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(57) Abrégé/Abstract:
The present invention relates to a method of protection against or treatment for infection by H. pylori comprising non-mucosal administration of an effective amount of one or more H.pylori antigens.

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(54) Title: IMMUNIZATION AGAINST AND TREATMENT FOR INFECTION BY *H.PYLORI*

(57) Abstract

The present invention relates to a method of protection against or treatment for infection by *H. pylori* comprising non–mucosal administration of an effective amount of one or more *H.pylori* antigens.

IMMUNIZATION AGAINST AND TREATMENT FOR INFECTION BY *H.PYLORI*

Technical field

The present invention relates to a method of protection against or treatment of an *H.pylori* infection, comprising non-mucosal administration of an effective amount of one or more *H.pylori* antigens.

Background of the Invention

H.pylori was isolated in 1982 by B. Marshall and J. Warren using microaerophilic conditions that had been developed to grow *Campylobacter jejuni*. *H.pylori* bacteria are S-shaped, gram negative bacilli 2-3.5 μm in length and 0.5-1 μm in width (Blaser, M.J., and J. Parsonnet. 1994. J. Clin. Invest. 94:4-8). It is now (only recently) known that infection with *H.pylori* is the most common infection in the world. In developing countries 80% of the population is infected by the bacterium at the age of 20, while in developed countries *H.pylori* infection increases with age from <20% in 30-year old people to >50% in 60-year olds (Axon, AT. 1995. Pharmacol. Ther. 9:585-588; Blaser and Parsonnet, 1994). The infection is transmitted by either the oro-fecal or the oro-oral route (Blaser and Parsonnet, 1994). Infection occurs during the first years of life and persists forever. Once established, the infection is chronic, possibly permanent. Risk factors for infection are crowding, poor hygiene and host-specific genetic factors.

The complete genome sequence of *H.pylori* has been published (Tomb, J.-F., *et al.* 1997. Nature 388:539-547). A brief review of this article and a general review of the biology of *H.pylori* can be found in Doolittle, R.F. 1997. Nature 388:515-516.

Chronic infection of the human gastroduodenal mucosae by *H.pylori* is frequently associated with chronic gastritis, peptic ulcer, and increases the risk of occurrence of gastric malignancies such as adenocarcinoma and low grade B cell lymphoma (Blaser and Parsonnet, 1994; Parsonnet, J., *et al.* 1991. N. Engl. J. Med. 325: 1127-1231; Parsonnet, J., *et al.* 1994. N. Engl. J. Med. 330: 1267-1271). Most of the infections remain asymptomatic,

whereas symptomatic, severe diseases correlate epidemiologically with the infection by a subset of *H.pylori* strains, called Type I (Blaser, M.J., *et al.* 1995. Cancer Res. 55:2111-2115; Covacci, A., et al. 1997. Trends Microbiol. 5:205-208; Covacci, A., *et al.* Proc. Natl. Acad. Sci. U.S.A. 90:5791-5795; Eck, M., *et al.* 1997. Gastroenterology. 112:1482-1486; Xiang, Z., *et al.* 1995. Infect. Immun. 63:94-98). This subset of strains is endowed with increased virulence due to the expression of a biologically active toxin (VacA), which is cytopathic to gastric epithelial cells *in vitro* and *in vivo* (Ghiara, P., *et al.* 1995. Infect. Immun. 63:4154-4160; Harris, P.R., *et al.* 1996. Infect. Immun. 64:4867-4871; Telford, J.L., *et al.* 1994. J. Exp. Med. 179:1653-1658), and also to the acquisition of a pathogenicity island (PAI), called *cag*, which contains a set of genes encoding several virulence factors (Censini, S., *et al.* 1996. Proc. Natl. Acad. Sci. USA. 93:14648-14653) which are responsible for the induction of the synthesis of the neutrophil chemotactic cytokine IL-8 by the gastric epithelial cells (Censini *et al.*, 1996).

H.pylori factors that have been identified so far include the flagella that are probably necessary to move across the mucus layer, see for example, Leying *et al.* 1992. Mol. Microbiol. 6:2863-2874; the urease that is necessary to neutralize the acid environment of the stomach and to allow initial colonization, see, for example, Cussac *et al.* 1992. J. Bacteriol. 174:2466-2473, Perez-Perez *et al.* 1992. J. Infect. Immun. 60:3658-3663, Austin, *et al.* 1992. J. Bacteriol. 174:7470-7473, WO 90/04030; the *H.pylori* cytotoxin (sometimes referred to as VacA, as it causes vacuolation), see, for example, WO 93/18150, Telford, J.L. *et al.* 1994. J. Exp. Med. 179:1653-1658, Cover *et al.* 1992. J. Bio. Chem. 267:10570-10575, Cover *et al.* 1992. J. Clin. Invest. 90:913-918, Leunk, 1991. Rev. Infect. Dis. 13:5686-5689; the *H.pylori* heat shock proteins (hsp), see, for example, WO 93/18150, Evans *et al.* 1992. Infect. Immun. 60:2125-2127, Dunn *et al.* 1992. Infect. Immun. 60:1946-1951, Austin *et al.*, 1992; and the cytotoxin-associated protein, CagA, see, for example, WO 93/18150, Covacci, A., *et al.* 1993. Proc. Natl. Acad. Sci. USA 90:5791-5795, Tummuru, M.K., *et al.* 1994. Infect. Immun. 61:1799-1809.

Currently, *H.pylori* strains can be partitioned into at least two major groups, which either express (Type I) or do not express (Type II) the cytotoxin (VacA) and the CagA proteins. Type I strains contain the CagA and toxin genes and produce active forms of these antigens. Type II strains lack the CagA locus and fail to express the cytotoxin. The association between the presence of the CagA gene and cytotoxicity suggests that the

production of the CagA gene is necessary for the transcription, folding, export or function of the cytotoxin. Epidemiological analysis indicate that Type I bacteria are associated with duodenal ulcerations, gastric ulceration and severe forms of active gastritis.

For a general review of the pathogenic role of *H.pylori* in peptic ulcer, see Telford, J.L., *et al.* 1994. TIBTECH 12:420-426.

H.pylori culture supernatants have been shown by different authors to contain an antigen with a molecular weight of 120, 128 or 130 kDa (Apel, *et al.*. 1988. Zentralblat für Bakteriol. Microb. Und Hygiene 268:271-276; Crabtree, *et al.* 1992. J. Clin. Pathol. 45:733-734; Cover, *et al.* 1990. Infect. Immun. 58:603-610; Figura, *et al.* 1990. *H.pylori, gastritis and peptic ulcer* (eds. Malfrtheiner, *et al.*), Springer Verlag, Berlin). Whether the difference in size of the antigen described was due to interlaboratory differences in estimating the molecular weight of the same protein, to the size variability of the same antigen, or to actual different proteins was not clear. This protein is very immunogenic in infected humans because specific antibodies are detected in sera of virtually all patients infected with *H.pylori* (Gerstenecker, *et al.* 1992. Eur. J. Clin. Microbiol. 11:595-601).

A protein known as NAP (neutrophil activating protein – Evans D.J., *et al.* 1995. *Gene* 153:123-127; WO 96/01272 & WO 96/01273, especially SEQ ID NO:6; see also WO 97/25429), which is found in both type I and II strains, appears to be protective when tested in the *H.pylori* mouse model (Marchetti, M., *et al.* 1995. Science. 267:1655-1658). NAP is a homodecamer of 15 kDa subunits, and it has been proposed that the multimeric complex has a ring-shaped structure which spontaneously forms hexagonal paracrystalline structures. The assembled protein appears to interact with glycosphingolipid receptors of human neutrophils.

A number of other *H.pylori* antigens are described in WO 98/04702, including ureaseB (SEQ ID NO: 4), HopX (SEQ ID NO: 21), HopY (SEQ ID NO: 21), 36 kDa (SEQ ID NO: 26), 42 kDa (SEQ ID NO: 25), and 17 kDa (SEQ ID NO: 27). Urease is also described in, for example, EP-B-0367644 (protein with urease activity), EP-A-0610322 (ureaseE, F, G, H and I), EP-A-0625053 (urease protein) and EP-A-0831892 (multimeric forms of urease). Other *H. pylori* antigens include the 54 kDa (SEQ ID NO: 2) and 50 kDa (SEQ ID NO: 1) proteins described in EP-A-0793676.

Discussions of various virulence factors of *H.pylori* can be found in, for example, WO 93/18150 and WO 96/33274.

Colonization of the mucosa of the stomach by *H.pylori* is today recognized as the major cause of acute and chronic gastroduodenal pathologies in humans (Blaser and Parsonnet, 1994; Covacci *et al.*, 1997). The recognition of the infectious nature of the illness is having a major impact in the treatment of the disease that is shifting from the treatment of symptoms by anti-H2 blockers to the eradication of the bacterial infection by antibiotic regimen.

In spite of the unquestionable successes that will be achieved with antibiotic treatment, it should be remembered that such treatment inevitably leads to the occurrence of resistant strains that in the long term will make antibiotics ineffective. This suggests that vaccination, which classically is the most effective way to prevent and control infectious diseases in a large population, could be used to prevent infection and possibly also to treat the disease.

The increasing importance of *H.pylori* in the induction of a wide variety of gastric pathologies has represented a major challenge for the development of efficacious prophylactic and/or therapeutic strategies. To better understand the interactions between the bacterium and the host, much effort has focused on the development of appropriate animal models of infection reproducing aspects of the natural human infection.

Several animal models of infection and disease have been developed aiming at studying the pathogenesis of infection and development of preventive and therapeutic strategies. Many of these models are highly impractical, since they employ monkeys (Dubois, A., *et al.* 1994. Gastroenterology. 106:1405-1417) or species that are kept under gnotobiotic (that is, germ-free) conditions, for example germ-free dogs or piglets (Krakowka, S., *et al.* 1987. Infect. Immun. 55: 2789-2796; Radin, M.J., *et al.* 1990. Infect. Immun. 58: 2606-2612). Colonization of gnotobiotic piglets (Krakowka *et al*, 1987) has been reported using *H.pylori* strains isolated from patients with gastroduodenal diseases. However piglets cannot be kept under germ-free conditions for more than 2 months (Radin *et al.*, 1990) mainly due to their nutritional needs.

Successful infection of specific pathogen-free (SPF) cats has been described using a strain isolated from conventional cats (Fox, J.G., *et al.* 1995. Infection and Immunity. 63: 2674-2681; Handt, L.K., *et al.* 1995. J. Clin. Microbiol. 33:2280-2289). Gnotobiotic beagle pups have also been infected with a human *H.pylori* isolate and kept under germ-free conditions

for 30 days. However, in this model no data are available on long term infections with *H.pylori* (Radin *et al.*, 1990). Other experimental animal models include athymic *nu/nu* or germ-free mice (Karita, M., *et al.* 1991. Am. J. Gastroenterol. 86:1596-1603).

The major drawbacks of these experimental infections, however, are the sophisticated and expensive housing systems required, and, more importantly, the peculiar immunological status of the gnotobiotic or immunodeficient hosts employed. More recently, *H.pylori*, freshly isolated from human gastroduodenal biopsies, have been adapted to persistently colonize the gastric mucosa of xenobiotic mice (Marchetti *et al.*, 1995). This model has proven particularly useful to assess the feasibility of either preventive (Manetti, R., *et al.* 1997. Infect. Immun. 65:4615-4619; Marchetti *et al.*, 1995; Marchetti, M., *et al.* 1998. Vaccine 16: 33-37; Radcliff, F.J., *et al.* 1997. Infect. Immun. 65:4668-4674) or therapeutic (Ghiara, P., *et al.* 1997. Infect. Immun. 65:4996-5002) vaccination, as well as for the *in vivo* screening of anti-*H.pylori* antimicrobials (Lee, A., *et al.* 1997. Gastroenterol. 112:1386-1397), and for studying the pathogenesis of infection (Sakagami, T., *et al.* 1996. Gut. 39:639-648). However, to evaluate gastric infection, mice have to be sacrificed; the pathological changes induced by the chronic infection and/or the effect of therapeutic or immunizing regimens cannot, therefore, be followed up in the same individual animal.

In International patent application WO 99/35907 (filed 15th January 1999), there is described for the first time an animal model which can reproduce symptoms which have been clearly associated with the acute phases of infection with *H.pylori* in humans (Marshall, B.J., *et al.* 1985. Med. J. Australia. 142:436-439; Mitchell, J.D., *et al.* 1992. Am. J. Gastroenterol. 87:382-386; Morris, A., and G. Nicholson. 1987. Am. J. Gastroenterol. 82:192-199; Sobala, G.M., *et al.* 1991, Gut 32:1415-1418). The invention described in WO 99/35907 is based on the discovery that *H.pylori* can persistently colonize the gastric mucosa of conventional xenobiotic dogs, and that this colonization causes acute symptoms, histopathological lesions and elicits specific immune responses. Thus, the animal model provided in WO 99/35907 is ideal for studying the efficacy of treatments for *H.pylori* infection.

As *H.pylori* is a mucosa-related infection, where the bacteria do not invade the surrounding host cells, attempts to develop vaccines or treatments against the disease have concentrated on mucosal administration, specifically oral administration into the gastro-intestinal tract.

