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(71) Applicant *(for all designated States except US):* TECHNOLOGY LICENCE COMPANY LIMITED [GB/GB]; 24 Finch Road, Douglas, Isle of Man (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only) :* WRIGHT, Bruce, William [GB/GB]; 48 Bush Close, Comberton, Cambridgeshire CB3 7EG (GB). COX, Peter, John [GB/GB]; Church Cottage, Church Road, Chevington, Bury St. Edmunds, Suffolk IP19 5QH (GB). NOYES, Alice, Margaret [GB/GB]; 68 High Street, Willingham, Cambrigeshire CB4 5ES (GB). WIDDOWS, Danny [GB/GB]; 14 Sherbourne Close, Cambridge CB4 1RT (GB).

(74) Agent: GILL JENNINGS & EVERY; 53/64 Chancery Lane, London WC2A 1HN (GB).

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(54) Title: MONOCLONAL ANTIBODIES AND THEIR USE

(57) Abstract

Monoclonal antibodies to the genus *Vibrio*, the labelled antibodies, compositions and kits containing them, and their use in diagnosis of antigen and treatment.

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MONOCLONAL ANTIBODIES AND THEIR USE

BACKGROUND OF THE INVENTION

Of current interest in the fields of analysis and diagnosis is the use of monoclonal antibodies to determine the presence of antigens in specimens such as urine, blood, water, milk, and the like.

More particularly, monoclonal antibodies specific for the antigens of Vibrio are desired which when used will rapidly diagnose the presence of such organisms in specimens.

Vibrio will be described with particular reference to Vibrio cholerae. Vibrio cholerae is described in Zinsser Microbiology (17th ed.) at pp. 750-4. V. cholerae is a facultatively anaerobic organism with an optimum temperature ranging from 18 to 37° C. Its metabolism is both respiratory and fermentative. Vibrio cholerae will grow on simple media providing a utilizable carbohydrate, inorganic nitrogen, sulfur, phosphorus, minerals, and adequate buffering. They grow best at pH 7.0 but tolerate alkaline conditions to pH 9.5, a property used in the design of isolation media. They are extremely sensitive to an acid pH, and a pH of 6.0 or less will sterilize cultures.

The important clinical features of cholera are the result of host reaction to an extracell-

ular enterotoxin. De and Chatterje were the first to describe the experimental model using ligated intestinal loops of rabbits, which led to the discovery of the enterotoxin and opened the way for research into the pathogenesis of other gastrointestinal diseases.

The cholera enterotoxin, or choleragen, is a complex molecule with a molecular weight of approximately 80,000 to 98,000. Choleragen is predominately protein (98 percent), with approximately one percent lipid and one percent carbohydrate. There are two major subunits of choleragen: A, which is responsible for biologic activity; and B, which is responsible for the binding of toxin to cell membranes. Subunit A consists of a single molecule of subunit A1 which contains the toxic activity, and a single molecule of subunit A2 which may serve as a stabilizer of the A complex before its action on the cell. One subunit A is noncovalently linked to four to six of the B subunits.

Gm1 ganglioside, an acidic glycolipid containing sialic acid and galactose, or other gangliosides of glycoproteins may be the natural receptors for the choleragen. The action of choleragen is to activate membrane-bound adenyl

cyclase, which converts to ATP to cyclic AMP. The resulting increase in cyclic AMP produces a net flow of fluid and electrolytes from the cells of the small intestine into the lumen. Whole choleragen does not enter the cell but rather remains associated with the cell membrane. Gill has shown that activation of adenyl cyclase requires nicotinamide adenine dinucleotide (NAD), ATP, and cellular protein. NAD is hydrolyzed by choleragen or its subunit to ADP-ribose and nicotinamide. The ADP-ribose is bound to the A subunit after hydrolysis. The mechanism of adenyl cyclase activation by the ribosylated subunit A has not been determined.

The enterotoxin is active in a number of other test systems, such as tissue culture cytotoxicity, fat cell lipase stimulation, and mouse foot edema. The mechanism of action of enterotoxin in these systems also seems to be a result of cyclic AMP stimulation. These various tests have proven to be useful in the elucidation of the enterotoxin mode of action and have provided insight into the physiology of enterotoxin stimulation. Enterotoxins in this application will be referred to as enterotoxins A1, A2, B1, and B2.

