	10.34±2.52	13.60±2.30	1.89±0.78	1.30±0.62	0.18±0.09	1	1
Complex Purified from the cell sonicate	0.72±0.15	1.23±0.24	0.09±0.02	1.72±0.84	0.12±0.06	1.3	0.68
Crude Triton X114 extract^	70.14±9.23	35.62±4.32	4.56±1.35	0.51±0.22	0.07±0.01	1	1

Antigenic Complex Purified from the Triton X114 extract	0.63±0.12	2.45±0.68	1.19±0.86	4.11±1.40	1.97±0.56	8.16	30.35

* Amidolytic activity using 2.0 mM Bz-L-Arg-*p*NA: 1 unit (U) = 1 μmol min-1 at 37 °C.

Amidolytic activity using 2.0 mM z-L-Lys-*p*NA: 1 unit (U) = 1 μmol min-1 at 37°C.

^ 330 ml of TX-114 treated and sonicated *P. gingivalis* extracts were used for complex purification.

5 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed on FPLC fractions by using a Novex™ electrophoresis system (Novex, San Diego, CA) with Novex 12% Tris-glycine pre-cast mini gels (Invitrogen, NSW, Australia). RgpA-Kgp complex proteins samples (20 μg) were precipitated by addition of trichloroacetic acid (TCA) to a final concentration of 10% v/v and incubated for 20 min at 4°C. Precipitated proteins were collected 10 by centrifugation (10 min, 13,000 g) and re-suspended in 20 μl of reducing sample buffer (10% w/v SDS, 0.05% w/v bromophenol blue, 25% v/v glycerol and 0.05% v/v 2-Mercaptoehtanol) and the pH adjusted with the addition of 10 μL of 1.5 M Tris/HCl, pH 8.0 and then heated for 5 min at 100°C. Samples were loaded onto the gels and electrophoresis was performed using a current of 30-50 mA and a potential difference of 125 V. After completion of electrophoresis the 15 gels were fixed in destain (methanol/water/acetic acid (45:45:10, v/v) for three minutes at room temperature. For Coomassie blue staining, gels were placed in Coomassie brilliant blue (CBB) (0.2% w/v CBB R250, 30% v/v ethanol, 0.5% v/v acetic acid) and heated in a microwave until boiling and then allowed to cool for five minutes. The stain was removed and destain was added and heated in a microwave until boiling and allowed to cool for five minutes. Protein bands 20 were visualised by rinsing gels in Milli Q water overnight. A typical SDS-PAGE Coomassie blue stained gel of the Triton X114 extracted complex is shown in Figure 2. Fourteen distinct bands (1 to 14) corresponding to approximate molecular masses of 75, 62, 57, 48, 45, 44, 39, 37, 34, 31, 27, 26, 17 and 15 kDa, respectively, were found. The proteins within these bands were identified using N-terminal sequencing and peptide mass fingerprinting techniques.

25 For N-terminal sequence analysis and Western blotting, proteins were transferred onto a PVDF membrane (Problott, Applied Biosystems) using a transblot cell (Bio-Rad). The PVDF membrane was wetted in 100% methanol and soaked in transfer buffer (10 mM CAPS, 10% v/v methanol, pH 11.5). Transfer was performed using a potential difference of 60 V for 90 min. For N-terminal sequencing membranes were stained with 0.1% (w/v) Coomassie brilliant blue

R250 in methanol/water/acetic acid for 30 sec and destained in 50% v/v methanol. Protein bands were excised and N-terminal sequences determined using a Hewlett Packard 10005A protein sequencer. For peptide mass fingerprinting analysis; Coomassie blue stained protein bands from SDS-PAGE were excised and subjected to in-gel trypsin digestion and subsequent peptide
5 extraction. Protein bands were excised from the Coomassie Blue stained SDS-PAGE gel and gel pieces were washed in 50 mM NH_4HCO_3/ethanol 1:1, reduced and alkylated with DTT and iodoacetamide, respectively and digested with sequencing grade modified trypsin (Promega) overnight at 37 °C as previously published Mortz et al. (1996). Electrophoresis 17:925-31]. The peptide extract containing 25 mM NH_4HCO_3 was then analysed by MALDI-TOF MS using an
10 Ultraflex TOF/TOF instrument (Bruker Daltonics) in positive ion and reflectron mode. A saturated solution of 4-hydroxy-α-cyanocinnamic acid (HCCA) was prepared in 97:3 v/v acetone/0.1% v/v aqueous TFA. A thin layer was prepared by pipetting and immediately removing 2 μL of this solution onto the 600 μm anchorchips of the target plate. Sample (0.5 μL) was deposited on the thin layers with 2.5 μL of 0.1% v/v aqueous TFA, and allowed to adsorb for
15 5 min, after which the sample solution was removed, and the thin layers washed once with 10 μL of ice-cold 0.1% v/v aqueous TFA for 1 min. Spectra were calibrated by close external calibration using a standard peptide mix. Proteins were identified by peptide mass fingerprinting against the *P. gingivalis* database (available from www.tigr.org) using an in-house Mascot search engine. Table 2 shows the peptide sequences used to identify the SDS-PAGE separated protein
20 bands of the antigenic complex. The SDS-PAGE of the complex (Figure 2) is annotated with the designation of the proteins identified by N-terminal sequencing and peptide mass fingerprinting. The complex was found to consist of: Kgp_{cat}, $RgpA_{cat}$, $RgpA_{A1}$, Kgp_{A1}, $RgpA_{A3}$, $RgpA_{A2}$, Kgp_{A2}, $HagA_{A1*}$, $HagA_{A1**}$, $HagA_{A3}$ and $HagA_{A2}$ as well as partially processed Kgp (residues 1 to 700 and residues 136 to 700). A schematic of the processed domains of RgpA, Kgp and HagA are
25 shown in Figure 3.

The Triton X114 extracted complex was analysed by size exclusion chromatography. Size exclusion chromatography was performed using a macrosphere GPC 300Å column (7μm, 250 x 4.6 mm, with exclusion limits of 7,500-1,200,000 Daltons; Alltech, NSW, Australia) installed in a Waters Delta 600 HPLC system (Waters, Australia). Chromatography was performed at a flow
30 rate of 0.5 mL/min in 0.05 M KH_2PO_4 containing 0.15 M Na_2SO_4 (pH 7.0). Material eluted from the column was detected by determining absorbance at 280 nm. A standard curve using molecular mass gel filtration standards (Amersham Pharmacia Biotech, Uppsala, Sweden) was used to determine the molecular mass of the eluted fractions. A typical size exclusion chromatogram of the purified Triton X114 extracted complex is shown in Figure 4. The major
35 peak (peak 1) eluted in the void volume of the column (>300 kDa; antigenic complex) and a second peak (peak 2) eluted with an average molecular mass of 223 kDa (the 223 kDa RgpA-Kgp complex).

Table 2. Identification Data for the proteins in the Arg-affinity purified Triton X114 extracted antigenic complex.

