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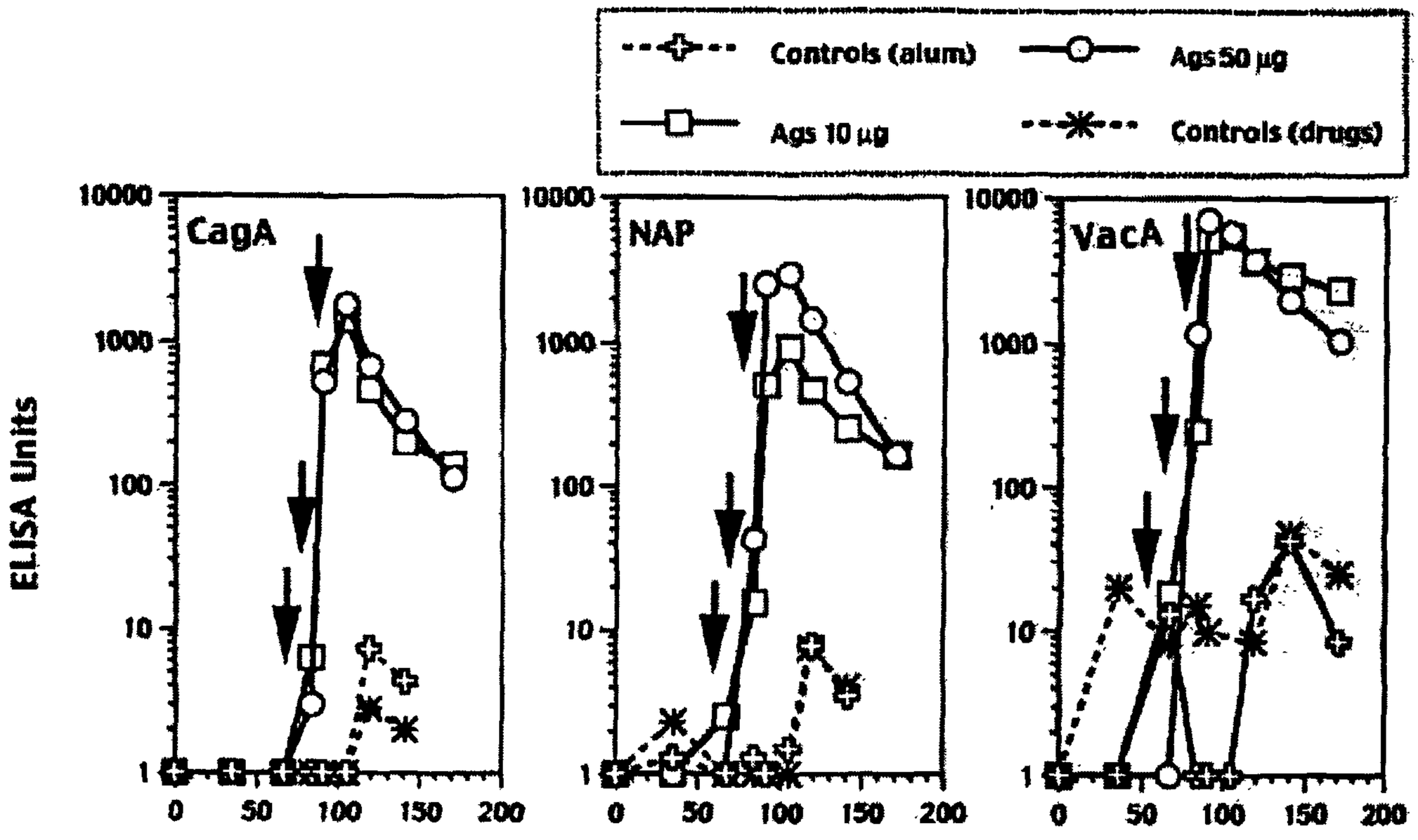
(54) Titre : VACCINATION CONTRE HELICOBACTER PYLORI AVEC UNE COMBINAISON DE PROTEINES CAGA, VACA ET NAP

(54) Title: HELICOBACTER PYLORI VACCINATION



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A sterile immunogenic preparation of three purified H.pylori antigens (CagA, VacA and NAP) adjuvanted with alum in an isotonic buffer solution for intramuscular injection. The antigens may be administered in conjunction with antibiotics and/or antisecretories. Urease breath testing, stool antigen testing, and/or immunological analysis may be used as correlate(s) of protection against H.pylori infection. Urea may be used to improve VacA solubility.

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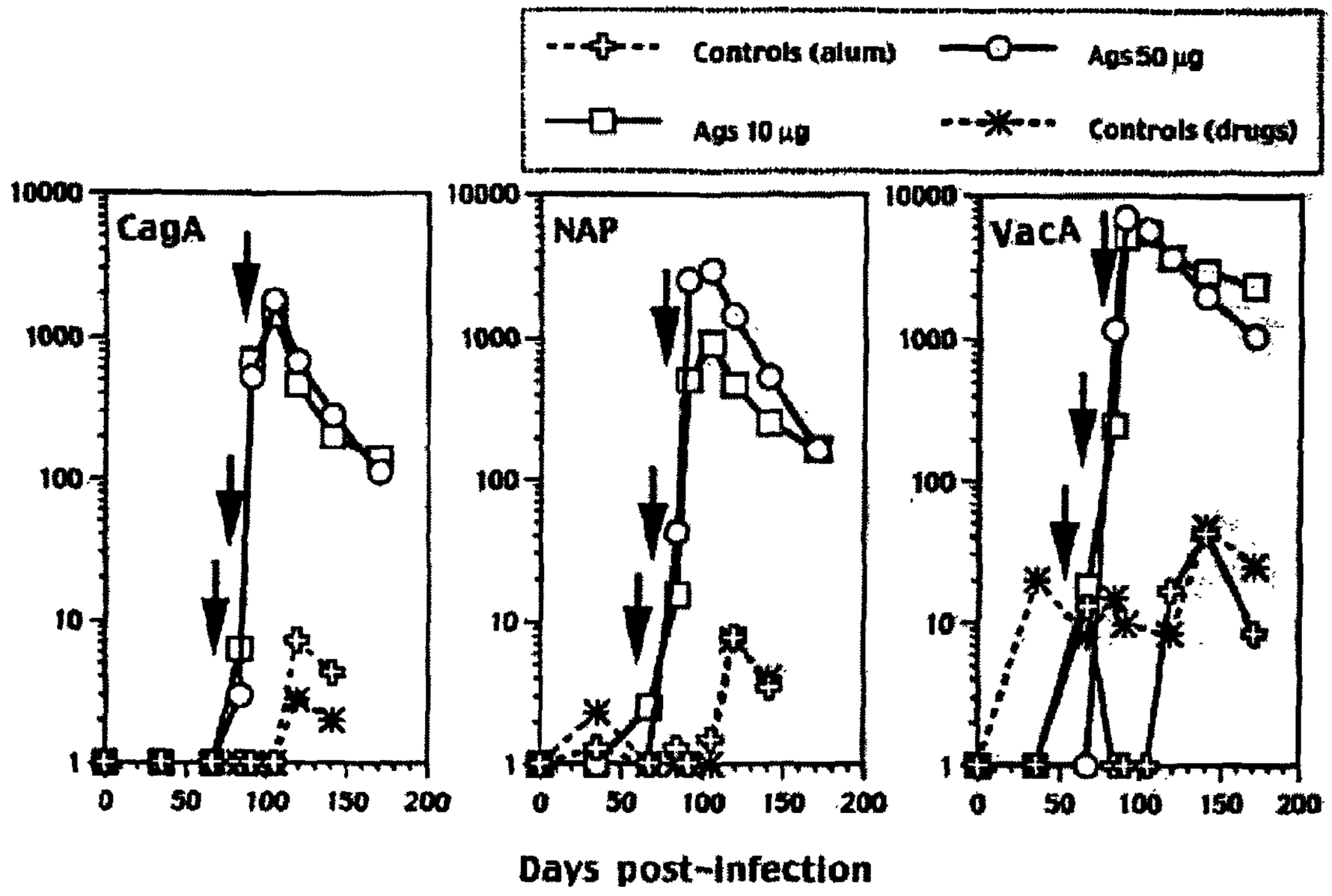

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HELICOBACTER PYLORI VACCINATION

All documents cited herein are incorporated by reference in their entirety.

TECHNICAL FIELD

This invention is in the field of vaccines against *Helicobacter pylori*.

BACKGROUND ART

Helicobacter pylori (HP) is a Gram-negative spiral bacterium which infects the human stomach. It is believed that over 50% of the world's population harbour the bacterium.

Because of the high prevalence of HP infection and its acquisition in childhood, global eradication of disease caused by HP can only be achieved by widespread vaccination. Prevention of HP infection in a given individual would be expected to decrease the likelihood of that individual subsequently developing gastroduodenal ulcer disease or gastric cancer.

Various antigenic proteins have been identified in HP [*e.g.* references 1 to 5], including urease, VacA, CagA, NAP, flagella proteins, adhesins *etc.* and many of these have been proposed for use in vaccines. Two complete HP genome sequences are also available [6,7].

The feasibility of prophylactic vaccination against HP infection has been demonstrated in both small and large animal models. A mouse model of infection [8] was developed based upon the ability to infect mice with HP strains freshly isolated from patients with peptic ulcer disease. Oral immunisation of mice with three recombinant HP antigens (VacA, CagA, and NAP), singly or in combination, together with mucosal adjuvants (*e.g.* enterotoxin LT from wild type *E.coli* or the non-toxic K63 mutant) was shown to protect against subsequent challenge with HP [9,10]. Moreover, VacA (native and recombinant form p95) protected against challenge with a type I ($VacA^+$) but not a type II ($VacA^-$) HP strain. Protection therefore appears to be antigen-specific.

It is an object of the invention to provide a HP vaccine for clinical use in humans.

DISCLOSURE OF THE INVENTION

The vaccine of the invention is a sterile preparation of three purified HP antigens, adjuvanted with alum, in an isotonic buffer solution for intramuscular injection. The three antigens in this formulation are CagA, VacA and NAP. Each of these is involved in infection pathogenesis and has demonstrated immunogenicity and prophylactic efficacy in preclinical testing.

The invention therefore provides a composition comprising: (a) *H.pylori* CagA, VacA and NAP proteins; (b) an aluminium salt adjuvant; and (c) a buffer solution.

The invention also provides a process for producing such a composition, comprising admixing *H.pylori* CagA, VacA and NAP proteins, an aluminium salt adjuvant, and a buffer solution. These five components may be mixed in any order; the preferred order of mixing the proteins is to add CagA to NAP, and then add VacA to the CagA/NAP mixture.

The proteins

CagA, VacA and NAP proteins can be produced in any suitable manner. They may be purified from HP but, more typically, they will be purified from a recombinant expression system.

Recombinant expression preferably utilises a bacterium, and most preferably utilises *E.coli*. The bacteria will generally contain plasmids which encode the proteins of the invention. It is preferred that the proteins are expressed separately, rather than co-expressing the proteins in the same bacterium. After purification of the separate proteins, they may then be combined during preparation of the compositions of the invention. Preferably, therefore, the proteins are expressed in different bacteria (*e.g.* by using plasmids in different bacteria, each plasmid directing the expression of one of the three antigens) rather than in the same bacterium.

CagA, VacA and NAP proteins are preferably each prepared in purified form prior to being combined to form the composition of the invention. The degree of purity for each antigen prior to combination is preferably ≥90% (w/w) for each antigen *i.e.* the amount of CagA, VacA or NAP is at least 90% by weight of the total amount of protein. More preferably, the degree of purity is at least 91% (*e.g.* ≥92%, ≥93%, ≥94%, ≥95%, ≥96%, ≥97%, ≥98%, ≥99%).

The proteins can, of course, be prepared by various means (*e.g.* native expression, recombinant expression, purification from *H.pylori* culture, chemical synthesis *etc.*) and in various forms (*e.g.* native, fusions *etc.*). They are preferably prepared in substantially pure form (*i.e.* substantially free from other bacterial or host cell proteins). The proteins may each be in solution or in dry form (*e.g.* lyophilised) prior to their combination, but it is preferred that they are in solution. The protein concentrations in the solutions are assessed and then the appropriate volume of each is used to give a desired concentration of each protein in the final mixture.

CagA antigen

CagA (cytotoxicity-associated antigen) is the protein that is actively injected into epithelial cells during *in vivo* HP infection. After tyrosine phosphorylation and binding to a host protein, CagA activates a signaling cascade, actin remodeling, IL-8 production and other responses [11]. CagA was identified as an immunodominant antigen, present in the majority of HP strains [12,13,14]. Most individuals infected with $CagA^+$ strains mount an antibody response against this antigen. Furthermore, most $CD4^+$ T lymphocytes infiltrating the gastric mucosa of infected individuals are specific for CagA. The theoretical mass of CagA is ~128kDa, with a size variability obtained via internal duplications which generates sequences already present in the antigen, without producing antigenic diversity [13]. The protein is otherwise relatively conserved in sequence variability [6,7].

Any suitable form of CagA can be used in accordance with the invention *e.g.* allelic and polymorphic forms [*e.g.* 15], variants, mutants, immunogenic fragments *etc.* Identifying the CagA gene in any given HP strain is straightforward, particularly in light of the available HP genomic sequences [*e.g.* refs. 6 & 7].

A preferred form of CagA is a 1147 residue protein having the sequence given in reference 13, but having a substitution of threonine-382 with alanine. This protein has a main protein band of about 100 kDa as shown by SDS-PAGE analysis.

VacA antigen

VacA (vacuolating toxin) is released *in vivo* from *H.pylori* as a high MW homo-oligomer. Each monomer consists of a 95kDa polypeptide which undergoes proteolytic processing to produce two fragments: one (p37) containing the enzymatic activity, and the other (p58) containing the region of binding to a gastric epithelial cell receptor [9,16]. The protein assembles to form hexa- or hepta-meric "flower-like" structures with high MW. The amino acid sequence of the VacA cytotoxin is well conserved, except for a part of the p58, called mid-region or "m", which expresses allelic variation [6,7,17].

Any suitable form of VacA can be used in accordance with the invention *e.g.* allelic and polymorphic forms [*e.g.* 15], variants, mutants, immunogenic fragments *etc.* Identifying the VacA gene in any given HP strain is straightforward, particularly in light of the available HP genomic sequences [*e.g.* refs. 6 & 7].

Although wild-type VacA is associated with vacuolation of the gastric mucosa, the VacA used in the compositions of the invention is preferably in a form which does not possess any vacuolating activity. This may be due, for instance, to mis-folding [18] or to partial or complete denaturation (*e.g.* by formaldehyde treatment [19]).

A preferred form of VacA is a 980 amino acid molecule beginning at its amino-terminus with the amino acid sequence NH_2-Met-Arg-Gly-Ser-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Gly-Ser- and continuing with residues 34 to 1001 of the sequence from reference 16. Each of the six Xaa residues can be the same or different as the others, and each can be any amino acid (*e.g.* Glu, Arg, or His). This antigen has a main protein band between 95-100 kDa as shown by SDS-PAGE analysis.

NAP antigen

NAP (neutrophil-activating protein) is a highly conserved antigen in all strains of *H.pylori* [6,7,20, 21,22]. It is a virulence factor important for the HP pathogenic effects at the site of infection and a candidate antigen for vaccine development. NAP protein activates human neutrophils and monocytes, and promotes their chemotaxis. The majority of HP-infected patients produce NAP-specific antibodies, suggesting an important role of this factor in immunity. This activity is potentiated by TNF-α and IFN-γ, is inhibited by pertussis toxin (suggesting that NAP activity is exerted through a G protein), and is sensitive to wartmannin (suggesting that NAP activity is exerted through a PI3-kinase). It has been also shown that vaccination of mice with NAP antigen induces protection against HP challenge [10]. NAP is a 17 kDa monomer, rich in alpha helices (80% of the structure), that assembles to form dodecameric structures and binds up to 40 atoms of iron per monomer [23].

Any suitable form of NAP can be used in accordance with the invention *e.g.* allelic and polymorphic forms, variants, mutants, immunogenic fragments *etc.* Identifying the NAP gene in any given HP strain is straightforward, particularly in light of the available HP genomic sequences [*e.g.* refs. 6 & 7]. NAP is preferably included in multimeric form.

A preferred form of NAP is a 144 amino acid protein having the sequence set out in reference 20, but with substitution of lysine-8 with arginine, leucine-58 with isoleucine, and aspartic acid-80 with glutamic acid [24]. This antigen has a main protein band of approximately 15 kDa as shown by SDS-PAGE analysis.

Alum adjuvant

The choice of the alum adjuvant was based on the observation that infected animals and humans exhibit a prominent Th1-type immune response, whereas a Th2-type response is more frequently encountered in individuals with mild HP infection [25]. Alum is recognised to be a strong inducer of Th2-type responses, both in animals and humans. Consequently, safety and adjuvanticity must be balanced between obtaining maximum immune stimulation with minimum side effects. Aluminium salts, including aluminium hydroxides (alum), are presently the only adjuvants approved by the FDA for use in humans. Billions of doses have been administered to children and infants, and their safety has been demonstrated with extensive clinical use. Although side effects include erythema, contact hypersensitivity, subcutaneous nodules, and granulomatous inflammation, little or no systemic toxicity is generally seen [26].