In addition to enterotoxin production, virulent V. cholerae must be able to adhere to the intestinal surfaces. Studies on adherence show that virulent cells penetrate the intestinal mucus and attach to the microvilli at the brush border of the epithelial cells. Motility may be involved in the adherence of V. cholerae, since nonmotile varieties are unable to attach to the intestine. The mechanism of intestinal adherence by virulent motile organisms has not been clearly determined. Attachment antigens in this application will be referred to as attachment antigens 1 and 2.

Classic asiatic cholera is one of the most devastating diseases known to man. The incubation period may be hours or days, with a mean of two to three days. The onset is abrupt, with vomiting and diarrhea. Fluid loss in severe cases approaches 15 to 20 liters per day. The voided fluid is watery without traces of odor or enteric organisms. It contains no protein but is high in sodium, potassium, bicarbonate, and chloride. Hypovolemic shock and metabolic acidosis are consequences of this fluid loss. The untreated case fatality rate is over 60 percent and higher attack rates are seen in

children. There are also significant differences in the incidence of hospitalized cases compared with milder forms of the disease with respect to the biotype of the infecting agent; a higher disease rate is associated with the cholerae biotype.

The nonagglutinable vibrios can cause isolated as well as focal outbreaks of diarrhea but the volume of fluid loss does not approach that of classic cholera, and the disease is usually self-limiting.

The primary defense in the control of cholera is the maintenance of adequate sewage treatment and water purification systems, together with the prompt detection and treatment of patients and carriers. In countries with adequate sanitation, cholera is limited to imported cases.

Bacterial diarrhea caused by Vibrio cholerae is a common and often serious condition manifesting as fluid loss from the bowel, leading in many cases to dehydration and occasionally to death. Epidemic infantile diarrhea is a serious problem that occurs in newborn nurseries and can result in high mortality rates. In fact, diarrhea is the most common cause of death in early childhood in many tropical and subtropical

countries. Dysentery is a severe form of diarrhea and can result in high mortality rates. Travelers' diarrhea (Turista) is the most common, and among the most feared, illness to threaten the traveler. Diarrhea is also commonly associated with contaminated food and water. Severe diarrhea often brings the patient to the hospital or the physician's office. Acute diarrhea in adults can be so fulminant as to cause hypovolemic shock and death from the outpouring of fluid into the upper small bowel before the first diarrheal stool occurs.

Highly specific divisions have been made among the Vibrio species. Several of the more commonly known Vibrio members include Vibrio cholerae, Vibrio parahemolyticus, and Vibrio anguillerum. Vibrio cholerae has serogroup O1, which subdivides on the basis of additional factors A, B, and C. Biotypes of serotype O1 are ogawa (A, B), inaba (A, C), and hikojima (A, B, C), plus a proposed serotype with only A. The extreme specificity of antigen-antibody reactions has made it possible to recognize differences between strains of a bacterial species that are indistinguishable on the basis of other phenotypic criteria.

Present treatment and diagnosis of Vibrio infections vary depending on the locus of the infection. It is estimated that in the United States and Europe many millions of cases of bacterial diarrhea occur annually of which several million are seen by a physician or admitted to a hospital. Because of the self-limiting nature of the adult disease, most people do not seek treatment. Of the people seeking treatment, bacterial diagnosis of diarrhea is presently made by stool culture techniques. These techniques are generally performed only in hospitals and are slow, requiring one to three days. During this time the patient is exposed, if treated, to the expense and potential hazard of inappropriate therapy. On the other hand, if not treated the patient is exposed to the hazard of a deteriorating condition pending the test result and initiation of therapy.

Thus, existing methods of detection of Vibrio with high accuracy in diarrhea are less than satisfactory in that they consume large amounts of expensive skilled labor and laboratory time, generally taking one and often several days before returning results. The ability of monoclonal antibodies specifically to bind

to antigens of Vibrio provides many opportunities for diagnosis and treatment. Such specificity is a most important requirement for proper and accurate analysis and/or diagnosis, particularly in diagnosing the presence of infectious diseases which require prompt treatment.