Thus, as it had previously been thought that local (mucosal) treatment at the site of the *H.pylori* infection was necessary, the thrust of research in this area has been to develop mucosa-associated anti-*H.pylori* antibodies by the mucosal administration of prophylactics/therapeutics (see, for example: Chen, M., *et al.* 1992. Lancet 339:1120-1121; Ferrero, R.L., *et al.* 1994. Infect. Immun. 62:4981-4989; Michetti, P., *et al.* 1994. Gastroenterology 107:1002-1011; Lee, A., *et al.* 1994. Infect. Immun. 62:3594-3597; Doidge, C., *et al.* 1994. Lancet 343:974-979; Marchetti *et al.*, 1995; Lee, C.K., *et al.* 1995. J. Infect. Dis. 172:161-172; Corthesy-Theulaz, I., *et al.* 1995. Gastroenterology 109:115-121; Cuenca, R., *et al.* 1996. Gastroenterology 110:1770-1775; Radcliff, F.J., *et al.* 1996. Vaccine 14:780-784; Stadtlander, C.T.K.H., *et al.* 1996. Dig. Dis. Sci. 41:1853-1862; Ferrero, R.L., *et al.* 1997. Gastroenterology 113:185-194; Weltzin, R., *et al.* 1997. Vaccine 15:370-376; Radcliff *et al.*, 1997; Ghiara *et al.*, 1997; Marchetti *et al.*, 1998).

However, it has been shown in the present invention that a systemic protective effect against challenge with infectious *H.pylori* can be unexpectedly achieved using a non-mucosally administrated *H.pylori* antigen-containing composition. Specifically, it has been shown that, for instance, intramuscular (i.m.) immunization with whole *H.pylori* cell lysate can protect dogs against challenge with infectious *H.pylori*, and that the i.m. route, as an example of a non-mucosal route, can, unexpectedly, be considered for vaccination against this bacterium. Such a method may also be useful therapeutically in treating an already established *H.pylori* infection.

Summary of the Invention

The present application is therefore based on the discovery that *H.pylori* antigen-containing compositions against *H.pylori* can be administered non-muscosally and still result in effective (systemic) protection from disease, despite the fact that the *H.pylori* bacteria are associated with the mucosa.

According to the present invention there is provided a method of protection against or treatment for infection by *H.pylori* comprising non-mucosal administration of an effective amount of one or more *H.pylori* antigens.

The term "comprising" means "including" as well as "consisting of".

Preferably, administration is parenteral, more preferably, intramuscular.

The one or more antigens administered may separately or in combination elicit a protective immune response in an animal, preferably, a mammal, more preferably a human.

At least one of the antigens may be a virulence factor of *H.pylori*. Examples of such virulence factors include VacA, CagA, NAP or urease. Other preferred antigens include HopX, HopY, 36kDa, 42kDa, and 17kDa (see WO 98/04702) or 50 kDa antigen (see EP-A-0793676). The virulence factors of *H. pylori* administered preferably include VacA, CagA, NAP and urease. More preferably, the virulence factors include VacA, CagA and NAP. Most preferably, the virulence factors include CagA and NAP.

The *H. pylori* antigens administered may consist of only virulence factors or of only specific combinations of virulence factors such as VacA, CagA, NAP and urease; VacA, CagA and NAP; or CagA and NAP.

The antigens administered may also include non-*H. pylori* antigens.

The one or more antigens is/are preferably purified antigen(s) or a whole cell immunogen.

The term "purified" means that at least one step of purification has been carried out such that a purified antigen is more pure than the same antigen in its natural context. There may, however, be present some impurities associated with such purified antigens. The term "purified" includes the situation where an antigen is "isolated". In this case, the antigen is generally not associated with any other substance which may adversely affect its ability to protect against or treat infection by *H.pylori*. Thus, the term "isolated" implies the highest degree of purification.

The antigens are obtained by various usual methods, i.e. by purification/isolation from cell culture, recombinant technology or by chemical synthesis. The whole cell immunogen is preferably prepared by extraction from *H.pylori* cells. The extraction may be carried out by lysis or sonication of the *H.pylori* cells or any other suitable method. The whole cell immunogen may be or may comprise inactivated *H.pylori* cells.

An adjuvant is preferably co-administered with the one or more *H.pylori* antigens. Preferably the adjuvant is aluminium hydroxide or MF59™ (see WO 90/14837; EP-B-0399843; Ott *et al.* Chapter 10 of *Vaccine Design: The subunit and adjuvant approach*, eds. Powell & Newman, Plenum Press 1995)

The one or more antigens may also be in association with one or more pharmaceutically acceptable excipients.

The present invention also provides the use of an effective amount of one or more *H.pylori* antigens in the manufacture of a medicament for non-mucosal administration for protection against or treatment for infection by *H.pylori*.

The present invention further provides a pharmaceutical composition for non-mucosal administration comprising one or more *H.pylori* antigens and one or more pharmaceutically acceptable excipients. This is preferably an immunogenic composition.

The one or more antigens of the pharmaceutical composition may separately or in combination elicit a protective immune response in an animal, preferably a mammal, more preferably, a human.

Preferably, at least one of the antigens is a virulence factor of *H.pylori*. Examples of such virulence factors are given above, along with examples of other preferred antigens.

The pharmaceutical composition preferably includes at least the virulence factors of VacA, CagA, NAP and urease. More preferably, the pharmaceutical composition includes at least the virulence factors of VacA, CagA and NAP. Most preferably, the pharmaceutical composition includes at least the virulence factors of CagA and NAP.

The *H. pylori* antigens of the pharmaceutical composition may consist of only virulence factors or of only specific combinations of virulence factors such as VacA, CagA, NAP and urease, VacA, CagA and NAP, or CagA and NAP.

The antigens of the pharmaceutical composition may also include non-*H. pylori* antigens.

The one or more antigens is/are preferably purified antigen(s) or a whole cell immunogen as described above.

The pharmaceutical composition may also further comprise an adjuvant. Preferably, the adjuvant is aluminium hydroxide or MF59™.

The pharmaceutical composition of the invention can be an immunogenic composition such as, but not limited to, a vaccine.

The present invention therefore also provides an immunogenic composition for non-mucosal administration comprising one or more *H.pylori* antigens and one or more pharmaceutically acceptable excipients.

Immunogenic compositions and vaccines according to the invention may either be prophylactic (*i.e.* to prevent infection) or therapeutic (*i.e.* to treat disease after infection). Such vaccines comprise antigen or antigens, usually in combination with "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 metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplets or liposomes), and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. Additionally, these carriers may function as immunostimulating agents ("adjuvants"). Furthermore, the antigen may be conjugated to a bacterial toxoid.

Preferred adjuvants to enhance effectiveness of the composition include, but are not limited to: (1) aluminium salts (alum), such as aluminium hydroxide, aluminium phosphate, aluminium sulphate, etc.; (2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides or bacterial cell wall components), such as for example (a) MF59™, containing 5% Squalene, 0.5% Tween™ 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE, although not required) formulated into submicron particles using a microfluidizer (b) SAF, containing 10% Squalene, 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), 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™); (3) saponin adjuvants, such as Stimulon™ may be used or particles generated therefrom such as ISCOMs (immunostimulating complexes); (4) Freund's complete and incomplete adjuvants (CFA & IFA); (5) cytokines, such as interleukins (e.g. IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, etc.), interferons (e.g. IFNγ), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), etc.; and (6)

other substances that act as immunostimulating agents to enhance the efficacy of the composition. Alum and MF59™ are preferred.

As mentioned above, muramyl peptides include, but are not limited to, 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-huydroxyphosphoryloxy)-ethylamine (MTP-PE), *etc.*

The immunogenic compositions (*eg.* the antigen, pharmaceutically acceptable carrier, and adjuvant) typically will contain diluents, such as water, saline, glycerol, ethanol, etc. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles.

Typically, the immunogenic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. The preparation also may be emulsified or encapsulated in liposomes for enhanced adjuvanticity effect, as discussed above.

Immunogenic compositions used as vaccines comprise an immunologically effective amount of the antigenic polypeptides, 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, 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.

The immunogenic compositions are administered non-mucosally, more specifically parenterally *eg.* by injection, either subcutaneously or intramuscularly. Transdermal or transcutaneous administration may also be used. Dosage treatment may be a single dose schedule or a multiple dose schedule. The vaccine may be administered in conjunction with other immunoregulatory agents.

According to the present invention, there is further provided the use of the pharmaceutical composition as described above or the immunogenic composition as described above for protection against or treatment for infection by *H. pylori*.

Brief Description of the Drawings

Figure 1 shows endoscopic examination of the antral region of protected dogs (*Fig. 1A*) and control (infected) dogs (*Fig. 1B*) 42 days after challenge with *H.pylori*.

Figure 2 shows histological examination (hematoxylin-eosin (HE) staining) of biopsies taken from the antral region of protected dogs (*Fig. 2A*, magnification = 10x) and control (infected) dogs (*Fig. 2B*, magnification = 10x; *Fig. 2C*, magnification = 40x; *Fig. 2D*, magnification = 100x) 42 days after challenge with *H.pylori*.

Figure 3 shows immunohistochemical examination of biopsies taken from the antral region of protected dogs (*Fig. 3A*) and control (infected) dogs (*Fig. 3B*) 42 days after challenge with *H.pylori* (magnification = 40x).

Figure 4 shows the schedule of the experiment to determine whether Beagle dogs may be protected against a challenge with *H. pylori* using purified antigens given i.m.

Figure 5 shows serum antibody titers to recombinant VacA (*Fig. 5A*), to CagA (*Fig. 5B*) and to NAP (*Fig. 5C*) (averages) before the first immunization, and after the second, third and fourth immunizations i.m. with purified antigens or with whole cell lysate. The horizontal arrow individuates the lower limit for positive ELISA results.

Figure 6 shows the titers of serum IgG antibody subclasses IgG1 and IgG2 to NAP (*Fig. 6A*) and CagA (*Fig. 6B*) (average titers per groups immunized i.m. with purified antigens or with whole cell lysate) at the same time points as above (*Fig. 5*).

Figure 7 shows serum IgA antibody titers to p95 (recombinant VacA) in Beagle dogs immunized i.m. with purified antigens (VacA, CagA and NAP) or with whole cell lysate (single dogs – *Figs. 7A to 7F*) before immunization and after the third immunization. (Similar serum IgA antibody titers were observed against CagA.)

Figure 8 shows the endoscopic image of protected (*Fig. 8A*) and non-protected (infected) (*Fig. 8B*) dogs taken about 8 weeks post-challenge.

Figure 9 shows the histology (HE staining, magnification = 10x) of gastric mucosa from protected (*Fig. 9A*) and non-protected (infected) (*Fig. 9B*) dogs taken about 8 weeks post-challenge. Note in *Fig. 9A* the normal mucosa and submucosa. Conversely, in *Fig. 9B*, a large lymphoid follicle, disrupting the structure of the mucosa and the submucosa can be noted.

Figure 10 shows the immunohistochemistry using anti-VacA monoclonal antibody taken about 8 weeks post-challenge (magnification = 10x). Note the negative staining in protected dog (*Fig. 10A*) and the heavily positive staining in non-protected (infected) dog (*Fig. 10B*).

Detailed Description of the Invention

Whole cell H. pylori *vaccine*

As an embodiment of the invention, the feasibility of immunizing conventional dogs with whole cell *H.pylori* vaccine (i.e. *H.pylori* cell lysate) was investigated. It is shown that such a preparation can elicit an immunity that provides a protective effect against a later challenge with infectious *H.pylori*.

Immunogenicity. The protective effect of a whole cell *H.pylori* vaccine was investigated in dogs challenged with infectious *H.pylori*. The details of the experimental procedures followed are set out later. As compared to time 0 and to control dogs, i.m. immunization induced very high titers of serum IgG antibodies as shown in Table 1. I.m. immunization with *H.pylori* lysate also induced production of antigen-specific serum IgA antibodies.

Symptoms. Control dogs #2 and #3 had diarrhoea during the first week after the last challenge. Control dog #2 also had vomiting. There were no symptoms of *H.pylori* infection in the i.m. group.

Urease test on gastric (antral) biopsy and on gastric lavage. This assay, performed on antral biopsies and gastric lavage taken 10 days after the last challenge, was positive in the control group, and negative in the i.m.group as shown in Table 2 below, even 24 h later. These data were also confirmed at day 42 post-challenge.

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Table 1

Immunogenicity of the H. pylori lysate given four times intramuscularly to Beagle dogs

Dog no.	Immunization route	Serum IgG titers to: H. pylori lysate Pre*	H. pylori lysate Post-4	CagA Pre	CagA Post-4	NAP Pre	NAP Post-4	Serum IgA titers to H. pylori lysate Pre	H. pylori lysate post-4
1	nil (control)	< 100§	< 100	< 100	< 100	< 100	< 100	< 20	< 20
2	nil (control)	< 100	< 100	< 100	< 100	< 100	< 100	< 20	< 20
3	nil (control)	< 100	< 100	< 100	< 100	< 100	< 100	< 20	< 20
4	intramuscular	< 100	≥ 25000	< 100	12800	< 100	≥ 12800	< 20	1280
5	intramuscular	< 100	≥ 25000	< 100	12800	< 100	≥ 12800	< 20	320
6	intramuscular	< 100	≥ 25000	< 100	6400	< 100	≥ 12800	< 20	640

*Pre: serum samples taken before the immunization; Post-4: serum sample taken after four immunizations, before the challenge.