The composition of the invention comprises an aluminium salt as adjuvant. Suitable aluminium salts include hydroxide, phosphate, hydroxyphosphate, oxyhydroxide, orthophosphate, sulphate *etc.* (*e.g.* see chapters 8 & 9 of ref. 27). Mixtures of different aluminium salts may also be used. The salt(s) may take any suitable form (*e.g.* gel, crystalline, amorphous *etc.*).

A preferred amount of aluminium salt is about 0.5mg per dose.

Aluminium hydroxides are the preferred salts for use according to the invention.

CagA, VacA and NAP are preferably adsorbed to the aluminium salt.

Formulation

The compositions of the invention may be formulated in unit dosage form.

VacA, CagA and NAP are preferably present at a concentration such that a single dose administered to a patient will contain between 10μg and 50μg of each of the three proteins. The amount of each protein per dose may be the same or different, so the total amount of the three proteins can vary anywhere between 30μg and 150μg.

A preferred composition comprises 10μg of each protein per dose (*i.e.* 30μg in total). Another preferred composition comprises 25μg of each protein per dose (*i.e.* 75μg in total).

A single dose of the composition will typically have a volume of about 500μl.

Compositions of the invention comprise a buffer solution. The composition is preferably buffered to a pH of between 6 and 8, more preferably between 6.5 and 7.5, and most preferably about 7. This will typically be achieved using a phosphate buffer, although other buffers (*e.g.* histidine buffer) may also be used.

Compositions of the invention may also include components which enhance protein solubility (*e.g.* denaturing agents, such as urea or guandinium hydrochloride). These are particularly useful for ensuring that VacA remains soluble (*i.e.* the amount should be sufficient to ensure that VacA remains soluble). Preferred compositions of the invention may therefore include a low level of urea *e.g.* between 2.9mg/dose and 4.1 mg/dose. These concentrations are not considered to be a safety concern – urea is normally present in blood at 60-200 mg/l, and has been administered in some clinical settings to induce hyperosmolality. Favourable safety data in rabbits using 3.75mg/dose and 7.5mg/dose have also been obtained. The urea may be added to the composition as a separate component; typically, however, it will be added together with VacA because it will already be present in the purified VacA composition.

The invention also provides a composition comprising VacA and urea.

Compositions of the invention may also include low levels of a preservative, such as phenoxyethanol (*e.g.* about 0.5%).

Compositions of the invention may include trace amounts of antibiotics, such as chloramphenicol.

Composition of the invention are preferably isotonic with respect to human tissue.

Compositions of the invention are preferably sterile. This may be achieved by any convenient means *e.g.* by filter sterilisation of the components prior to mixing.

The composition may comprise components in addition to those specified herein. For example, the composition may include components in addition to (a), (b) and (c), but it may consist of (or consist essentially of) components (a), (b) and (c).

Route and method of administration

Once formulated, the compositions of the invention can be administered to a patient. The patients to be treated can be animals; in particular, human subjects can be treated.

The comparative immunogenicity and prophylactic efficacy of vaccination by different routes (intragastric, intramuscular, and intranasal) was examined in the Beagle model [28] using either whole cell HP lysate or a combination of CagA, VacA and NAP. Alum adjuvant was used in each case. Antigen doses ranged from 10 through 250μg per antigen. It was found that the intramuscular route of immunisation is superior to the intragastric and intranasal routes.

It is therefore preferred that the compositions of the invention are adapted for administration by the intramuscular route. Other possible parenteral routes of administration for direct delivery of the compositions include subcutaneous injection and intravenous injection. The compositions can also be

administered into a lesion, or by oral and pulmonary administration, suppositories, transdermal or transcutaneous applications [*e.g.* reference 29] and hyposprays.

The compositions are preferably prepared as injectables, either as liquid solutions or suspensions or, alternatively, as solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection. Any substances in the composition should preferably be compatible with intramuscular injection. Administration will typically require injection using a needle *e.g.* a 1-1½ inch (2.5-4 cm; 21-25 gauge) needle. The composition is preferably located within a syringe.

As an alternative, the composition may be administered by needle-free means [*e.g.* reference 30].

Dosage treatment may be a single dose schedule or a multiple dose schedule, which may include booster doses. The composition is preferably intramuscularly administered to a patient three times in a single course of treatment, optionally followed by a fourth (booster) dose. Administration is preferably to the upper arm (M. deltoideus). Where a treatment comprises more than one administration, it is convenient to alternate the left and right arms.

The composition is preferably stored in a refrigerator (*e.g.* between 2°C and 8°C) prior to administration to a patient.

Immunogenic compositions and medicaments

The compositions of the invention are preferably immunogenic composition, and are more preferably vaccine compositions.

Vaccines according to the invention may either be prophylactic (*i.e.* to prevent infection) or therapeutic (*i.e.* to treat infection), but will typically be prophylactic.

The invention also provides a composition of the invention for use as a medicament. The medicament is preferably able to raise an immune response in a mammal against CagA, VacA and NAP (*i.e.* it is an immunogenic composition) and is more preferably a vaccine.

The invention also provides the use of a composition of the invention in the manufacture of a medicament for raising an immune response in a mammal against the CagA, VacA and NAP. The medicament is preferably a vaccine.

The invention also provides a method for raising an immune response in a mammal comprising the step of administering an effective amount of a composition of the invention. The immune response is preferably protective. The method may raise a booster response.

The mammal is preferably a human. Where the vaccine is for prophylactic use, the human is preferably a child (*e.g.* a toddler or infant); where the vaccine is for therapeutic use, the human is preferably an adult. A vaccine intended for children may also be administered to adults *e.g.* to assess safety, dosage, immunogenicity, *etc.*

These uses and methods are preferably for the prevention and/or treatment of a disease caused by *Helicobacter pylori* (*e.g.* chronic gastritis, duodenal and gastric ulcer disease, gastric adenocarcinoma).

Assessing vaccine efficacy

To assess efficacy as an immunogenic composition or as a vaccine, compositions of the invention may be tested in animal models of *H.pylori* infection [*e.g.* see pages 530-533 of reference 1]. The presence or absence of *H.pylori* infection can be assessed using one or more invasive (*e.g.* endoscopy with biopsy, culture, urease testing) and/or non-invasive (*e.g.* urease breath test, stool antigen) approaches.

To assess prophylactic efficacy in a human subject, it is preferred to use one, two or all of the following non-invasive methods: the urease breath test (UBT), stool antigen shedding, and/or analysis of immune response. The presence of *H.pylori* antigens in stools indicates active infection, as does a positive result in UBT. The appearance of anti-*H.pylori* antibodies indicates that the composition of the invention has provoked an immune response. Prophylactic efficacy can therefore be assessed by continued negative results in stool antigen or UBT assays, and immunogenicity can be assessed by the devlopment of a positive immune response (antibody or cellular) in any biological fluid. These methods are preferably used singly or in combination to give a correlate of protection, optionally in combination with invasive methods such as biopsy.

The UBT is widely used to detect and/or diagnose *H.pylori* infection [*e.g.* refs. 31 & 32]. It typically involves the measurement of labelled CO_2 following oral administration of isotopically-labelled urea. UBT has been used to monitor H.pylori eradication by antibiotic therapy, but it has not previously been used to monitor prophylactic efficacy.

The presence of *H.pylori* antigens in stools has also been used to monitor *H.pylori* therapy [*e.g.* ref. 33], but this test has not been used to monitor prophylactic efficacy or the efficacy of therapeutic immunisation. The test generally measures antigens using polyclonal sera, so is not specific to any particular *H.pylori* antigens. It is also possible, however, to measure particular antigens (*e.g.* CagA, VacA) which are *H.pylori*-specific.

Immunological testing has been widely used for monitoring both infection and vaccine immunogenicity. Serological testing is typical. For the compositions of the invention, the presence of antibodies against the antigens in the composition (*i.e.* against CagA, VacA and/or NAP) indicates that it has successfully provoked an immune response. The antibodies may be of any type (*e.g.* IgA, IgG, IgM *etc.*), and may be measured in any biological fluid, but it is preferred to test IgG in serum. The test is preferably semi-quantitative or quantitative, with quantitative ELISA being the most preferred way of assessing serological response.

The same tests can be used to monitor the therapeutic efficacy of a composition of the invention, although efficacy will be determined differently. For example, rather than monitoring for the failure of a positive UBT response to appear, the loss of a positive response will be monitored.

Compositions of the invention

The invention provides a composition comprising: (a) *H.pylori* CagA, VacA and NAP proteins; (b) an aluminium salt adjuvant; and (c) a buffer solution, wherein CagA, VacA and NAP are each present at a concentration of between 20 µg/ml and 100 µg/ml.

The invention also provides a composition comprising: (a) *H.pylori* CagA, VacA and NAP proteins; (b) an aluminium salt adjuvant; (c) a buffer solution; and (d) urea.

The invention also provides a composition in unit dosage form comprising (a) *H.pylori* CagA, VacA and NAP proteins; (b) an aluminium salt adjuvant; and (c) a buffer solution, wherein CagA, VacA and NAP are each present at a concentration of between 10 µg/dose and 50 µg/dose.

The invention also provides a kit comprising a composition of the invention and an antisecretory agent and/or an antibiotic effective against *Helicobacter pylori*.

Two preferred compositions of the invention consist essentially of the following components per dose (*e.g.* per 0.5ml dose) and have a pH in the range 7.0 to 8.0:

Component	**Amount per final dose**	
	First composition	**Second composition**
Aluminium hydroxide adjuvant	0.5 mg	0.5 mg
NAP	10 µg	25 µg
CagA	10 µg	25 µg
VacA	10 µg	25 µg
Sodium phosphate ($NaH_2PO_4.H_2O$)	10 mM	10 mM
Sodium chloride (NaCl)	2.13 – 2.77 mg	2.13 – 2.77 mg
Urea	2.9 – 4.1 mg	2.9 – 4.1 mg
H_2O	Up to 0.5 mL	Up to 0.5 mL

Further components of the composition

The composition of the invention will typically, in addition to the components mentioned above, comprise one or more 'pharmaceutically acceptable carriers', which include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolised macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, trehalose (WO00/56365) and lipid aggregates (such as oil droplets or liposomes). Such carriers are well known to those of ordinary skill in the art. The vaccines may also contain diluents, such as water, saline, glycerol, *etc.* Additionally, auxiliary substances, such as wetting or emulsifying agents,

pH buffering substances, and the like, may be present. A thorough discussion of pharmaceutically acceptable excipients is available in *Remington's Pharmaceutical Sciences*.

Immunogenic compositions used as vaccines comprise an immunologically effective amount of antigen, as well as any other of the above-mentioned components, as needed. By 'immunologically effective amount', it is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the individual to be treated, age, the taxonomic group of individual to be treated (*e.g.* non-human primate, primate, *etc.*), the capacity of the individual's immune system to synthesise antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials. Dosage treatment may be a single dose schedule or a multiple dose schedule (*e.g.* including booster doses). The vaccine may be administered in conjunction with other immunoregulatory agents.

The vaccine may be administered in conjunction with other immunoregulatory agents.

The composition may include other adjuvants in addition to (or in place of) the aluminium salt. Preferred adjuvants to enhance effectiveness of the composition include, but are not limited to: (1) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (see below) or bacterial cell wall components), such as for example (a) MF59™ (WO90/14837; Chapter 10 in ref. 27), containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing MTP-PE) formulated into submicron particles using a microfluidizer, (b) SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) Ribi™ adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™); (2) saponin adjuvants, such as QS21 or Stimulon™ (Cambridge Bioscience, Worcester, MA) may be used or particles generated therefrom such as ISCOMs (immunostimulating complexes), which ISCOMS may be devoid of additional detergent *e.g.* WO00/07621; (3) Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA); (4) cytokines, such as interleukins (*e.g.* IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12 (WO99/44636), *etc.*), interferons (*e.g.* gamma interferon), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), *etc.*; (5) monophosphoryl lipid A (MPL) or 3-O-deacylated MPL (3dMPL) *e.g.* GB-2220221, EP-A-0689454; (6) combinations of 3dMPL with, for example, QS21 and/or oil-in-water emulsions *e.g.* EP-A-0835318, EP-A-0735898, EP-A-0761231; (7) oligonucleotides comprising CpG motifs [Krieg *Vaccine* 2000, 19, 618-622; Krieg *Curr opin Mol Ther* 2001 3:15-24; Roman *et al.*, *Nat. Med.*, 1997, 3, 849-854; Weiner *et al.*, *PNAS USA*, 1997, 94, 10833-10837; Davis *et al.*, *J. Immunol.*, 1998, 160, 870-876; Chu *et al.*, *J. Exp. Med.*, 1997, 186, 1623-1631; Lipford *et al.*, *Eur. J. Immunol.*,

1997, 27, 2340-2344; Moldoveanu *et al., Vaccine*, 1988, 16, 1216-1224, Krieg *et al., Nature,* 1995, 374, 546-549; Klinman *et al., PNAS USA,* 1996, 93, 2879-2883; Ballas *et al., J. Immunol.,* 1996, 157, 1840-1845; Cowdery *et al., J. Immunol.,* 1996, 156, 4570-4575; Halpern *et al., Cell. Immunol.,* 1996, 167, 72-78; Yamamoto *et al., Jpn. J. Cancer Res.,* 1988, 79, 866-873; Stacey *et al., J. Immunol.,* 1996, 157, 2116-2122; Messina *et al., J. Immunol.,* 1991, 147, 1759-1764; Yi *et al., J. Immunol.,* 1996, 157, 4918-4925; Yi *et al., J. Immunol.,* 1996, 157, 5394-5402; Yi *et al., J. Immunol.,* 1998, 160, 4755-4761; and Yi *et al., J. Immunol.,* 1998, 160, 5898-5906; International patent applications WO96/02555, WO98/16247, WO98/18810, WO98/40100, WO98/55495, WO98/37919 and WO98/52581] *i.e.* containing at least one CG dinucleotide, with 5-methylcytosine optionally being used in place of cytosine; (8) a polyoxyethylene ether or a polyoxyethylene ester *e.g.* WO99/52549; (9) a polyoxyethylene sorbitan ester surfactant in combination with an octoxynol (*e.g.* WO01/21207) or a polyoxyethylene alkyl ether or ester surfactant in combination with at least one additional non-ionic surfactant such as an octoxynol (*e.g.* WO01/21152); (10) an immunostimulatory oligonucleotide (*e.g.* a CpG oligonucleotide) and a saponin *e.g.* WO00/62800; (11) an immunostimulant and a particle of metal salt *e.g.* WO00/23105; (12) a saponin and an oil-in-water emulsion *e.g.* WO99/11241; (13) a saponin (*e.g.* QS21) + 3dMPL + IL-12 (optionally + a sterol) *e.g.* WO98/57659; (14) other substances that act as immunostimulating agents to enhance the efficacy of the composition.

Muramyl peptides include N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-*sn*-glycero-3-hydroxyphosphoryloxy)-ethylamine MTP-PE), *etc.*

Further antigens

Further antigens which can be included in the composition of the invention include:

- further antigens from *H.pylori* such as HopX [*e.g.* 34], HopY [*e.g.* 34] and/or urease.
- a protein antigen from *N.meningitidis* serogroup B, such as those in refs. 35 to 41, with protein '287' (see below) and derivatives (*e.g.* 'ΔG287') being particularly preferred.
- an outer-membrane vesicle (OMV) preparation from *N.meningitidis* serogroup B, such as those disclosed in refs. 42, 43, 44, 45 *etc.*
- a saccharide antigen from *N.meningitidis* serogroup A, C, W135 and/or Y, such as the oligosaccharide disclosed in ref. 46 from serogroup C [see also ref. 47].
- a saccharide antigen from *Streptococcus pneumoniae* [*e.g.* 48, 49, 50].
- an antigen from hepatitis A virus, such as inactivated virus [*e.g.* 51, 52].
- an antigen from hepatitis B virus, such as the surface and/or core antigens [*e.g.* 52, 53].
- an antigen from hepatitis C virus [*e.g.* 54].
- an antigen from *Bordetella pertussis*, such as pertussis holotoxin (PT) and filamentous haemagglutinin (FHA) from *B.pertussis*, optionally also in combination with pertactin and/or agglutinogens 2 and 3 [*e.g.* refs. 55 & 56].
- a diphtheria antigen, such as a diphtheria toxoid [*e.g.* chapter 3 of ref. 57] *e.g.* the CRM_{197} mutant [*e.g.* 58].