A wide variety of isotopic and nonisotopic immunoassays have been utilized in conjunction with monoclonal antibodies to test for the presence of an antigenic substance. At the present time, agglutination, immuno-fluorescent, chemiluminescent or fluorescent immunoassay, immuno-electron microscopy, radiometric assay systems, radio immunoassays, and enzyme-linked immunoassays are the most common techniques used with the monoclonal antibodies. Other techniques include bioluminescent, fluorescence polarization, and photon-counting immunoassays.

When utilizing the enzyme-linked immunoassay procedure (EIA), it is necessary to bind, or conjugate, the monoclonal antibody with an enzyme capable of functioning in such assay; such as alkaline phosphatase.

The enzyme-linked monoclonal antibody can then be used in the known enzyme-linked immunosorbent assay procedure to determine the presence

of an antigenic substance.

After the specific antigen is identified, the serotype of the infecting organism can be determined, and appropriate treatment can then be initiated to rapidly and efficiently eliminate the disease.

The production of monoclonal antibodies is now a well-known procedure first described by Kohler and Milstein (Eur. J. Immunol. 6, 292 (1975)). While the general technique of preparing hybridomas and the resultant monoclonal antibodies is understood, it has been found that preparing a specific monoclonal antibody to a specific antigen is difficult, mainly due to the degree of specificity and variations required in producing a particular hybridoma.

SUMMARY OF THE INVENTION

The present invention provides novel monoclonal antibodies for use in accurately and rapidly diagnosing samples for the presence of Vibrio antigens and/or organisms.

Briefly stated, the present invention comprises monoclonal antibodies specific for the antigens or species of Vibrio; in particular, the antigens or species of Vibrio cholerae; the antigens or species of Vibrio parahemolyticus;

the antigens or species of Vibrio anguillerum, and the toxins of Vibrio cholerae, as well as a monoclonal antibody broadly cross-reactive with an antigen for each species of the genus Vibrio.

The invention also comprises labeled monoclonal antibodies for use in diagnosing the presence of the Vibrio antigens, each comprising a monoclonal antibody against one of the above-mentioned antigens to Vibrio or to a particular species thereof and linked thereto an appropriate label. The label can be chosen from the group consisting of a radioactive isotope, enzyme, fluorescent compound, chemiluminescent compound, bioluminescent compound, ferromagnetic atom, or particle, or any other label.

The invention further comprises the process for diagnosing the presence of Vibrio antigens or organisms in a specimen comprising contacting said specimen with the labeled monoclonal antibody in an appropriate immunoassay procedure.

Additionally, the invention is also directed to a therapeutic composition comprising a monoclonal antibody for an antigen of Vibrio and a carrier or diluent, as well as kits containing at least one labeled monoclonal antibody to

an antigen of a Vibrio.

DETAILED DESCRIPTION

The monoclonal antibodies of the present invention are prepared by fusing spleen cells, from a mammal which has been immunized against the particular Vibrio antigen, with an appropriate myeloma cell line, preferably NS0 (uncloned), P3NS1-Ag4/1, or Sp2/0 Ag14. The resultant product is then cultured in a standard HAT (hypoxanthine, aminopterin, and thymidine) medium. Screening tests for the specific monoclonal antibodies are employed utilizing immunoassay techniques which will be described below.

The immunized spleen cells may be derived from any mammal, such as primates, humans, rodents (i.e., mice, rats, and rabbits), bovine, ovine, canine, or the like, but the present invention will be described in connection with mice. The mouse is first immunized by injection of the particular Vibrio antigen chosen generally for a period of approximately eleven weeks. When the mouse shows sufficient antibody production against the antigen, as determined by conventional assay, it is given a booster injection of the appropriate Vibrio antigen, and then killed so that the immunized spleen may be remov-

ed. The fusion can then be carried out utilizing immunized spleen cells and an appropriate myeloma cell line.