§Titers are expressed as the last serum dilution giving an optical density equal or higher than 0.2. Titers < 100 (IgG) and <20 (IgA) are considered as negative.

Table 2

Group	*dog #*	*biopsy*	*gastric lavage*
Control	1	+	+
	2	+	+
	3	+	+
i.m.	1	-	-
	2	-	-
	3	-	-

Endoscopy, histology and immunohistochemistry results. Protected dogs showed normal mucosa at endoscopic examination at 42 days post-challenge (Fig. 1A), with a bright and smooth surface, without signs of hyperemia or edema. Conversely, in the control (infected) dogs, the gastric mucosa was heavily red and edematous, and had a rippled surface with appearance of plicae (Fig. 1B), suggestive of the nodular (follicular) gastritis observed previously and described in patent application WO 99/35907.

Histologically, the gastric mucosa of protected dogs conserved an intact structure, both at the surface and at the submucosa (Fig. 2A), whereas in infected dogs there was appearance of hyperemia (Fig. 2B, arrows), edema (Fig. 2B, asterisk), inflammatory cellular infiltrates (Figure 2B, arrowheads and Fig. 2C). *H.pylori* was also easily identified in the mucous layer (Fig. 2D).

All these data were confirmed by immunohistochemistry using an anti-VacA monoclonal antibody, which heavily stained epithelial cells of infected dogs (Fig. 3B, arrows), but not those of protected dogs (Fig. 3A).

Conclusions. Taken together these data show that i.m. immunization with *H.pylori* lysate can protect dogs against challenge with infectious *H.pylori*, and that the i.m. route can be considered for vaccination against this bacterium.

Purified H. pylori *antigen vaccine*

As a further embodiment of the invention, the feasibility of immunizing conventional dogs with purified *H.pylori* antigens was also investigated. It is shown that such a preparation can elicit an immunity that provides a protective effect against a later challenge with infectious *H.pylori*.

Immunogenicity. The protective effect of purified *H.pylori* antigens (specifically VacA, CagA and NAP) was investigated in dogs challenged with infectious *H.pylori*. The details of the experimental procedures followed are set out later. Immunization with these antigens induced very strong serum IgG antibody response specific for each antigen after only two doses. Titers increased after the third dose. Doses of 50 and 10 μg of antigens were as good as 250 μg to induce high titers of antigen-specific antibodies. Comparatively, lower antibody titers were detectable in dogs immunized with *H. pylori* lysate (see Fig. 5).

Immunization with these antigens also induced high titers of antigen-specific serum IgG1 and IgG2 antibodies, suggesting that this immunization induces both Th1- and Th2-type immune response, unlike what has already been observed in infected mice and dogs, in which a preponderant Th1-type immune response is evident (see Fig. 6).

Dogs immunized in this manner also had detectable titers of antigen-specific IgA antibodies in the serum (see Fig. 7).

Endoscopy, histology and immunohistochemistry results. In protected animals (see Table 3 – which gives the conclusive results on protection, based on all parameters taken together, including endoscopy (gastroscopy), histology and immunohistochemistry), gastric mucosa was normal at endoscopic investigation, at histology, and at immunohistochemistry (see Figs. 8A, 9A and 10A, respectively). Non-protected (infected) animals showed hyperemic, heavily flogistic aspect of the gastric mucosa at endoscopy (Fig. 8B), with a diffuse infiltration with mononuclear cells aggregated in lymphoid follicle structures (Fig. 9B) disrupting the normal glandular structure. Fig. 10B shows a strong positivity at immunohistochemistry using an anti-VacA monoclonal antibody.

Conclusions. Taken together these data show that i.m. immunization with *H. pylori* antigens can protect dogs against challenge with infectious *H.pylori*.

Table 3

Intramuscular immunization with purified antigens protects dogs against challenge with *Helicobacter pylori*

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

Experimental

***H.pylori* strains.** SPM326s, a streptomycin-resistant derivative of the mouse-adapted *H.pylori* Type I (CagA+/VacA+) strain SPM326 (Marchetti *et al.*, 1995), was grown as previously described (Marchetti *et al.*, 1995) and used to challenge the dogs. The CCUG strain of *H.pylori* is well known in the art.

Animals. Six 4-6 months-old xenobiotic beagle dogs, all female (Morini s.a.s., S. Polo D'Enza, Italy), were selected on the basis of the absence of detectable serum IgG against *Helicobacter spp.* in Western blot (WB) analysis using total bacterial lysate as antigen (see below). The six dogs selected were housed in standard conditions and maintained on a diet of dry food (MIL, Morini s.a.s.) and tap water *ad libitum*. Upon arrival in our animal facilities, an additional WB analysis on sera confirmed their *H.pylori* status. The dogs were housed in individual cages and allowed to adapt for a month to the new environment. During the month of adaptation, two tests were carried out on fecal samples to assess the presence of intestinal parasites or common enteric pathogenic bacteria.

Preparation of *H.pylori* lysate. Two pellets of *H.pylori* CCUG strain from two 5 liter fermenters (Olivieri, R., *et al.* 1993. J. Clin. Microbiol. 31:160-162) were obtained. After each pellet was resuspended in 50 ml of sonication buffer (50 mM $Na_2HPO_4.2H_20$, 300 mM NaCl, pH 7.8), the two resuspended pellets were mixed. The OD_{530nm} of the combined resuspension was measured to determine bacterial concentration (= 3.2×10^{10} CFU/ml). The resuspension was diluted with sonication buffer to bring the concentration to 2×10^{10} CFU/ml. Before sonication, bacteria had the classical spiral form when viewed under the microscope. Sonication of the resuspended bacteria was then carried out on ice: 2 cycles of 4 minutes, and 2 cycles of 5 minutes, waiting one minute between each cycle. After sonication, all the bacteria appeared broken when viewed under the microscope. Protein concentration was then determined using the Bradford method (= 57.5 mg/ml protein). Aliquots of the cell lysate were then prepared and frozen at -80°C until use.

Immunization. Three dogs were immunized on day 0 intramuscularly (i.m.) with the prepared *H.pylori* lysate (the equivalent of 10^{10} CFU *H.pylori* (= 28 mg/dose)) adsorbed onto 1 mg aluminium hydroxide (Chiron Behring GmbH & Co., Marburg, Germany; Lot No. 277345) in 1 ml volume. Immunizations were repeated on days 7, 14, and 22. Serum samples were taken on day 0, 21 (post-3) and 43 (post-4). The other three dogs, as a control group, were treated identically but saline was used in place of the *H.pylori* lysate.

Challenge with infectious *H.pylori*. The dogs were then challenged on days 49, 51 and 53 with the mouse-adapted *H.pylori* SPM326s strain as follows: 24 h before each challenge the dogs were fasted. 2 h before bacteria inoculation, dogs received 10 mg/kg of cimetidine i.m. (Tagamet® 200; Smith Kline & French, USA). At the moment of challenge, the dogs were anesthetized with a mixture of 40 μg/kg of medetomidine chloridrate (Domitor®; Centralvet-Vetem s.p.a., Milano, Italy) and 5 mg/kg of ketamine (Ketavet®, Gellini, Latina, Italy) intravenously (i.v.); then a gastric lavage was performed with 100 ml of 0.2M $NaHCO_3$ sterile solution followed by oral challenge with 3 ml of a freshly prepared suspension of 10^9 CFUs in sterile saline of the *H.pylori* strain SPM326s, grown under microaerobic conditions (see below), prepared immediately before the inoculation procedure. At the end of the bacterial inoculation, 200 μg/kg of the anesthetic antagonist atipamezole (Antisedan®; Centralvet-Vetem s.p.a., Milano, Italy) was administered and then dogs were again treated with cimetidine and fed after 2 h.

Post-challenge follow-up. Ten and 42 days after the last challenge gastric endoscopies were performed using a 4.9-mm-diameter Pentax pediatric bronchoscope (Pentax Technologies, Zaventem, Belgium). At the same time, gastric biopsies were taken during the endoscopies using flexible pinch-biopsy forceps at the antrum, corpus fundus, and cardias for urease testing and for microbiological, histopathological and immunohistochemical analyses. Before each endoscopy the whole instrument and the flexible forceps were soaked in 4% glutaraldehyde for 45 minutes and then rinsed in sterile saline. To avoid cross-contamination among biopsies taken at different sites, the forceps were washed with tap water and lightly flame-sterilized before the collection of each bioptic sample. The above experimental protocol was approved by the Scientific and Ethical Commitee of the University of Pisa and received official authorization (DM No. 21/97-C) from the Italian Ministry of Health (Department of Veterinary Health, Food and Nutrition).

Rapid urease test. Antral biopsies and liquid from gastric lavage were incubated for up to 24 h in 1 ml of a 10% urea solution in distilled water added with two drops of a 1% phenol red solution (Sigma Chemical Co., St. Louis, MO, USA) in sodium phosphate buffer, pH 6.5. A positive test is indicated by change of color (from orange to dark pink) in the medium; the time necessary for the color change is recorded. At time 0, endoscopy was

carried out on the six dogs and antral biopsies were taken for the urease test. In all six dogs, the urease test was negative at time 0.

Histopathology and Transmission Electron Microscopy. Samples for histological, immunohistochemical and ultrastructural examination were taken from the biopsies at sites adjacent to those utilized for microbiological analysis. The samples were fixed in 10% buffered formalin and embedded in paraffin. 3 μm sections were stained with hematoxylin-eosin (H & E) and Alcian and Periodic acid Schiff's (PAS) staining using standard procedures for histopathological examination. Similar sections were also employed for immunohistochemical analyses using the Avidine-Biotine-Complex (ABC)-peroxidase technique with a monoclonal antibody (Mab) specific for *H.pylori* (Biogenesis Ltd, Poole, England, UK) or an anti-VacA mouse monoclonal antibody (C1G9) obtained by immunizing Balb/c mice with purified native *H.pylori* VacA (Burroni and Telford, unpublished observations). Biotinylated horse anti-mouse antibody was used as secondary antibody. The reaction was developed with 3-1-diaminobenzidine-chlorhydrate (DAB) (Sigma) for identification and location of bacterial antigen. For electron microscopic examination, other samples were fixed in Karnowsky, post-fixed in OsO_4, and embedded in Epon-Araldite (Polysciences Inc., Warrington, PA, USA). Semi-thin sections were stained with toluidine blue for evaluation of cell damage, whereas ultra-thin sections were stained with uranyl acetate and lead citrate, and then examined with a Philips EM 301 transmission electron microscope (TEM) operating at 80 KV.

Detection of anti-*H.pylori* antibodies. SDS-PAGE of *H.pylori* (strain SPM326s) and WB analysis of sera were performed according to previously published procedures (Marchetti *et al.*, 1995). Briefly, dog sera were diluted 1:200 and incubated for 2 h at room temperature. Then, horseradish peroxidase (HRP)-conjugated rabbit anti-dog IgG antibody (Nordic Immunological Laboratories, Tilburg, The Netherlands) was added at 1:2,000 dilution for 2 h, and the reaction was developed using 4-α-chloronaphtol as substrate. Detection of antibody against *H.pylori* by ELISA was carried out on 96-well plates coated overnight at 4°C with the prepared CCUG strain lysate used for immunization (5 μg/well) or with purified native CagA or NAP (0.2 μg/well). Coated wells were blocked with PBS containing 5% non-fat milk. Twofold serial dilutions of the sera were incubated at 37°C for 2 h and then washed with PBS. Antigen specific IgG titers were determined using a 1:4,000 dilution of HRP-conjugated goat anti-dog IgG antibody (Bethyl Laboratories, Inc.,

Montgomery, TX, USA) for 2 h at 37°C. Antigen bound antibodies were revealed by adding o-phenylenediamine dihydrochloride (Sigma) as a substrate. Antibody titers were determined as previously described (Ghiara *et al.*, 1997).

Intramuscular (i.m.) immunization of dogs with purified *H. pylori* antigens. VacA and CagA were expressed and purified as described in Ghiara, *et al.* 1997. NAP was expressed and purified as described in earlier patent applications.

As shown in Table 4, groups of 4 dogs were immunized i.m. with:

(i) a mixture of recombinant VacA, CagA, and NAP (250, 50 or 10 μg of each antigen) adsorbed onto aluminium hydroxide (1 mg dose);

(ii) CCUG lysate (prepared as discussed above), at 25 or 5 mg per dose adsorbed onto aluminium hydroxide (1 mg dose);

(iii) aluminium hydroxide alone (1 mg dose).

Dogs were immunized 4 times at weekly intervals. Challenge with *H. pylori* was carried out 4 weeks later as already described. Samples (blood, biopsies, *etc.* – as previously described) were taken before immunization, after the second, third and fourth immunizations, and then 2-3 weeks and 8 weeks after the last challenge.