- a tetanus antigen, such as a tetanus toxoid [*e.g.* chapter 4 of ref. 57].
- a saccharide antigen from *Haemophilus influenzae* B [*e.g.* 47].
- an antigen from *N.gonorrhoeae* [*e.g.* 35, 36, 37].
- an antigen from *Chlamydia pneumoniae* [*e.g.* 59, 60, 61, 62, 63, 64, 65].
- an antigen from *Chlamydia trachomatis* [*e.g.* 66].
- an antigen from *Porphyromonas gingivalis* [*e.g.* 67].
- polio antigen(s) [*e.g.* 68, 69] such as IPV or OPV.
- rabies antigen(s) [*e.g.* 70] such as lyophilised inactivated virus [*e.g.*71, RabAvert™].
- measles, mumps and/or rubella antigens [*e.g.* chapters 9, 10 & 11 of ref. 57].
- influenza antigen(s) [*e.g.* chapter 19 of ref. 57], such as the haemagglutinin and/or neuraminidase surface proteins.
- an antigen from *Moraxella catarrhalis* [*e.g.* 72].
- an antigen from *Streptococcus agalactiae* (group B streptococcus) [*e.g.* 73, 74].
- an antigen from *Streptococcus pyogenes* (group A streptococcus) [*e.g.* 74, 75, 76].
- an antigen from *Staphylococcus aureus* [*e.g.* 77].

The composition may comprise one or more of these further antigens.

Where a saccharide or carbohydrate antigen is used, it is preferably conjugated to a carrier protein in order to enhance immunogenicity [*e.g.* refs. 78 to 87]. Preferred carrier proteins are bacterial toxins or toxoids, such as diphtheria or tetanus toxoids. The CRM_{197} diphtheria toxoid is particularly preferred. Other suitable carrier proteins include the *N.meningitidis* outer membrane protein [*e.g.* ref. 88], synthetic peptides [*e.g.* 89, 90], heat shock proteins [*e.g.* 91], pertussis proteins [*e.g.* 92, 93], protein D from *H.influenzae* [*e.g.* 94], toxin A or B from *C.difficile* [*e.g.* 95], *etc.* Where a mixture comprises capsular saccharides from both serogroups A and C, it is preferred that the ratio (w/w) of MenA saccharide:MenC saccharide is greater than 1 (*e.g.* 2:1, 3:1, 4:1, 5:1, 10:1 or higher). Saccharides from different serogroups of *N.meningitidis* may be conjugated to the same or different carrier proteins.

Any suitable conjugation reaction can be used, with any suitable linker where necessary.

Toxic protein antigens may be detoxified where necessary (*e.g.* detoxification of pertussis toxin by chemical and/or genetic means [56]).

Where a diphtheria antigen is included in the composition it is preferred also to include tetanus antigen and pertussis antigens. Similarly, where a tetanus antigen is included it is preferred also to include diphtheria and pertussis antigens. Similarly, where a pertussis antigen is included it is preferred also to include diphtheria and tetanus antigens.

Antigens are preferably adsorbed to an aluminium salt.

Antigens in the composition will typically be present at a concentration of at least 1μg/ml each. In general, the concentration of any given antigen will be sufficient to elicit an immune response against that antigen.

Where urea is included in the composition of the invention, it is preferred not to include active urease as an antigen.

As an alternative to using proteins antigens in the composition of the invention, nucleic acid encoding the antigen may be used [*e.g.* refs. 96 to 104]. Protein components of the compositions of the invention may thus be replaced by nucleic acid (preferably DNA *e.g.* in the form of a plasmid) that encodes the protein.

Further anti-Helicobacter agents

Compositions of the invention may be administered in conjunction with an antisecretory agent and/or an antibiotic effective against *Helicobacter pylori*. These components offer rapid relief from any existing *H.pylori* infection, thereby complementing the longer timescale of immunotherapy.

These may be administered in the same composition as the protein antigens, but will typically be administered separately. They may be administered at the same time as the protein antigens, but they will generally follow a separate administration protocol *e.g.* daily. They may be administered by the same route as the protein antigens, but they will generally be administered orally. They may be administered over the same timescale as the protein antigens, but they will generally be administered from shortly before (*e.g.* up to 5 to 14 days before) the first dose of protein antigen up to shortly after (*e.g.* up to 5 to 14 days after) the last dose of protein antigen.

Preferred antisecretory agents are proton pump inhibitors (PPIs), H2 receptor antagonists, bismuth salts and prostaglandin analogs.

Preferred PPIs are omeprazole (including S- and B- forms, Na and Mg salts *etc.* [*e.g.* 105,106]), lansoprazole, pantoprazole, esomeprazole, rabeprazole, the heterocyclic compounds disclosed in reference 107, the imidazo pyridine derivatives of reference 108, the fused dihydropyrans of reference 109, the pyrrolidine derivatives of reference 110, the benzamide derivatives of reference 111, the alkylenediamine derivatives of reference 112 *etc.*

Preferred H2-receptor antagonists are ranitidine, cimetidine, famotidine, nizatidine and roxatidine.

Preferred bismuth salts are the subsalicylate and the subcitrate, and also bismuth salts of antibiotics of the moenomycin group [113].

Preferred prostaglandin analogs are misoprostil and enprostil.

Preferred antibiotics are tetracycline, metronidazole, clarithromycin and amoxycillin.

Other suitable anti-*H.pylori* agents are disclosed in, for instance, reference 114.

Definitions

The term "comprising" means "including" as well as "consisting" *e.g.* a composition "comprising" X may consist exclusively of X or may include something additional *e.g.* X + Y.

The term "about" in relation to a numerical value *x* means, for example, *x*±10%.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows the efficacy of prophylactic oral immunisation with *H.pylori* antigens [9,10] using LTK63 as adjuvant. Protection is assessed as the absence of colonies after plating of stomachs from mice which received the indicated treatments. Data are from different experiments.

Figure 2 shows the protection of beagle conventional dogs against *H.pylori* infection following immunisation with whole-cell lysates by different routes. Figure 2A shows immunogenicity in the dogs (the four bars in each graph are, from left to right: control, intragastric, intranasal, intramuscular). Figure 2B summarises protection results. Protection was assessed as the absence of detectable bacteria by: rapid urea text, histology, immunohistochemistry, and gastric macroscopic & microscopic studies.

Figure 3 shows the immunogenicity (3A; average titres per group) and protection conferred (3B) by intramuscular immunisation with purified VacA, CagA or NAP antigens or with whole cell lysate. Protection was assessed as described for Figure 2.

Figure 4 shows the immunogenicity of a mixture of CagA, VacA and NAP in beagles. Animals were immunised with either 10μg (squares) or 50μg (circles) of each antigen, adjuvanted with alum. The arrows show the dates of immunisation.

Figure 5 shows the gastric biopsy results from a tolerance study in beagles.

Figures 6 to 13 show safety data for human administration over days 1 to 6: (6) erythema; (7) induration; (8) malaise; (9) myalgia; (10) headache; (11) arthralgia; (12) fatigue; (13) fever. Mild reactions (transient to mild discomfort) are shown as empty bars; moderate reactions (no limitation in normal daily activity) are shown as grey bars; severe reactions (unable to perform normal daily activity) are shown as black bars. The horizontal axis shows percentages.

Figures 14 to 19 show immunogenicity data for human administration. Figures 14 & 15 show antibody responses (serum IgG antibody GMT) in the monthly (14) and weekly (15) groups. Figures 16 & 17 show the percentage of subjects in the monthly (16) and weekly (17) groups with antibodies against all three antigens in the composition. Figures 18 & 19 show the cellular proliferative response to the three antigens in the monthly (18) and weekly (19) groups. In all cases the horizontal shows the number of months after the first immunisation.

MODES FOR CARRYING OUT THE INVENTION

HP3 composition

Three compositions were produced for stability studies:

Composition name	Components (0.5ml dose)
'HP3'	25 µg/dose of each antigen (VacA, NAP, CagA); 3.75 mg/dose urea; aluminium hydroxide adjuvant 0.5 mg/dose in isotonic sodium phosphate buffer; 0.5% phenoxyethanol
'HP3 placebo'	3.75 mg/dose urea; aluminium hydroxide adjuvant 0.5 mg/dose in isotonic sodium phosphate buffer; 0.5% phenoxyethanol
'HP3 alum control'	Aluminium hydroxide adjuvant 0.5 mg/dose; NaCl 4.25mg/dose; 10mM phosphate buffer; 0.5% phenoxyethanol

Stability

The stability of HP3 lots was monitored for up to 3 months at both 4°C and 37°C.

Physico-chemical stability was assessed by measuring pH. There was no significant change in pH over the time period tested at either 4°C or at 37°C.

Physico-chemical stability was also assessed by assaying the antigens by Western blot. There was no significant change in antigenic identity over the time period tested at either 4°C or at 37°C.

Immunological stability was assessed by using the stored vaccines in immunisations. Groups of mice were immunised once intraperitoneally, serum samples were taken at day 28 and tested by ELISA for titration of VacA-, CagA-, and NAP-specific antibodies. The data obtained indicate that the immunogenicity of the three antigens is satisfactory for up to 3 months at 4°C. After 5 weeks of storage at 37°C, the immunogenicity of CagA was the same as for the composition stored at 4°C, whereas VacA and NAP immunogenicity was slightly reduced (but still effective).

On the basis of the results obtained under stress conditions (37°C), the HP3 composition can be regarded as stable.

Experimental studies – immunogenicity

HP3 was administered to rabbits either as a single intramuscular dose or as six doses administered once per week for six weeks. Rabbits had consistently detectable low IgG titres to all three antigens 15 days after a single immunisation. Progressively higher levels of IgG were detected in the multiple dose study starting on day 15. Levels increased by day 29 and persisted through necropsy and recovery (days 38 and 50, respectively). Untreated control animals did not mount an antibody response.

A similar study was performed in mice, and HP3 was again found to be consistently immunogenic at all doses tested (25µg or less of each antigen per dose) following a single immunisation.

Experimental studies – prophylactic efficacy

Oral immunisation of mice with recombinant or native HP antigens (VacA, CagA, NAP, and others) together with mucosal adjuvants conferred protection against subsequent challenge with *H.pylori* that had been freshly isolated from patients with peptic ulcer disease [Figure 1 herein; references 9 & 10]. The immunologic mechanism underlying the observed prophylactic efficacy appears to involve MHC class II-restricted $CD4^+$ cell responses, but not B cell responses [115].

Unlike mice (which remain asymptomatic following HP infection), beagle dogs develop symptomatic infection with HP and can therefore be assessed both clinically and histologically following infection [28,116]. Using this dog model, it was determined that immunogenicity of whole-cell lysates (with aluminium hydroxide adjuvant) was greater when the lysates were administered intramuscularly compared to intranasal and intragastric administration. This intramuscular immunization also conferred protection against challenge with *H.pylori* (Figure 2).

Intramuscular injection of VacA, CagA, and NAP antigens (10, 50 or 250 µg/dose of each antigen, with aluminium hydroxide adjuvant was similarly immunogenic, and conferred protection from subsequent infection (Figure 3). In these experiments, there were no histologic or immunohistologic signs of infection in any (0/8) of the animals receiving 10µg or 50µg of each antigen.

Experimental studies – therapeutic efficacy

Chronic *H.pylori* infection was eradicated in mice given intragastric recombinant VacA and CagA together with mucosal adjuvants [117]. There was no recurrence of infection for at least three months and the mice were subsequently resistant to infection with later challenge with HP. This suggests that vaccination with these recombinant antigens induced specific immunological memory in addition to causing eradication of established infection.

Beagle dogs infected with HP and then immunized with 10 or 50 µg of a combination of VacA + CagA + NAP (with aluminium hydroxide adjuvant) mounted a dose-dependent antigen-specific antibody response (Figure 4). They did not show eradication of infection by mucosal urease testing at 7 and 11 weeks following immunisation. At 17 weeks, however, 2 of 4 animals treated with either dosage had negative mucosal urease tests, whereas the tests in all 4 control animals remained strongly positive. Additionally, gastric inflammatory scores showed reduced inflammation in the antigen-treated animals and no change in inflammation in the controls receiving adjuvant only.

In other experiments, beagle dogs were infected with HP and then treated intramuscularly with 10, 50, and 250 µg of antigens or bacterial lysate.

Experimental studies – therapeutic efficacy in combination with proton pump inhibitor

Fourteen beagle dogs were experimentally infected with *H.pylori* SPM326 by intragastric administration. Three control dogs received the same treatment, but with saline substituted for bacteria.

These seventeen dogs were divided into the following experimental groups:

Group	*n*	Immunisation	PPI
# 1	4	HP3	omeprazole
# 2	4	HP3	none
# 3	3	HP3 alum control	omeprazole
# 4	3	HP3 placebo	none
control	3	none	none

Immunisations were given intramuscularly three times, at monthly intervals. Omeprazole was administered orally, daily, starting two days before the first dose of vaccine, ending two weeks after the last dose of vaccine.

No adverse clinical signs, nor body weight or temperature variations, were observed through the experimental period.

Efficacy was assessed by immunohistochemistry and histopathology on bioptic samples.

Preliminary results were obtained with biopsies taken 3 weeks after the administration of the last dose of vaccine.

In both immunised groups (#1 and #2), 3 out of 4 dogs became *Helicobacter pylori*-negative by immunohistochemistry, and their inflammation score was reduced compared with that observed in the pre-vaccination biopsies. No significant differences were found between the two groups.

Conversely, in both infected, control groups (#3 and #4), 3 out of 3 dogs remained *Helicobacter pylori*-positive by immunohistochemistry, and their inflammation score was higher than that of vaccinated groups.

Preclinical studies – toxicology

Four toxicology studies were conducted to support the administration of up to 6 doses of HP3 as frequently as once per week. The third and fourth studies were designed to conform to good laboratory practice (GLP). In the GLP studies, local (injection site) and systemic toxicity were evaluated on the basis of clinical signs, physical examinations, dermal scoring, body weights and temperatures, food consumption, ophthalmoscopy, clinical pathology (serum chemistry, hematology, coagulation including fibrinogen), and full macroscopic postmortem and histopathological examinations.

In addition to the toxicology studies, pertinent safety information can also be drawn from efficacy studies conducted in two other species. Immunogenicity and challenge studies were performed in mice and beagle dogs with HP3 antigens. In mice, there were no deaths attributed to HP3 formulations nor any apparent toxicity based on clinical signs. In dogs, there were no HP3 treatment-related deaths, clinical signs, changes in body weights, or clinical pathology findings.

Irritation study

A single dose intramuscular irritation study (code 3391.24) was performed in male NZW Rabbits. The objective of this study was to evaluate the potential for local irritant effects of the three antigens,

alum and formulation excipients, including urea, in rabbits. On Day 1, twelve rabbits received three 0.5 ml intramuscular injections to the paravertebral muscle of the test and control articles as follows:

Group	N	Site 1	Site 2	Site 3
1	6 males	HP3	HP3 placebo	Saline
2	6 males	HP3, but with 7.5mg/dose urea	HP3 alum control	Saline

Clinical signs, body weights, dermal irritation, hematology, coagulation, and serum chemistry were evaluated. Three animals per group were necropsied on days 3 and 15. A macroscopic postmortem examination was conducted and injection sites, stomach, duodenum and macroscopic lesions were examined for histopathology.

There were no deaths or treatment-related effects on body weight, hematology, coagulation, or serum chemistry. Very slight erythema was seen in two animals given HP3 (Group 2, site 1). Well-defined erythema was seen in one animal given the alum control (Group 2, site 2), which diminished and was resolved completely by Day 5. There were no dermal observations in any other animals. Apparent bruising at the test sites correlated with erythema in two animals.

Injection site histopathology in animals necropsied on day 3 consisted of acute inflammation/focal necrosis attributed to needle trauma. In animals euthanized on day 15, the injection site lesions consisted of small focal clusters or accumulations of macrophages. These were typical sequelae following acute inflammation and focal necrosis seen two weeks prior. No differences in the size or character of the inflammatory components between groups or injection sites could be detected on histologic examination.

Conclusion: Under the conditions of the study, *H. pylori* antigens (HP3) adjuvanted with alum and containing low (3.75 mg/dose) or high (7.5 mg/dose) urea were well tolerated when administered to rabbits as a single intramuscular injection. Findings in skin (erythema) and muscle (bruising/inflammation/necrosis) were comparable across groups and sites. Local reactogenicity of formulations with or without HP3 antigens was of a low order of magnitude and was similar to either alum in saline or the HP3 placebo formulation (no antigens).

Tolerance study

A tolerance study (code 7795) was performed in beagle dogs infected with *H.pylori*.