Protein Band	Assigned designation	Identifying peptide	Observed mass
1	Kgp (42-700)	^{42}QFDASFSFNEVELTK^{56} (SEQ ID No:4)	1761.86
		^{61}GGTFASVSIPGAFPTGEVGSPEVPAVRK^{87} (SEQ ID No:5)	2286.3
		^{88}KLIAVPVGATPVVR^{101} (SEQ ID No:6)	1419.93
		^{89}LIAVPVGATPVVR^{101} (SEQ ID No:7)	1291.83
		^{104}SFTEQVYSLNQYGSEK^{119} (SEQ ID No:8)	1879.89
		^{130}SDDPEKVPFVYNAAAYAR^{147} (SEQ ID No:9)	2012.99
		^{148}KGFVGQELTQVEMLGTMR^{165} (SEQ ID No:10)	2024.03
		^{169}IAALTINPVQYDVVANQLK^{187} (SEQ ID No:11)	2070.17
		^{190}NNIEIEVSFQGADEVATQR^{208} (SEQ ID No:12)	2120.03
		^{209}LYDASFSPYFETAYK^{223} (SEQ ID No:13)	1801.85
		^{229}DVYTDHGDLYNTPVR^{243} (SEQ ID No:14)	1764.84
		^{254}EALKPWLTWK^{263} (SEQ ID No:15)	1271.73
		^{267}GFYLDVHYTDEAEVGTTNASIK^{288} (SEQ ID No:16)	2430.15
		^{295}YNDGLAASAAPVFLALVGDTDVISGEK^{321} (SEQ ID No:17)	2693.38
		^{328}VTDLYYSAVDGDYFPEMYTFR^{348} (SEQ ID No:18)	2552.11
		^{381}VLLIAGADYSWNSQVGQPTIK^{401} (SEQ ID No:19)	2260.21
		^{402}YGMQYYYNQEHGYTDVYNYLK^{422} (SEQ ID No:20)	2712.18
		^{602}TNTYTLPASLPQNQASYSIQASAGSYVAISK^{632} (SEQ ID No:21)	3231.66
		^{633}DGVLYGTGVANASGVATVSMTK^{654} (SEQ ID No:22)	2098.07
		^{655}QITENGNYDVVITR^{668} (SEQ ID No:23)	1621.83
		^{677}QIQVGEPSPYQPVSNLTATTQGQK^{700} (SEQ ID No:24)	2571.31

Protein Band	Assigned designation	Identifying peptide	Observed mass
2	Kgp (136-700)	^{136}VPFVYNAAAYAR^{147} (SEQ ID No:25)	1341.77
		^{149}GFVGQELTQVEMLGTMR^{165} (SEQ ID No:26)	1896.02
		^{169}IAALTINPVQYDVVANQLK^{187} (SEQ ID No:11)	2070.27
		^{190}NNIEIEVSFQGADEVATQR^{208} (SEQ ID No:12)	2120.13
		^{209}LYDASFSPYFETAYK^{223} (SEQ ID No:13)	1801.93
		^{229}DVYTDHGDLYNTPVR^{243} (SEQ ID No:14)	1764.91
		^{254}EALKPWLTWK^{263} (SEQ ID No:15)	1271.78
		^{267}GFYLDVHYTDEAEVGTTNASIK^{288} (SEQ ID No:16)	2430.28
		^{295}YNDGLAASAAPVFLALVGDTDVISGEK^{321} (SEQ ID No:17)	2693.54
		^{328}VTDLYYSAVDGDYFPEMYTFR^{348} (SEQ ID No:18)	2552.25
		^{349}MSASSPEELTNIIDK^{363} (SEQ ID No:27)	1634.88
		^{381}VLLIAGADYSWNSQVGQPTIK^{401} (SEQ ID No:19)	2260.32
		^{402}YGMQYYYNQEHGYTDVYNYLK^{422} (SEQ ID No:20)	2712.38
		^{633}DGVLYGTGVANASGVATVSMTK^{654} (SEQ ID No:22)	2098.16
		^{655}QITENGNYDVVITR^{668} (SEQ ID No:23)	1621.9
		^{677}QIQVGEPSPYQPVSNLTATTQGQK^{700} (SEQ ID No:24)	2571.47

Protein Band	Assigned designation	Identifying peptide	Observed mass
3	Kgp_{cat}	^{1}DVYTDHGDLYNTPVR^{15} (SEQ ID No:14)	1765.02
		^{26}EALKPWLTWK^{35} (SEQ ID No:15)	1271.86
		^{39}GFYLDVHYTDEAEVGTTNASIK^{60} (SEQ ID No:16)	2430.44
		^{67}YNDGLAASAAPVFLALVGDTDVISGEK^{93} (SEQ ID No:17)	2693.71
		^{100}VTDLYYSAVDGDYFPEMYTFR^{120} (SEQ ID No:18)	2552.43
		^{125}MSASSPEELTNIIDK^{135} (SEQ ID No:27)	1635
		^{153}VLLIAGADYSWNSQVGQPTIK^{173} (SEQ ID No:19)	2260.47
		^{174}YGMQYYYNQEHGYTDVYNYLK^{194} (SEQ ID No:20)	2712.57
		^{374}TNTYTLPASLPQNQASYSIQASAGSYVAISK^{404} (SEQ ID No:21)	3232.08
		^{405}DGVLYGTGVANASGVATVSMTK^{426} (SEQ ID No:22)	2098.30
		^{427}QITENGNYDVVITR^{440} (SEQ ID No:23)	1621.99
		^{449}QIQVGEPSPYQPVSNLTATTQGQK^{472} (SEQ ID No:24)	2571.62
4	$RgpA_{cat}$	^{228}YTPVEEK^{234} (SEQ ID No:28)	865.44
		^{269}VAEDIASPVTANAIQQFVK^{287} (SEQ ID No:29)	2001.19
		^{293}EGNDLTYVLLIGDHK^{307} (SEQ ID No:30)	1686.98
		^{319}SDQVYGQIVGNDHYNEVFIGR^{339} (SEQ ID No:31)	2410.24
		^{412}CYDPGVTPK^{420} (SEQ ID No:32)	1036.53
		^{421}NIIDAFNGGISLANYTGHGSETAWGTSHFGTTHVK^{455} (SEQ ID No:33)	3661.11
		^{493}DGKPTGTVAIIASTINQSWASPMR^{516} (SEQ ID No:34)	2501.4
		^{517}GQDEMNEILCEK^{528} (SEQ ID No:35)	1465.73
		^{536}TFGGVTMNGMFAMVEK^{551} (SEQ ID No:36)	1719.9
		^{559}MLDTWTVFGDPSLLVR^{574} (SEQ ID No:37)	1850.06

Protein Band	Assigned designation	Identifying peptide	Observed mass
5	$RgpA_{A1}$	^{101}IWIAGQGPTK^{110} (SEQ ID No:38)	1070.69
		^{122}YHFLMKK^{128} (SEQ ID No:39)	966
		^{111}EDDYVFEAGK^{120} (SEQ ID No:40)	1172.62
		^{129}MGSGDGTELTISEGGGSDYTYTVYR^{153} (SEQ ID No:41)	2616.31
		^{160}EGLTATTFEEDGVAAGNHEYCVEVK^{184} (SEQ ID No:42)	2726.43
		^{196}DVTVEGSNEFAPVQNLTGSAVGQK^{219} (SEQ ID No:43)	2447.39
6	Kgp_{A1}	^{101}MWIAGDGGNQPAR^{113} (SEQ ID No:44)	1372.8
		^{114}YDDFTFEAGK^{123} (SEQ ID No:45)	1192.65
		^{114}YDDFTFEAGKK^{124} (SEQ ID No:46)	1320.77
		^{124}KYTFTMR^{130} (SEQ ID No:47)	946.57
		^{132}AGMGDGTDMEVEDDSPASYTYTVYR^{156} (SEQ ID No:48)	2730
		^{161}IKEGLTATTFEEDGVAAGNHEYCVEVK^{187} (SEQ ID No:49)	2967.7
		^{163}EGLTATTFEEDGVAAGNHEYCVEVK^{187} (SEQ ID No:42)	2726.54
		^{199}DVTVEGSNEFAPVQNLTGSSVGQK^{222} (SEQ ID No:43)	2463.48
		^{373}GRIQGTWRQK^{382} (SEQ ID No:50)	1230.6
7	$HagA_{A1*/A1**}$	^{112}HFGCTGIFR^{120} (SEQ ID No:51) **($HagA_{A1*}$ peptide confirmed by LIFT ms/ms)**	1094.56
		^{95}TIDLSAYAGQQVYLAFR^{111} (SEQ ID No:52) ($HagA_{A1*}$ sequence)	1916.57
		^{186}DVTVEGSNEFAPVQNLTGSAVGQK^{209} (SEQ ID No:43) ($HagA_{A1*}$ sequence)	2447.92
		^{121}LYLDDVAVSGEGSSNDYTYTVYR^{143} (SEQ ID No:53) ($HagA_{A1*}$ sequence)	2587.85
		$^{820/1272}$PQSVWIER$^{827/1279}$ (SEQ ID No:54) ($HagA_{A1**}$ sequence)	1013.56
		$^{1079/1531}$TVVTAPEAIRGTR$^{1091/1543}$ (SEQ ID No:55) ($HagA_{A1**}$ sequence)	1370.73