Table 4

IMMUNIZATION PROTOCOL

All antigens given intramuscularly with alum as adjuvant

GROUP	DOGS	ROUTE	IMMUNOGEN	DOSE	ADJUVANT (mg)	VOLUME
1	4	control (IM)	nil (saline)	-	AlOH (1 mg)	1 ml
2	4	IM	VacA + CagA + NAP	250 μg each (tot. 750 μg)	AlOH (1 mg)	1 ml
3	4	IM	VacA + CagA + NAP	50 μg each (tot. 150 μg)	AlOH (1 mg)	1 ml
4	4	IM	VacA + CagA + NAP	10 μg each (tot. 30 μg)	AlOH (1 mg)	1 ml
5	4	IM	CCUG lysate	25 mg	AlOH (1 mg)	1 ml
6	4	IM	CCUG lysate	5 mg	AlOH (1 mg)	1 ml

CLAIMS:

1. Use of an effective amount of purified *H.pylori* antigens comprising one or more of vacuolating cytotoxin (VacA), cytotoxin-associated protein (CagA) and neutrophil activating protein (NAP) virulence factors in the manufacture of a medicament for intramuscular delivery to a human subject for protection against or treatment of infection by *H.pylori*.

2. Use according to claim 1, wherein said medicament further comprises a pharmaceutically acceptable excipient.

3. Use according to claim 1 or claim 2, wherein the antigens separately or in combination elicit a protective immune response in said human subject.

4. Use according to any one of claims 1 to 3, wherein the medicament additionally comprises *H.pylori* urease.

5. Use according to any one of claims 1 to 4, wherein said medicament further comprises an adjuvant.

6. Use according to claim 5, wherein the adjuvant is aluminium hydroxide.

7. Use of an effective amount of purified *H.pylori* antigens comprising one or more of vacuolating cytotoxin (VacA), cytotoxin-associated protein (CagA) or neutrophil activating protein (NAP) virulence factors intramuscularly for protection against or treatment of infection by *H.pylori* in a human subject in need thereof.

8. Use according to claim 7, in combination with a pharmaceutically acceptable excipient.

9. Use according to claim 7 or claim 8, wherein the antigens separately or in combination elicit a protective immune response in said human subject.

10. Use according to any one of claims 7 to 9, in combination with *H.pylori* urease.

11. Use according to any one of claims 7 to 10, in combination with an adjuvant.

12. Use according to claim 11, wherein the adjuvant is aluminium hydroxide.

13. A commercial package comprising a pharmaceutically effective amount of purified *H.pylori* antigens comprising one or more of vacuolating cytotoxin (VacA), cytotoxin-associated protein (CagA) and neutrophil activating protein (NAP) virulence factors together with instructions for intramuscular delivery to a human subject for protection against or treatment of infection by *H.pylori*.

