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(54) Title: ANTIBODY TREATMENT OF HELICOBACTER PYLORI

(57) Abstract

The invention provides an antibody with specific activity to urease. Also provided is a pharmaceutical composition for use in a method of treatment for gastrointestinal disorders in an animal. The invention also provides methods for detecting and inhibiting H. pylori urease activity.
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ANTIBODY TREATMENT OF HELICOBACTER PYLORI

BACKGROUND OF THE INVENTION

Helicobacter pylori, formerly called Campylobacter pylori, has become identified as a primary cause of chronic gastroduodenal disorders in adults, particularly, gastritis, dyspepsia, and peptic ulcers. H. pylori is a gram-negative, microaerophilic, spiral bacterium found in the mucous layer of the stomach and attached to the surface of gastric epithelium.

Attempts have been made to eradicate this organism from the upper gastrointestinal tract, for example by employing antimicrobial agents. However, there are significant drawbacks to existing standard therapeutic agents to treat and eradicate H. pylori. Treatment of H. pylori disorders has proven to be difficult once infection has been established.

It has been suggested that H. pylori secretes one or more virulence substances that permits it to escape the bactericidal properties of gastric acid, thereby colonizing gastric epithelium, damaging epithelial cells, and inducing inflammation of mucosal tissues (W. L. Peterson, New Engl. J. Medicine, 324, 1043 (1991)). Research to eradicate H. pylori has included efforts to prevent the initial colonization by H. pylori in the gastrointestinal tract. In one attempt to induce mucosal immunity, an oral vaccine containing killed H. pylori induced anti-H. pylori antibodies in the gastrointestinal secretions and sera in mice (S. J. Czinn et al., Infection and Immun., 59, 2359-2363 (1991)).

Use of antibodies developed against the H. pylori organism itself does not adequately address eradication of colonized H. pylori. Such antibodies do not effectively inhibit specific H. pylori virulence factors. For example, H. pylori urease is an extracellular, cell-bound enzyme with a molecular weight of about 600,000. Urease catalyzes the hydrolysis of urea found in the gastric juices of the stomach to
carbon dioxide and ammonia. H. pylori exhibits strong urease activity. Antibodies developed against the H. pylori organism do not directly neutralize the ureolytic activity of the organism.

The wide range of potential virulence factors associated with H. pylori and the difficulties in eradicating the organism from gastric tissue has contributed to the present lack of treatments for H. pylori-caused disorders. Therefore, a need exists for an effective method of treating health problems, including gastrointestinal disorders caused by H. pylori.

**SUMMARY OF THE INVENTION**

The present invention provides a method for inhibiting urease activity in the gastrointestinal tract of a mammal, particularly humans. The method involves administering to the mammal an effective amount of an antibody having specific activity to a urease enzyme found in the gastrointestinal tract of the mammal, to inhibit in vivo activity of the urease enzyme. Preferably, the antibody is effective in reducing the ammonia concentration in the gastrointestinal of the mammal.

The invention also provides a method for treating gastric disorders caused by H. pylori in a mammal, most particularly a human. The method includes administering an effective amount of an antibody that exhibits specific activity to the urease enzyme produced by H. pylori. The invention further provides an in vitro method of detecting urease, particularly urease produced by H. pylori.

In a preferred embodiment, the present invention employs an antibody to Jack Bean urease or antibody to H. pylori urease, an H. pylori urease subunit, or a recombinant H. pylori urease fragment. The antibody is capable of inhibiting the activity of
urease associated with H. pylori in vivo in a mammal, particularly the ureolytic activity of urease to hydrolyze urea to ammonia in the human gastrointestinal tract. The reactivity of the antibody with urease in vivo is effective to substantially inhibit growth of H. pylori in the gastrointestinal tract, and substantially inhibit H. pylori colonization of the mucosa of the gastrointestinal tract.

A composition useful in the present invention preferably includes an antibody (polyclonal, monoclonal, or chimeric) with specific activity to a urease enzyme, as for example, H. pylori urease. The composition includes an amount of the antibody effective to inhibit, neutralize or significantly reduce the in vivo activity of urease. Preferably, the antibody is combined with a pharmaceutically-acceptable carrier such as water, saline, and the like.

In a method of treating H. pylori gastritis in a patient according to the invention, the patient is administered an effective therapeutic amount of the foregoing pharmaceutical composition. Use of the composition effectively reduces the ammonia concentration in the gastrointestinal tract of the patient, thus preventing or inhibiting colonization and/or growth of H. pylori.

**BRIEF DESCRIPTION OF THE DRAWINGS**

FIGURES 1a and 1b are graphs showing bovine serum antibody response to Jack Bean urease for Cow W-19 and Cow W-23, respectively, vaccinated with Jack Bean urease.

FIGURE 2 is a graph showing bovine colostrum antibody response to Jack Bean urease for Cow W-23 vaccinated with Jack Bean urease.

FIGURE 3 is a graph showing urease inhibition employing immune serum containing anti-urease antibody.
FIGURES 4a and 4b are photographs showing immunoreactivity patterns of anti-urease and anti-\textit{H. pylori} antibodies with 8-25% Gradient PhastGel-SDS reducing conditions. FIGURE 4a shows Gel B: Double stained (Coomassie and silver stain) polyacrylamide gel (PAGE gel); FIGURE 4b shows Gel A: PAGE immunoblot (A-1 = versus rabbit anti-Jack Bean urease; A-2 = versus rabbit anti-\textit{H. pylori} cells); Lane identification: #1 = \textit{H. pylori} - culture 1;

#2 = \textit{H. pylori} - culture 2; #3 = Jack Bean urease; 
# 4 = BioRad molecular weight standards (18,500 - 106,000 daltons); #5 = same as Lane #4; #6 = same as Lane #3; #7 = same as Lane #2; #8 = same as Lane #1.