Protein Band	Assigned designation	Identifying peptide	Observed mass
8	$RgpA_{A2}/Kgp_{A2}/HagA_{A2}$	^{100}TGTNAGDFTVVFEETPNGIN^{119} (SEQ ID No:56) (2083)	2083
		^{80}YYYAVNDGFPGDHYAVMISK^{99} (SEQ ID No:57) (2310)	2310.23
9	$RgpA_{A3}$	^{1}PQSVWIER^{8} (SEQ ID No:54)	1014.59
		^{61}IKEGLTETTFEEDGVATGNHEYCVEVK^{87} (SEQ ID No:49)	3055.65
		^{97}CVNVTVNSTQFNPVK^{111} (SEQ ID No: 58) **(confirmed BY LIFT ms/ms)**	1706.97
		^{96}KCVNVTVNSTQFNPVK^{111} (SEQ ID No:59)	1835.09
		^{63}EGLTETTFEEDGVATGNHEYCVEVK^{87} (SEQ ID No:42)	2814.46
	$Kgp_{A3}/HagA_{A3}$	^{1}PQSVWIER^{8} (SEQ ID No:54)	1014.59
		^{63}EGLTETTFEEDGVATGNHEYCVEVK^{87} (SEQ ID No:42)	2814.46
		^{61}IKEGLTETTFEEDGVATGNHEYCVEVK^{87} (SEQ ID No:49)	3055.65

(2) Preparation of Antibodies

Polyclonal antiserum to the complex was raised in mice by immunising with the O_2-inactivated complex subcutaneously. The mice were immunised at day 0 with 25μg of protein in incomplete
5 Freund's adjuvant and day 30 with 25μg of protein in incomplete Freund's adjuvant. Immunisations were carried out using standard procedures. Polyclonal antisera having a high titre against *P. gingivalis* was obtained. If desired the antibodies directed specifically against *P. gingivalis* can be obtained using standard procedures.

EXAMPLE 2

Methods and compounds for vaccine formulations related to antigenic complex.

This embodiment of the present invention is to provide complex protein to be used in as an immunogen in a prophylactic and/or therapeutic vaccine for active immunisation to protect against or treat infections caused by *P. gingivalis*. For vaccine purposes, an antigen of *P. gingivalis* comprising a bacterial protein should be immunogenic, and induce functional antibodies directed to one or more surface-exposed epitopes on intact bacteria, wherein the epitope(s) are conserved amongst strains of *P. gingivalis*.

Protective efficacy of immunisation with the antigenic complex in animal models.

The protective efficacy of the antigenic complex was evaluated in two internationally accepted animal models of *P. gingivalis*-infection i.e the lesion model and the periodontitis model. For the lesion model of disease, the maximum sizes of the lesions developed were statistically analyzed using the Kruskal-Wallis test and Mann-Whitney U-Wilcoxon rank sum test with a Bonferroni correction for type 1 error [Norusis MJ (1993). SPPS for Windows: Base systems user's guide. Release 6.0 Chicago, Il, USA: SPSS Inc]. For the periodontitis model, the bone loss (mm^2) data were statistically analyzed using One-Way analysis of variance and Dunnett's T3 test [Norusis MJ (1993). SPPS for Windows: Base systems user's guide. Release 6.0 Chicago, Il, USA: SPSS Inc]. Effect sizes, represented as Cohen's *d* were calculated using the effect size calculator provided on-line by Evidence-Based Education UK web site at http://www.cemcentre.org/ebeuk/research/effectsize/default.htm. According to Cohen [Cohen J (1969). Statistical Power Analysis for the Behavioural Sciences. New York: Academic Press] a small effect size is $d \geq 0.2$ and < 0.5, moderate $d \geq 0.5 < 0.8$ and large $d \geq 0.8$

(1). Murine lesion model of *P. gingivalis* infection.

This model is loosely based on the methods described by Kesavalu *et al* (1992) [Infect Immun 60:1455-1464]. A typical experiment is outlined below. The murine lesion model protocols were approved by the University of Melbourne Ethics Committee for Animal Experimentation. BALB/c mice 6-8 weeks old (10 mice/group) were immunized subcutaneously (scruff of the neck, 100 µL) with 25 µg of the Triton X114 extracted antigenic complex, 25 µg of sonication extracted RgpA-Kgp complex or phosphate buffered saline (pH 7.4) emulsified in Freund's adjuvant (IFA). After 30 days mice were boosted with antigen or PBS (subcutaneous injection, emulsified in IFA) and then 12 days later bled from the retrobulbar plexus. Two days after bleeding, mice were challenged with 7.5×10^9 viable cells of *P. gingivalis* strain ATCC 33277 by subcutaneous injection (100 µl) in the abdomen, and the lesions sizes measured over 14 days.

The *P. gingivalis* inocula were prepared in PG buffer in the anaerobic workstation as described by O'Brien-Simpson *et al* [O'Brien-Simpson N et al. (2000). Infect Immun 68:4055-4063]. The number of viable cells in the inocula was verified by enumeration on horse blood agar plates. Lesion sizes were statistically analyzed using the Kruskal-Wallis test and the Mann-Whitney U-Wilcoxon rank sum test with a Bonferroni correction for type 1 error. The average lesion size of mice immunized with the antigenic complex extracted via Triton X114 or sonication was significantly ($p < 0.01$; $p < 0.05$, respectively) smaller than that of the PBS/IFA control group, indicating that immunization of mice with complex protects against *P. gingivalis* infection (Figure 5). Furthermore, the Triton X114 extracted complex was more effective in protecting mice against *P. gingivalis*-induced lesions as indicated by the larger effect size of $d = -1.85$ (99.9% CI: -3.18, -0.32) compared to $d = -1.32$ (95% CI: -2.08, -0.10). Although, there was no significant difference in the lesion sizes of mice immunised with the Triton X114 or sonication extracted complex, the Triton X114 extracted complex when used as a vaccine was more effective in providing protection with an effect size of $d = -0.42$ (95% CI: -1.37, 0.49) compared to the sonication extracted complex. Moreover, only fifty percent of the mice immunised with the Triton X114 extracted complex developed *P. gingivalis*-induced lesions, whereas 70% of the mice immunised with sonication extracted complex developed lesions.

(2). Murine periodontitis model of *P. gingivalis* infection.

The murine periodontitis experiments were based on the model of Baker *et al* 1994 [Arch Oral Biol 39:1035-40] and were approved by the University of Melbourne Ethics Committee for Animal Experimentation. BALB/c mice 6-8 weeks old (10 mice per group) were immunized subcutaneously (s.c. 100 μL) with either 25 μg of the Triton X114 extracted complex or phosphate buffered saline (PBS), pH 7.4 emulsified in incomplete Freund's adjuvant (IFA). After 30 days the mice were boosted with antigen (s.c. injection, emulsified in IFA) and then bled 12 days later from the retrobulbar plexus. After bleeding mice received kanamycin (Sigma, New South Wales, Australia) at 1mg/mL in deionised water *ad libitum* for 7 days. Three days after the antibiotic treatment mice were orally inoculated four times, two days apart with 1×10^{10} viable *P. gingivalis* W50 cells (25μL) in PG buffer containing 2% wt/vol carboxymethylcellulose (CMC, Sigma, New South Wales, Australia), a control group was sham-infected with PG buffer containing 2% wt/vol CMC alone. Two weeks later mice received another four doses (2 days apart) of 1×10^{10} viable *P. gingivalis* W50 cells (25μL) in PG buffer containing 2% wt/vol CMC. The number of viable cells in each inoculum was verified by enumeration on HB agar. Twenty-eight days after the second oral challenge mice were killed and the maxillae removed.