Dogs were infected with *H.pylori* using three oral administrations (10^9 cfu each) administered every other day [117]. Following infection, 2 animals/sex/group were given intramuscular injections of either CagA+VacA+NAP (10µg or 50µg of each antigen per dose) or the alum control. A fourth group was treated with a conventional regimen including antibiotics and a proton pump inhibitor (clarithromycin 250mg, metronidazole 250mg, bismuth citrate 60mg, omeprazole 20mg). Serological

and endoscopic evaluations were performed 7, 11, 17, and 27 weeks following the first administration:

Group	Number of Animals		Treatment	Route of Administr'n	Treatment Days
	Males	Females			
1	2	2	1.0 mg alum	Intramuscular	1, 8, 15
2	2	2	50 µg each antigen	Intramuscular	1, 8, 15
3	2	2	10 µg each antigen	Intramuscular	1, 8, 15
4	2	2	Antibiotics + PPI	b.i.d. oral	daily 1–15

Animals in groups 2 and 3 exhibited an antibody response against each of the three antigens. A dose-response was most pronounced for the NAP component (Figure 4). Vaccination with either antigen dose did not cause any adverse effects in terms of clinical signs, body weight, injection site reactions, body temperature, hematology, or serum chemistry as compared to the control group.

Evaluation of gastric biopsies by rapid urea test at 7 and 11 weeks post-vaccination revealed persistent *H. pylori* infection in all animals given adjuvant or antigen. In animals given conventional antibiotic treatment, 1/4 and 2/4 were positive for infection at weeks 7 and 11, respectively. Evaluation of gastric biopsies by immunohistochemistry using an anti-VacA-specific monoclonal antibody confirmed infection in all control animals at both timepoints. In treated groups immunohistochemistry results were variable, with 2 or 3 animals in each group scored as negative. Results are summarised in Figure 5.

At 17 weeks, *H. pylori* infection was detected by rapid urease test in 4/4 in group 1, 2/4 in group 2, 2/4 in group 3, and 2/4 in group 4. In contrast to the week 7 and 11 assessments, the immunohistochemical studies confirmed the rapid urea test results.

Conclusion: The results of these studies suggest that a mixture of VacA, CagA and NAP given intramuscularly induces partial eradication of *H. pylori* infection and has a beneficial effect on the histological severity of post-infection gastritis. In addition, there was no evidence that the enhanced immune response elicited by the antigens was associated with any gastrontestinal or systemic adverse effects.

GLP safety and tolerance study (single dose)

A single dose safety and tolerability study (code UBAW-154) was performed in rabbits. The objective of this study was to evaluate the safety and tolerability of a single dose of HP3 administered intramuscularly to NZW rabbits. A secondary immunogenicity assessment was also included as a study parameter. The study consisted of three groups of 4/sex/group. Each animal either received an alum/saline mixture (Group 1), an alum/HP3 placebo formulation (Group 2), or the HP3 (Group 3). A single intramuscular dose (0.5 mL) was injected into the left quadriceps muscle on day 1 of the study. Two animals/sex/group were euthanised for a comprehensive macroscopic necropsy and tissue collection on days 3 and 15.

Group	Treatment	Day 3 Necropsy	Day 15 Necropsy
1	Alum control	2/sex	2/sex
2	HP3 Placebo	2/sex	2/sex
3	HP3	2/sex	2/sex

Potential toxicity was evaluated based on clinical and injection site observations, body weights, physical examinations (body temperature, respiratory rate, heart rate, and capillary refill time) ophthalmic examinations, food consumption, clinical pathology (hematology, coagulation, and serum chemistry parameters), terminal organ weights, and macroscopic & microscopic evaluation of selected tissues. Serum was collected from all animals for analysis of antibody titres to HP3.

There were no deaths, no treatment-related adverse effects on any antemortem study parameters, and no relevant changes in terminal organ weights. The only dermal observation was for male number 5 (Group 2) which had a "very slight" erythema score at 24 hours post-dose that resolved by the 48-hour observation. Macroscopic postmortem findings at the injection site consisted of purple discoloration in 1/2 Group 1 females and 1/2 Group 3 males. With the exception of injection sites, there were no microscopic alterations that could be attributed to treatment. Any abnormalities noted (minor inflammatory or degenerative changes) were of the type/incidence/severity considered to be background in this strain and age of rabbit [118]. Microscopic injection site findings were minimal-to-mild and noted as follows:

Group Number		1 (Alum)				2 (HP3 Placebo)				3 (HP3)			
Finding	Day	3		15		3		15		3		15	
	Number M/F	2	2	2	2	2	2	2	2	2	2	2	2
Per-acute hemorrhage		0	0	1	0	0	0	0	0	0	0	0	0
Granulomatous inflammation		1	1	0	0	0	0	0	0	0	0	0	0
Acute inflammation		0	0	0	0	0	0	0	0	1	0	0	0
Interstitial hemorrhage		1	1	0		0		0		1		0	

Based on the similarities in the histopathology regardless of treatment, the single intramuscular injection of HP3 was well tolerated by male and female rabbits. Any observations on day 3 were gone by day 15, indicating recovery or reversibility.

Analysis of day 15 serum samples for anti-NAP, CagA, and VacA antibodies indicated that low but measurable levels of IgG to all three antigens were found in all four group 3 rabbits (see above). Control rabbits were negative for antibodies.

Conclusion: Under the conditions of the study, a single 0.5 ml intramuscular injection of HP3 was well tolerated and immunogenic in male and female NZW rabbits. The local reactogenicity of HP3 was of a low order of magnitude and was similar to either the alum control or the placebo.

GLP safety and tolerance study (multiple dose)

A single dose safety and tolerability study (code UBAW-155) was performed in rabbits. The objective of this study was to evaluate the safety and tolerability of multiple (6) doses of HP3, once per week for six weeks by intramuscular injection to NZW rabbits. A secondary immunogenicity assessment was also included as a study parameter. The study consisted of three groups of 6/sex/group. Each animal either received the alum control, the placebo, or HP3. The dose volume was 0.5 mL alternately injected into the right and left quadriceps muscles on days 1, 8, 15, 22, 29, and 36 of the study. Three animals/sex/group were euthanised for a comprehensive macroscopic necropsy and tissue collection on days 38 and 50:

Group	Treatment*	Number		Day 38 Necropsy		Day 50 Necropsy	
		M	F	M	F	M	F
1	Alum/Saline	6	6	3	3	3	3
2	Alum/HP3 Placebo	6	6	3	3	3	3
3	HP3 Vaccine	6	6	3	3	3	3

Potential toxicity was evaluated based on the following parameters: daily clinical signs, dermal injection site observations (24 and 48 hours post-dose for each dose), body weights, physical examinations (body temperature, respiratory rate, heart rate, and capillary refill time), ophthalmic examinations, food consumption, clinical pathology (hematology, coagulation, and serum chemistry parameters), terminal organ weights, full macroscopic postmortem examination, and microscopic evaluation of selected tissues:

Bone marrow	Injection site	Spleen
Eyes with optic nerve	Kidneys	Thymus
Femorotibial joint	Liver	Urinary bladder
Femur	Lung	Lesions
Heart	Lymph nodes	

Observations of "very slight" dermal erythema at 24 hours post-dose were sporadic and resolved by the 48-hour observation. There were no apparent differences in the incidence or severity of dermal observations between the three groups.

There were no deaths and no treatment-related adverse effects on any antemortem study parameters (including body temperatures). There were some statistically-significant differences between groups in a few hematology, serum chemistry and coagulation parameters, however, all values were within the range of normal for this age and strain of rabbit, the changes were of small magnitude, and there was no consistent relationship to duration of dosing.

Macroscopic postmortem findings at the injection site consisted of discoloration (red/purple/tan) of the quadriceps in a few group 1 and 3 males and females. These sites of discoloration corresponded to several histologic findings, which are summarized in the following table:

Group Number		1 (Alum)				2 (HP3 Placebo)				3 (HP3)			
Finding	Day	38		50		38		50		38		50	
	Number M/F	3	3	3	3	3	3	3	3	3	3	3	3
Spleen													
-Follicular hyperplasia		0	0	1	1	1	1	0	1	3	3	3	3
Grade 1		0	0	1	1	1	1	0	1	1	1	3	2
Grade 2		0	0	0	0	0	0	0	0	2	2	0	1
Injection site, Right		0		0		0		0		0		0	
Per-acute hemorrhage			1		0		0		0		2		1
Myofiber lysis			1		0		0		0		2		0
-Eosinophil infiltration			0		0		0		0		0		0
Injection site, Left													
-Chronic inflammation		1	0	0	1	0	0	0	0	1	1	0	0
-Interstitial hemorrhage		1	0	0	0	0	0	0	0	0	1	0	0
-Per-acute hemorrhage		0	0	0	0	0	0	0	0	0	0	1	1
-Myofiber lysis		0	0	1	0	0	0	0	0	0	0	1	1
-Eosinophil infiltration		0	0	0	1	0	0	0	0	0	1	0	0
-Proteinaceous debris		0	0	1	0	0	0	0	0	0	0	0	0
-Granulomatous inflammation		0	0	0	0	0	0	1	0	0	0	0	0

Two animals, one in group 1 and one in group 3, had a whitish discoloration at the injection sites noted at necropsy, but there were no correlating microscopic lesions.

Microscopic examination of the injection sites revealed that any inflammation seen in the alum controls (group 1) and HP3 placebo controls (group 2) was comparable to the HP3 vaccine injection sites. Mild granulomatous inflammation was noted in one male in group 2. The macrophage cytoplasm was distended with a granular amphophilic material, putatively alum. Granulomatous inflammation associated with i.m. administration of aluminium-based adjuvants has been reported in several species [119,120].

HP3-related microscopic alterations were noted in the spleen of all group 3 animals at both days 38 and 50. Follicular hyperplasia (B-cell dependent peri-arteriolar regions) occurred with increased incidence and severity when compared to groups 1 or 2. A slight increase in the average severity of lymphoid hyperplasia was noted for both sexes on day 38 compared to day 50. Such findings may be related to the immunological response of the rabbits to the HP3 vaccine.

With the exception of injection sites and spleen, there were no microscopic alterations that could be attributed to treatment. Any other abnormalities noted were of the type/severity/incidence considered to be background in this strain and age of rabbit [118].

Serum was collected from all animals for analysis of antibody titres to HP3. All 12 rabbits immunised with HP3 had detectable antibody titres to each of the three antigens by day 15. IgG antibody titres in all group 3 rabbits were higher on day 29 and were sustained at the same level on days 38 and 50 (See above). All control rabbits gave negative results.

Conclusion: Under the conditions of the study, administration of six 0.5ml intramuscular injections of HP3 on a once-per-week schedule was well tolerated and immunogenic in male and female NZW rabbits. The local reactogenicity of HP3 was of a low order of magnitude and was similar to either alum in saline or the placebo formulation.

Human administration

A typical human immunisation will use three intramuscular injections of up to 25µg each of NAP, CagA, and VacA antigens with alum adjuvant. The animal toxicology studies utilised a high human dose of HP3 in rabbits weighing up to approximately 4 kg. An adult body weight of 60 kg can be used as a conservative estimate. Therefore, on a body weight basis, each dose given to these rabbits would be at least 15 times higher than in a human adult. Also, the triple human regimen was exceeded by an additional three doses in the multiple-dose rabbit study.

Based on these toxicity and immunogenicity results, it can thus be expected that an immunotherapeutic (once per week for three weeks) or a prophylactic (once per month for three months) clinical regimen of intramuscular injections of 10µg/dose or 25 µg/dose of CagA, VacA and NAP will be immunogenic and well tolerated in humans. Any local effects should be comparable to those seen with alum adjuvant and systemic effects should be consistent with other intramuscular administrations of protein antigens adjuvanted with alum.

For human use, a typical vaccine is a sterile preparation of purified CagA, VacA and NAP, with aluminium hydroxide adjuvant, in an isotonic buffer solution for intramuscular injection. The *H.pylori* antigens are expressed in genetically-engineered *E.coli* cells, utilising plasmid vector expression systems. Because of the relative insolubility of the VacA antigen, the vaccine will include urea in the amount of 2.9-4.1 mg/dose. The vaccine is provided in a pre-mixed format in syringes containing the antigens and the adjuvant. These syringes should be stored refrigerated between 2-8°C until ready for administration. The vaccine should be shaken before use. The vaccination site should be disinfected with a skin disinfectant (*e.g.* 70% alcohol). Before vaccination, the skin must be dry again. The content of pre-mixed single-dose vaccine in the syringe (0.5 ml) is applied intramuscularly into alternating sides of the upper arm (M. deltoideus). using a 1 to 1½ inch needle.

Two alternative vaccine compositions for human use have the following components in a single 0.5 ml dose and have a pH in the range 6.5 to 7.5:

Component	**Amount per final dose**	
	Low dose	**High dose**
Aluminium hydroxide adjuvant	0.5 mg	0.5 mg
NAP	10 µg	25 µg
CagA	10 µg	25 µg
VacA	10 µg	25 µg
Sodium phosphate ($NaH_2PO_4.H_2O$)	10 mM (0.69 mg)	10 mM (0.69 mg)
Sodium chloride (NaCl)	2.13 – 2.77 mg	2.13 – 2.77 mg
Urea	2.9 – 4.1 mg	2.9 – 4.1 mg
H_2O	Up to 0.5 mL	Up to 0.5 mL

Trace amounts of chloramphenicol may also be present.

Human testing — safety and immunogenicity

These two compositions (and a placebo in which antigens were omitted) were tested in humans in a randomised, controlled, single-blind, dose-ranging, and schedule-optimising study with the aim of evaluating safety and immunogenicity in healthy adults. Two test populations were used: one negative for *H.pylori* infection (57 patients) and the other positive for *H.pylori* infection (56 patients). Compositions were administered as 0.5ml doses from pre-filled syringes.

The 57 HP–negative volunteers were split into seven groups to receive the high (H; 25µg of each antigen) or low (L; 10µg of each antigen) dose vaccine, or the placebo (P; no antigen) with two different administration schedules. The first dose was given at time zero. In groups 1 to 5, three subsequent doses were given at 1, 2 and 4 months ('monthly' groups). In groups 6 & 7, two subsequent doses were given at 1 and 2 weeks ('weekly' groups):

Group	***n***	**First dose**	**Second dose**	**Third dose**	**Fourth dose**
1	7	L	L	L	P
2	7	H	H	H	P
3	7	L	L	P	L
4	8	H	H	P	H
5	9	P	P	P	P
6	9	L	L	L	—
7	10	H	H	H	—

Demographic data for the 57 volunteers were as follows:

Parameter		**Monthly doses (*n* = 38)**	**Weekly doses (*n* = 19)**	**All patients (*n* = 57)**
Age	mean (years)	29.9	28.9	29.6
	standard dev^{n}	6.3	5.7	6.1
	range	20-40	20-40	20-40
Sex	(% male)	53	37	47
Ethnicity		100% caucasian	100% caucasian	100% caucasian

Safety

The following safety parameters were monitored:

- Local and systemic reactions (up to day 6 post-injection).
- Adverse and serious events (for entire study period).
- Standard lab parameters *i.e.* serum chemistries and renal function (Na, K, Cl, HCO_3, urea, creatinine), complete blood count (WBC and differential, Hb, haematocrit, platelets), liver function (ALT, AST, alkaline phosphatase, bilirubin, prothrombin time, total protein, albumin).

Data on erythema, induration, malaise, myalgia, headache, arthralgia, fatigue and fever are shown, in that order, in Figures 6 to 13. Figures 6 & 7 show local reactions, whereas figures 8 to 13 show systemic reactions. Short-lasting pain was reported by around 89% of non-placebo subjects,

compared to 78% of placebo subjects. Pain was predominantly mild and resolved after injection. Systemic reactogenicity results are summarised in the following table:

Adverse event (frequency ≥ 5%)	**Monthly** (*n* = 29)	**Weekly** (*n* = 19)	**Placebo** (*n* = 9)
Any adverse event	14	15	7
Administration site reactions and general disorders	8	11	5
Gastrointestinal symptoms	3	3	2
Infections	3	3	0
Musculo-skeletal symptoms	2	0	0
Nervous system disturbances*	2	6	0
Skin and subcutaneous tissue manifestations	2	0	1

* headache, dizziness, akinesia, disturbances of alertness

The frequency and severity of local and systemic reactions were as expected in this population. Adverse events were mild in nature, transitory (lasting from a few hours up to an average two days), and were well in agreement with previous observations during clinical studies with aluminium hydroxide adjuvant. No serious adverse events related to the administration of the composition occurred in the volunteers. Local reactions were not frequent, except for local pain at the injection site in all groups. Induration and erythema occurred more often in the 'weekly' groups. The most frequently reported solicited systemic reactions among all groups, of any severity, were fatigue, headache and malaise. Local and systemic post-immunisation reactions were usually mild and resolved within 24-72 hours. Administration of the composition does not significantly alter laboratory parameters. Compositions of the invention are therefore safe for human administration.

Immunogenicity

The following immunogenicity parameters were monitored:

- Serum IgG specific for CagA, VacA and NAP.
- Proliferative responses driven by CagA, VacA and NAP.

Immune responses are shown in Figure 14 to 19. These data show that the composition is immunogenic both at antibody and cellular level in all vaccination groups. More than 85% of subjects mounted a significant antibody response to CagA, VacA and NAP after the third immunisation. The majority of subjects maintained antibody titres above the cut-off limits to all three antigens months after the 3rd dose. The majority of the subjects exhibited a significant antigen-specific cellular proliferative response (particularly CagA and VacA). The composition induces antigen-specific memory, with the antibody response being boostable and significant proliferative responses to at least two of the antigens detectable up to >3 months after the third immunisation

It will be understood that the invention has been described by way of example only and modifications may be made whilst remaining within the scope and spirit of the invention.