14. The commercial package of claim 13 additionally comprising a pharmaceutically acceptable excipient.

15. The commercial package of claim 13 or claim 14 additionally comprising *H.pylori* urease.

16. The commercial package of any one of claims 13 to 15 further comprising an adjuvant.

17. The commercial package of claim 16 wherein the adjuvant comprises aluminium hydroxide.

FIG. 1A

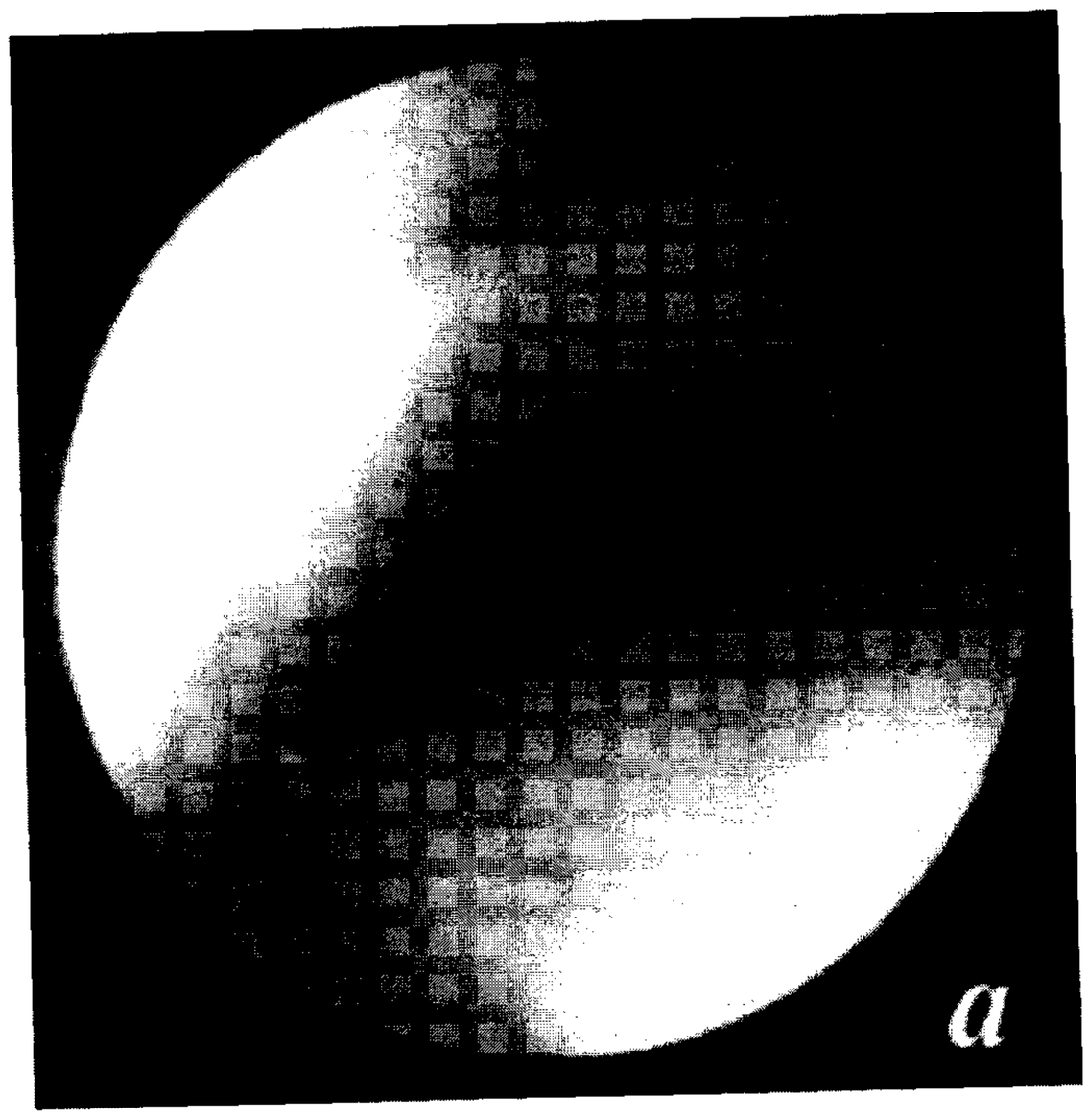


FIG. 1B

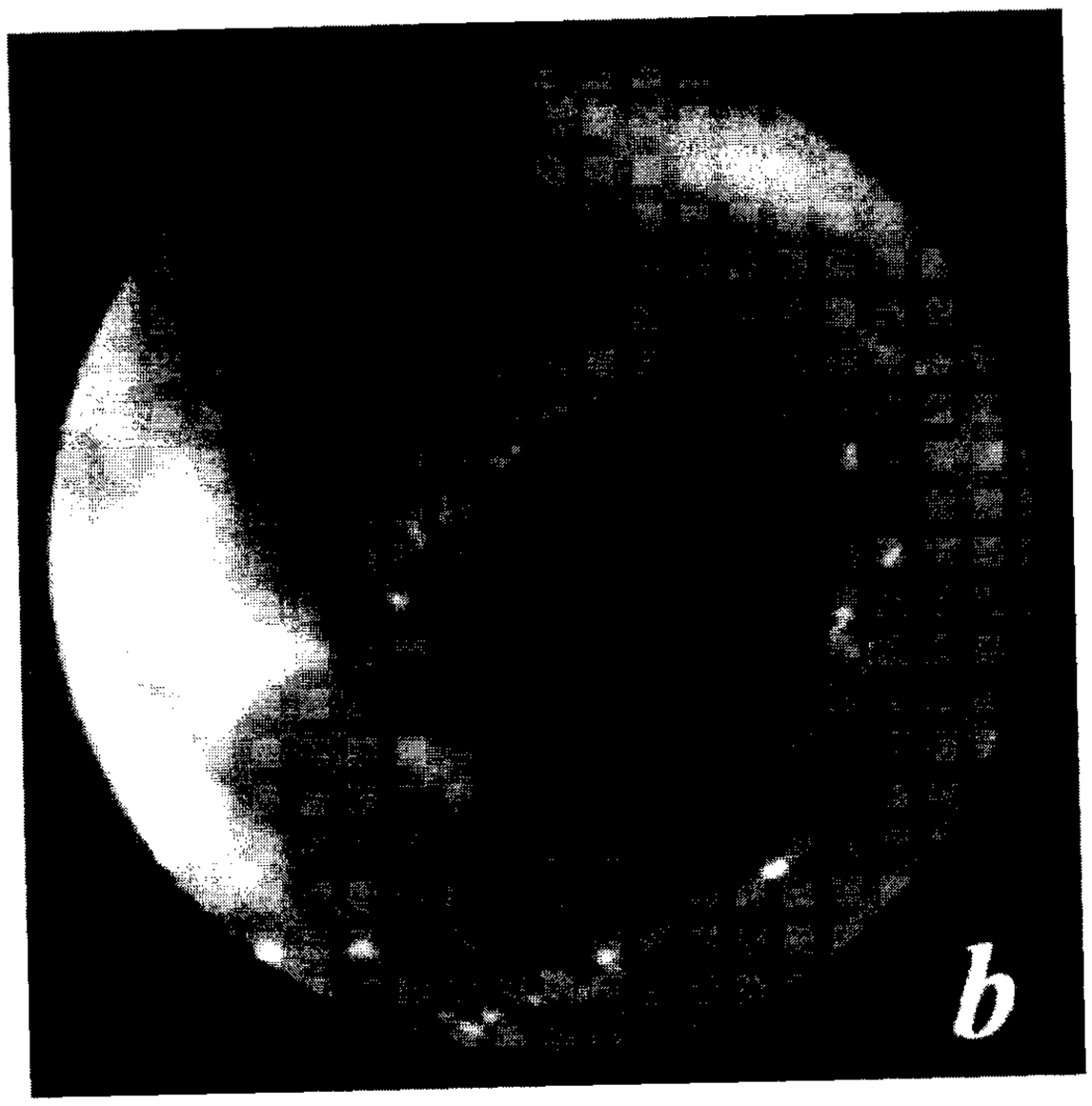


FIG. 2A

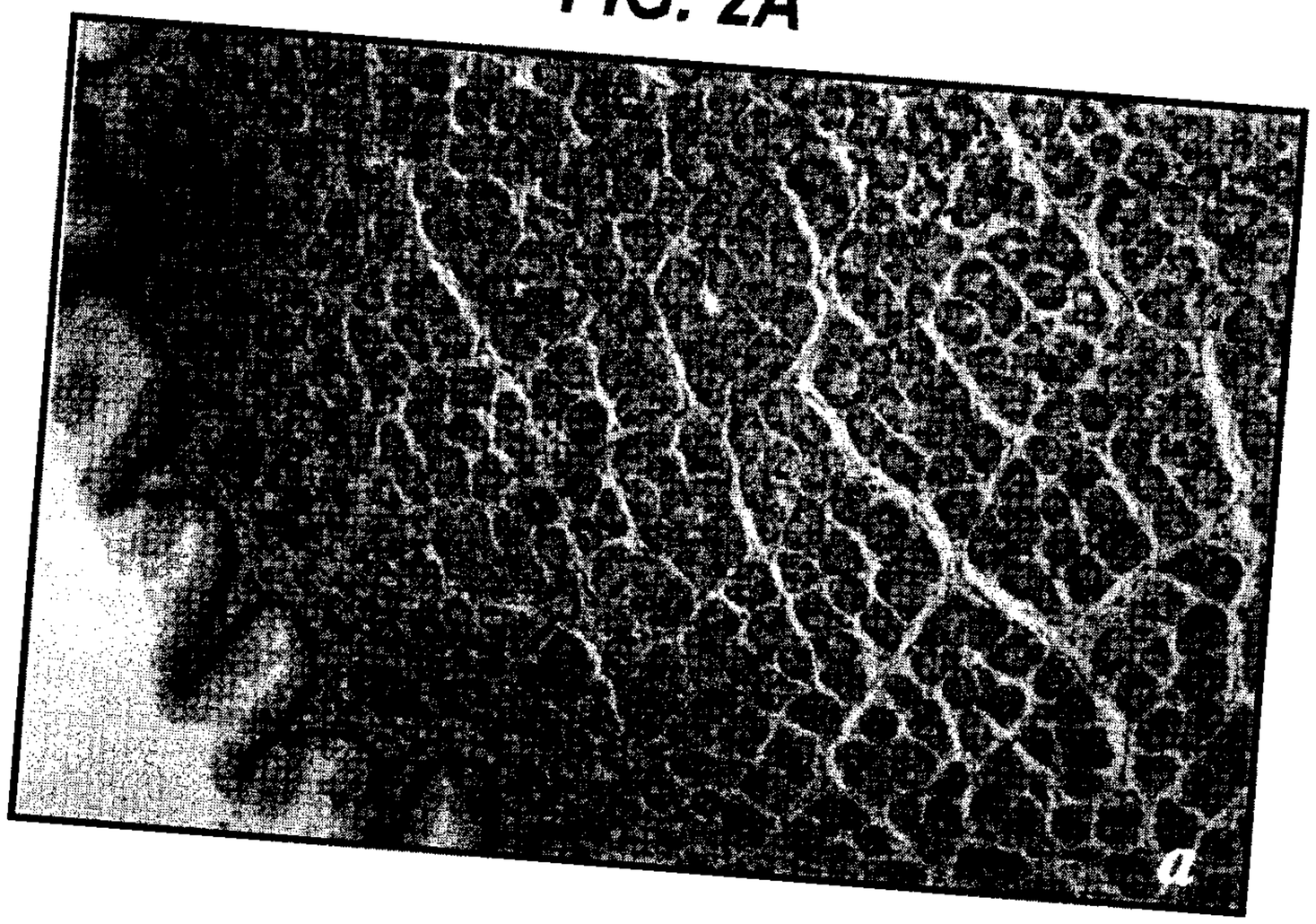


FIG. 2B



FIG. 2C



FIG. 2D



FIG. 3A



FIG. 3B



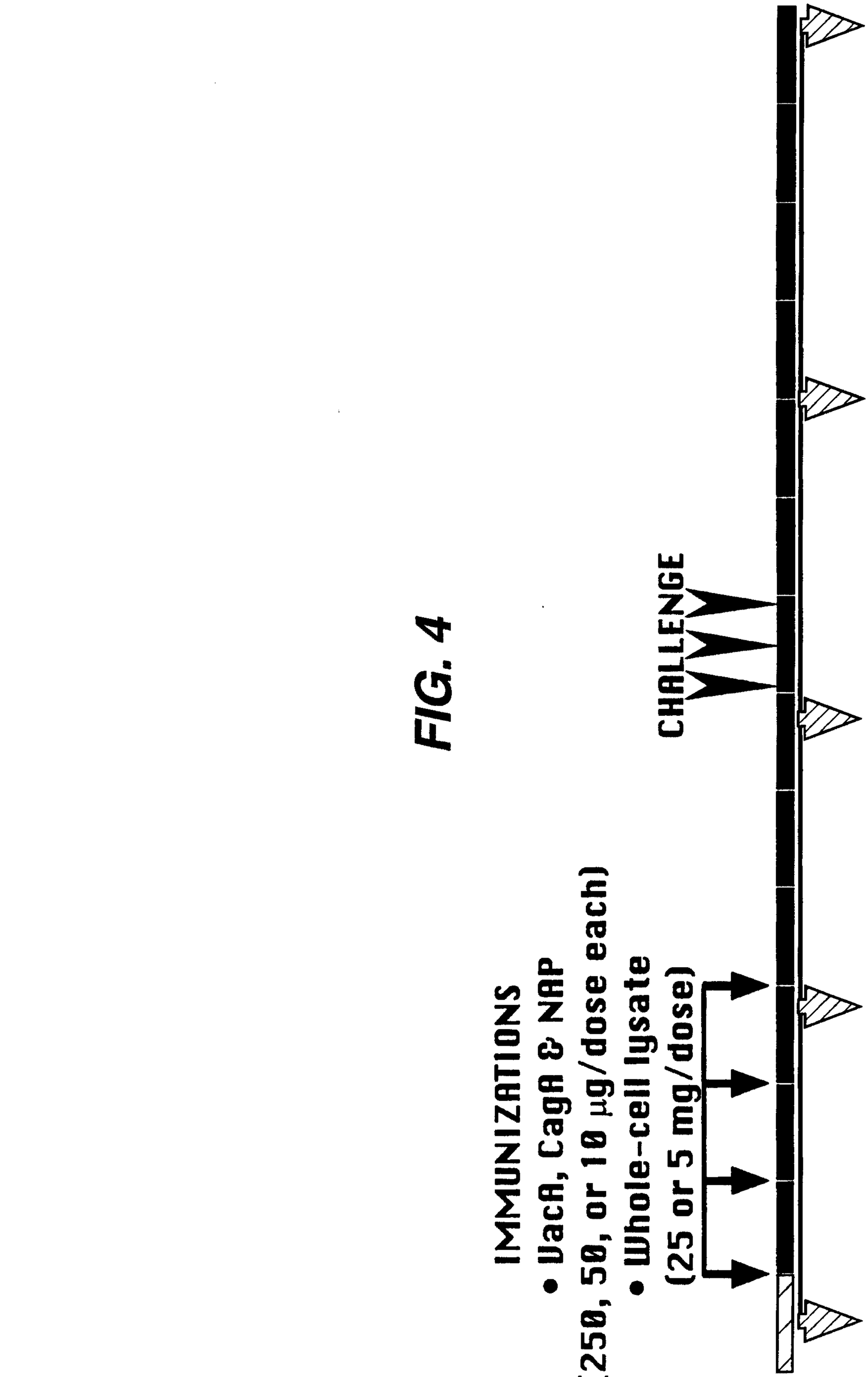

IMMUNIZATIONS
• UacA, CagA & NAP
(250, 50, or 10 µg/dose each)
• Whole-cell lysate
(25 or 5 mg/dose)
CHALLENGE
SAMPLES
BIOPSIES, SERUM, SALIVA, FAECES, ETC.

FIG. 4

FIG. 5A

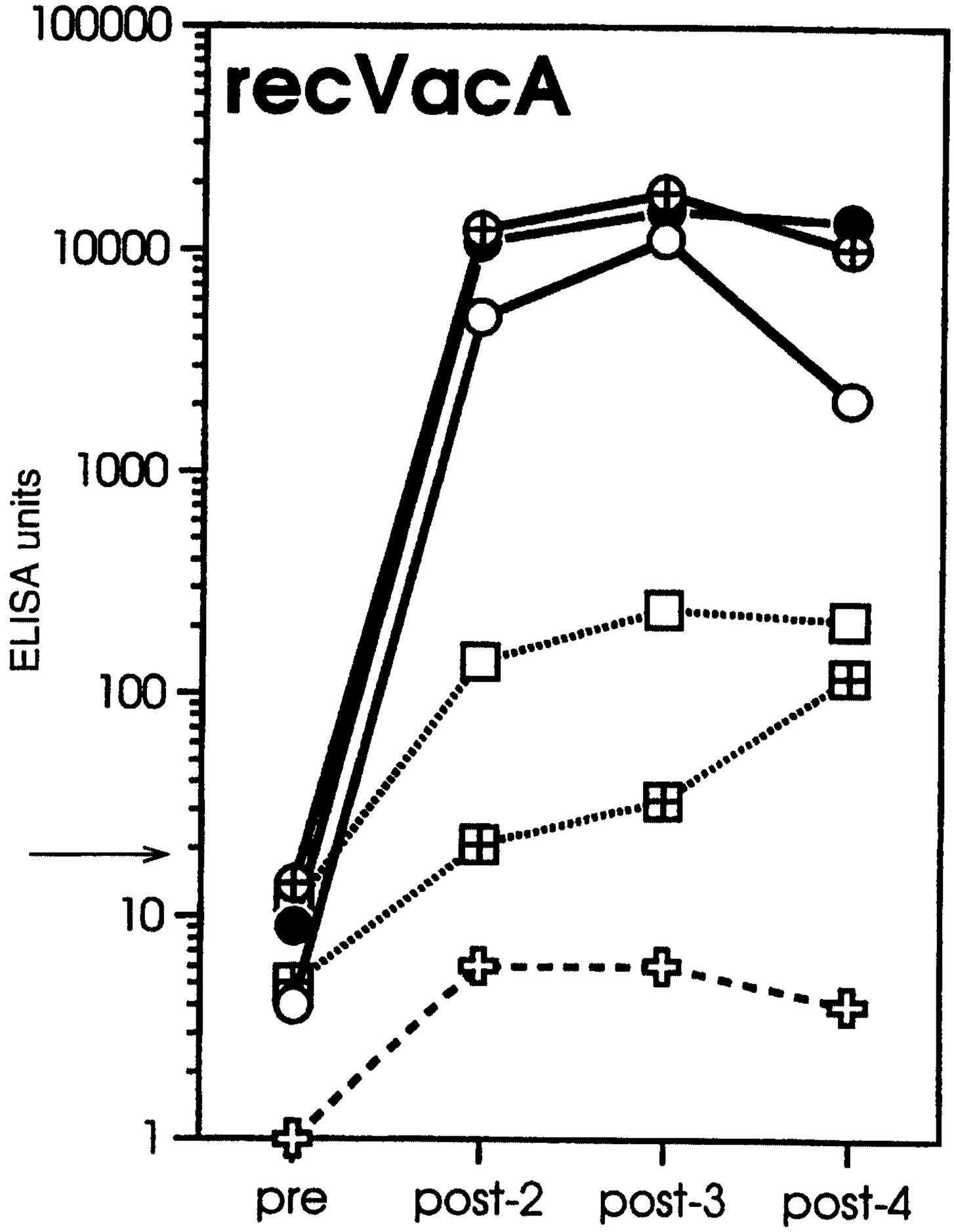

recVacA
ELISA units
100000
10000
1000
100
10
1
pre
post-2
post-3
post-4
Controls
Antigens 250 µg
Antigens 50 µg
Antigens 10 µg
Lysate 25 mg
Lysate 5 mg

FIG. 5B

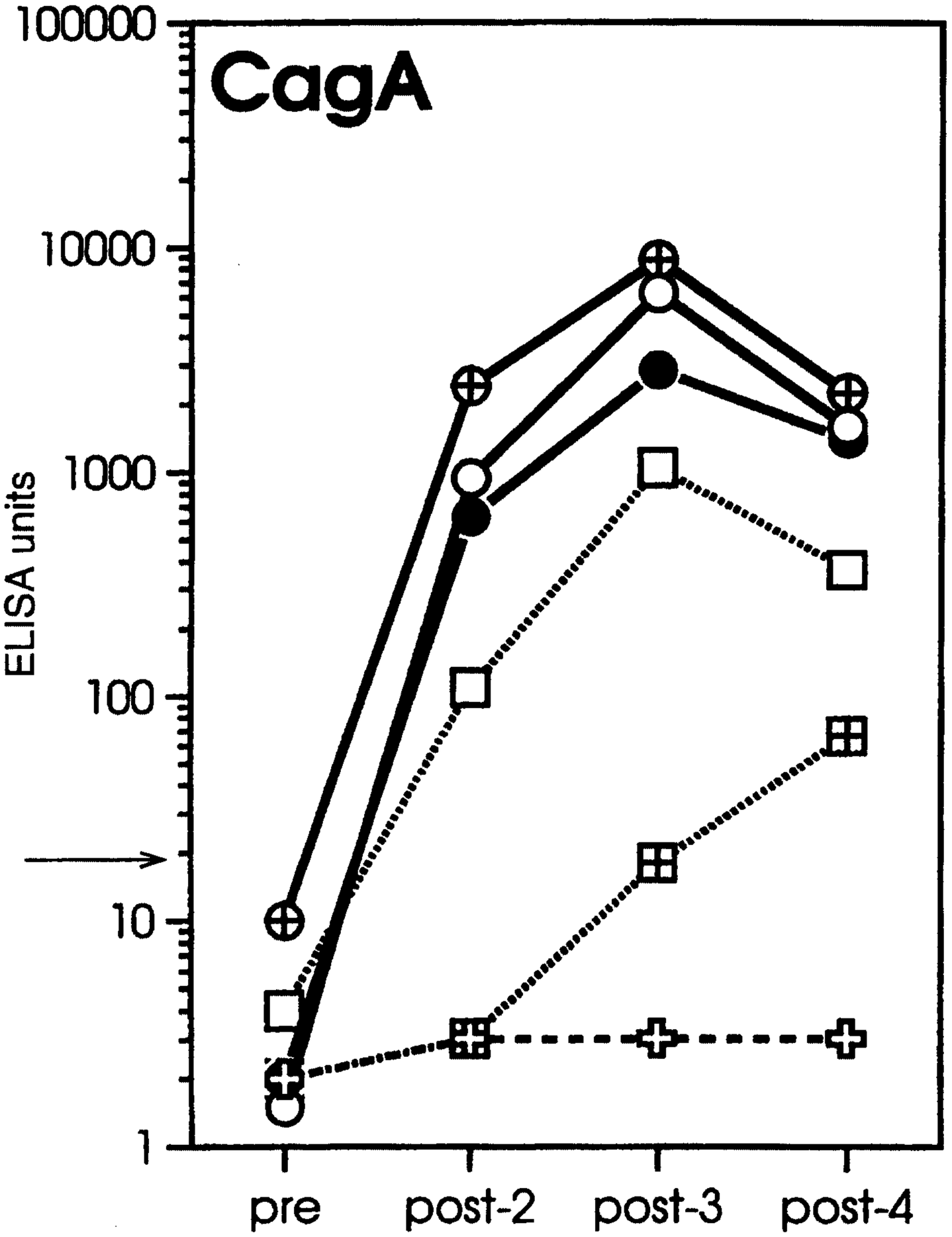
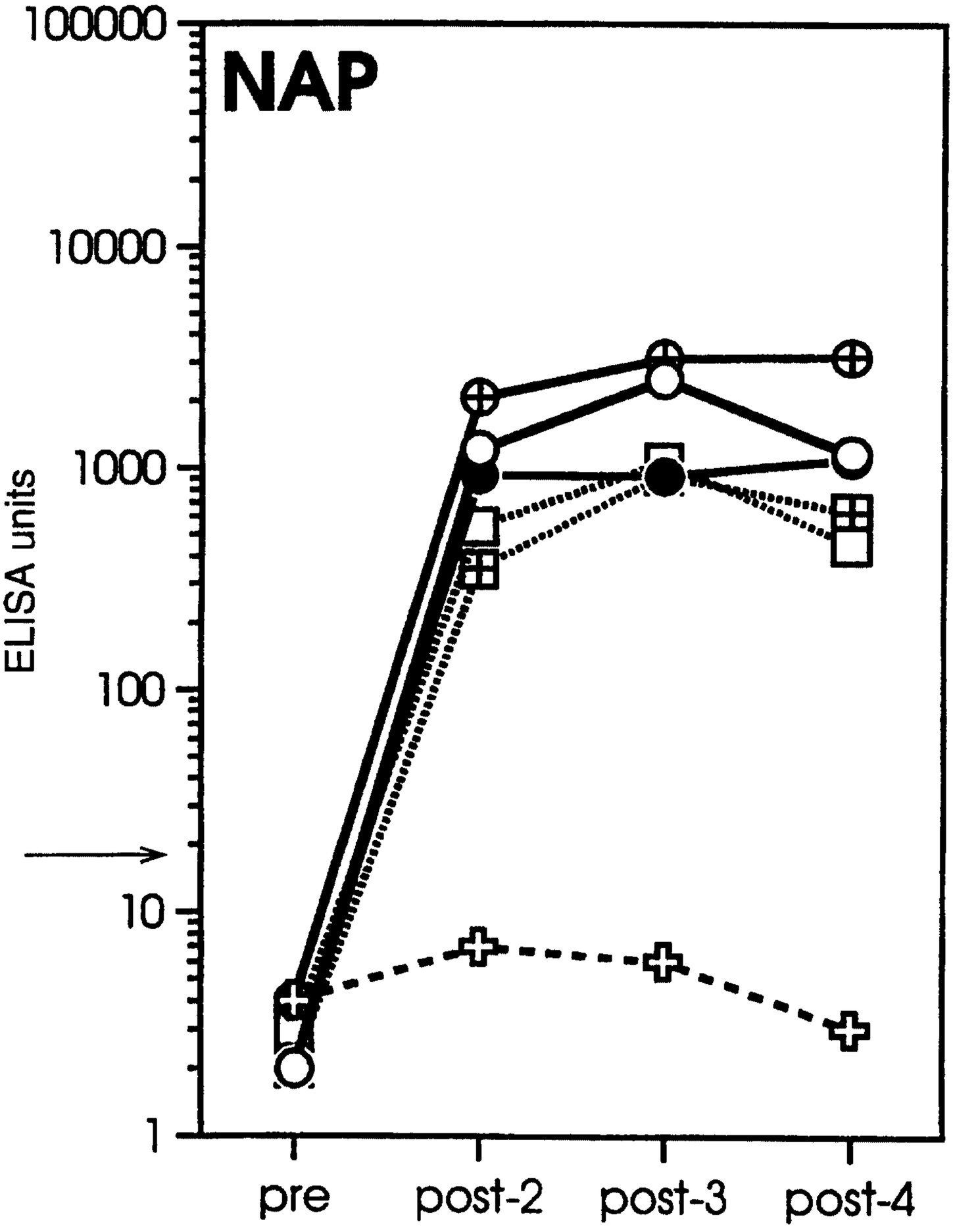