Maxillae were boiled (1 min) in deionised water, mechanically defleshed and immersed in 2% wt/vol potassium hydroxide (16 hours, 25°C). The maxillae were then washed (2 x deionised water) and immersed in 3% wt/vol hydrogen peroxide (6 hours, 25°C). After washing (2 x

deionised water) the maxillae were stained with 0.1% wt/vol aqueous methylene blue and a digital image of the buccal side was captured with a Sound and Vision digital camera (Scitech Pty. Ltd, Melbourne, Australia) mounted on a dissecting microscope using Adobe Photoshop version 4.0 to assess horizontal bone loss. Horizontal bone loss is loss occurring in a horizontal plane, perpendicular to the alveolar bone crest that results in a reduction of the crest height. Maxillae were aligned so that the buccal and lingual molar cusps were superimposed. A micrometer scale in plane with the maxillae was digitally imaged at the same time so that measurements could be standardised for each image. The area from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) for each tooth was measured using Scion Image Beta 4.02 (Scion Corporation, Frederick, MD) imaging software downloaded from the Scion Corporation website (http://www.scioncorp.com/index.htm). Bone loss measurements were determined twice in a random and blinded protocol by two standardised examiners. Figure 6 shows that the Triton X114 extracted complex provided significant ($p < 0.001$) protection from *P. gingivalis*-induced bone loss compared to control infected group, as well as, being significantly more effective ($d = -2.45$, 99.9% CI: -4.73, -0.93) in providing protection against *P. gingivalis*-induced periodontitis compared to the non-specific highly immunogenic protein diphtheria toxoid.

These data show clearly that the antigenic complex extracted using the Triton X114 methodology is far superior to the sonication extraction method in providing protection against *P. gingivalis*-induced lesions and that the Triton X114 extracted complex also confers protection against bone loss in animal models of disease.

EXAMPLE 3

In one illustration of the antigenic complex having the properties desirable of a vaccine antigen, the protein was purified from *P. gingivalis* using the method described herein in Example 1. Mice were immunized with the purified inactivated Triton X114 and sonication extracted complex (25 µg) with adjuvant (IFA) two times at four week intervals. The purified complex was inactivated by air oxidation. Blood from the immunized mice was drawn 32 days after the last immunization and the immune sera were pooled. The pooled immune sera were assayed against the complex by an enzyme linked immunosorbent assay (ELISA) and a Western blot. ELISAs were performed in triplicate in wells of flat-bottom polyvinyl microtitre plates (Dynatech laboratories, McLean, Va) coated with 10 µg/ml of P. gingivalis whole cells in 0.1 M phosphate-buffered saline (PBS), pH 7.4, overnight at 4 °C. After removal of coating solution, 2% (w/v) skim milk powder in PBS, pH 7.4, containing 0.1% (v/v) Tween 20 was added to wells to block the uncoated plastic for 1 h at room temperature. After washing four times with PBS, pH 7.4 containing 0.1% v/v Tween 20 (PBST), serial dilutions of mouse sera in PBS, pH 7.4 containing 0.5% v/v skim milk (SK-PBS) were added to each well and incubated for 16 h at room temperature. After washing six times (PBST), a 1/2000 dilution of goat antisera to mouse IgM,

IgA, IgG1, IgG2a, IgG2b, or IgG3 (Sigma, NSW, Australia) were added in SK-PBS and allowed to bind for 2 h at room temperature. Plates were washed six times (PBST) and a 1/5,000 dilution of horseradish peroxidase-conjugated rabbit anti-goat immunoglobulin in SK-PBS was added to each well. After washing (6 times, PBST), 100 μl of ABTS substrate [(0.9 mM 2,2′-azino-bis (3-ethylbenz-thiazoline-6) sulfonic acid) in 80 mM citric acid containing 0.005% (v/v) hydrogen peroxide, pH 4.0] was added to each well. The optical density at 415 nm (OD415) was measured using a BioRad microplate reader (BioRad microplate reader, model 450). ELISA titers were determined as the reciprocal of the dilution at which absorbance was double the background level, with each titer representing the mean ± standard deviation of three values. The results, shown in Figure 7, demonstrate that immunisation with inactivated complex extracted using the Triton X114 methodology elicit higher titer antibodies compared to the sonication extraction method. The Triton X114 extracted complex induced higher IgG, IgG1, IgG2a, IgG2b and IgG3 antibodies compared to the sonication extracted complex, with the predominant antibody being IgG1 (equivalent to IgG4 in humans), which has been shown to be the antibody that is involved in a protective immune response [O'Brien-Simpson et al. (2000). Infect Immun 68:4055-406; O'Brien-Simpson et al (2000) Infect Immun 68: 2704-2712].

The purified Triton X114 extracted complex and the sonication extracted RgpA-Kgp complex were subjected to SDS-PAGE and electrophorectically transferred onto PVDF membrane as described above. After sectioning the membrane the molecular weight standards were stained with 0.1% wt/vol CBB R250. The remaining sections were blocked for 1 hour at 20°C with 5% wt/vol non-fat skim milk powder in TN buffer (50mM Tris-HCl, pH 7.4, 100mM NaCl). Sections were subsequently incubated with either anti-complex (Triton X114 extracted) antisera or anti-RgpA-Kgp complex (sonication extracted) antisera diluted 1:50 with TN buffer. After 16 hours at 20°C the sections were washed (4x TN buffer containing 0.05% vol/vol Tween 20, 10 mins) and then incubated for an hour at 20°C with horseradish peroxidase-conjugated goat immunoglobulin (Ig) directed against mouse Ig (1/400 dilution) (Sigma, NSW, Australia). After washing (4 x TN buffer containing 0.05% vol/vol Tween 20, 10 mins) bound antibody was detected with 0.05% wt/vol 4-chloro-1-napthol in TN buffer containing 16.6% vol/vol methanol and 0.015% wt/vol H2O2. Colour development was stopped by rinsing the membranes with Milli Q water. The anti-complex antisera (Triton X114 extracted) had a strong immunoreactive response to proteins of molecular weights 44, 39 and 30 kDa corresponding to the antigenic complex proteins RgpAA1, KgpA1 and HagAA1*/** (Figure 8). The anti-RgpA-Kgp complex antisera (sonication extracted) also had a strong immunoreactive response to proteins of molecular weights 44, and 39 kDa corresponding to the antigenic complex proteins RgpAA1 and KgpA1 but had a very weak response to the HagAA1*/** adhesin (Figure 8). The immunoreactive 45 kDa protein band was found not to be the RgpAcat proteinase domain as the complex antisera did not recognise the RgpB proteinase, which has 97% sequence identity to the

RgpA proteinase, suggesting that the immunoreactive band detected at 45 kDa was also derived from the adhesins. These data indicate that the complex extracted using the Triton X114 method produces a strong antibody response directed towards the A1 adhesins of RgpA; Kgp and HagA polyproteins. These protein share a high degree of sequence similarity and each contain the
5 previously described protective peptide epitopes ABM1, ABM2 and ABM3 (WO 98/49192). These results suggest that the large cell surface complexes on P. gingivalis are composed of non-covalently associated, processed domains of all three polyproteins, RgpA, Kgp and HagA. The superiority of the Triton X114-extracted complex in protection may, therefore, relate to the vaccine antigen more closely resembling the form of the proteins on the cell surface.

10 Additional evidence supporting the immunogenicity of the antigenic complex comes from a study of the human immune response in which 86% of 43 patients with adult periodontitis had specific IgG in their sera to the complex.