The fused cells yielding an antibody which give a positive response to the presence of the particular Vibrio antigen are removed and cloned utilizing any of the standard methods. The monoclonal antibodies from the clones are then tested against standard antigens to determine their specificity for the particular Vibrio antigen. The monoclonal antibody selected, which is specific for the particular Vibrio antigen or species, is then bound to an appropriate label.

Amounts of antibody sufficient for labeling and subsequent commercial production are produced by the known techniques, such as by batch or continuous tissue culture or culture in vivo in mammals, such as mice.

The monoclonal antibodies may be labeled with a multitude of different labels, such as enzymes, fluorescent compounds, luminescent compounds, radioactive compounds, ferromagnetic labels, and the like. The present invention will be described with reference to the use of an enzyme labeled monoclonal antibody. Some

of the enzymes utilized as labels are alkaline phosphatase, glucose oxidase, galactosidase, peroxidase, or urease, and the like.

Such linkage with enzymes can be accomplished by any one of the conventional and known methods, such as the Staphylococcal Protein A method, the glutaraldehyde method, the benzoquinone method, or the periodate method.

Once the labeled monoclonal antibody is formed, testing is carried out employing one of a wide variety of conventional immunoassay methods. The particular method chosen will vary according to the monoclonal antibody and the label chosen. At the present time, enzyme immunoassays are preferred due to their low cost, reagent stability, safety, sensitivity, and ease of procedure. One example is enzyme-linked immunosorbent assay (EIA). EIA is a solid phase assay system which is similar in design to the radiometric assay, but which utilizes an enzyme in place of a radioactive isotope as the immunoglobulin marker.

Fluorescent-immunoassay is based on the labeling of antigen or antibody with fluorescent probes. A nonlabeled antigen and a specific antibody are combined with identical fluorescently

labeled antigen. Both labeled and unlabeled antigen compete for antibody binding sites. The amount of labeled antigen bound to the antibody is dependent upon, and therefore a measurement of, the concentration of nonlabeled antigen. Examples of this particular type of fluorescent-immunoassay would include heterogenous systems such as Enzyme-Linked Fluorescent Immunoassay, or homogeneous systems such as the Substrate Labeled Fluorescent Immunoassay. The most suitable fluorescent probe, and the one most widely used is fluorescein. While fluorescein can be subject to considerable interference from scattering, sensitivity can be increased by the use of a fluorometer optimized for the probe utilized in the particular assay and in which the effect of scattering can be minimized.

In fluorescence polarization, a labeled sample is excited with polarized light and the degree of polarization of the emitted light is measured. As the antigen binds to the antibody its rotation slows down and the degree of polarization increases. Fluorescence polarization is simple, quick, and precise. However, at the present time its sensitivity is limited to the micromole per liter range and upper nano-

mole per liter range with respect to antigens in biological samples.

Luminescence is the emission of light by an atom or molecule as an electron is transferred to the ground state from a higher energy state. In both chemiluminescent and bioluminescent reactions, the free energy of a chemical reaction provides the energy required to produce an intermediate reaction or product in an electronically excited state. Subsequent decay back to the ground state is accompanied by emission of light. Bioluminescence is the name given to a special form of chemiluminescence found in biological systems, in which a catalytic protein or enzyme, such as luciferase, increases the efficiency of the luminescent reaction. The best known chemiluminescent substance is luminol.

A further aspect of the present invention is a therapeutic composition comprising one or more of the monoclonal antibodies to the particular Vibrio antigen or species, as well as a pharmacologically acceptable carrier or diluent. Such compositions can be used to treat humans and/or animals afflicted with some form of Vibrio infection and they are used in amounts effective to cure; an amount which will vary

widely dependent upon the individual being treated and the severity of the infection.

One or more of the monoclonal antibodies can be assembled into a diagnostic kit for use in diagnosing for the presence of an antigen, antigens, or species of Vibrio in various specimens. It is also possible to use the broadly cross-reactive monoclonal antibody which can identify the genus Vibrio alone or as part of a kit containing antibodies that can identify other bacterial genera or species of Vibrio and/or other bacteria.