CagA
100000
10000
1000
100
10
1
ELISA units
pre
post-2
post-3
post-4
Controls
Antigens 250 µg
Antigens 50 µg
Antigens 10 µg
Lysate 25 mg
Lysate 5 mg

FIG. 5C


NAP
100000
10000
1000
100
10
1
ELISA units
pre
post-2
post-3
post-4
Controls
Antigens 250 µg
Antigens 50 µg
Antigens 10 µg
Lysate 25 mg
Lysate 5 mg

NAP

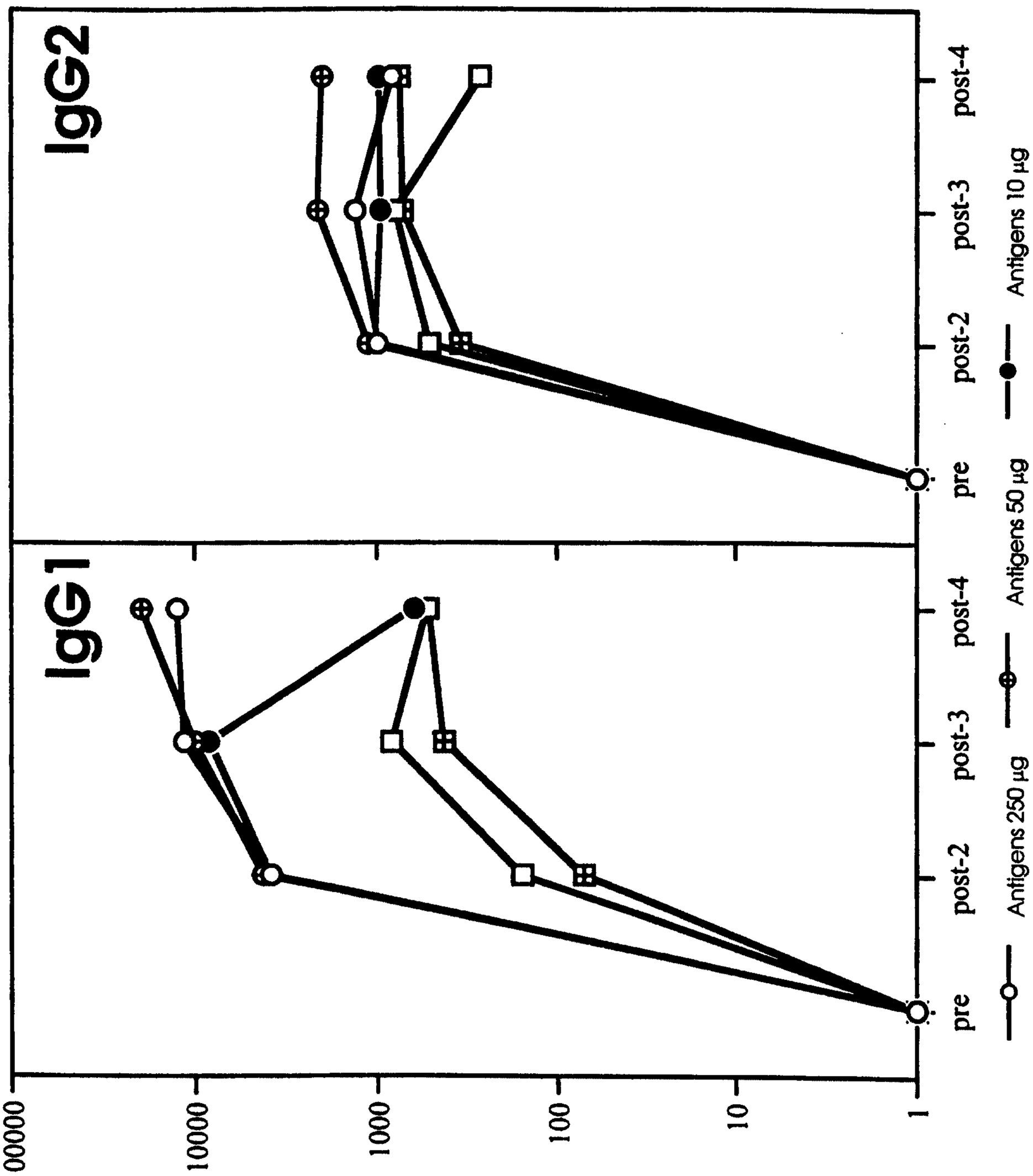

IgG2
IgG1
pre
post-2
post-3
post-4
Antigens 10 µg
Antigens 50 µg
Antigens 250 µg
Lysate 5 mg
Lysate 25 mg
100000
10000
1000
100
10
1
ELISA units