EXAMPLE 4

The following is an example of a proposed toothpaste formulation containing anti-(complex)
15 antibodies.

Ingredient	% w/w
Dicalcium phosphate dihydrate	50.0
Glycerol	20.0
Sodium carboxymethyl cellulose	1.0
Sodium lauryl sulphate	1.5
Sodium lauroyl sarconisate	0.5
Flavour	1.0
Sodium saccharin	0.1
Chlorhexidine gluconate	0.01
Dextranase	0.01
Goat serum containing anti-Antigenic Complex antibodies	0.2
Water	balance

EXAMPLE 5

The following is an example of a proposed toothpaste formulation.

Ingredient	% w/w
Dicalcium phosphate dihydrate	50.0
Sorbitol	10.0
Glycerol	10.0
Sodium carboxymethyl cellulose	1.0
Sodium lauryl sulphate	1.5
Sodium lauroyl sarconisate	0.5
Flavour	1.0
Sodium saccharin	0.1
Sodium monofluorophosphate	0.3
Chlorhexidine gluconate	0.01
Dextranase	0.01
Bovine serum containing anti-Antigenic Complex antibodies	0.2
Water	balance

EXAMPLE 6

5 The following is an example of a proposed toothpaste formulation.

Ingredient	% w/w
Dicalcium phosphate dihydrate	50.0
Sorbitol	10.0
Glycerol	10.0
Sodium carboxymethyl cellulose	1.0
Lauroyl diethanolamide	1.0
Sucrose monolaurate	2.0
Flavour	1.0
Sodium saccharin	0.1
Sodium monofluorophosphate	0.3
Chlorhexidine gluconate	0.01
Dextranase	0.01
Bovine milk Ig containing anti-Antigenic Complex antibodies	0.1
Water	balance

EXAMPLE 7

The following is an example of a proposed toothpaste formulation.

Ingredient	% w/w
Sorbitol	22.0
Irish moss	1.0
Sodium Hydroxide (50%)	1.0
Gantrez	19.0
Water (deionised)	2.69
Sodium Monofluorophosphate	0.76
Sodium saccharine	0.3
Pyrophosphate	2.0
Hydrated alumina	48.0
Flavour oil	0.95
anti-Antigenic Complex mouse monoclonal antibody	0.3
sodium lauryl sulphate	2.00

EXAMPLE 8

The following is an example of a proposed liquid toothpaste formulation.

Ingredient	% w/w
Sodium polyacrylate	50.0
Sorbitol	10.0
Glycerol	20.0
Flavour	1.0
Sodium saccharin	0.1
Sodium monofluorophosphate	0.3
Chlorhexidine gluconate	0.01
Ethanol	3.0
Equine Ig containing anti-Antigenic Complex antibodies	0.2
Linolic acid	0.05
Water	balance

EXAMPLE 9

The following is an example of a proposed mouthwash formulation.

Ingredient	% w/w
Ethanol	20.0
Flavour	1.0
Sodium saccharin	0.1
Sodium monofluorophosphate	0.3
Chlorhexidine gluconate	0.01
Lauroyl diethanolamide	0.3
Rabbit Ig containing anti-Antigenic Complex antibodies	0.2
Water	balance

EXAMPLE 10

The following is an example of a proposed mouthwash formulation.

Ingredient	% w/w
Gantrez S-97	2.5
Glycerine	10.0
Flavour oil	0.4
Sodium monofluorophosphate	0.05
Chlorhexidine gluconate	0.01
Lauroyl diethanolamide	0.2
Anti-Antigenic Complex mouse monoclonal antibody	0.3
Water	balance

5

EXAMPLE 11

The following is an example of a proposed lozenge formulation.

Ingredient	% w/w
Sugar	75-80
Corn syrup	1-20
Flavour oil	1-2
NaF	0.01-0.05
Anti-Antigenic Complex mouse monoclonal antibody	0.3
Mg stearate	1-5
Water	balance

EXAMPLE 12

The following is an example of a proposed gingival massage cream formulation.

Ingredient	% w/w
White petrolatum	8.0
Propylene glycol	4.0
Stearyl alcohol	8.0
Polyethylene Glycol 4000	25.0
Polyethylene Glycol 400	37.0
Sucrose monostearate	0.5
Chlorhexidine gluconate	0.1
Anti-Antigenic Complex mouse monoclonal antibody	0.3
Water	balance

5 **EXAMPLE 13**

The following is an example of a proposed chewing gum formulation.

Ingredient	% w/w
Gum base	30.0
Calcium carbonate	2.0
Crystalline sorbitol	53.0
Glycerine	0.5
Flavour oil	0.1
Anti-Antigenic Complex mouse monoclonal antibody	0.3
Water	balance

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Claims

1. A purified multimeric complex from *P. gingivalis*, the complex comprising at least one domain from each of RgpA, Kgp and HagA, and having a molecular weight greater than about 300 kDa.

5 2. A complex according to claim 1 wherein the complex has a molecular weight greater than about 500 kDa.

3. A complex according to claim 1 or claim 2 wherein the complex has a molecular weight greater than about 800 kDa.

4. The complex according to any on of claims 1 to 3 wherein the enzymatic activity of the
10 complex is inactivated.

5. A method of obtaining a purified multimeric complex from *P. gingivalis*, the complex comprising at least one domain from each of RgpA, Kgp and HagA, and having a molecular weight greater than about 300 kDa the method comprising detergent extraction of the complex from whole *Porphyromonas gingivalis* cells.

15 6. A method according to claim 5 wherein the complex is subjected to further purification using ion exchange or ultrafiltration and diafiltration methods.

7. A method according to claim 5 or claim 6 wherein the detergent is Triton X114.

8. A method according to any one of claims 5 to 7 wherein the *Porphyromonas gingivalis* is a virulent strains.

20 9. A method according to any one of claims 5 to 8 wherein the *P. gingivalis* has high arginine and/or lysine proteolytic activity.

10. A method according to any one of claims 5 to 9 wherein the enzymatic activity of the complex is inactivated.

11. A method according to claim 10 wherein the inactivation is by oxidation.

25 12. A composition for use in eliciting an immune response directed against *Porphyromonas gingivalis*, the composition comprising an effective amount of the complex according to any one of claims 1 to 4 and a suitable adjuvant and /or acceptable carrier.

13. An antibody preparation comprising antibodies specifically directed against the complex according to any one of claims 1 to 4.

14. A method of treating a subject suffering from *Porphyromonas gingivalis* infection, the method comprising administering to the subject an amount of the antibody preparation according to claim 13.

15. A method of reducing the prospect of *Porphyromonas gingivalis* infection in an
5 individual and/or severity of disease, the method comprising administering to the individual an amount of the composition according to claim 12 effective to induce an immune response in the individual directed against *Porphyromonas gingivalis*.

16. A method of treatment of a patient human and/or animal either suffering from *Porphyromonas gingivalis* infection, the method comprising active vaccination of said
10 patient with a composition according to the claim 12.

17. A method of treatment of a patient human and/or animal either suffering from *Porphyromonas gingivalis* infection, the method comprising passive vaccination of said patient with an antibody preparation according to claim 13.

18. The use of the antibody preparation according to claim 13 in the preparation of a
15 medicament for the treatment of *Porphyromonas gingivalis* infection.