FIGURE 5 is a graph showing bacterial counts in \textit{H. pylori}-infected gnotobiotic piglets (0 = untreated, control group; \(\Delta\) = group treated with Jack Bean urease-specific antibody preparation).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for detecting and treating gastric disorders caused by urease activity, such as that associated with \textit{H. pylori}, or the like, by employing an antibody that is capable of specifically reacting, \textit{in vitro} or \textit{in vivo}, with urease, such as urease associated with \textit{H. pylori}. As used herein, the term “specific activity to” means the antibody reacts only with urease. For example, an antibody with specific activity to \textit{H. pylori} urease reacts with urease secreted by or present at the surface of the \textit{H. pylori} bacterium, but does not react with other cellular components of \textit{H. pylori}. That is, the antibody is substantially unreactive with the \textit{H. pylori} bacterium itself. More specifically, the antibody inhibits the function of ureases.

The anti-urease antibody according to the present invention may be raised to a number of different urease enzymes or polypeptide fragments or proteins.
associated with recombinant segments or fragments of such enzymes. As is understood by those skilled in the art, urease is a globulin complex produced by a variety of plants and microorganisms. In the present invention, antibodies raised to plant ureases such as Jack Bean urease (genus Canavalia), a bacterial source urease, a mammalian urease, a related urease produced by recombinant genetic procedures, and the like, or a mixture of such ureases, can be employed. Preferably, the antibody is raised in response to Jack Bean urease or \textit{H. pylori} urease.

To produce the urease antibody of interest, a mammal, such as a rabbit, mouse, cows, horses, sheep, goats, and the like, is immunized with an effective amount of an antigen of interest from a variety of sources such as cultured media supernate, or purified antigen such as Jack Bean urease or \textit{H. pylori} urease. Anti-urease antibodies obtained from the immunized animal, preferably from the milk, colostrum or serum, are subsequently screened and selected for specific activity to the urease of interest, as for example, \textit{H. pylori} urease.

As will be understood by those skilled in the art, spleen cells from immunized animals can be fused with a suitable myeloma cell line to produce hybridoma cells. A selected hybridoma cell line that secretes monoclonal antibodies reactive with the urease of interest can serve as a source of the desired antibodies and may be clonally expanded.

According to the invention, the anti-urease antibody is administered, preferably orally or parentally, to a patient, preferably a human, in an amount effective to:
(i) inhibit urease activity in the gastrointestinal tract of the patient, preferably to decrease the in vivo urease activity, such as that of H. pylori, by about 10 to 100%, more preferably about 50 to 100%.

(ii) decrease the in vivo concentration of ammonia in the gastrointestinal tract of a patient by about 20 to about at least 95%, more preferably by about 50 to about at least 95%; and/or

(iii) eradicate bacterial infection and/or inhibit urease activity, such as that caused by H. pylori, in the gastrointestinal tract, including the human gastric mucosa (i.e., gastric and esophagus epithelium).

The anti-urease antibody can be administered, for example, orally, by suppository, by injection, by infusion, or intravenously, or by other suitable means and techniques known in the art. It is also envisioned that the antibody may be combined with another suitable compatible, and optionally synergistic, therapeutic agent that cooperatively reduces urease activity.

Patient treatment using the method of the present invention involves administering therapeutic amounts of the anti-urease antibody. The antibody may be formulated with conventional pharmaceutically-acceptable parenteral vehicles for administration by injection. These vehicles comprise substances that are essentially nontoxic and nontherapeutic such as water, saline, Ringer's Solution, dextrose solution, Hank's Solution, and the like. It is to be understood that antibody formulations may also include small amounts of adjuvants such as buffers and preservatives to maintain isotonicity, physiological pH and stability.

As indicated by the above formulation, the antibody may be administered parenterally. The antibody can also be delivered intravenously, as a bolus. The
antibody can be administered to a patient on a periodic or continuous basis.

Preferably, the antibody is administered orally to the patient. In such application, the antibody is a constituent in a suitable liquid media such as water and the like, or a feed in an appropriate dry format such as a tablet or other oral form understood by those skilled in the art. As described hereinabove, the oral composition can include suitable compatible adjuvants.

The specific urease antibody is contained in an immunoglobulin fraction provided to a patient. In such form, the immunoglobulin concentration provided to the patient is about 1 gram per day. Typically amounts from about 1 to 20 gram per day will be used. For example, about 1 to 2 grams of immunoglobulin could be given to a patient 3 to 4 times per day. The doses of the antibody formulation to be administered will depend upon the patient and the patient's medical history. However, the dose should be sufficient to deplete a substantial portion, usually more than about 50%, of the ammonia production resulting from in vivo urease activity, such as that associated with H. pylori. Dosages of the specific antibody for adult humans envisioned by the present invention and considered to be therapeutically effective will range from between about 0.1 to 500 mg. However, it is to be understood that doses can readily be adjusted to provide appropriate amounts of the antibody to any patient, including children.