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CLAIMS

1. A composition in unit dosage form comprising (a) *H.pylori* CagA, VacA and NAP proteins; (b) an aluminium salt adjuvant; and (c) a buffer solution, wherein CagA, VacA and NAP are each present at a concentration of between 10 µg/dose and 50 µg/dose.

2. A composition comprising: (a) *H.pylori* CagA, VacA and NAP proteins; (b) an aluminium salt adjuvant; (c) a buffer solution; and (d) urea.

3. The composition of claim 1, wherein CagA, VacA and NAP are each present at a concentration of 10 µg/dose.

4. The composition of claim 2, wherein CagA, VacA and NAP are each present at a concentration of 20 µg/ml.

5. The composition of claim 1, wherein CagA, VacA and NAP are each present at a concentration of 25 µg/dose.

6. The composition of claim 2, wherein CagA, VacA and NAP are each present at a concentration of 50 µg/ml.

7. The composition of any preceding claim, wherein the alum salt is an aluminium hydroxide.

8. The composition of claim 7, wherein the aluminium hydroxide has a concentration of 1 mg/ml.

9. The composition of any preceding claim, wherein the buffer solution is a phosphate buffer.

10. The composition of any preceding claim, buffered to a pH of between 6 and 8.

11. The composition of any preceding claim, wherein the composition is isotonic.

12. The composition of any preceding claim, wherein the composition is sterile.

13. The composition of any preceding claim, adapted for intramuscular administration.

14. The composition of claim 13, adapted for administration as an injectable.

15. The composition of any one of claims 2 to 14, wherein urea is present in an amount sufficient to ensure that VacA remains soluble.

16. The composition of any preceding claim, further comprising an antigen selected from the group consisting of:
    - a protein antigen from *N.meningitidis*;
    - an outer-membrane vesicle (OMV) preparation from *N.meningitidis*;
    - a saccharide antigen from *N.meningitidis*;
    - a saccharide antigen from *Streptococcus pneumoniae*;

– an antigen from hepatitis A, B and/or C virus;
– an antigen from *Bordetella pertussis*;
– a diphtheria antigen;
– a tetanus antigen;
– a protein antigen from *Helicobacter pylori*;
– a saccharide antigen from *Haemophilus influenzae*;
– an antigen from *N.gonorrhoeae*;
– an antigen from *Chlamydia pneumoniae*;
– an antigen from *Chlamydia trachomatis*;
– an antigen from *Porphyromonas gingivalis*;
– polio antigen(s);
– rabies antigen(s);
– measles, mumps and/or rubella antigens;
– influenza antigen(s);
– an antigen from *Moraxella catarrhalis*;
– an antigen from *Streptococcus agalactiae*;
– an antigen from *Streptococcus pyogenes*; and
– an antigen from *Staphylococcus aureus*.

17. The composition of any preceding claim, being an immunogenic composition.

18. The composition of any preceding claim, wherein said composition is a vaccine composition.

19. The composition of any preceding claim, further comprising an antisecretory agent and/or an antibiotic effective against *Helicobacter pylori*.

20. The composition of claim 19, wherein the antisecretory agent is a proton pump inhibitor, a H2 receptor antagonist, a bismuth salt or a prostaglandin analog.

21. A kit comprising a syringe, a needle, and the composition of any preceding claim.

22. The kit of claim 21 wherein the composition is within the syringe.

23. The kit of claim 21 or claim 22, further comprising an antisecretory agent and/or an antibiotic effective against *Helicobacter pylori*.

24. The kit of claim 23, wherein the antisecretory agent is a proton pump inhibitor, a H2 receptor antagonist, a bismuth salt or a prostaglandin analog.

25. A process for producing the composition of any one of claims 1 to 20, comprising the step of admixing *H.pylori* CagA, VacA and NAP proteins, an aluminium salt, and a buffer solution.

26. The use of (a) the composition of any one of claims 1 to 18 and (b) an antisecretory agent and/or an antibiotic effective against *Helicobacter pylori*, in the manufacture of a medicament for raising an immune response in a mammal against CagA, VacA and NAP.

27. The use of claim 26, wherein the medicament is for the prevention and/or treatment of an infection and/or disease caused by *Helicobacter pylori* at any age.

28. A process for monitoring the efficacy of a composition of any one of claims 1 to 20, wherein one or more of the following tests is performed on a patient to whom the composition has been administered: urease breath test, stool antigen shedding, and/or immunological (*e.g.* serological) analysis.

29. The process of claim 28, wherein the process monitors prophylactic efficacy.

30. The process of claim 28, wherein the process monitors therapeutic efficacy.

31. The use of urease breath testing, stool antigen testing, and/or immunological (*e.g.* serological) analysis as correlate(s) of protection against *H.pylori* infection.

FIGURE 1

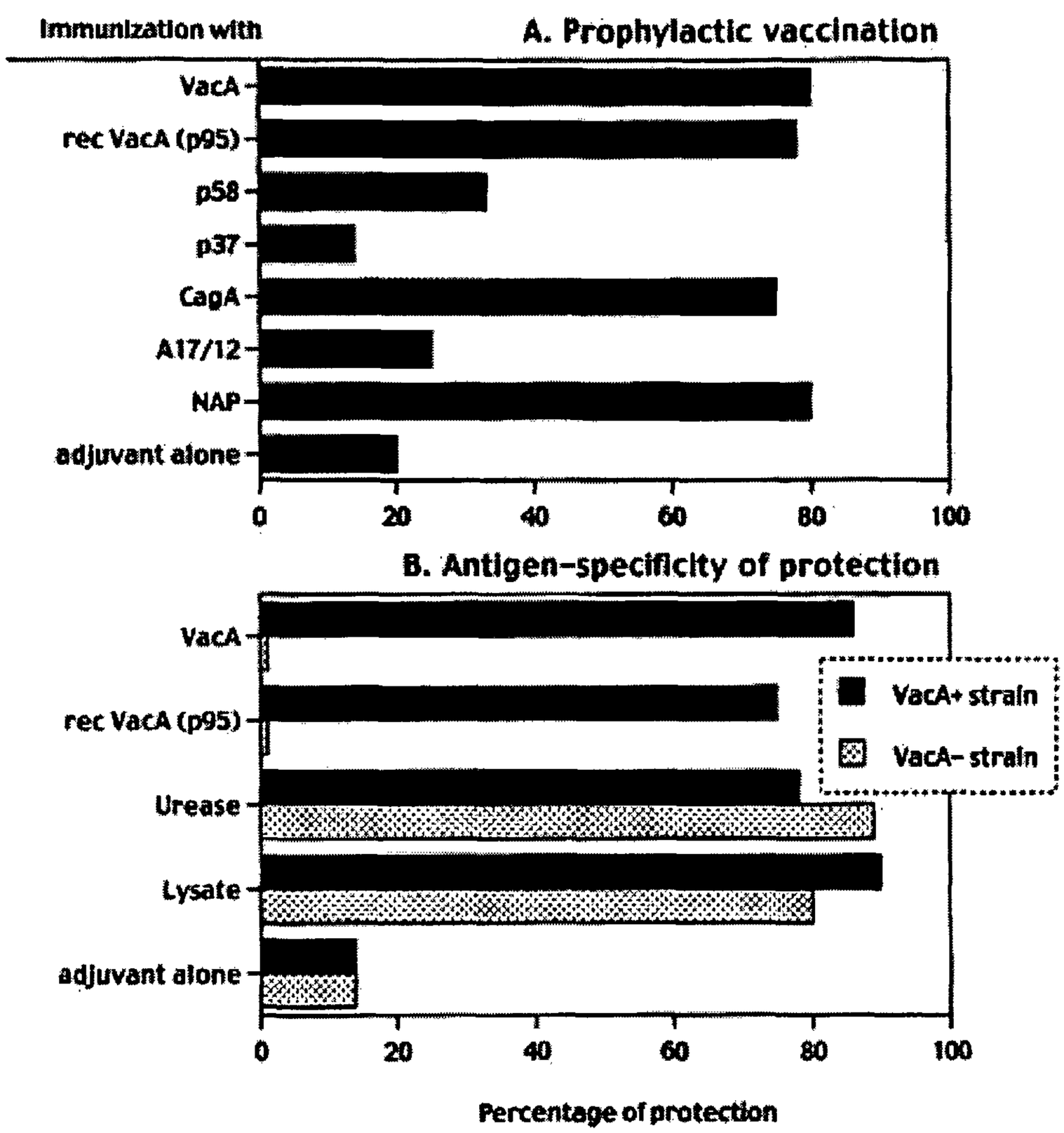
Immunization with
A. Prophylactic vaccination
VacA
rec VacA (p95)
p58
p37
CagA
A17/12
NAP
adjuvant alone
0
20
40
60
80
100
B. Antigen-specificity of protection
VacA
rec VacA (p95)
Urease
Lysate
adjuvant alone
VacA+ strain
VacA- strain
0
20
40
60
80
100
Percentage of protection

FIGURE 2A

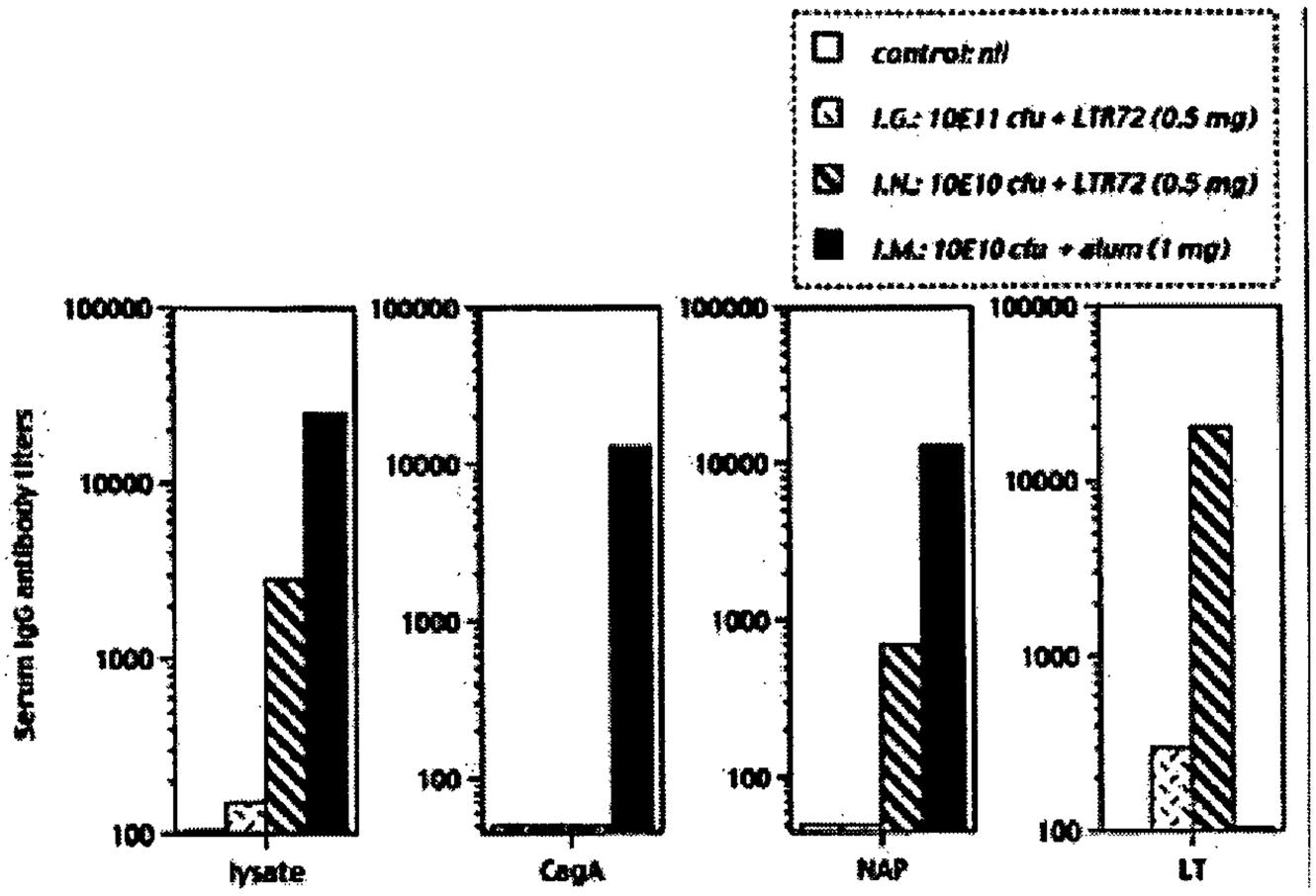

FIGURE 2B

Immunization	Dog #	Protection
nil	1	-
	2	-
	3	-
Intragastric	4	-
	5	-
	6	-
	7	+
Intramuscular	8	+
	9	+
	10	+
Intranasal	11	+
	12	-
	13	-

FIGURE 3

FIGURE 3A

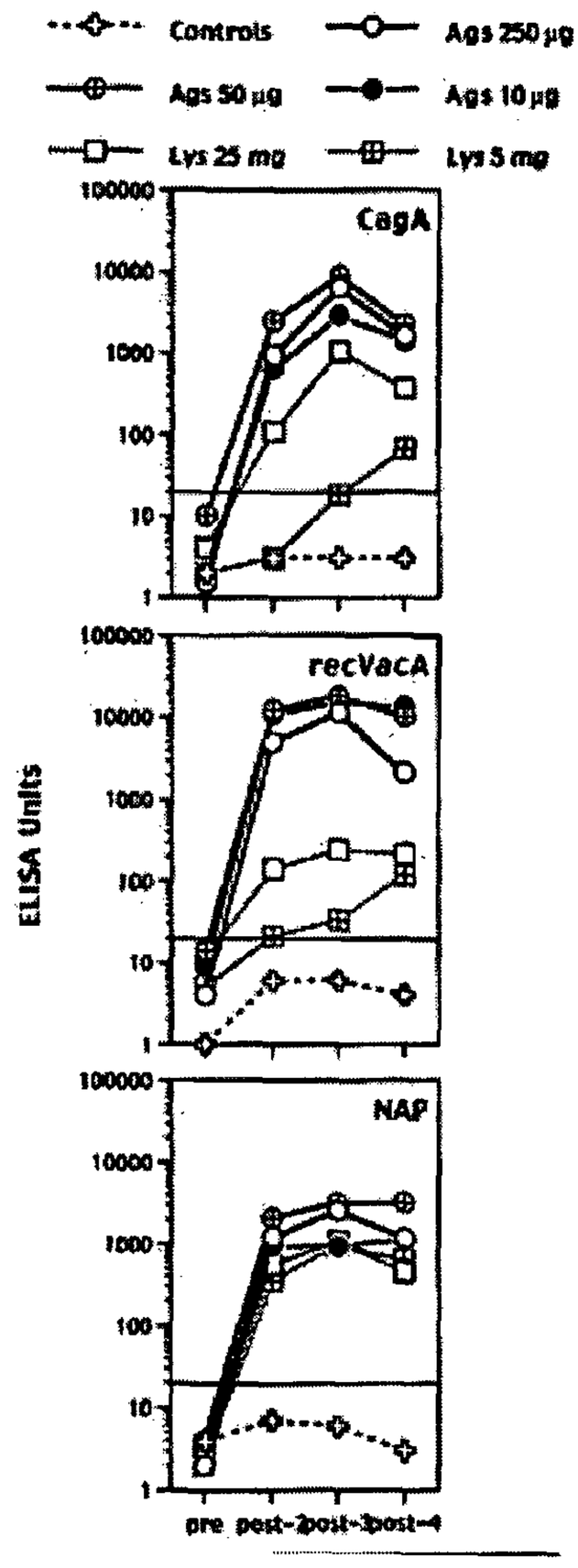

FIGURE 3B

Group	Dog #	Protection
Control	1	–
	2	–
	3	–
	4	–
VacA/CagA/NAP	5	+
250 mg each	6	+
	7	+
	8	–
VacA/CagA/NAP	9	+
50 mg each	10	+
	11	+
	12	+
VacA/CagA/NAP	13	+
10 mg each	14	+
	15	+
	16	+
whole-cell lysate	17	–
25 mg	18	+
	19	+
	20	+
whole-cell lysate	21	+
5 mg	22	+
	23	–
	24	+