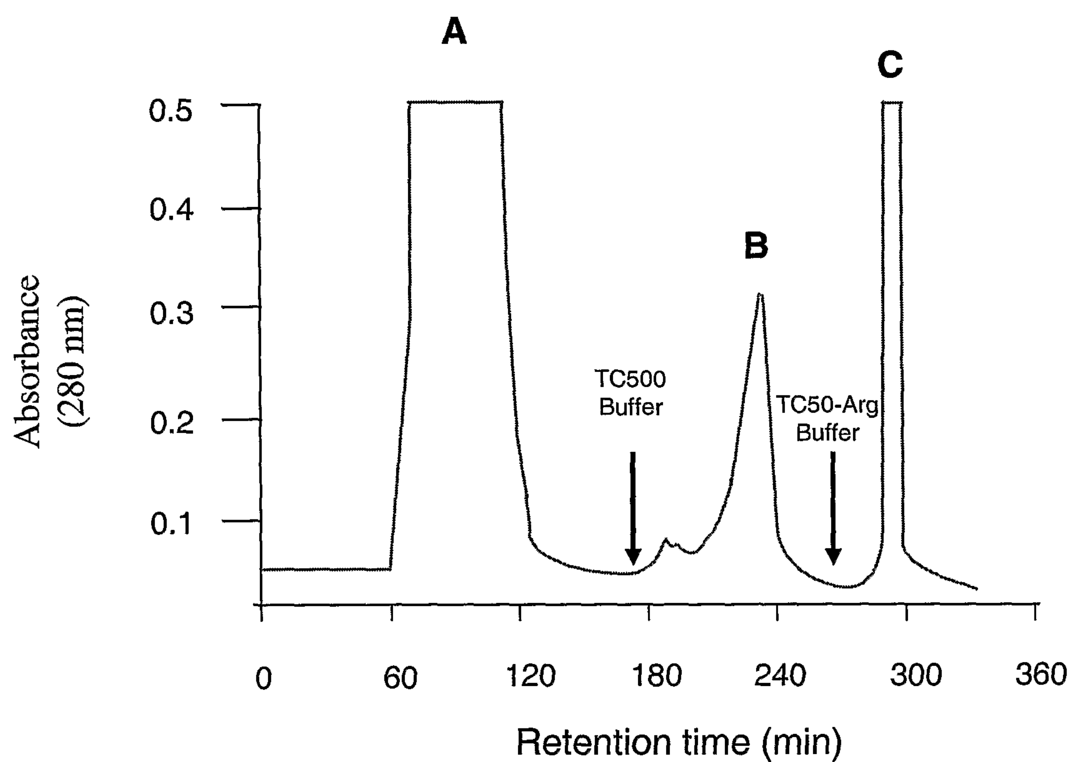


Figure 1.

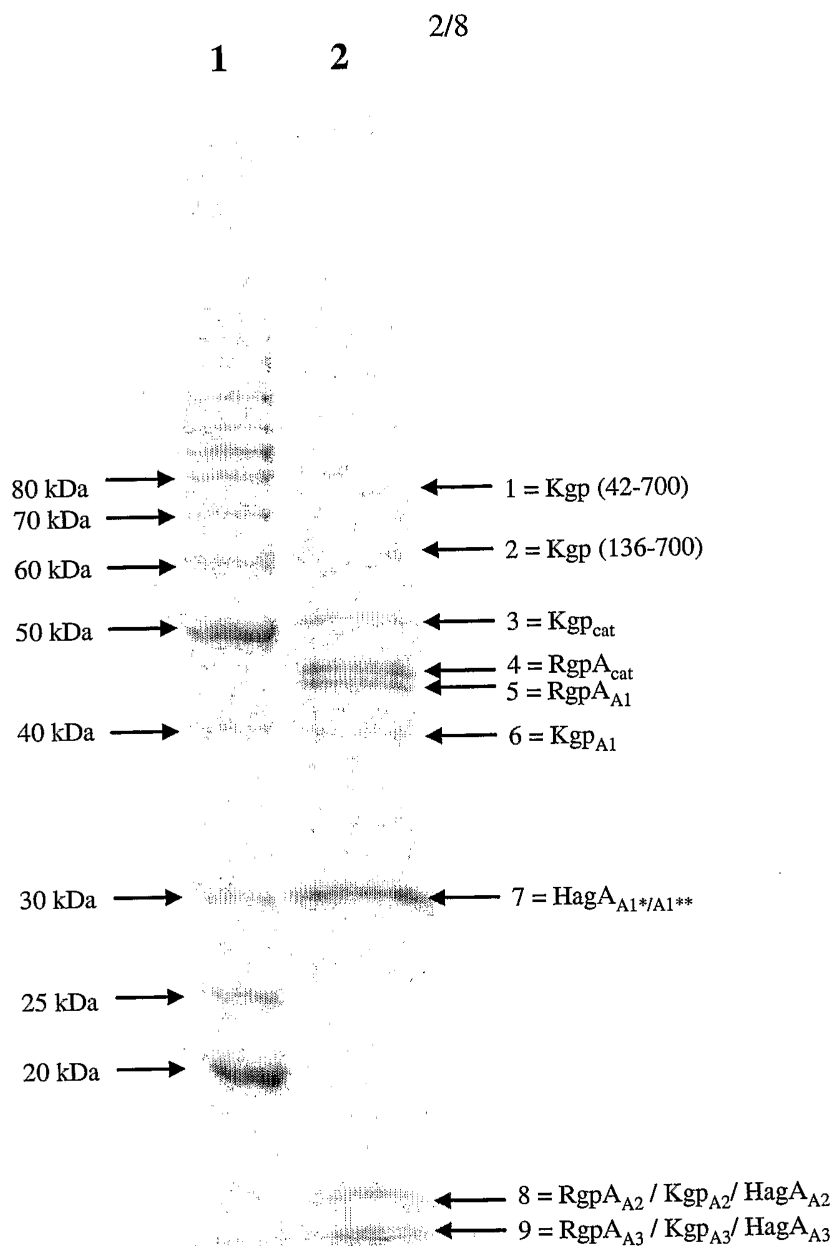
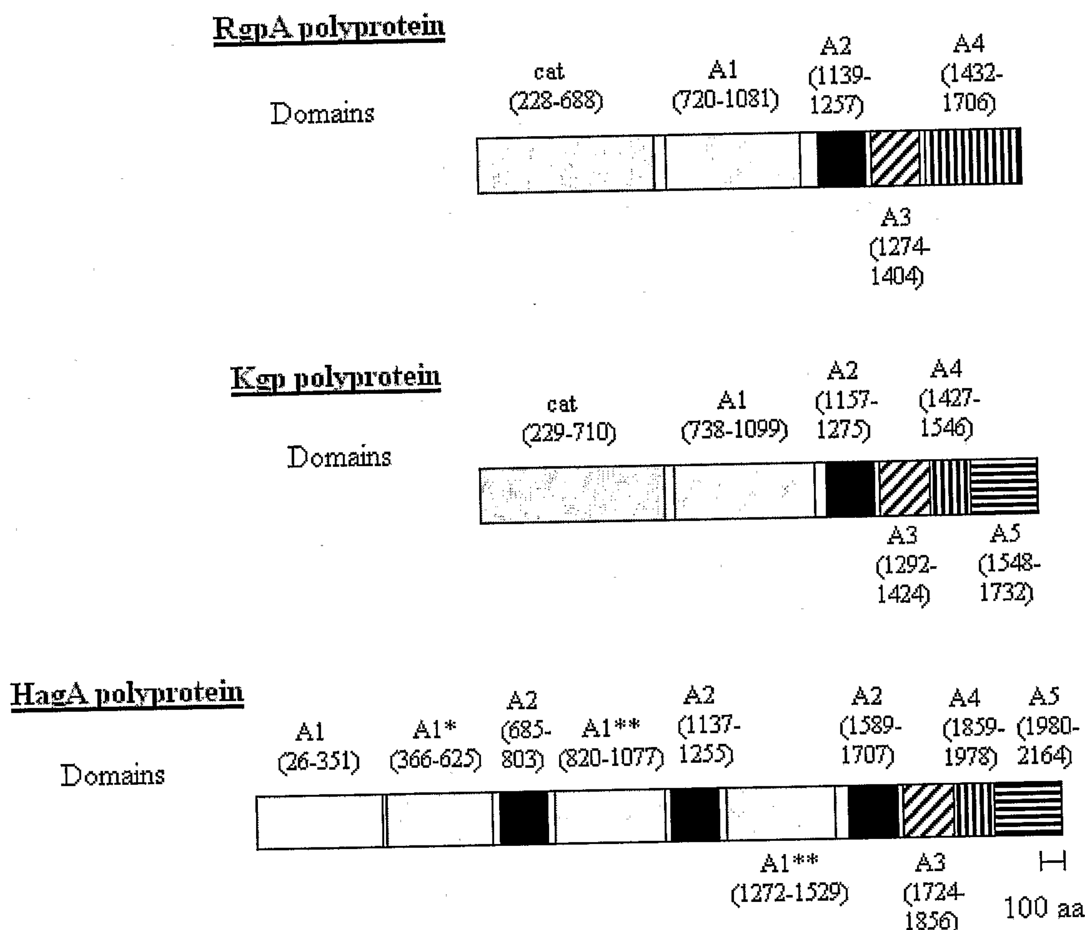


Figure 2.