The antibody of the present invention may also be used for in vitro detection of urease activity, such as that associated with H. pylori, in a body sample, such as a fluid, tissue, cell extract, and the like, that is obtained from the animal for testing. For example, the antibody may be immobilized on a solid phase support, as for example, immunomagnetic beads, a resin test plate such as polyvinylchloride, polystyrene and the like, or a nitrocellulose carrier and the like,
in an amount effective to bind with a fluid, tissue, or other sample that evidences the presence of urease, such as that associated with \textit{H. pylori}. An example of a useful composition for detecting urease associated with \textit{H. pylori} in a sample \textit{in vitro} is one that includes about 1 to 100 $\mu$g of the antibody per $2 \times 10^8$ immunomagnetic beads.

In a method for detecting urease in a sample \textit{in vitro}, the anti-urease antibody may be combined with the sample, and after a suitable reaction time, the antibody that is bound to the sample is detected by a suitable method known in the art. In one example of such an assay, the antibody may be labeled and immobilized onto a solid phase carrier according to techniques known in the art, and then reacted with the urease in the sample, or fragment thereof, that includes the epitopal-binding site(s) for the antibody, to form a complex between the labeled antibody and the enzyme (i.e., protein). The carrier with the bound antibody/protein complex would then be washed to remove unbound materials, and the labeled antibody in the complex detected by conventional methods known in the art. Alternately, a labeled second antibody that will react with the urease may be combined with the antibody-urease complex, wherein the second antibody binds to the urease in the antibody/urease complex to form an immobilized antibody/urease/labeled second antibody complex, and the labeled second antibody in the complex is then detected. As another example, the sample may be immobilized onto a solid phase carrier according to conventional methods in the art, and reacted with labeled antibody. The carrier with the bound enzyme antibody complex would then be washed to remove unbound material and the labeled antibody in the complex would be detected.

The label that is used may be any label that may be suitably bound to the antibody or second antibody, and which will allow for the reaction of the
antibody with urease in the sample. The label may be, for example, a radioactive isotope, an enzyme, a dyestuff, a fluorescent group, or any combination thereof. The detection method may also include quantifying, by known techniques, the amount of labeled antibody that is bound to the sample.

The invention will be further described by reference to the following detailed examples, wherein the methodologies are as described below. These examples are not meant to limit the scope of the invention that has been set forth in the foregoing description. Variation within the concepts of the invention are apparent to those skilled in the art. The disclosures of the cited references are incorporated by reference herein.

**EXAMPLE 1**

**Antibody production in Rabbits**

High titered antisera in rabbits to Jack Bean urease and *H. pylori* whole organisms was produced. This antisera was then used to inhibit either ammonia production by Jack Bean urease or by *H. pylori* cell-associated urease.

**Methods.** New Zealand White rabbits were inoculated subcutaneously with either urease (E.C. 3.5.1.5, from Jack Beans) or a formalin-treated suspension of *H. pylori* whole cells (ATCC #43504). Vaccines for inoculation at weeks 0 and 3 were equal volume emulsions of antigen with Freunds Complete Adjuvant. Additional inoculations at week 7 were emulsions made with Freunds Incomplete Adjuvant. At week 9, rabbits previously immunized with urease were inoculated with native urease in saline (no adjuvant emulsion). Concentrations for immunization of rabbits with urease were increased over the course of this study--at weeks 0 and 3, rabbits each received 7.5 units; at weeks 7 and 9, rabbits each
received 15 units. The rabbits receiving the *H. pylori* cells were vaccinated with $2 \times 10^9$ cells at week 0 and with $1 \times 10^9$ cells at week 3. The animals immunized with *H. pylori* received two inoculations. Table 1 below summarizes the immunization schedule.

### TABLE 1. Immunization Schedule

<table>
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<tr>
<th>Urease Immune Rabbits</th>
<th>H. pylori Immune Rabbits</th>
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<tr>
<td>Week 0 7.5 Units Urease</td>
<td>Week 0 $2 \times 10^9$ <em>H. pylori</em> cells</td>
</tr>
<tr>
<td>Week 3 7.5 Units Urease</td>
<td>Week 3 $1 \times 10^9$ <em>H. pylori</em> cells</td>
</tr>
<tr>
<td>Week 7 15.0 Units Urease</td>
<td></td>
</tr>
<tr>
<td>Week 9 15.0 Units Urease</td>
<td></td>
</tr>
<tr>
<td>Freunds Complete Adjuvant</td>
<td>Freunds Complete Adjuvant</td>
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<tr>
<td>Freunds Complete Adjuvant</td>
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</tr>
<tr>
<td>Freunds Incomplete Adjuvant</td>
<td>Saline (No adjuvant)</td>
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</table>

Serum was taken before the initial inoculations--at week 9 from the urease immunized rabbits and at week 5 from the *H. pylori* immunized rabbits. The immune serum was compared with the preimmunization serum by an EIA method (*The Enzyme Linked Immunosorbent Assay (ELISA)*, A. Voller, D.B. Bidwell and A. Barlett (1979), the disclosure of which is incorporated by reference herein). Briefly, EIA antigens were coated onto polystyrene 96-well plates by overnight incubation in pH 9.6 carbonate buffer. Optimal coating concentrations were previously determined by titration experiments. Following coating overnight, excess antigens were removed and the wells washed with a phosphate buffered saline solution containing a detergent (PBS-Tween, 20 mm phosphate, pH 7.4 with 0.9% sodium chloride and 0.05% Tween 20). Each serum sample tested was diluted in PBS-Tween and dilutions were incubated on the assay plate. After 30 minutes incubation at room temperature, the assay plate was washed and a dilution of the detection antibody was incubated on the assay plate for 15 minutes
at room temperature, then washed as before. The detection antibody used was Peroxidase Conjugated Affinity Purified Goat Anti-Rabbit IgG (Jackson ImmunoResearch Labs, West Grove, PA). Following detection antibody incubation for 15 minutes, the assay plate was washed and incubated with a peroxidase substrate (TMB, Kirkegaard & Perry, Gaithersburg, MD). After 5 minutes incubation, the reaction was quenched by addition of phosphoric acid, and absorbance at 450 nm was measured.