FIGURE 4

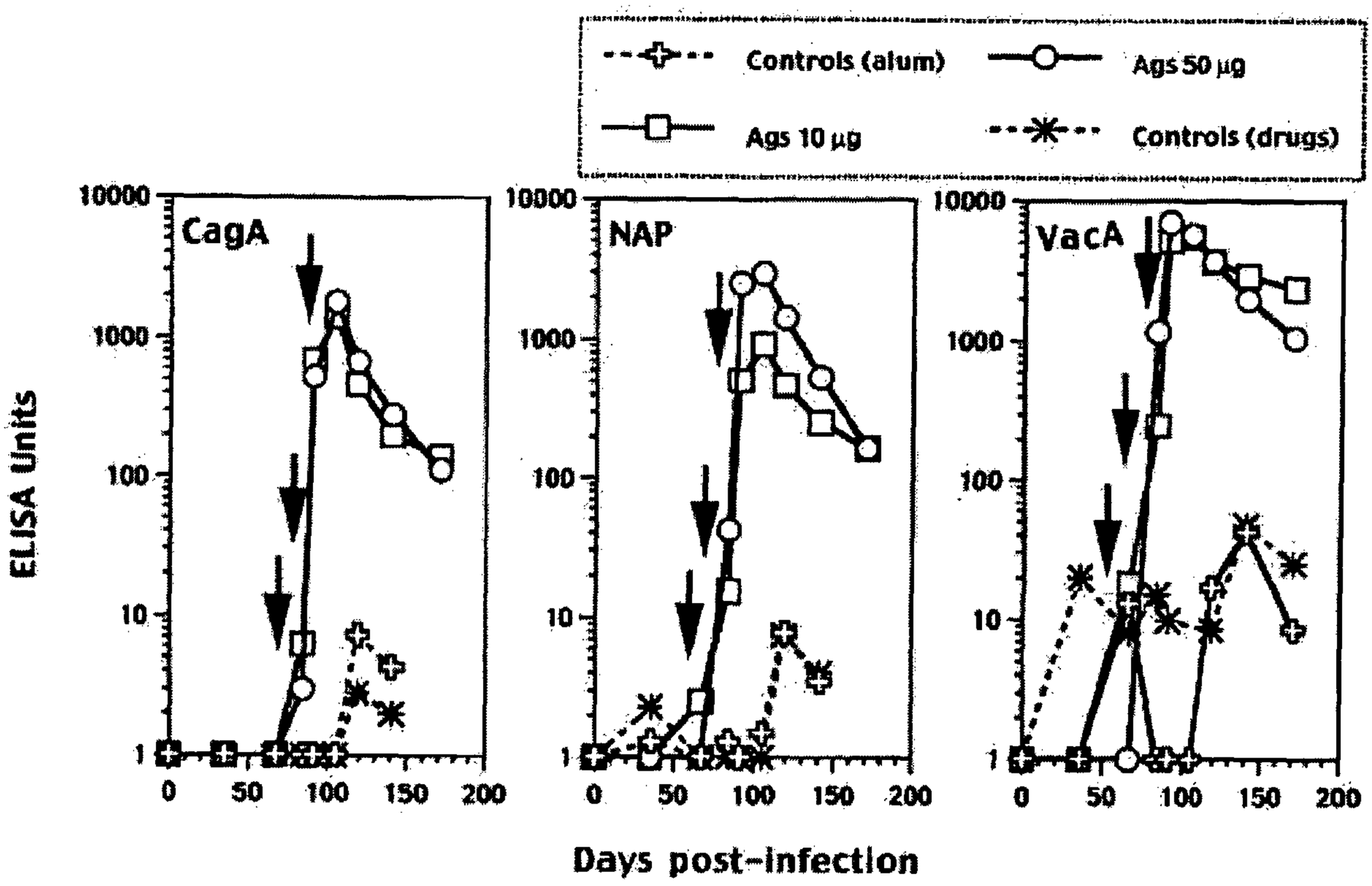
Controls (alum)
Ags 50 µg
Ags 10 µg
Controls (drugs)
CagA
NAP
VacA
ELISA Units
10000
1000
100
10
1
0
50
100
150
200
Days post-infection

FIGURE 5

		pre-vaccination				week 7 post-vaccination				week 11 post-vaccination				week 17 post-vaccination				week 27 post-vaccination			
group	dog n.	antrum inflammation	antrum follicles	urease	immunohistochemistry	antrum inflammation	antrum follicles	urease	immunohistochemistry	antrum inflammation	antrum follicles	urease	immunohistochemistry	antrum inflammation	antrum follicles	urease	immunohistochemistry	antrum inflammation	antrum follicles	urease	immunohistochemistry
1 alum	10	3	3	+	+	3	3	+	+	3	3	+	+	3	3	+	+	2	1	+	+
	11	1	0	+	+	3	0	+	+	2	0	+	+	2	0	+	+	2	1	+	+
	14	0	0	+	-	2	0	+	+	3	3	+	+	3	3	+	+	1	1	+	+
	8	3	3	+	+	3	0	+	+	2	0	+	+	0	0	+	+	2	1	+	+
2 HP3 50 µg	3	0	0	+	-	1	0	+	+	2	1	+	-	0	0	-	-	2	0	+	-
	1	2	2	+	+	1	0	+	+	0	0	+	-	0	0	-	-	1	1	+	+
	6	3	3	+	+	2	1	+	-	2	2	+	+	2	0	+	+	0	0	+	+
	7	2	0	+	+	0	0	+	-	0	0	+	-	0	0	+	+	2	2	+	-
3 HP3 10 µg	2	3	3	+	+	3	3	+	+	1	0	+	+	0	0	-	-	0	0	+	+
	12	2	1	+	+	1	0	+	-	0	0	+	-	0	0	+	+	2	2	+	-
	15	3	3	+	+	1	1	+	+	0	0	+	+	0	0	+	+	1	0	+	-
	9	3	3	+	+	0	0	+	-	2	2	+	-	1	0	-	-	2	0	+	-
4 PPI + antib.	4	3	2	+	+	1	0	-	+	0	0	-	-	0	0	-	-	3	0	+	-
	5	2	0	+	+	3	3	-	-	1	0	-	-	0	0	-	-	1	0	+	+
	13	3	3	+	+	3	3	-	-	0	0	+	+	3	3	+	+	1	0	+	+
	16	3	3	+	+	0	0	+	+	2	2	+	+	1	0	+	+	1	0	+	+