FIG. 6A

CagA

IgG2

IgG1

ELISA units

100000
10000
1000
100
10
1

pre post-2 post-3 post-4

Antigens 10 µg
Antigens 50 µg
Lysate 5 mg
Antigens 250 µg
Lysate 25 mg

FIG. 6B

FIG. 7C

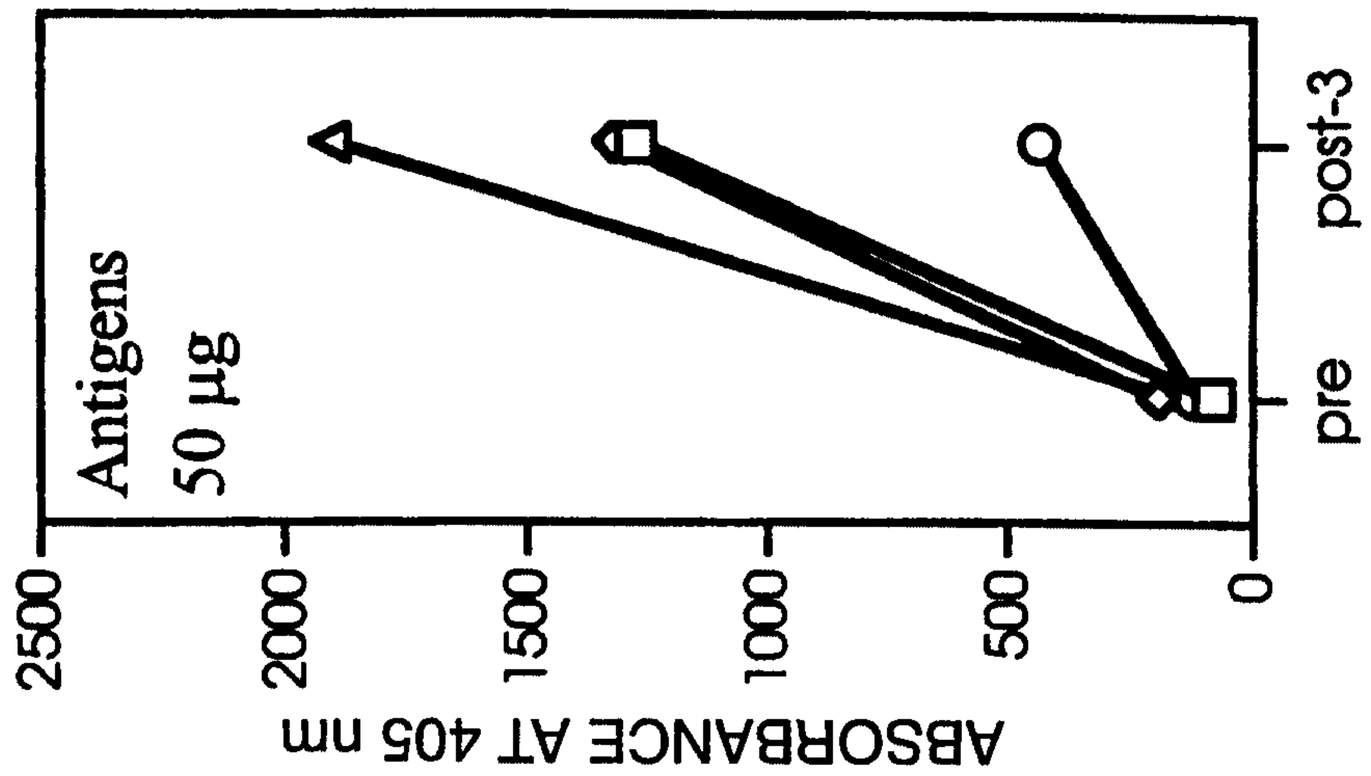


FIG. 7B

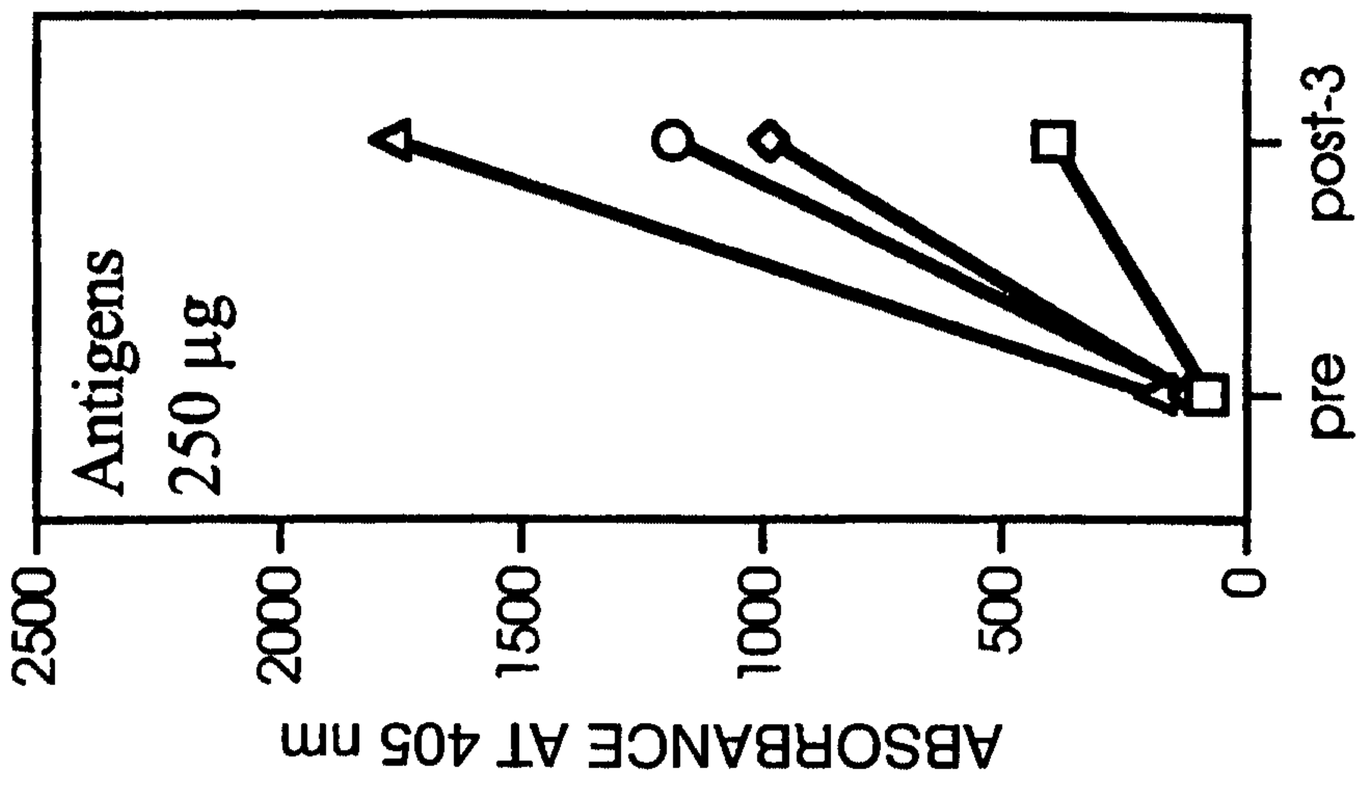


FIG. 7A

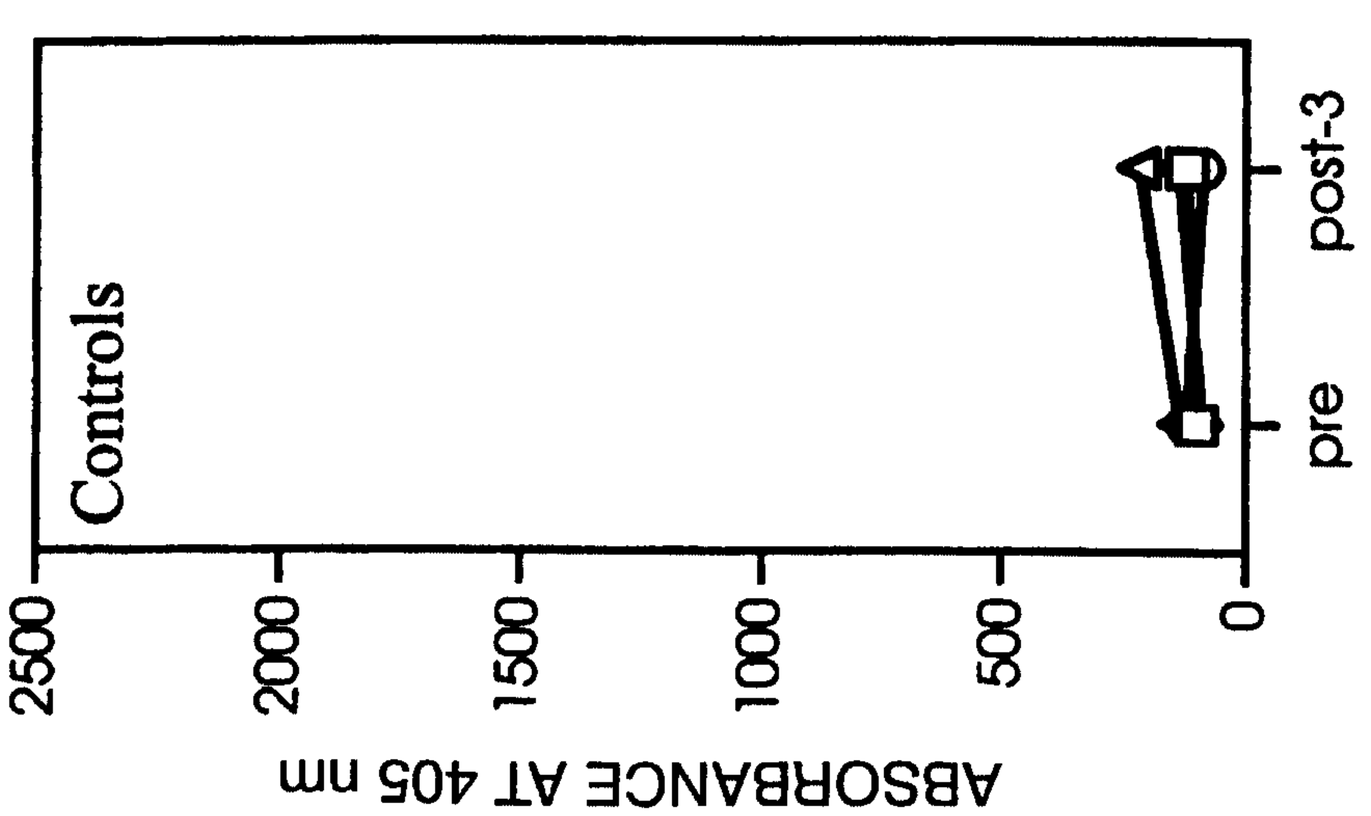


FIG. 7F

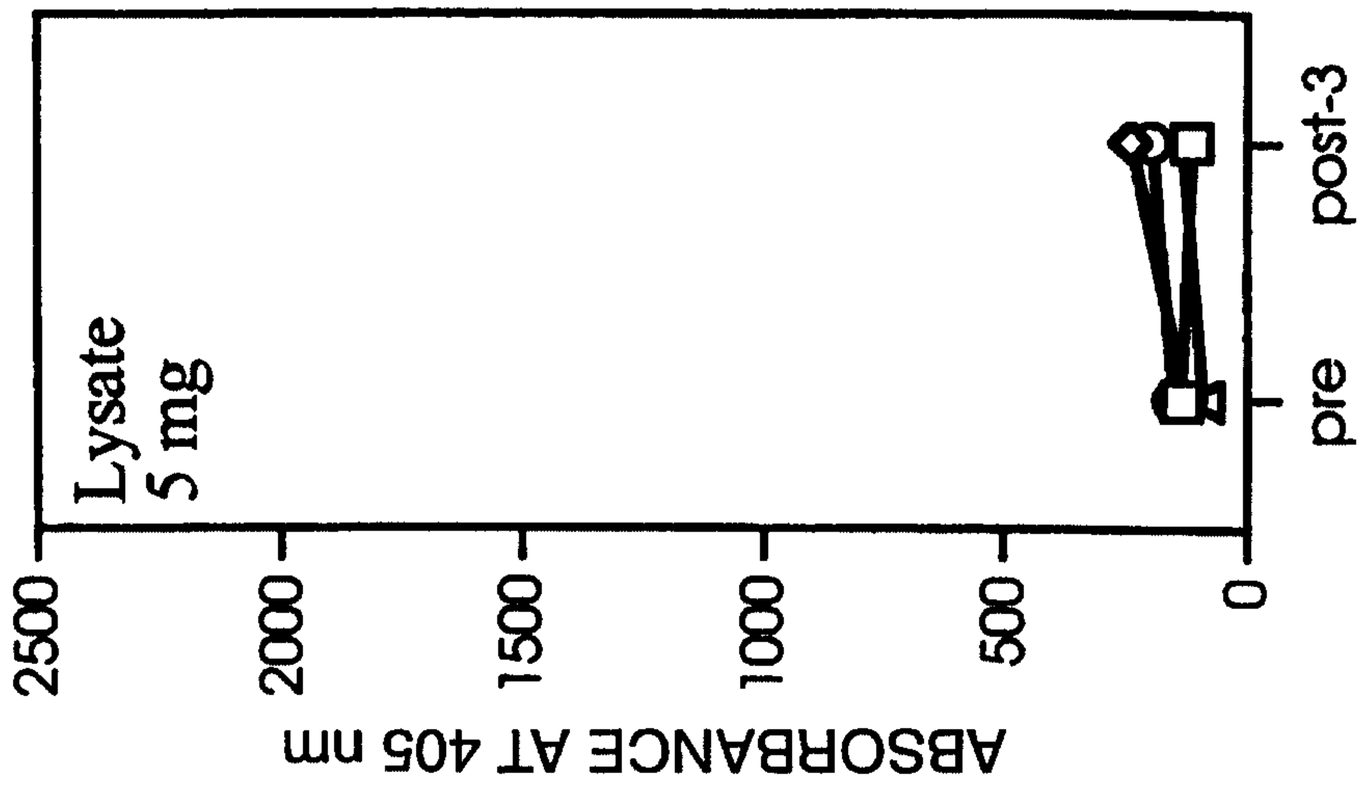


FIG. 7E

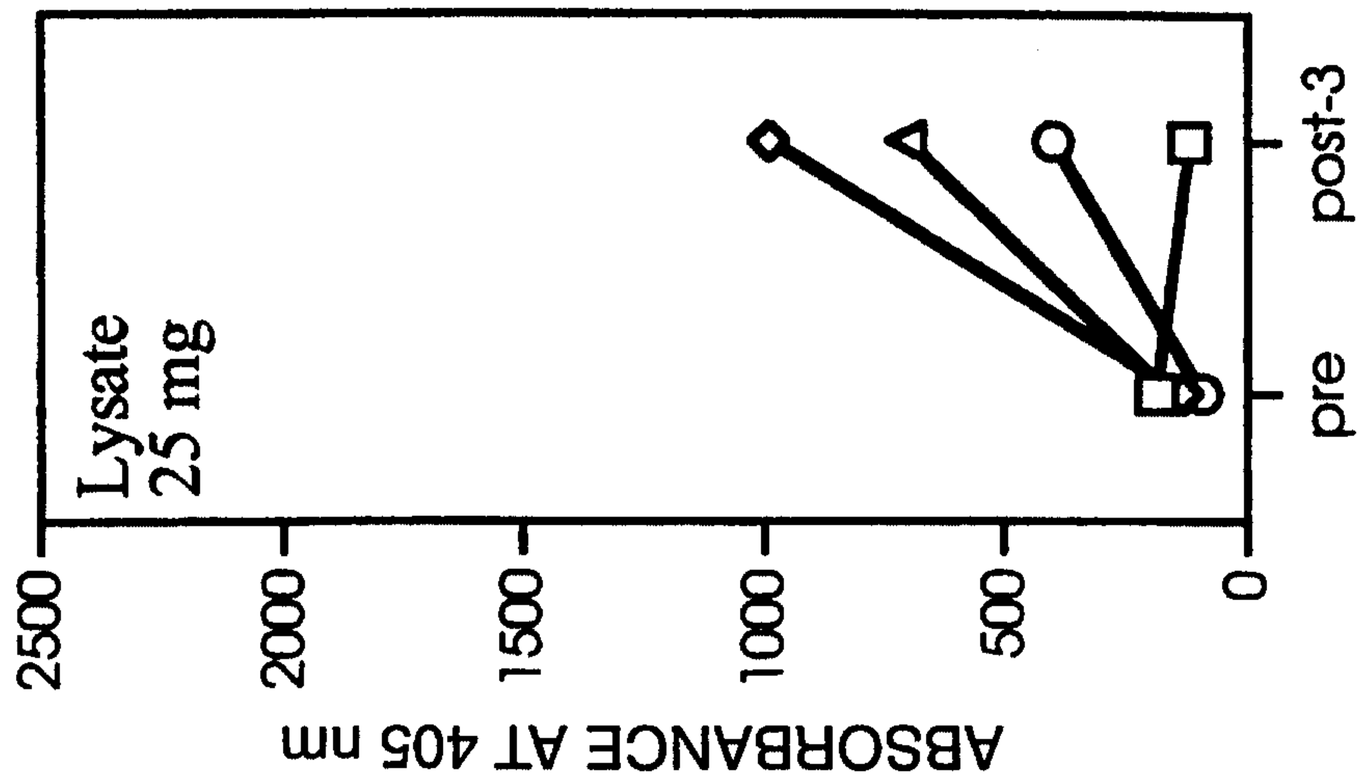


FIG. 7D

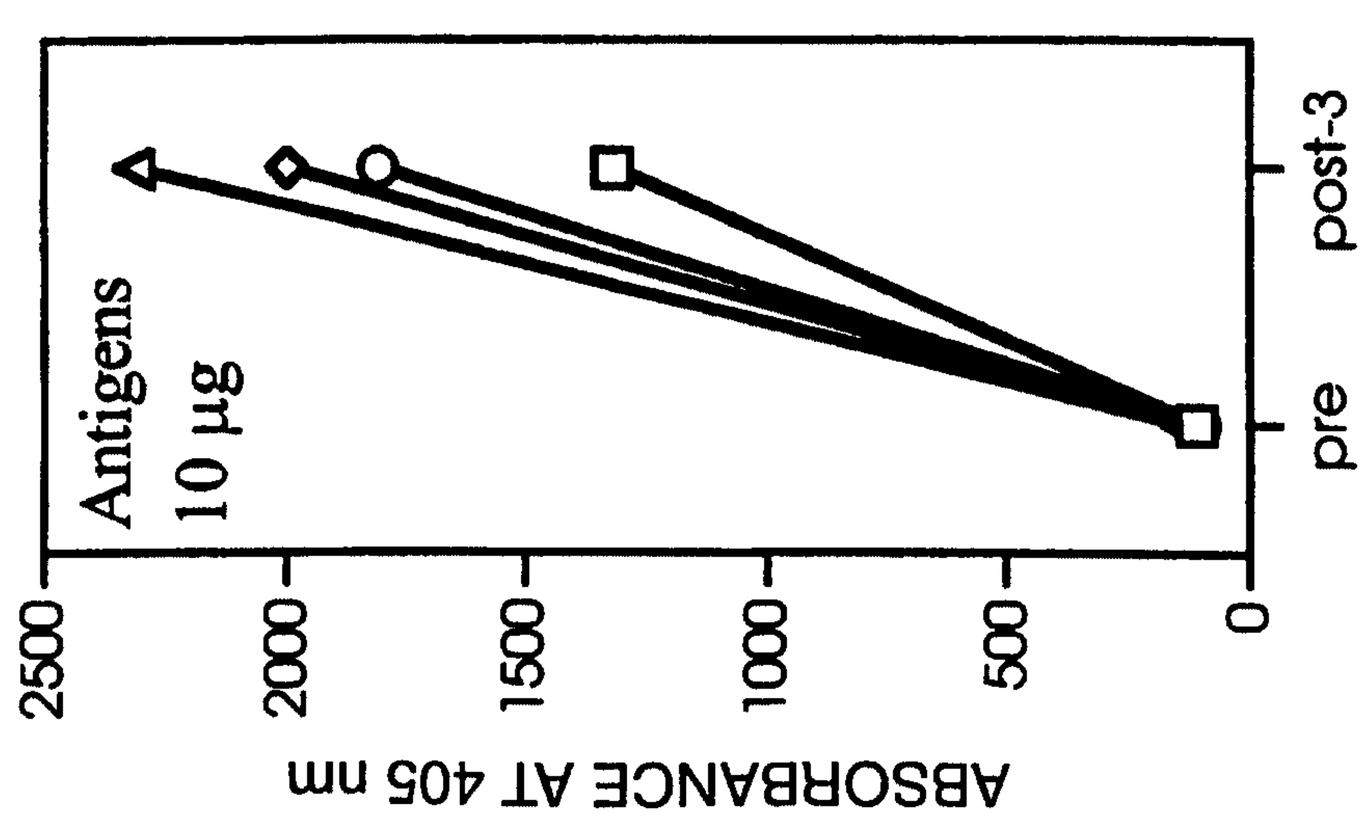


FIG. 8A

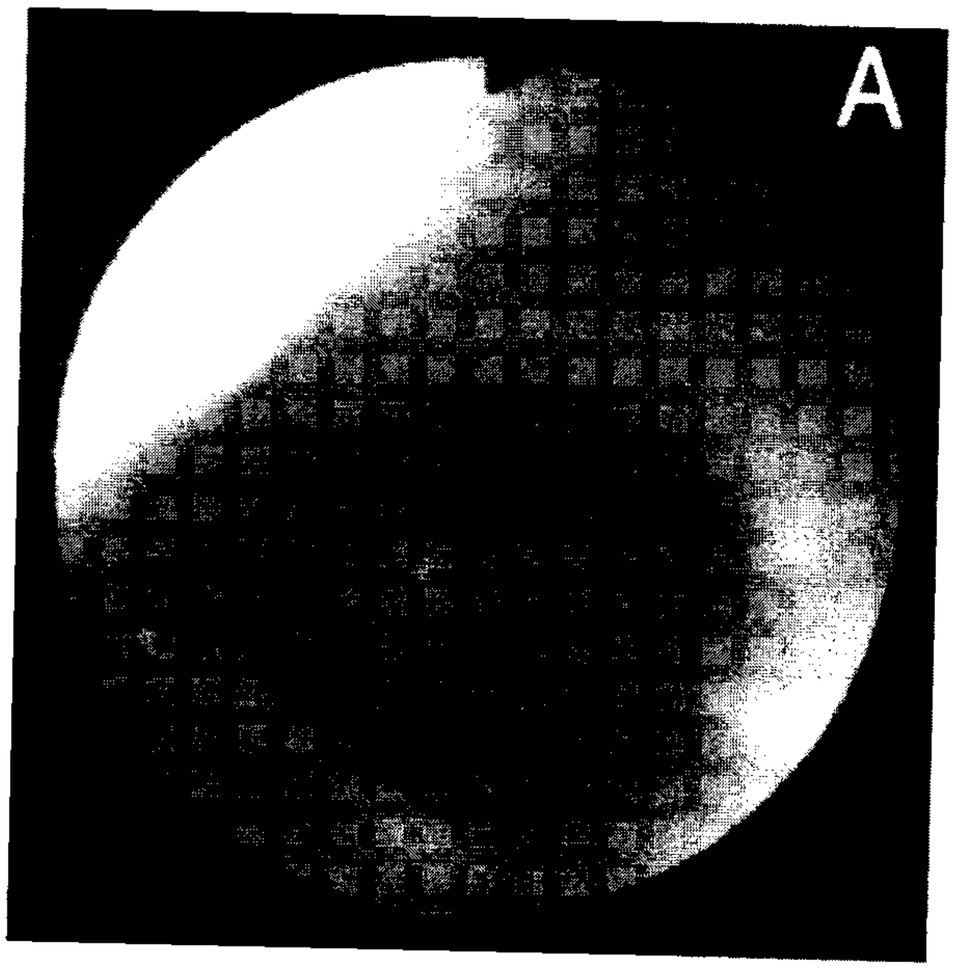


FIG. 8B

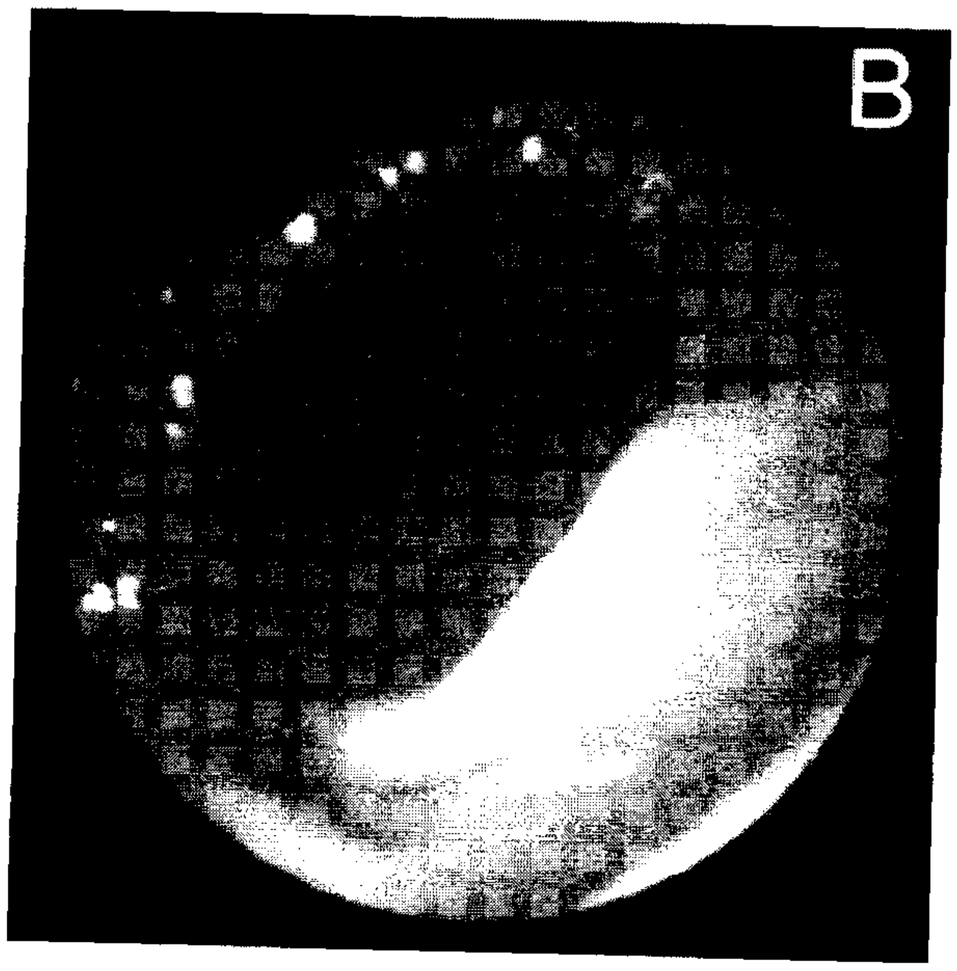




FIG. 9B



FIG. 9A

FIG. 10A

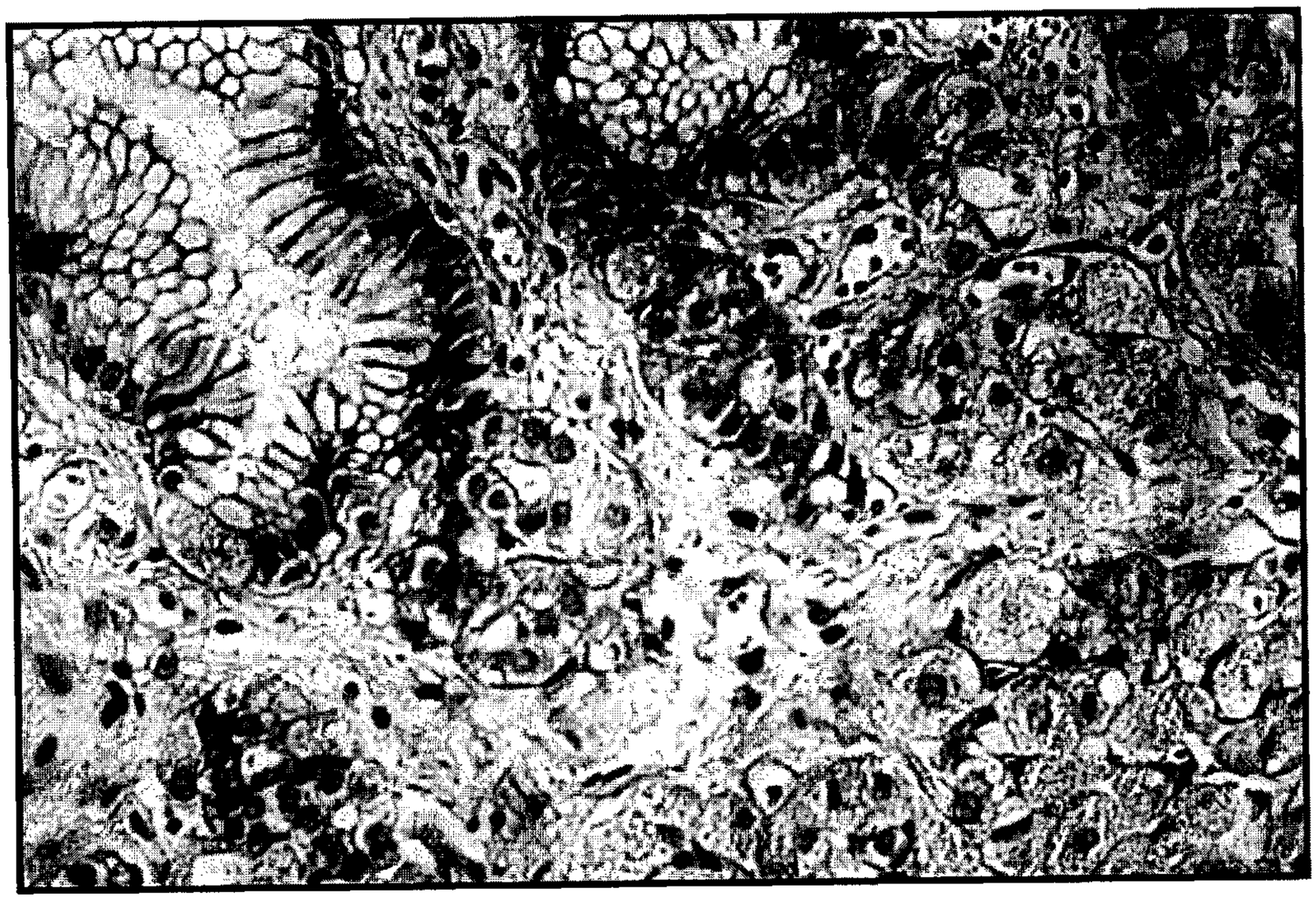


FIG. 10B