Results. The titers for the nonimmune rabbit sera and individual rabbits is given below in Table 2. Titer is the last dilution of sera giving an absorbance value greater than the assay background control.

| Table 2. EIA Titer against nonimmune rabbit sera and individual rabbits. |
|-----------------------------|-----------------------------|-----------------------------|
| Jack Bean & H. pylori Urease | Titer against EIA Solid Phase Antigen | Cells |
| Anti Urease Rabbit #1 | 1:512,000 | 1:800 |
| Anti Urease Rabbit #2 | 1:1,024,000 | 1:1,600 |
| Anti Urease Rabbit #3 | 1:256,000 | 1:900 |
| Anti H. pylori Rabbit #6 | 1:6,400 | 1:1,024,000 |
| NonImmune Rabbit Sera | 1:100 | 1:100 |

**Example 2**

**Antibody production in Cows**

High titered antisera was produced in cows inoculated with Jack Bean urease.

**Methods.** Pregnant Holstein cows were inoculated subcutaneously with urease (E.C. 3.5.1.5, from Jack Beans) as an equal volume emulsion with Freunds Incomplete Adjuvant. Antigen concentration in the
vaccine was increased over the course of immunization. Table 3 below summarizes the immunization schedule.

### TABLE 3. Immunization Schedule

<table>
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<tr>
<th>Cow W-19</th>
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<tr>
<td>Week 0</td>
<td>80 Units Urease</td>
<td></td>
<td>Freunds Incomplete Adjuvant</td>
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<td>Week 2</td>
<td>160 Units Urease</td>
<td></td>
<td>Freunds Incomplete Adjuvant</td>
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<tr>
<td>Week 4</td>
<td>320 Units Urease</td>
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<td>Freunds Incomplete Adjuvant</td>
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<tr>
<th>Cow W-23</th>
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<tr>
<td>Week 0</td>
<td>20 Units Urease</td>
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<td>Freunds Incomplete Adjuvant</td>
</tr>
<tr>
<td>Week 3</td>
<td>80 Units Urease</td>
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</tr>
<tr>
<td>Week 5</td>
<td>160 Units Urease</td>
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<td>Freunds Incomplete Adjuvant</td>
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Serum was taken before the initial inoculation and before each subsequent inoculation. Serum was also taken within 24 hours of calving. After calving, a sample from each of the first six colostral milkings were taken.

Immune and non-immune serum and colostrum samples were compared by an EIA method similar to that used in Example 1. The results of the EIA tests were graphed as to sample absorbance versus IgG concentration, as shown in FIGURES 1a, 1b and 2.

Results. The specific activity of an immune sample was approximated by comparing the specific activity of an immune sample to the specific activity of a non-immune sample at equal IgG levels. The specific activities of the immune samples, calculated according to the ratio of non-immune IgG:immune IgG, are shown in Table 4 below.

### TABLE 4. Specific Activities of immune samples.

<table>
<thead>
<tr>
<th>Cow W-19</th>
<th>Serum Week 0</th>
<th>Serum Week 2</th>
<th>Serum Week 4</th>
<th>(Calving) Week 5</th>
<th>Colostrum Week 5</th>
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<tr>
<td>1</td>
<td>23</td>
<td>71</td>
<td>---</td>
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<tr>
<td>Cow W-23</td>
<td>1</td>
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<td>14</td>
<td>14</td>
<td>22</td>
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EXAMPLE 3

Inhibition of Urease Produced by Helicobacter pylori
Urease Inhibition by Specific Antibodies

Microbial urease was measured by a microbiological test employing a urea-based agar system used for detecting urease-producing bacteria (Difco Manual, Tenth Edition, Difco Laboratories, Inc., Detroit, MI). This system produces a pH-related color change in the agar as the urease produced by the bacteria hydrolyzes urea contained in the agar. Inhibition of the microbial urease results in a reduced color intensity.

Helicobacter pylori has been reported by Shahamat, et al., J. Clinical Microbiology, 29, 2835-2837 (1991) to grow well under described conditions in serum-supplemented liquid media. This experiment utilizes a test method which modifies the liquid media method to a serum-supplemented, urea-based, agar system. Incorporation of either immune or control rabbit serum to the serum-supplemented, urea-based, agar system provides both the essential nutrient components for H. pylori growth and functionally active IgG to produce inhibition of H. pylori urease. Specific antibodies in the immune rabbit serum to urease inhibit the ability of H. pylori urease to hydrolyze urea in the agar, thus limiting the color change. Conversely, control rabbit serum have little, if any, specific antibody against urease, and as a result, the enzyme is allowed to react with the urea in the agar.

Methods. H. pylori (ATCC #43504) was maintained in two separate liquid media preparations at 37°C for a maximum of four days per passage in an atmosphere of 10% CO₂. Media #1 consisted of standard Brucella Broth (BB) + 10% fetal calf serum (FCS) and media #2 consisted of
BB + 0.16% Bacto Agar + 10% FCS. *H. pylori* in both media preparations tested gram negative and were positive for urease, catalase, and oxidase by methods standard in the art. Fourth-passage cultures of each media preparation were used to inoculate the serum-supplemented, urea agar plates (described below) in an inhibition test.

Sterile-filtered normal rabbit serum (NRS) or immune rabbit serum to urease (Jack Bean; Sigma #U-0251) was incorporated into Bacto urea agar to achieve a final serum concentration of 10% in the agar.

Sterile-filtered 1 M boric acid, an urease inhibitor, served as the positive control. The entire surface of one NRS supplemented urea agar plate was flooded with the boric acid solution and then excess was removed. The remaining liquid was allowed to permeate into agar for 30 minutes prior to streaking with bacteria.

NRS-supplemented urea agar test plates consisted of the following: 1) one loop full of BB + 10% FCS *Helicobacter pylori* culture streaked on boric acid primed plate; 2) one loop full of BB + 10% FCS *Helicobacter pylori* culture streaked on plate; and 3) one loop full of BB + 0.16% agar + 10% FCS *Helicobacter pylori* culture streaked on plate.

Urease-immune, serum-supplemented, urea agar test plates consisted of the following: 4) one loop full of BB + 10% FCS *Helicobacter pylori* culture streaked on plate; 5) one loop full of BB + 0.16% agar + 10% FCS *Helicobacter pylori* culture streaked on plate; and 6) no bacteria (plate left blank to serve as a control).

All plates were incubated at 37°C in a moist atmosphere of 10% CO₂. The extent of red color development as a measure of urease activity was assessed after an overnight and a five-day incubation. Higher intensity of red color in the agar indicated a higher level of urease activity.
Results. Data summarized in Table 5 below, show inhibition (as measured by color intensity) of urease by boric acid and urease immune rabbit serum as compared to control rabbit serum (NRS) after overnight and 5-day incubation.

TABLE 5. Inhibition of urease.

<table>
<thead>
<tr>
<th>Agar - Inoculum</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Overnight</td>
</tr>
<tr>
<td>1) NRS - BB + 10% FCS w/Boric Acid</td>
<td>-</td>
</tr>
<tr>
<td>2) NRS - BB + 10% FCS</td>
<td>+++</td>
</tr>
<tr>
<td>3) NRS - BB + 0.16% agar + 10% FCS</td>
<td>+++</td>
</tr>
<tr>
<td>4) Urease antisera - BB + 10% FCS</td>
<td>+</td>
</tr>
<tr>
<td>5) Urease antisera - BB + 0.16% agar + 10% FCS</td>
<td>+</td>
</tr>
<tr>
<td>6) Urease antisera - no bacteria</td>
<td>-</td>
</tr>
</tbody>
</table>

EXAMPLE 4

Inhibition of Jack Bean urease by using antibodies immune to urease

Methods. A pH increase caused by ammonia production from the urease enzyme was detected by measuring the optical density of the reaction solution containing a colored indicator for pH. Optimal levels of antibody and urease were arrived at by titration experiments. Comparison of non-immune serum from one rabbit with immune serum from the same rabbit was accomplished by the following technique.

1. The serum was diluted 1:50 final in 2 mm phosphate with 16 mM saline, pH 7.3.
2. The urease (E.C.3.5.1.5, Jack Bean) made soluble at 3000 units/ml was diluted 1:300 to a final concentration of 10 units/ml.
3. Equal volumes (200 µl) of serum and enzyme were combined and incubated under refrigeration for 48 hours.
4. At the time of assay (e.g., 48 hrs), a 1:50 dilution of the serum:enzyme solution was made in 2 mm phosphate/saline.

5. The 1:50 diluted serum:enzyme was then serially diluted 1:2 into 2% urea, resulting in a final volume of 100 µl per reaction well.

6. The serum:enzyme:urea solution was allowed to stand at room temperature for 5 minutes before the pH detection reagent, 100 µg/ml Phenol Red, was added.

7. The absorbance of the solution was read by a spectrophotometer device at 540 nm.

Results. As seen in FIGURE 3, the absorbance values are a plot of the solutions’ absorbance versus the log of the dilution.

The immune serum significantly inhibits the activity of urease when compared with non-immune serum (background).

EXAMPLE 5

**Immunoreactivity of anti-urease and H. pylori antibodies.**

Gel Electrophoresis. Whole **H. pylori** (cultures 1 and 2) or Jack Bean urease (Sigma) was mixed with 10% sodium dodecylsulfate (SDS), 1.0 M dithiothreitol (DTT), and sample buffer (0.1 M Tris-HCl and 0.01 M Disodium EDTA, pH 8.00). Each preparation was heated for 20 minutes at 100°C to derivitize the sample. Tracking dye (0.05% bromophenol blue in 50% glycerol) was added to each cooled sample. In addition, a prestained molecular weight standard (Bio Rad low range; Bio Rad Laboratories, Richmond, CA) was run to provide molecular weight determinations. Samples were loaded at 1.0 µl each on 8-25% Gradient PhastGels (Pharmacia LKB,
Piscataway, NJ) and separated under SDS conditions on
the Pharmacia PhastSystem™. Two gels were run in
duplicate: gel A was immunoblotted (see method described
below) and gel B was doublestained with silver stain
(Bio Rad Laboratories, Richmond, CA) and sensitive
Coomassie Brilliant Blue stain R-250 (10x R-250 diluted
1:10 with a 10% acetic acid/2.2% ammonium sulfate
solution).

**Immunoblot.** Gel A was transferred immediately after
separation to a glass plate with gel side up. The gel
surface was wetted with a few drops of transfer buffer
(6.25 mm Tris + 0.25 M glycine 4:1 with methanol,
pH 8.00) using a wetted test tube. A piece of 0.2 µm
nitrocellulose (Hoefer Scientific Instruments,
San Francisco, CA) was wetted in transfer buffer and
placed on the wetted gel. Trapped air was removed by
gently rolling wetted test tube over nitrocellulose
surface. A piece of transblot paper (Bio Rad
Laboratories) was saturated in transfer buffer and
placed on nitrocellulose surface, and trapped air was
removed. Three dry pieces of transblot paper were
placed on the sandwich. A 500 gram weight was placed on
the glass plate and the blot was allowed to sit
undisturbed for 3 hours at room temperature.

The nitrocellulose was removed from the gel
surface and the molecular weight standard position was
highlighted with an ink pen to preserve the integrity.
The nitrocellulose was blocked for 30 minutes at room
temperature with 1% ovalbumin. The blot was washed a
minimum of three times with wash buffer (phosphate
buffered saline (PBS) + 0.05% Tween 20 (Bio Rad
Laboratories)). The nitrocellulose was cut in half
between molecular weight standards (FIGURE 4b, lanes 4
and 5) and placed in two separated incubation
containers. Blot A-1 received immune rabbit antiserum
to Sigma Jack Bean urease and blot A-2 received immune
rabbit antiserum to whole cell *H. pylori*. Both serums were diluted to roughly 100 µg IgG/ml (1:200) with PBS + 0.05% Tween 20 + 0.1% BSA + 0.02% sodium azide and allowed to incubate for 2 hours at room temperature on the rocker platform. Blots were washed a minimum of three times with wash buffer. Blots were then incubated for 30 minutes at room temperature with a secondary antibody of peroxidase labelled goat anti-rabbit antibody (Jackson Immunoresearch Labs). Blots were washed a minimum of three times with wash buffer and visualized with a precipitating TMB substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

**Results.** FIGURE 4 shows the distinct and separate immunoreactivity patterns of anti-urease and anti-*H. pylori* antibodies against respective agents.

**EXAMPLE 6**

**Pig model study of activity of anti-urease antibodies**

To test the effect of Jack Bean urease specific bovine antibodies against *Helicobacter pylori*, an animal model supporting infection by *H. pylori* was used. The animal model was the gnotobiotic pig, a monogastric non-primate model for *H. pylori* infection, as described by Krakowka et al., *Infect. Immun.* 55:2789-2796 (1987). Following oral challenge with *H. pylori*, these animals develop an asymptomatic infection largely restricted to the stomach. In general, the infection persists for at least four weeks after challenge when the animals are maintained under gnotobiotic conditions, except for the challenge organism. Histological lesions resulting from the infection resemble lesions identified in human gastritis.

To evaluate the therapeutic impact of anti-Jack Bean urease bovine antibodies, a cow was immunized by
four intramuscular injections of a liquid preparation of purified Jack Bean urease (Sigma Chemical Co., St. Louis, MO) emulsified in Freunds Incomplete Adjuvant to enhance immunogenicity. The injections (2 ml) were administered bi-weekly over a two week period, according to an escalating dosage scheme of 80 µg, 160 µg, 320 µg and 640 µg of the purified Jack Bean urease.

The first colostrum was collected from the cow at the time of calving. Calving was 17 days following the final injection of antigen. Four liters of this colostrum was the source of specific anti-Jack Bean urease IgG. Colostral whey was prepared based on the technique described in Butler, in *Veterinary Immunol. and Immunopathology*, Vol. 4 (1983). The first fat was removed following centrifugation at 5000 x g. The de-fatted colostrum was treated with concentrated lactic acid to adjust the pH to 4.6 to cause the isoelectric precipitation of the casein proteins. Casein was removed by centrifugation at 1200 x g for 30 minutes.

Following removal of casein, the solution was clarified by filtration using a 0.5-1.8µ charged depth filter (Cuno zeta plus 30SP filter).

The filtration liquor was then subjected to a repetitive absorption batch treatment using a DEAE ion exchange resin (Indion DEAE, Phoenix Chemicals, Ltd.) under low salt (7.6 millimolar, pH 6.5) conditions. The conditions used in the treatment cause binding of contaminating milk proteins (i.e., casein, β-lactoglobulin) but not IgG to the resin. The treatment continued until >95% of the detectable protein in the solution migrated in SDS-PAGE on a 12.5% gel under reducing conditions showing molecular weights of 25,000 and 50,000, characteristic of IgG immunoglobulin heavy and light chains, respectively, with no other bands being detected by Coomassie blue staining. The solution was then clarified by centrifugation and by use of the charged depth filter, and sterilized by filtering
through a 0.2 micron filter (Nalgene) to remove bacteria from the solution.

The filtered solution was tested for bovine antibody specific for Jack Bean urease by a standard antigen-specific ELISA assay. The sterility of the antibody solution was established by a standard liquid broth culture method (USP Standard Methods). The antibody solution contained >95% IgG, with a high level of activity against Jack Bean urease.

An infection of *H. pylori* was established in gnotobiotic piglets to test the effect of the Jack Bean urease specific bovine antibody preparation against *H. pylori*. The use of gnotobiotic piglets ensured that a defined microbial mono-flora was established. Eleven gnotobiotic piglets were obtained by a standard technique described in Waxler et al., *Am. J. Vet. Res.* 27:300-307 (1966), by exteriorizing the uterus aseptically from an anaesthetized pregnant sow which was at term. The piglets were then placed in pentub isolation units with partitions.

At three days of age, the piglets were orally infected with *H. pylori* by administering 2-4 ml of a preparation containing $10^9$ viable bacteria (strain 26695) in peptone water to each piglet by oral gavage (Krakowka et al., *Infect. Immun.* 55:2789-2796 (1987); Eaton et al., *Infect. Immun.* 57:1119-1125 (1989)). The bacterial infection was allowed to establish itself with no interventions over a consecutive seven day period.

On the seventh day of the *H. pylori* infection, the antibody treatment phase was initiated to assess the therapeutic activity of the prepared antibody solution against *H. pylori*. Six of the piglets ("treatment group") were orally administered an aliquot (25 ml) of the liquid antibody preparation containing 30.6 mg/ml of the purified IgG anti-Jack Bean urease bovine antibodies, placed in their feeding dishes. The treatment group received 25 ml of the antibody
preparation, three times daily (765 mg/feeding, or 2.3 g of IgG per day) at about 4 hours apart, and immediately prior to three daily standard feedings of Similac plus iron, 100-150 ml/feeding (Ross Laboratories, Columbus, Ohio). A total of twenty-one treatments were given to each piglet over a continuous seven day period. The five control piglets did not receive any supplemental feeding to the standard feeding.

To measure the amount of bacterial suppression by the Jack Bean urease-specific bovine antibody preparation, the piglets were euthanized immediately at the end of the treatment period to obtain the stomach tissue for culture. The gastric mucosa was obtained by exteriorizing and removing the stomach, stripping out the muscular tunic, and weighing and homogenizing the remaining tissue in sterile Brucella broth (10% fetal calf serum) (Difco Laboratories). The homogenate was plated onto a suitable agar for bacterial re-isolation and counting, and the plates were incubated at 37°C for 2-4 days, as described in Eaton et al., Infect Immun. 57:1119-1125 (1989). Bacterial suppression was then measured by using standard bacterial plate counts and determining the number of bacterial colony forming units (cfu) recovered per gram of stomach tissue. The bacterial colonies were examined for characteristics of morphology, and assayed for urease, catalase, and oxidase enzyme activity, as described in Eaton et al, Infect Immun. 57:1119-1125 (1989). H. pylori was isolated from each of the treatment and control group piglets, and the assay results show that H. pylori was the only bacteria present in each of the stomach tissue samples. The results of the culture and re-isolation of H. pylori from the control and treatment groups are shown below in Table 6 and depicted in Figure 5.
TABLE 6. Culture and re-isolation of *H. pylori* from infected gnotobiotic piglets treated and untreated with anti-Jack Bean urease bovine-derived IgA

<table>
<thead>
<tr>
<th>Group</th>
<th>Piglet No.</th>
<th>Quantitative Count (cfu/g x 10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected, treated</td>
<td>93-2081</td>
<td>3.53</td>
</tr>
<tr>
<td></td>
<td>93-2082</td>
<td>21.40</td>
</tr>
<tr>
<td></td>
<td>93-2083</td>
<td>8.42</td>
</tr>
<tr>
<td></td>
<td>93-2089</td>
<td>28.30</td>
</tr>
<tr>
<td></td>
<td>93-2090</td>
<td>4.09</td>
</tr>
<tr>
<td></td>
<td>93-2091</td>
<td>13.10</td>
</tr>
<tr>
<td>Infected, untreated</td>
<td>93-2084</td>
<td>5.90</td>
</tr>
<tr>
<td>(control)</td>
<td>93-2085</td>
<td>31.70</td>
</tr>
<tr>
<td></td>
<td>93-2086</td>
<td>18.00</td>
</tr>
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</tr>
<tr>
<td></td>
<td>93-2088</td>
<td>22.30</td>
</tr>
</tbody>
</table>

The majority of the piglets in the treatment group had markedly reduced bacterial counts compared to the piglets in the untreated control group. The bacterial counts in the infected, untreated control group were less than 15 x 10^6 per gram in only one of the five piglets (20%). By comparison, four of six pigs in the infected, treatment group (67%) showed bacterial counts of less than 15 x 10^6 per gram. These results indicate that the Jack Bean urease-specific bovine antibody preparation effectively suppressed the growth of *H. pylori* in the piglets.
WHAT IS CLAIMED IS:

1. A method of inhibiting urease activity in the gastrointestinal tract of a mammal, comprising:
   (a) administering to the mammal, an effective amount of an antibody having specific activity to a urease found in the gastrointestinal tract to inhibit \textit{in vivo} activity of said urease.

2. The method according to claim 1, wherein the antibody is administered orally to the mammal.

3. The method according to claim 1, wherein the antibody is effective in reducing the ammonia concentration in the gastrointestinal tract.

4. The method according to claim 1, wherein the antibody is an antibody to \textit{Helicobacter pylori}.

5. A method of treating a gastrointestinal disorder associated with \textit{Helicobacter pylori}, in a patient, comprising:
   (a) administering to the patient an effective amount of antibody exhibiting specific activity to \textit{Helicobacter pylori} urease effective to inhibit \textit{in vivo} activity of said urease.

6. The method according to claim 5, wherein the antibody is effective in inhibiting colonization of \textit{H. pylori} in the gastric mucosa of the patient.

7. The method according to claim 5, wherein the antibody is effective in inhibiting the growth of \textit{H. pylori} in the gastrointestinal tract of the patient.
8. The method according to claim 5, wherein the antibody is effective in reducing the ammonia concentration in the gastrointestinal tract.

9. The method according to claim 5, wherein the antibody is an antibody to Jack Bean urease.

10. The method according to claim 5, wherein the gastrointestinal disorder is an ulcer.

11. A pharmaceutical composition for use in the treatment of a gastrointestinal disorder in a patient, comprising:
(a) an antibody in combination with a pharmaceutically acceptable carrier, the antibody having specific activity to Helicobacter pylori urease.

12. A method for producing an antibody exhibiting specific activity to Helicobacter pylori urease, comprising:
(a) immunizing a mammal with a suspension of Jack Bean urease;
(b) obtaining a sample of biological fluid from the mammal; and
(c) isolating an antibody fraction from the biological fluid sample that specifically reacts with H. pylori urease.

13. The method according to claim 12, wherein detection of the specific activity of the antibody with H. pylori urease, comprises determining urease inhibition by a color change in a serum- or colostrum-supplemented urea-based agar system.
14. A method for reducing ammonia concentration in the gastrointestinal tract of a mammal, comprising:
(a) administering to the mammal an effective amount of antibody exhibiting specific activity to urease associated with *H. pylori* to inhibit *in vivo* activity of said urease.

15. A method for inhibiting or eliminating colonization of *Helicobacter pylori* in the gastric mucosa of a mammal, comprising:
(a) administering an effective amount of an antibody specifically reactive with *H. pylori* urease.

16. The method according to claim 15, wherein the antibody is an antibody to Jack Bean urease.

17. A method for detecting *H. pylori* urease activity in a body sample, comprising:
(a) obtaining a sample of a body material from a patient;
(b) adding to the sample, an immobilized antibody with specific activity to *H. pylori* urease to form a complex of the immobilized antibody bound to the urease of the sample; and
(c) adding a labeled anti-species antibody reactive with the immobilized antibody to detect the complex.

18. The method according to claim 17, wherein the body material is gastric fluid or fecal material.

19. A method according to claim 17, wherein the labeled anti-species antibody carries a radioactive or color-producing enzyme label.
20. A method for diagnosing of an *H. pylori* gastrointestinal disorder in a patient, comprising:
   (a) obtaining a sample of a body material from the patient;
   (b) adding to the sample, a precipitating antibody which is immunospecific for *H. pylori* urease; and
   (c) detecting the presence of a precipitant comprising the antibody bound to the *H. pylori* urease of the sample.

21. Use of an antibody for the manufacture of a medicament for inhibiting *in vivo* activity of urease in the gastrointestinal tract of a mammal, the antibody having specific activity to a urease found in the gastrointestinal tract.

22. Use of an antibody for the manufacture of a medicament for treating a gastrointestinal disorder associated with *Helicobacter pylori* in a patient, the antibody having specific activity to a urease associated with *Helicobacter pylori*.

23. Use of an antibody for the manufacture of a medicament for detecting urease associated with *Helicobacter pylori* in a sample of body material, the antibody having specific activity to a urease associated with *Helicobacter pylori*.

24. The use according to claims 21, 22 or 23, wherein the antibody has specific activity to Jack Bean urease.
FIG. 5

- INFECTED & UNTREATED: ○ ○ ○
- INFECTED & TREATED: △ △ △ △△

COLONY FORMING UNITS x 10^6/g

15 x 10^6
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 A61K39/395 C07K15/28 G01N33/577

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 A61K C07K G01N

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tr>
<td>X</td>
<td>US.A.4 837 017 (LEVEEN ET AL.) 6 June 1989 see page 7, left column, line 24 - line 37; claims 1,2,5,7</td>
<td>12 1-11, 13-24</td>
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<td>Y</td>
<td>POULTRY SCIENCE vol. 67, 1988, POULTRY SCIENCE, INC., CHAMPAIGN, ILL, GB, pages 434 - 439 J.L. PIMENTEL AND M.E. COOK 'Improved growth in the progeny of hens immunized with Jack bean urease' see page 438, right column, line 19 - line 28 see page 439, left column, line 12 - line 21</td>
<td>1-11, 13-24</td>
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</tbody>
</table>

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

*T* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another document or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"E" document member of the same patent family

2. Date of the actual completion of the international search 9 December 1993

Date of mailing of the international search report 22-12-1993

Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HU Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016

Authorized officer Hornig, H
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<td>THE LANCET&lt;br&gt;vol. 336, no. 8708, 21 July 1990, THE LANCET LTD., LONDON, GB;&lt;br&gt;pages 186 - 187&lt;br&gt;M.J. PALLEN AND C.L. CLAYTON 'Vaccination against Helicobacter pylori urease'&lt;br&gt;see page 186, right column, line 25 - line 28&lt;br&gt;see page 186, right column, line 36 - line 39</td>
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<td>WO.A,93 07273 (INSTITUT PASTEUR) 15 April 1993</td>
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### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. **X** Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

   **Remark:** Although claims 1-10, 14-16 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. **☐** Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. **☐** Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. **☐** As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. **☐** As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. **☐** As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. **☐** No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- **☐** The additional search fees were accompanied by the applicant's protest.
- **☐** No protest accompanied the payment of additional search fees.

Form PCT.ISA.210 (continuation of first sheet (1)) (July 1992)
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