WO 03/018054 PCT/IB02/03768

CA 02458854 2004-02-27

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FIGURE 6

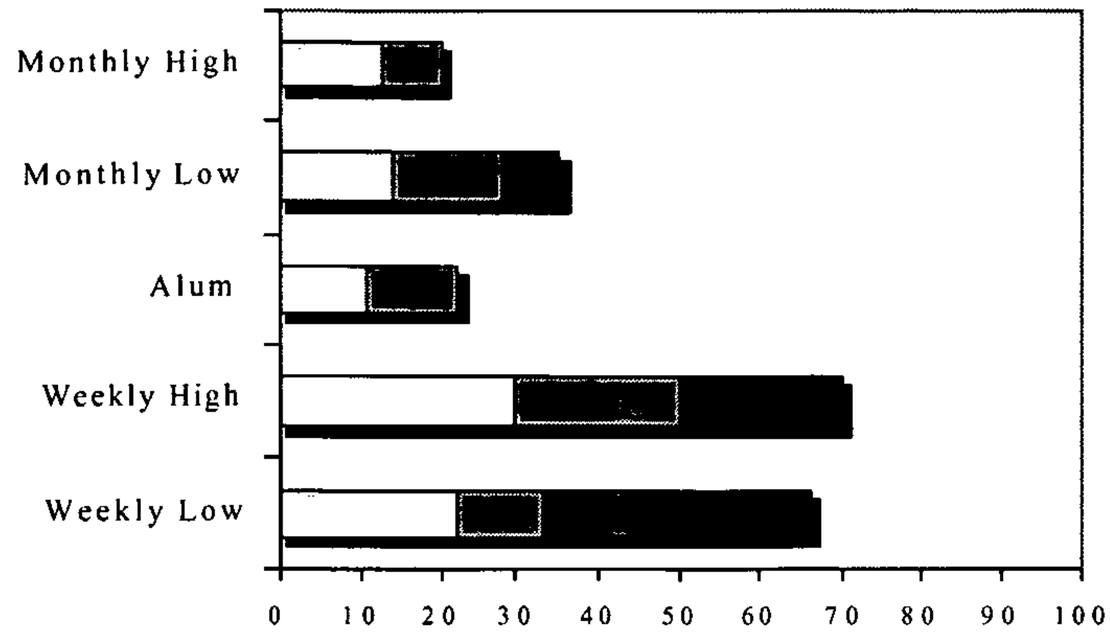


FIGURE 7

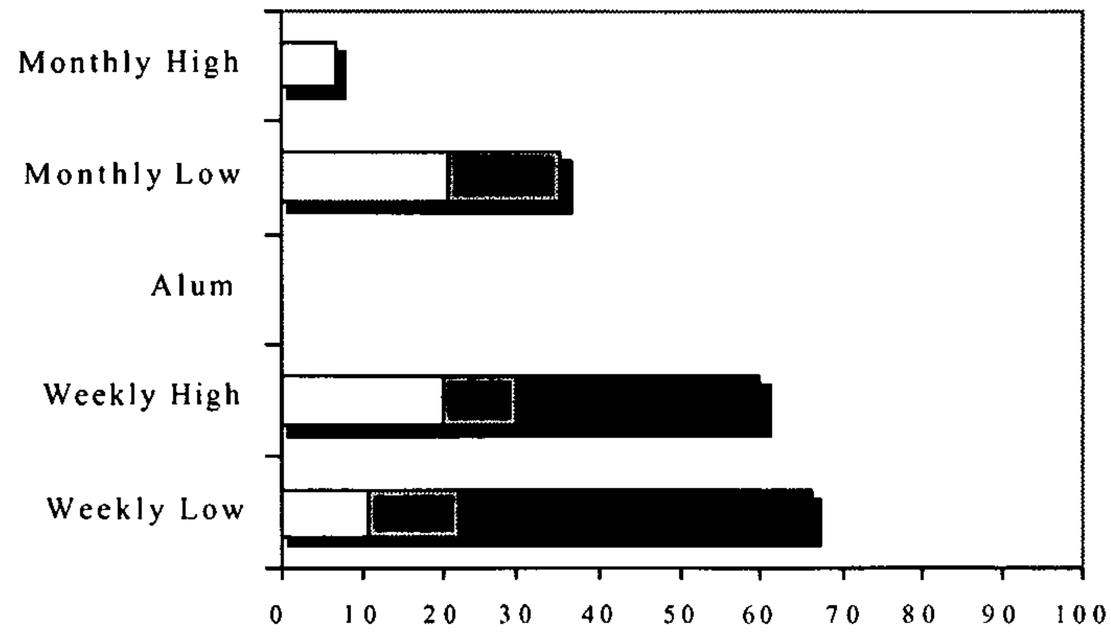


FIGURE 8

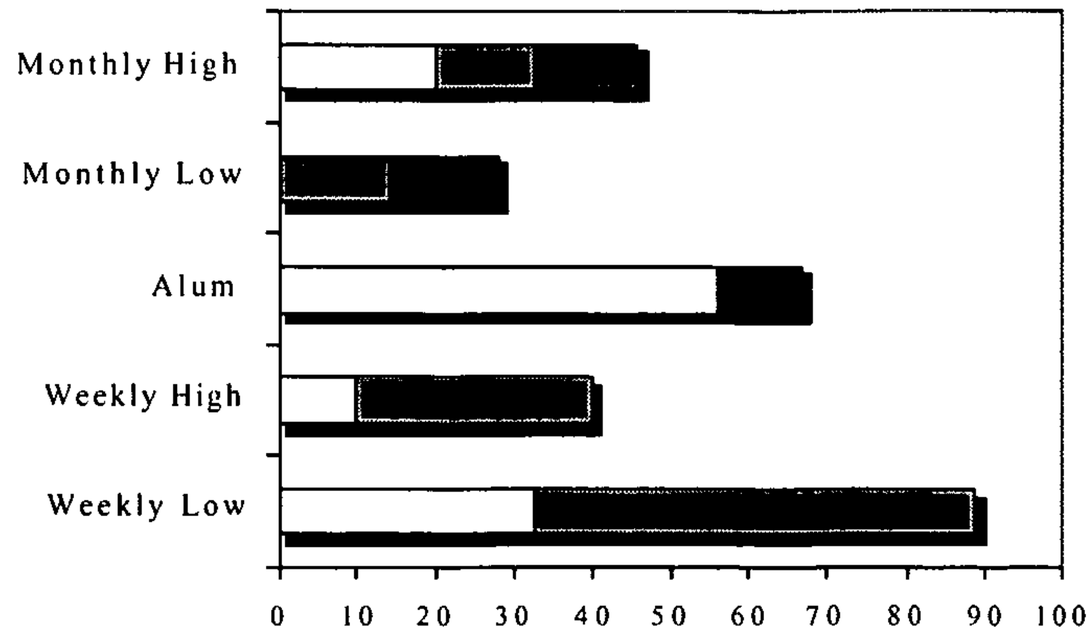


FIGURE 9

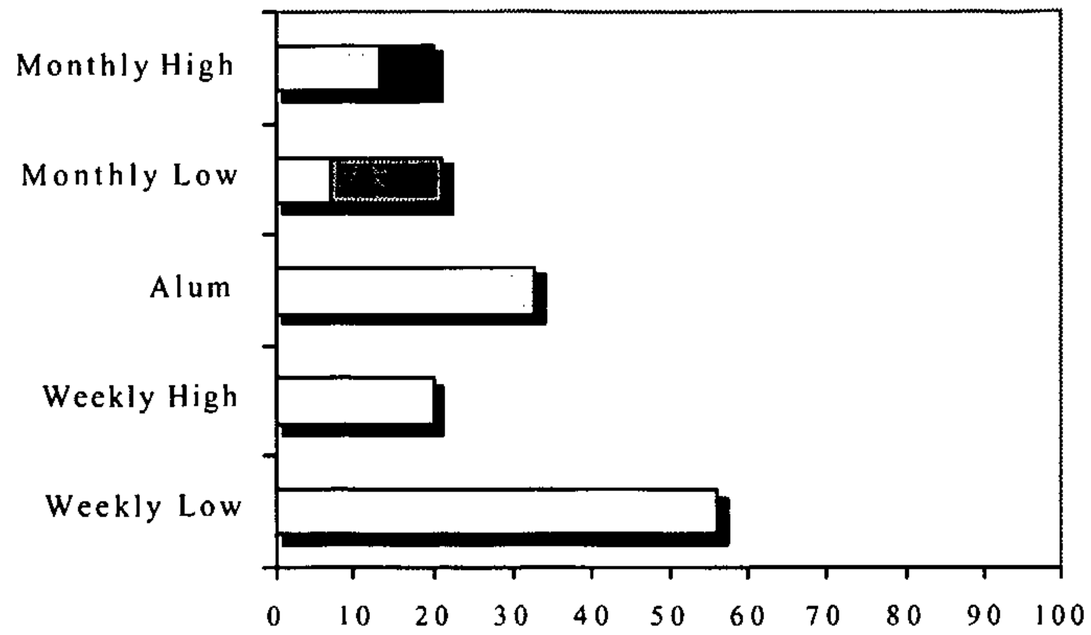


FIGURE 10

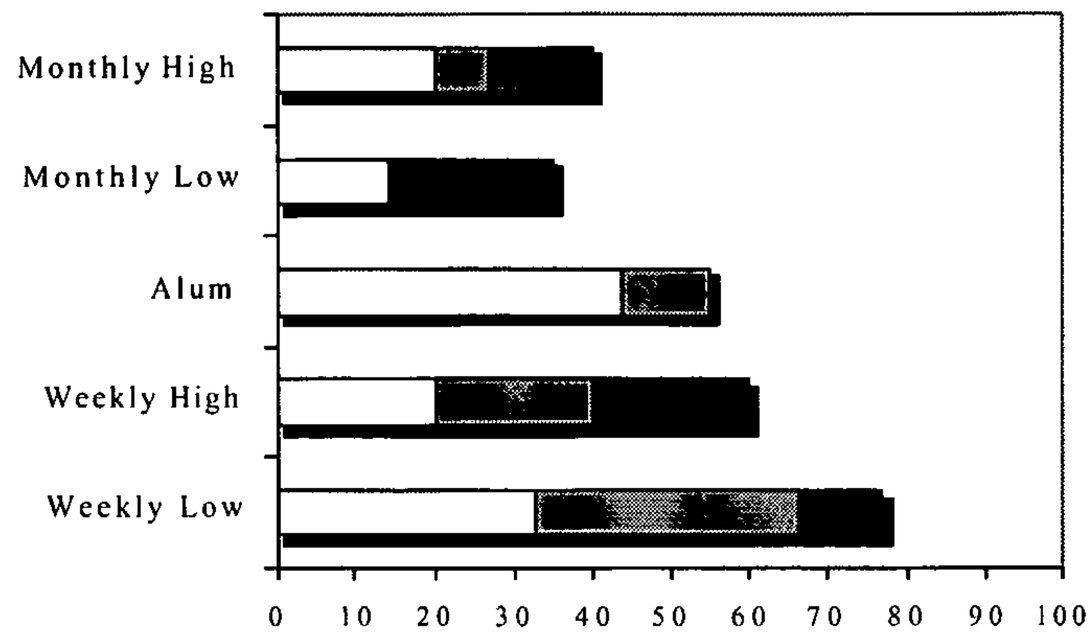


FIGURE 11

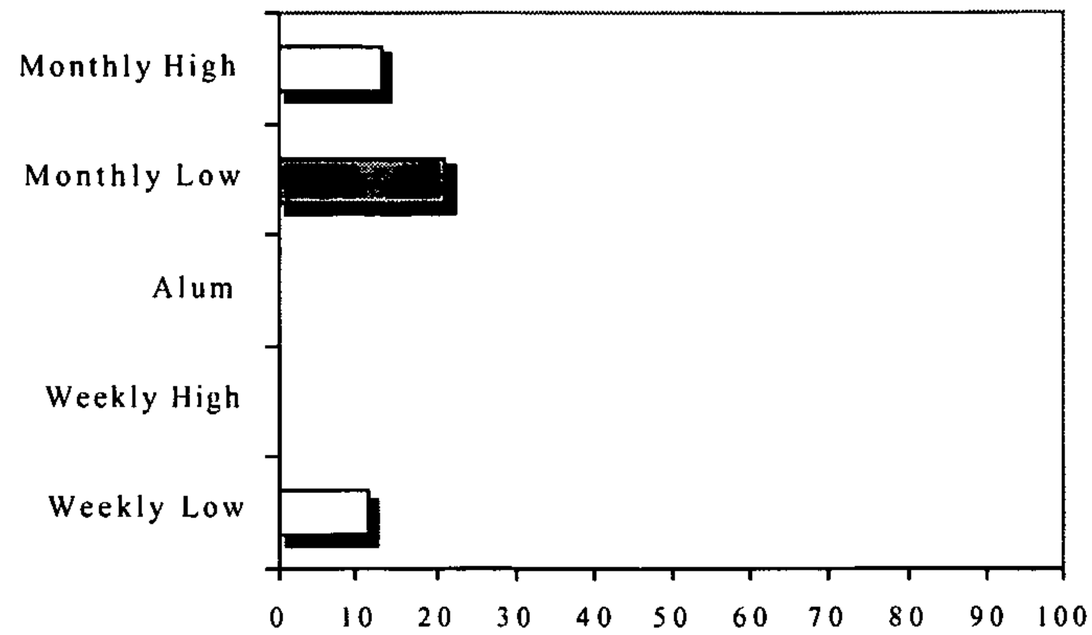


FIGURE 12

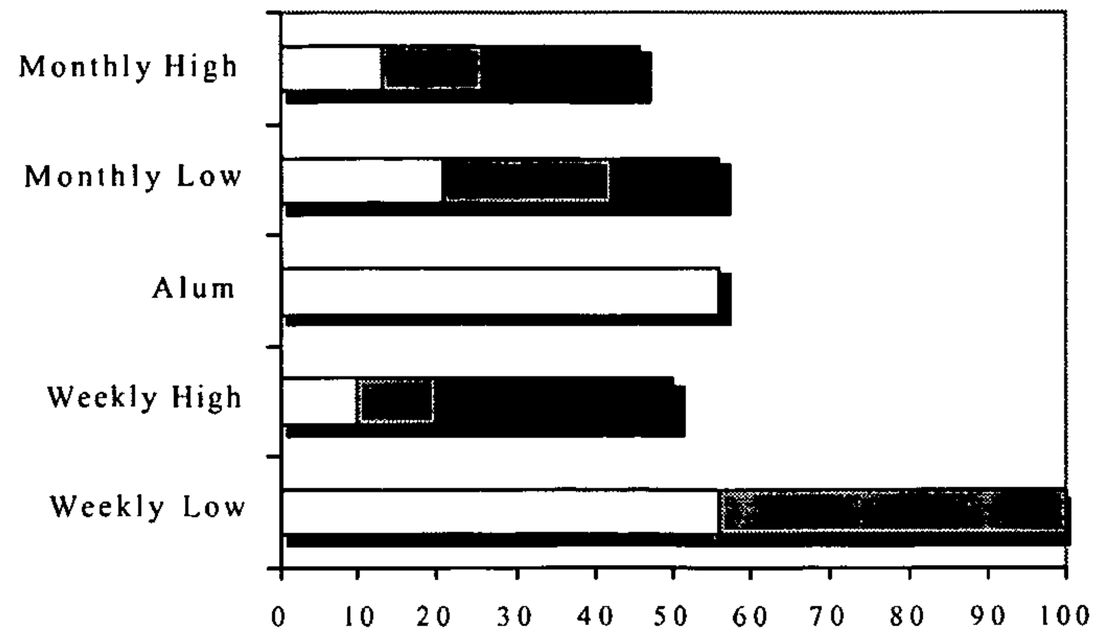


FIGURE 13

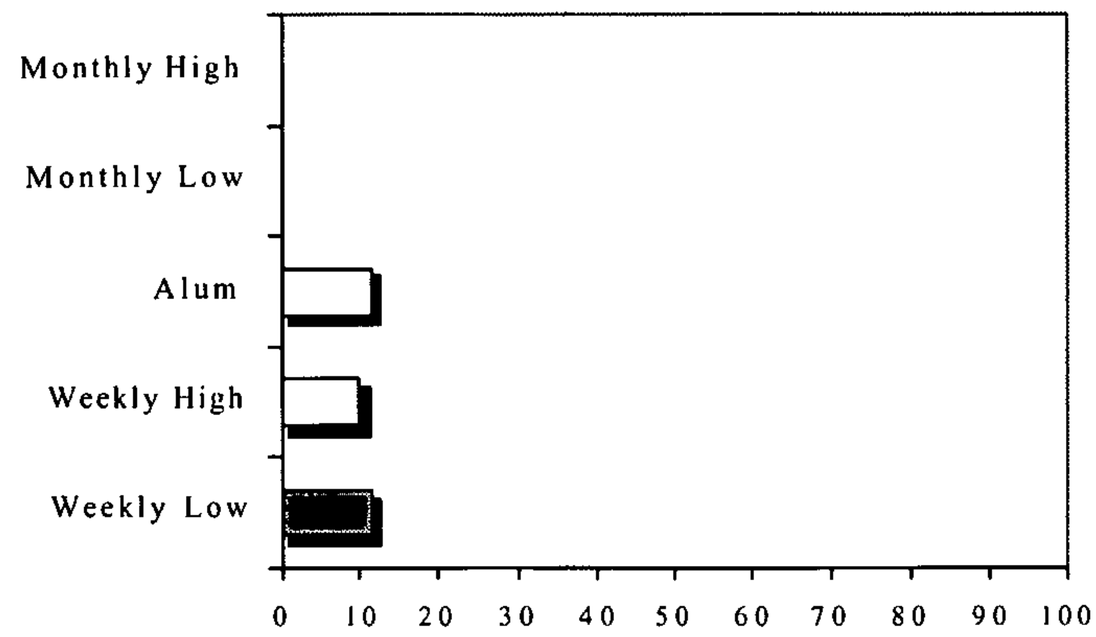


FIGURE 14

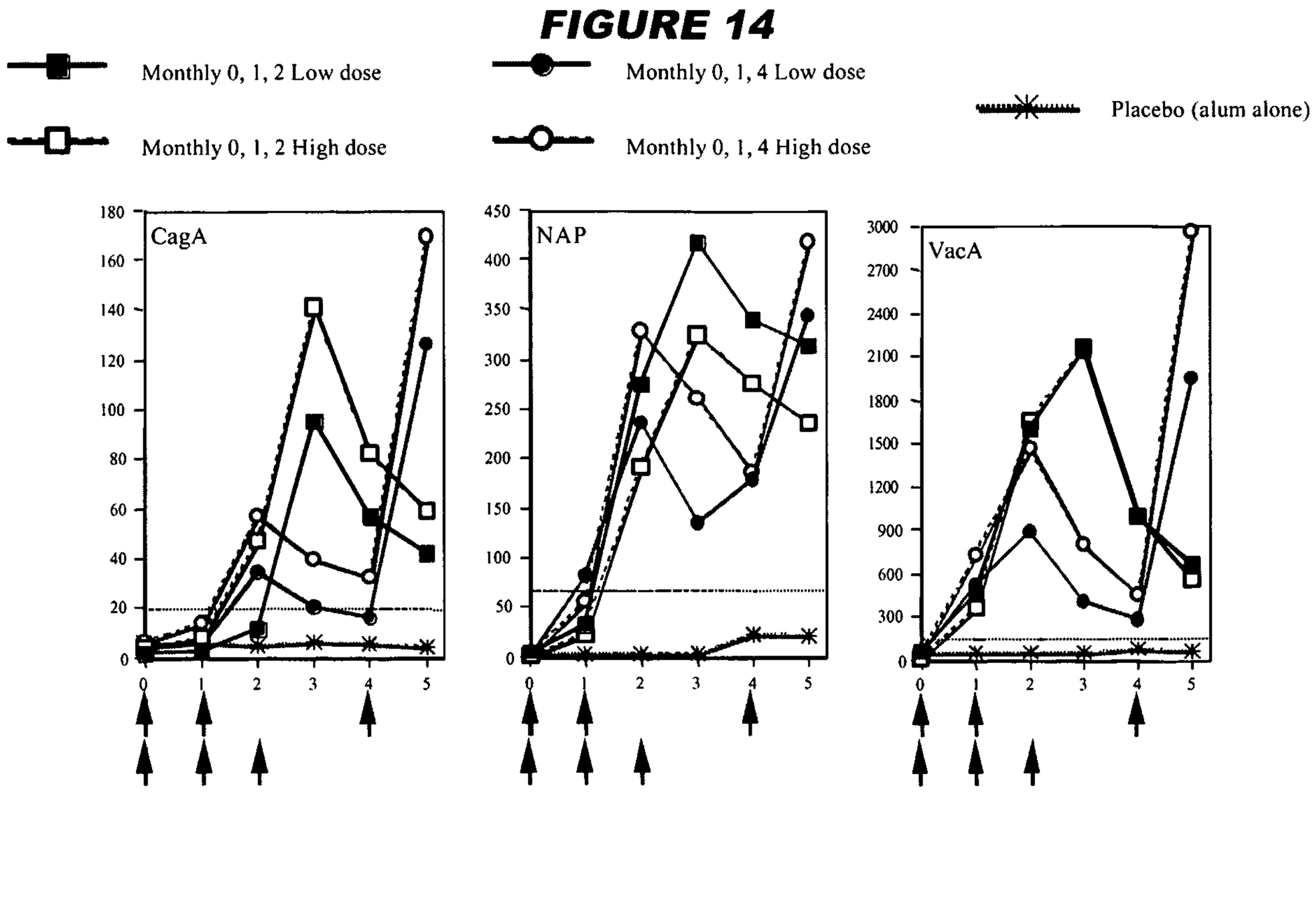


FIGURE 15

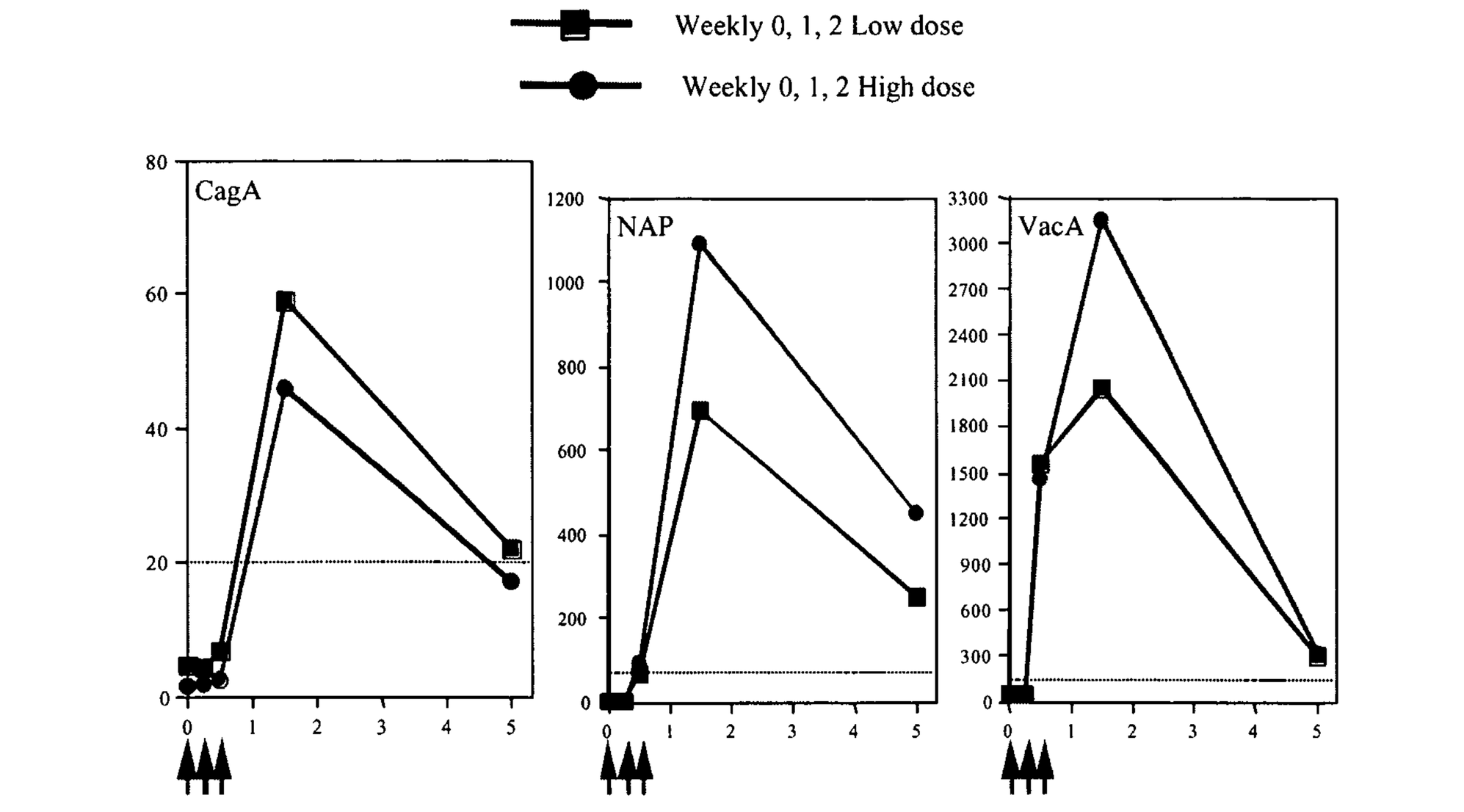


FIGURE 16

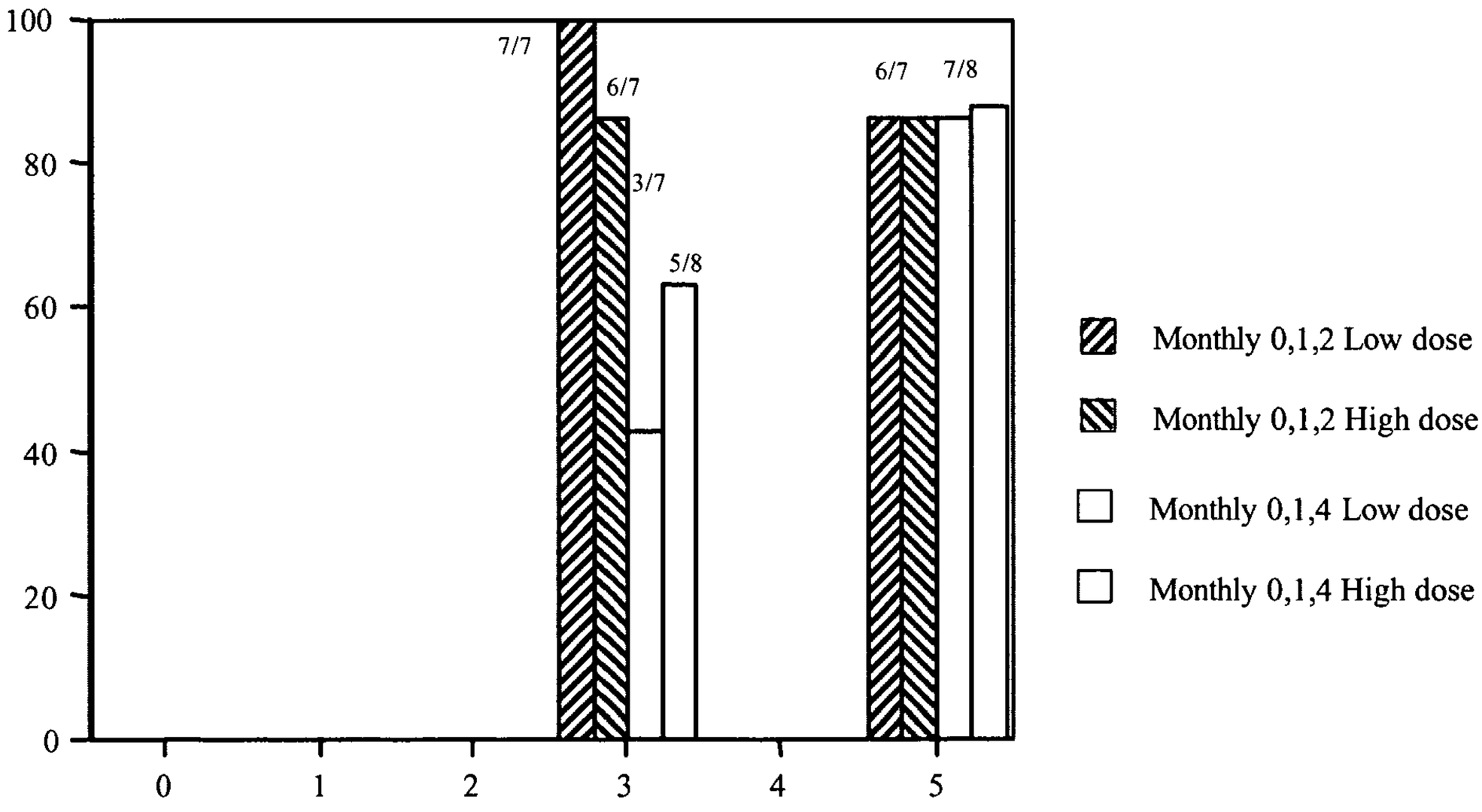


FIGURE 17

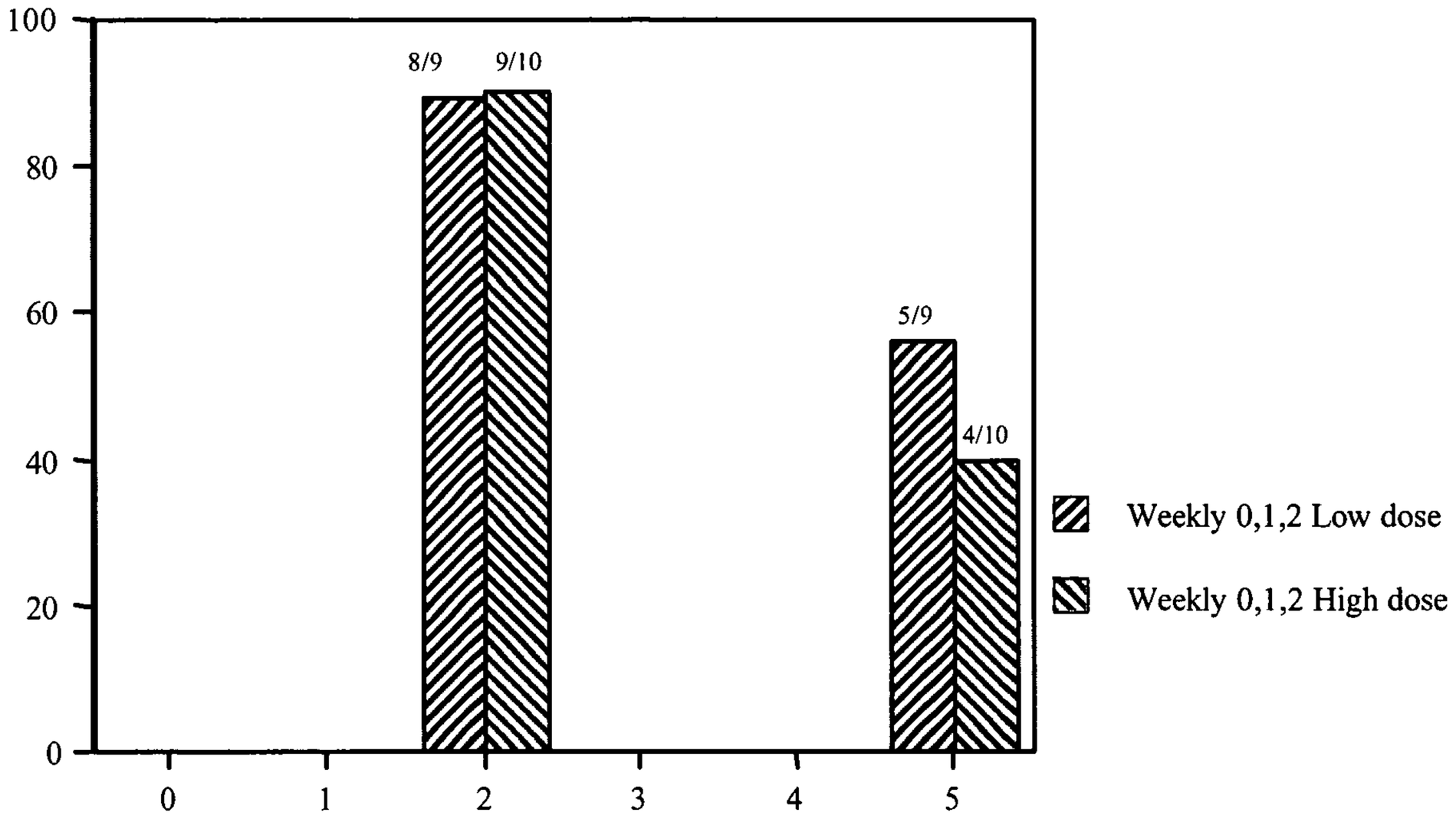


FIGURE 18

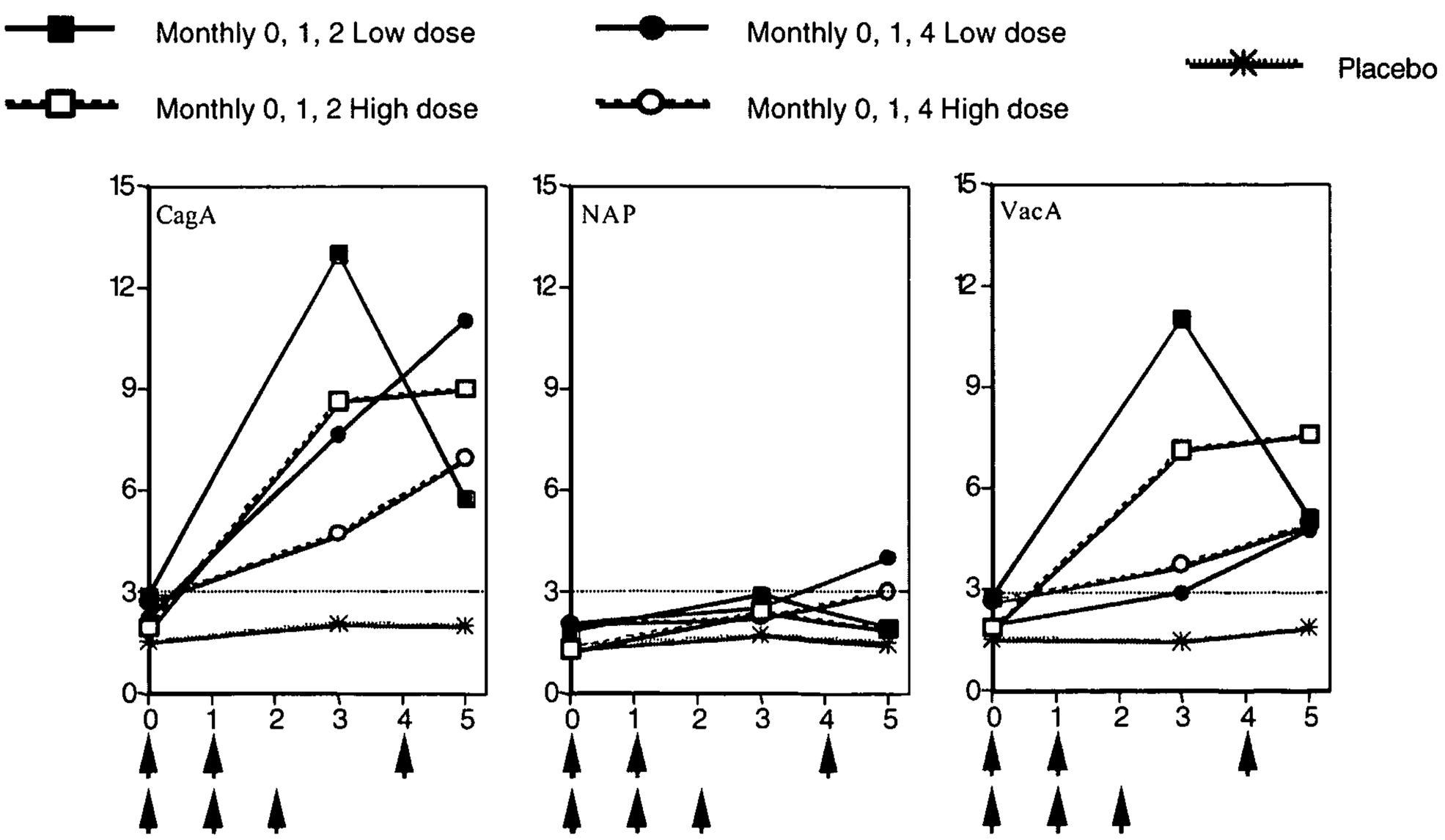
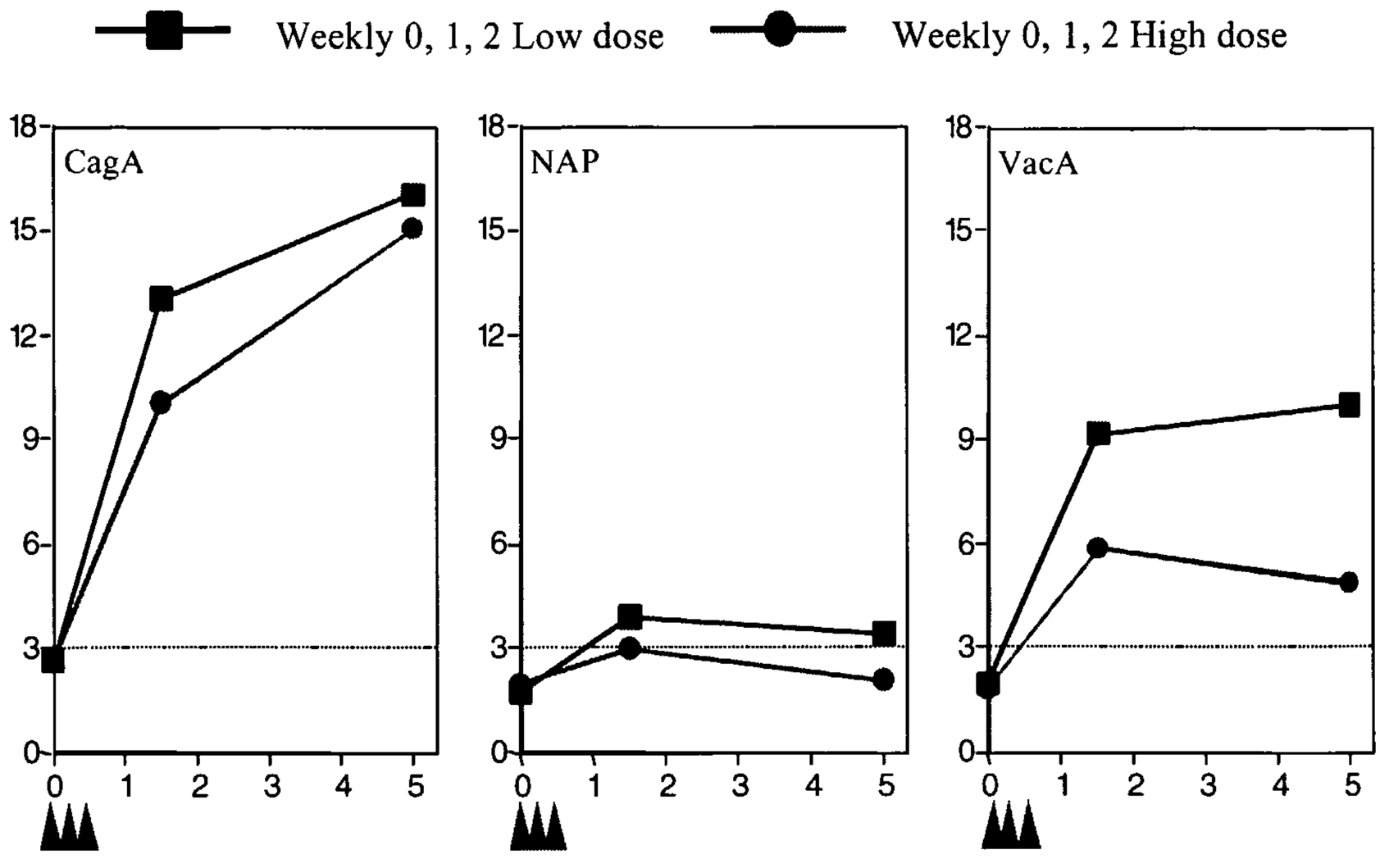
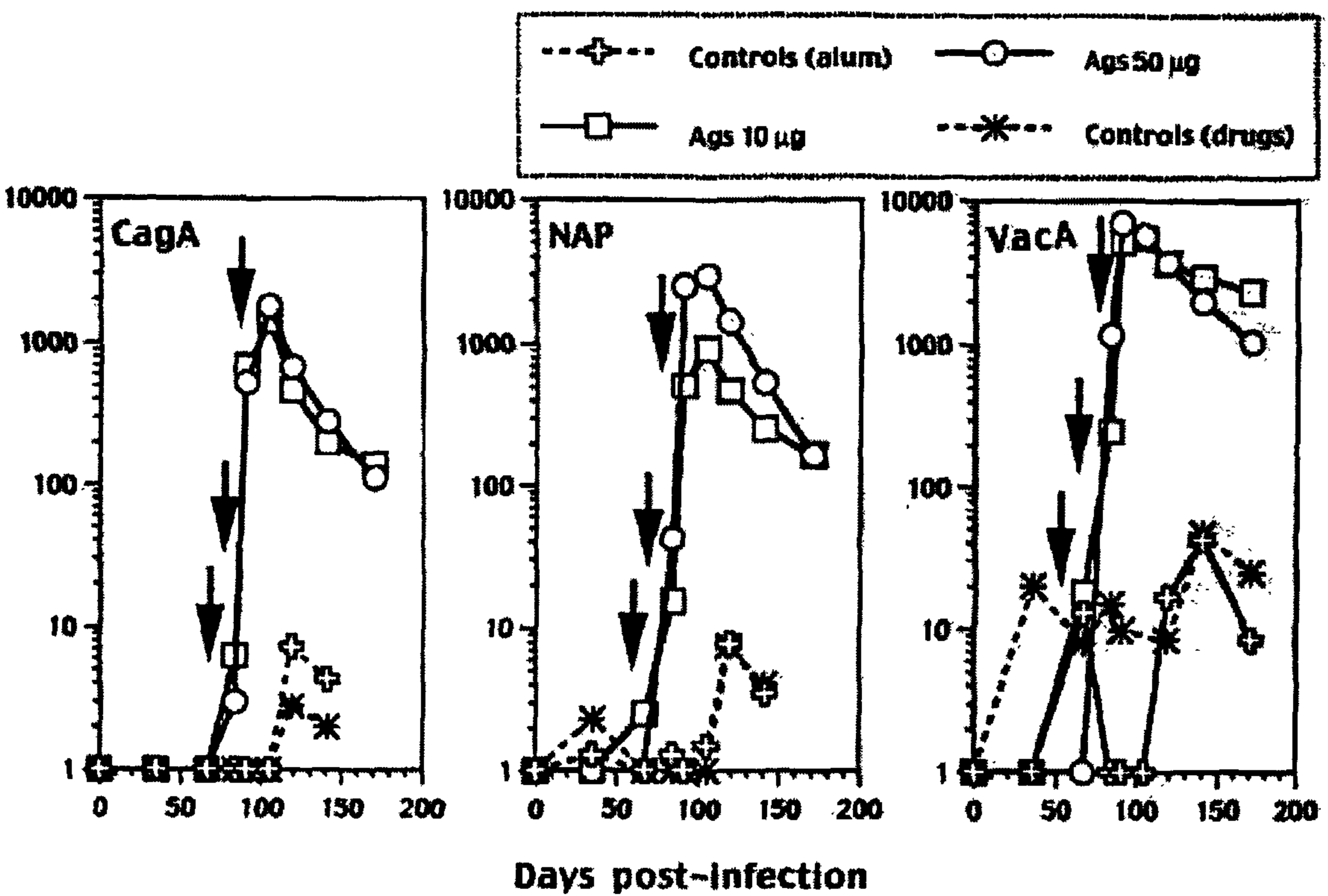


FIGURE 19


Weekly 0, 1, 2 Low dose
Weekly 0, 1, 2 High dose
CagA
NAP
VacA
18
15
12
9
6
3
0
0 1 2 3 4 5


Controls (alum)
Ags 50 µg
Ags 10 µg
Controls (drugs)
CagA
NAP
VacA
10000
1000
100
10
1
0
50
100
150
200
ELISA Units
Days post-infection