N-terminal sequence of the processed domains of RgpA, Kgp and HagA

RgpA polyprotein

$RgpA_{cat}$ YTPVEEKQ (SEQ ID No:60)

$RgpA_{A1}$ SGQAEIVL (SEQ ID No:61)

$RgpA_{A2}$ ADFTETFE (SEQ ID No:62)

$RgpA_{A3}$ PQSVWIER (SEQ ID No:54)

$RgpA_{A4}$ ANEAKVVL (SEQ ID No:63)

Kgp polyprotein

Kgp_{cat} DVYTDHGD (SEQ ID No:64)

Kgp_{A1} ANEAKVVL (SEQ ID No:63)

Kgp_{A2} ADFTETFE (SEQ ID No:62)

Kgp_{A3} PQSVWIER (SEQ ID No:54)

Kgp_{A4} AEVLNEDF (SEQ ID No:65)

Kgp_{A5} TVVTAPEA (SEQ ID No:66)

HagA polyprotein

$HagA_{A1}$ GGPKTAPS (SEQ ID No:67)

Hag_{A1*} APAPYQER (SEQ ID No:68)

$HagA_{A1**}$ PQSVWIER (SEQ ID No:54)

$HagA_{A2}$ ADFTETFE (SEQ ID No:62)

$HagA_{A3}$ PQSVWIER (SEQ ID No:54)

$HagA_{A4}$ AELLNEDF (SEQ ID No:69)

$HagA_{A5}$ TVVTAPE (SEQ ID No:70)

5 0 .5

Figure 3.

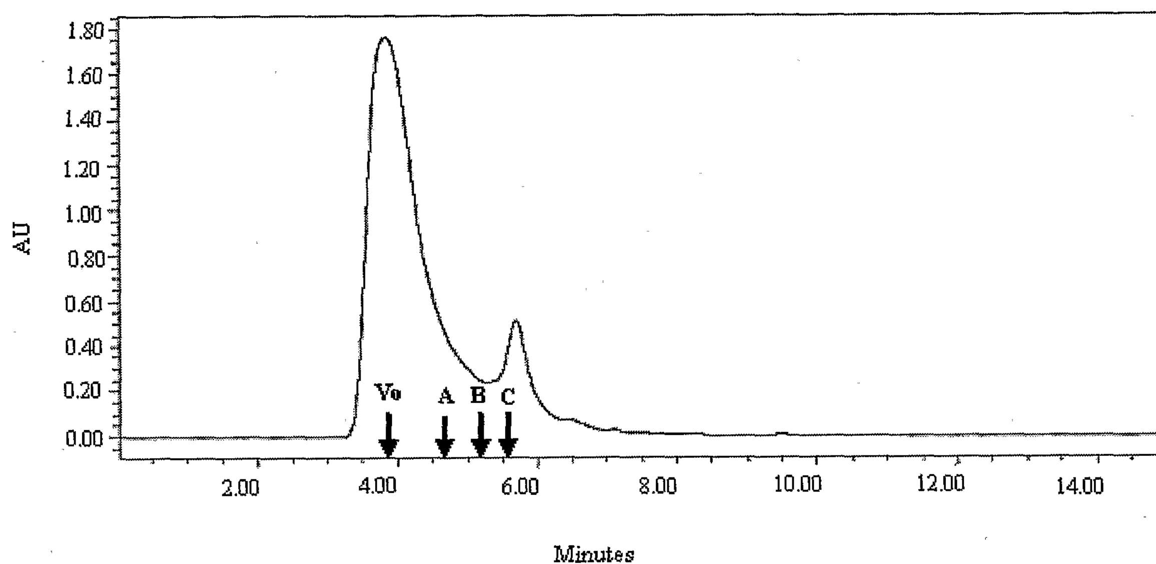


Figure 4.

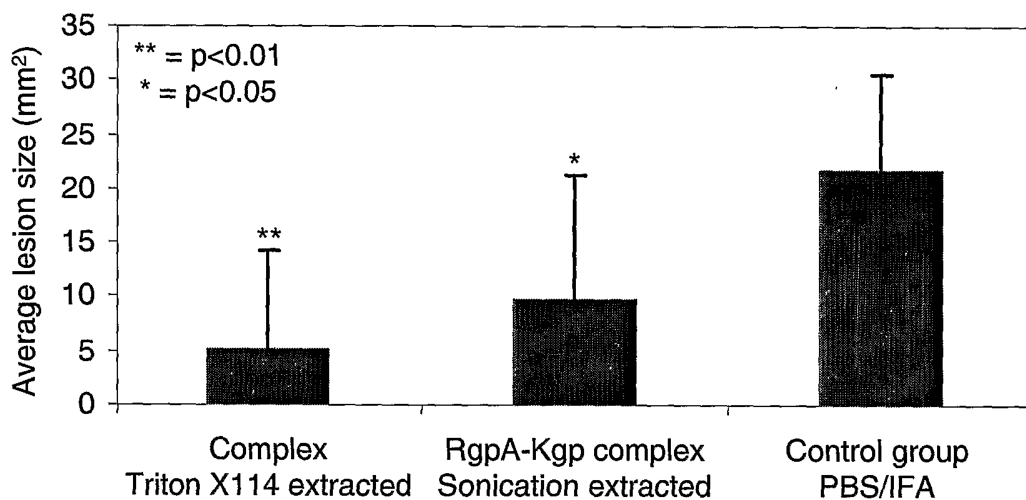


Figure 5.