In the past there have been difficulties in developing rapid kits because of undesirable cross-reactions of specimens; such as feces, with antiserum. The use of monoclonal antibodies can eliminate these problems and provide highly specific and rapid tests for diagnosis. For example, the incidence of significant diarrhea and diarrheal illness is so high that estimates of market size for such a kit is difficult to make, but a "same day" test could be expected to be used at least as often as stool cultures. Large use of such tests in developing countries might be anticipated because of more frequent and severe diarrhea, dysentery, and other related

illnesses.

Additionally, conjugated or labeled monoclonal antibodies for antigens and/or species of Vibrio and other gram-negative bacteria can be utilized in a kit to identify such antigens and organisms in blood samples taken from patients for the diagnosis of possible Vibrio or other gram-negative sepsis. The monoclonal test is an advance over existing procedures in that it is more accurate than existing tests; it gives "same day" results, provides convenience to the patient and improves therapy as a result of early, accurate diagnosis; and it reduces labor costs and laboratory time required for administration of the tests.

In addition to being sold individually, the kit could be included as a component in a comprehensive line of compatible immunoassay reagents sold to reference laboratories to detect the species and serotypes of Vibrio.

One preferred embodiment of the present invention is a diagnostic kit comprising at least one labeled monoclonal antibody against a particular Vibrio antigen or species, as well as any appropriate stains, counterstains, or reagents. Further embodiments include kits

containing at least one control sample of a Vibrio antigen and/or a cross-reactive labeled monoclonal antibody which would detect the presence of any of the Vibrio organisms in a particular sample. Specific antigens or species to be detected in this kit include the characteristic antigens and toxins of Vibrio cholerae and the antigen or species of Vibrio parahemolyticus, Vibrio anguillerum.

Monoclonal diagnostics which detect the presence of Vibrio antigens can also be used in periodic testing of water sources, food supplies and food processing operations. Thus, while the present invention describes the use of the labeled monoclonal antibodies to determine the presence of a standard antigen, the invention can have many applications in diagnosing the presence of antigens by determining whether specimens such as urine, blood, stool, water, milk, and the like contain the particular Vibrio antigen. More particularly, the invention could be utilized as a public health and safety diagnostic aid, whereby specimens such as water or food could be tested for possible contamination.

The invention will be further illustrated in connection with the following examples which

are set forth for purposes of illustration only and not by way of limitation.

The monoclonal antibodies of the present invention were prepared generally according to the method of Kohler and Milstein, supra.

In the Examples:

DMEM = Dulbeccos Modified Eagles Medium

FCS = Foetal Calf Serum

PBS = Phosphate Buffered Saline

TSB = Tryptone Soya Broth

CFA = Complete Freunds Adjuvant

Example 1

A. Animal Immunisation

Balb/c mice were injected with prepared Vibrio cholerae enterotoxin. They were given one intraperitoneal injection per week for three weeks and, after another week, one intravenous injection (5 µg prepared antigen/dose/mouse). The mice were bled approximately six days after the last injection and the serum tested for antibodies by assay. The conventional assay used for this serum titer testing was the enzyme-linked immunosorbent assay system. When the mice showed antibody production after this regimen, generally a positive titer of at least 10,000, a mouse was selected as a fusion donor and given a booster injection intraperitoneally, three days prior to splenectomy.

B. Cell Fusion

Spleen cells from the immune mice were harvested three days after boosting, by conventional techniques. First, the donor mouse selected was killed and surface-sterilised by immersion in 70% ethyl alcohol. The spleen was then removed and immersed in approximately 2.5 ml DMEM to which had been added 3% FCS. The spleen was then gently homogenised in a LUX homogenising tube until all cells had been released from the membrane, and

the cells were washed in 5 ml 3% FCS-DMEM. The cellular debris was then allowed to settle and the spleen cell suspension placed in a 10 ml centrifuge tube. The debris was then rewashed in 5 ml 3% FCS-DMEM. 50 ml suspension were then made in 3% FCS-DMEM.

The myeloma cell line used was NS0 (uncloned), obtained from the MRC Laboratory of Molecular Biology in Cambridge, England. The myeloma cells were in the log growth phase, and rapidly dividing. Each cell line was washed using, as tissue culture medium, DMEM containing 3% FCS.

The spleen cells were then spun down at the same time that a relevant volume of myeloma cells were spun down (room temperature for 7 minutes at 600 g), and each resultant pellet was then separately resuspended in 10 ml 3% FCS-DMEM. In order to count the myeloma cells, 0.1 ml of the suspension was diluted to 1 ml and a haemacytometer with phase microscope was used. In order to count the spleen cells, 0.1 ml of the suspension was diluted to 1 ml with Methyl Violet-citric acid solution, and a haemacytometer and light microscope were used to count the stained nuclei of the cells.

10^8 spleen cells were then mixed with 5×10^7 myeloma cells, the mixture washed in serum-free DMEM high in glucose, and centrifuged, and all the liquid removed. The resultant cell pellet was placed in a 37°C water-bath. 1 ml of a 50 w/v solution of polyethylene glycol 1500 (PEG) in saline Hepes, pH approximately 7.5, was added, and the mixture gently stirred for approximately 1.5 minutes. 10 ml serum-free tissue culture medium DMEM were then slowly added, followed by up to 50 ml of such culture medium, centrifugation and removal of all the supernatant, and resuspension of the cell pellet in 10 ml of DMEM containing 18% by weight FCS.

10 µl of the mixture were placed in each of 480 wells of standard multiwell tissue culture plates. Each well contains 1.0 ml of the standard HAT medium (hypoxanthine, aminopterin and thymidine) and a feeder layer of Balb/c macrophages at a concentration of 5×10^4 macrophages/well.

The wells were kept undisturbed and cultured at 37°C in 9% CO_2 air at approximately 100% humidity. The wells were analysed for growth, utilising the conventional inverted microscope procedure, after about 5 to 10 days. In those wells in which growth was present in the inhibiting HAT medium, screening tests for the specific monoclonal antibody were made utilising the conventional enzyme immunoassay screening method described below.

C. Cloning

From those wells which yielded antibody against the antigen, cells were removed and cloned using the standard agar method. In the agar method, a freshly-prepared stock solution of sterile 1.2% agar in double distilled water with an equal volume of double-strength DMEM and additives was maintained at 45 C. This solution (10 ml) was then aliquoted into 10 cm Petri dishes, to form a base layer. An overlay of equal volumes of agar and cells in 18% FCS-DMEM was spread evenly over the base. The cells were allowed to multiply for approximately 10 days at 37 C, 7-9% CO_2, 95% RH. Viable separate colonies were picked off the agar surface and placed into 60 wells of a 96-well microtitre tray in 18% FCS-DMEM. After a further period of growth, the supernatants were assayed for specific antibody by the standard enzyme immunosorbent assay.

Alternatively, the limiting dilution method was used. In limiting dilution, dilutions of cell suspensions in 18% FCS-DMEM + Balb/c mouse macrophages were made to achieve one cell/well and one-half cell/well

in a 96-well microtitre plate. The plates were incubated for 7-14 days at 37 C, 97% RH, 7-9% CO_2 until semi-confluent. The supernatants were assayed for specific antibody by the standard enzyme immunoabsorbent assay.

The clones were assayed by the enzyme immunoassay method to determine antibody production.

D. Monoclonal Selection

The monoclonal antibodies from the clones were screened by the standard techniques for binding to the enterotoxin, prepared as in the immunisation, and for specificity. Specificity to the beta sub-unit of cholera toxin was observed, as were negative reactions to E. coli labile toxin and boiled strains of E. coli. The EIA immunoassay noted above may be used.

E. Antibody Production and Purification

Balb/c mice were primed with pristane, for at least 7 days, and injected intraperitoneally with 10^7 cells of the monoclonal antibody-producing line. The ascitic fluid was harvested when the mice were swollen with fluid but still alive. The cells were then centrifuged at 1200 g for approximately 10 minutes, the cells discarded, and the antibody-rich ascites collected and stored at -20 C.

Purification was accomplished using the ammonium sulphate/DEAE method. Specifically, 10 ml of the ascites fluid were filtered through glass wool and centrifuged at 30,000 g for 10 minutes. The ascites was then stirred at +4°C, and an equal volume of cold, saturated ammonium sulphate added slowly. The mixture was stirred for 30 minutes after the addition was complete. The precipitate was harvested by centrifugation at 10,000 g for 10 minutes. The precipitate was dissolved in a minimum volume of cold phosphate/EDTA buffer (20 mM sodium phosphate, 10 mM EDTA, pH 7.5, + 0.02% sodium azide). The solution was dialysed versus 2x1000 ml of the same

buffer, at +4°C. The dialysed, redissolved precipitate was centrifuged at 30,000 g for 10 minutes and applied to a 10 ml column of DEAE-cellulose, previously equilibrated in phosphate/EDTA buffer. The monoclonal antibody was eluted with phosphate/EDTA buffer.

F. Enzyme-Monoclonal Linkage

The monoclonal antibody specific against Vibrio cholerae enterotoxin, prepared as above, was linked to an enzyme, viz. highly-purified alkaline phosphatase, using the one-step glutaraldehyde method. Monoclonal antibody was dialysed with alkaline phosphatase (Sigma Type VII-T) against 2 x 1000 ml of PBS pH 7.4, at +4°C. After dialysis, the volume was made up to 2.5 ml with PBS and 25 µl of a 20% solution of glutaraldehyde in PBS was added. The conjugation mixture was left at room temperature for 1.5 hours. After this time, glutaraldehyde was removed by gel filtration on a Pharmacia PD-10 (Sephadex G-25M) column, previously equilibrated in PBS. The conjugate was eluted with 3.5 ml PBS and then dialysed against 2 x 2000 ml of Tris buffer (50 mM Tris, 1 mM magnesium chloride, pH 8.0 plus 0.02% sodium azide) at +4°C. To the dialysed conjugate was added 1/10th its own volume of 10% BSA in Tris buffer. The conjugate was then sterile-filtered through a 0.22 µm membrane filter into a sterile amber vial, and stored at +4°C.

G. Monoclonal Antibody Conjugate Testing

The enzyme immunoassay method was used for testing. This method comprises coating the wells of a standard polyvinyl chloride (PVC) microtitre tray with the antigen, followed by addition of monoclonal antibody enzyme conjugate, and finally addition of the enzyme substrate, para-nitrophenyl phosphate.

In this case, the monoclonal antibodies were found to be specific for the enterotoxin. The monoclonal antibody was tested and shown to be of the Class IgG_1.

If deemed necessary, the particular epitopic site to which the antibody attaches to the antigen can also be determined. The same enzyme immunoassay method can also be used to determine whether diagnostic specimens such as urine, blood, stool, water or milk contain the antigen. In such cases, the antibody can first be bound to the plate.

Example 2

The procedure of Example 1 was repeated, to give a monoclonal antibody broadly cross-reactive with each species of the genus Vibrio.

Tests using the present invention are superior to existing tests, based on the following advantages: (i) greater accuracy; (ii) same day results, within an hour or two; (iii) reduction in amount of skilled labour required to administer laboratory procedures, resulting in reduced labour costs; (iv) reduction in laboratory time and space used in connection with tests, resulting in reduced overhead expenses; and (v) improved therapy based upon early, precise diagnosis.

While the invention has been described in connection with certain preferred embodiments, it is not intended to limit the scope of the invention to the particular form set forth but, on the contrary, it is intended to cover such alternatives, modifications, and equivalents as may be included within the spirit and scope of the invention as defined by the appended claims.

CLAIMS

1. A monoclonal antibody specific to the antigen or species of Vibrio parahemolyticus.

2. A monoclonal antibody specific to the antigen or species of Vibrio anguillerum.

3. A monoclonal antibody specific to an enterotoxin of Vibrio.

4. A monoclonal antibody specific to an enterotoxin of Vibrio cholera.

5. A monoclonal antibody broadly cross-reactive with an antigen of all species of the genus Vibrio.

6. A monoclonal antibody according to claims 1 to 5, which is labelled.

7. A monoclonal antibody according to claim 6, wherein the label is a radioactive isotope, enzyme, fluorescent compound, bio-luminescent compound, chemi-luminescent compound, or ferromagnetic atom or particle.

8. A monoclonal antibody according to claim 7, wherein the label is an enzyme capable of being used in an enzyme-linked immunoassay procedure, a fluorescent compound or probe capable of being used in an immuno-fluorescent, fluorescent, enzyme-fluorescent, fluorescence-polarisation or photon-counting immunoassay procedure, a chemi-luminescent compound capable of being used in a luminescent or enzyme-linked immunoassay procedure, or a bio-luminescent compound capable of being used in a bio-luminescent immunoassay procedure.

9. A monoclonal antibody according to claim 8, wherein the label is an enzyme selected from alkaline phosphatase, glucose oxidase, galactosidase and peroxidase, fluorescein, a chemi-luminescent compound selected from luminol and luminol derivatives, or a bio-luminescent compound selected from luciferase and luciferase derivatives.

10. A monoclonal antibody according to any preceding claim, for use in treating Vibrio infections.

11. A process for diagnosing for the presence of an antigen of Vibrio in a specimen, which comprises contacting the specimen with a monoclonal antibody according to any of claims 6 to 9 in an immunoassay procedure appropriate to the label.

12. A therapeutic composition which comprises a monoclonal antibody according to any of claims 1 to 10 and a pharmaceutically-acceptable carrier or diluent.

13. A kit for diagnosing for the presence of a gram-negative bacterial infection, which comprises a monoclonal antibody according to any of claims 1 to 9 and, as a control, a known Vibrio antigen.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 86/00411

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

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II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	G 01 N; A 61 K; C 12 P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Biological Abstracts, Reviews, Reports and Meetings, ref. 30001664 R. Gerlich et al.: "Murine monoclonal antibodies against vibrio anguillarum serotype I and II", see the on-line print-out & Z, Angew. Ichthyol., 1985, 1(2), 92-93 (German).	2
Y		1,3-13
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X	Chemical Abstracts, vol. 104, no. 5, 3 February 1986 (Columbus, Ohio, US) N.A. Stockbine et al.: "Characterization of monoclonal antibodies against shiga-like toxin from Escherichia coli" see page 440, abstract no. 32812y, & Infect. Immun., 1985, 15(3), 695-700 (Eng)	1,3,4
Y		2,5-13
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X	Chemical Abstracts, vol. 104, no. 1, 6 January 1986 (Columbus, Ohio, US) T. Holme et al.: "Monoclonal antibodies	./.

* Special categories of cited documents: [10]

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

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

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

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10th October 1986	26 NOV 1986
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. VAN MOL

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	against group- and type-specific antigens of vibrio cholerae oil", see page 399, abstract no. 4312s, & Monoclonal Antibodies Bact., 1985, 1, 167-189 (Eng.)	3,4
Y		1,2,5-13
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Y	Chemical Abstracts, vol. 103, no. 13, 30 September 1985 (Columbus, Ohio, US) B. Gustafsson et al.: "Immunological characterization of Vibrio cholerae O:1 lipopolysaccharide, O-side chain, and core with monoclonal antibodies", see page 464, abstract no. 103069d, & Infect. Immun., 1985, 49(2), 275-280 (Eng.)	1-13
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Y	Chemical Abstracts, vol. 103, no. 7, 19 August 1985 (Columbus, Ohio, US) C.V. Sciortino et al.: "Monoclonal antibodies to outer membrane antigens of vibrio cholerae", see page 440, abstract no. 52465v, & Infect. Immun. 1985, 49(1), 122-131 (Eng.)	1-13
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Y	Chemical Abstracts, vol. 98, no. 1, 3 January 1983 (Columbus, Ohio, US) B. Gustafsson et al.: "Monoclonal antibodies against vibrio cholerae lipopolysaccharide", see page 309, abstract no. 3381j, & Infect. Immunol. 1982, 38(2), 449-454 (Eng.)	1-13
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Y	US, A, 4461829 (A.C. GREENQUIST) 24 July 1984 see claims 1-3; column 2, lines 8-40; column 10, lines 17-26 and 51	6-9,11

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/GB 86/00411 (SA 13888)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 03/11/86

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 4461829	24/07/84	CA-A- 1190461	16/07/85