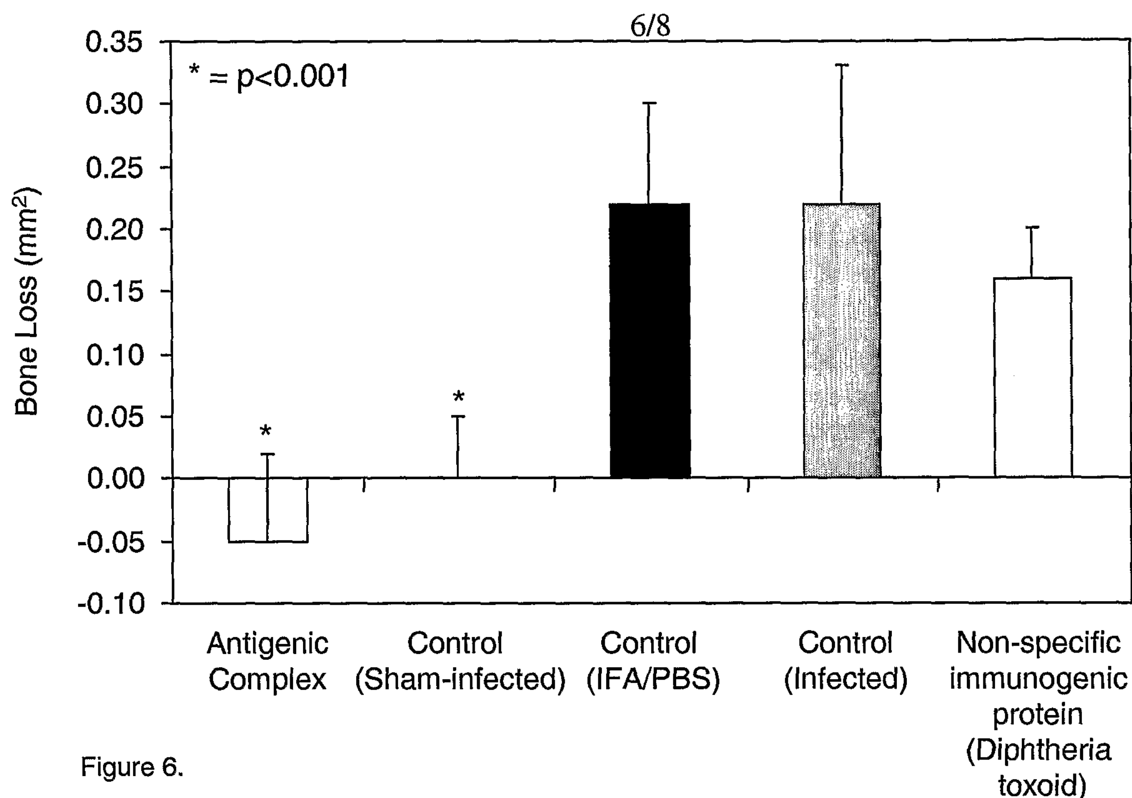


Figure 6.

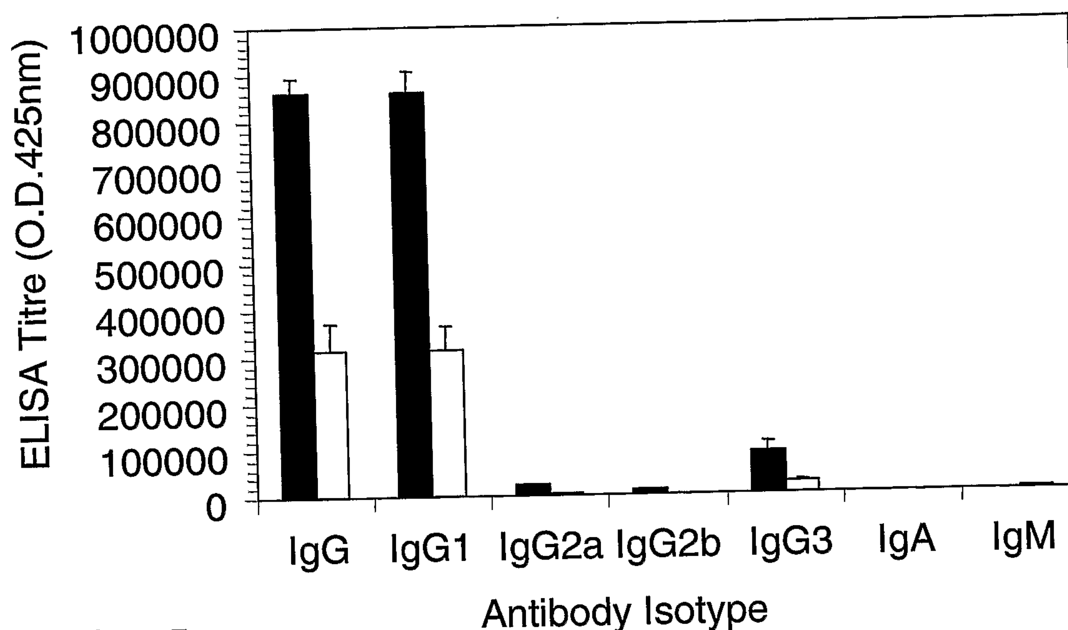


Figure 7.

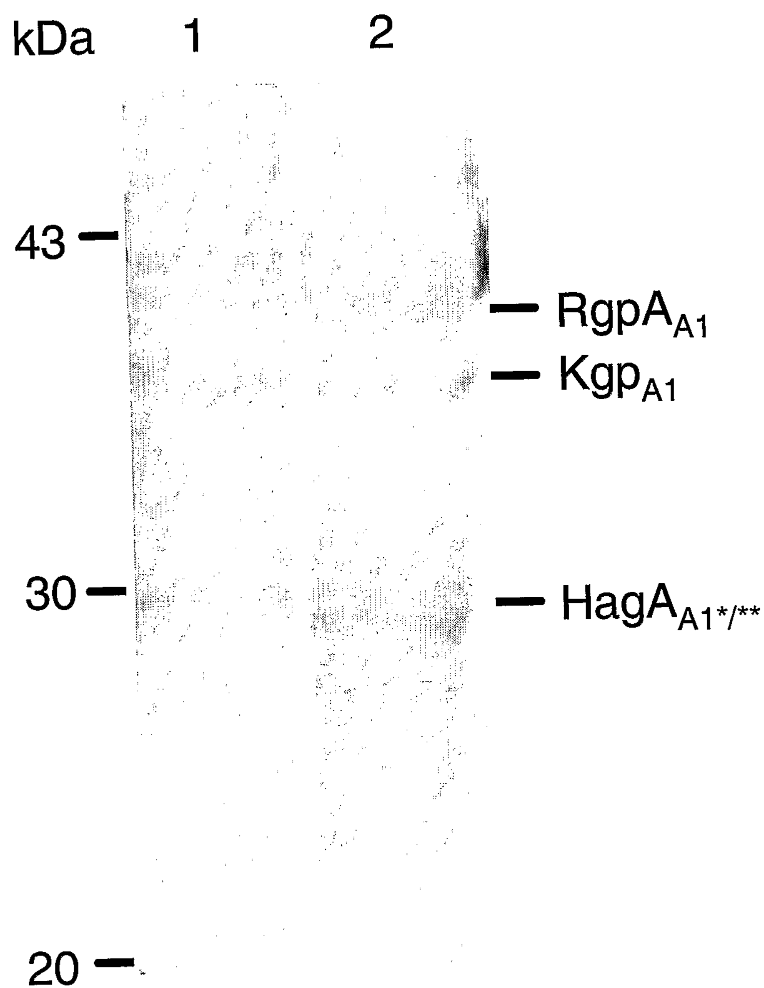


Figure 8.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2005/001463

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. 7: C07K 14/195, 16/12; A61K 38/16, 39/02, 39/40; A61P 1/02.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
File Medline, WPIDS, CA, Biosis: Keywords porphyromonas(w)gingivalis, p(w)gingivalis, complex, multimer?, rgpa?, arg?(w)specific, kgp?, lys?(w)specific, haga?, hemagglutinin, haemagglutinin, hemaglutinin, haemaglutinin, adhesin, endopeptidase, proteinase, protease.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PIKE, R. et al., Lysine- and Arginine-specific Proteinases from *Porphyromonas gingivalis*: Isolation, Characterization, and Evidence for the Existence of Complexes with Hemagglutinins, Journal of Biological Chemistry (1994), 269(1), 406-411.	1-18
A	BHOGAL, P. S. et al., A Cell-associated Protein Complex of *Porphyromonas gingivalis* W50 Composed of Arg- and Lys-specific Cysteine Proteinases and Adhesins, Microbiology (1997), 143, 2485-2495.	1-18
A	O'BRIEN-SIMPSON, N. M. et al., RgpA-Kgp Peptide-Based Immunogens Provide Protection Against *Porphyromonas gingivalis* Challenge in a Murine Lesion Model, Infection and Immunity (2000), 68(7), 4055-4063.	1-18

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 October 2005	1 NOV 2005
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **MARIE-ANNE FAM** Telephone No : (02) 6283 2254

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2005/001463

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 1997/016542 A (THE UNIVERSITY OF MELBOURNE et al.) 9 May 1997	1-18

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU2005/001463

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	1997016542	AU	72673/96	CA	2235224	EP	858504
		NZ	320015	ZA	9609130		

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX