PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number:
C07K 15/00, C12N 5/00, 7/02 C12N 15/00, C12Q 1/70	A1	WO 92/07001
		(43) International Publication Date: 30 April 1992 (30.04.92)

(21) International Application Number: PCT/US91/07571

(22) International Filing Date: 9 October 1991 (09.10.91)

(30) Priority data:
596,811 12 October 1990 (12.10.90) US

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(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: HEPATITIS C VIRUS ANTIBODY

(57) Abstract

The present invention relates to polyclonal and monoclonal antibodies which selectively bind to antigens associated with hepatitis C viral particles or HCV aggregated antigens isolated from infected patients and to processes which may be used to isolate hepatitis C virus from infected patients including a procedure by which HCV is partially purified. The use of these antibodies in diagnostic immunological assays is also described.

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HEPATITIS C VIRUS ANTIBODY

The present invention relates to antibodies which selectively bind to antigens associated with hepatitis C viral particles isolated from infected patients and to processes for isolating hepatitis C virus from plasma including a procedure by which HCV is partially purified.

BACKGROUND OF THE INVENTION

It has been established that hepatitis C virus or HCV (HCV has historically been referred to as non-A non-B hepatitis or NANBH) may be transmitted by contaminated blood and blood products. In transfused patients, as many as 10% will contract post-transfusion hepatitis. Of those patients contracting the disease, approximately 90% will be diagnosed as having HCV. The prevention of the transmission of this form of hepatitis by blood and blood products requires reliable, sensitive and specific diagnostic and prognostic assays which may be used to identify HCV carriers as well as contaminated blood and blood products.

The development of these needed diagnostic and prognostic assays has been burdened by the unavailability of HCV-specific immunoreactive reagents. Progress in developing needed reagents has been particularly hampered

by difficulties in identifying and isolating HCV antigens and antibodies. See, for example, Wands, et al., U.S. Patent 4,870,076, Hellings, Vox Sang, 51, Supp. 1:63-66 (1986), Linke, et al. [need cite: Production of Monoclonal Antibodies Specific for Non-A, Non B Hepatitis Infected Liver] Wands, et al., Proc. Nat'l. Acad. Sci., 83:6608-6612 (1986), Ohori, et al., J. Med. Virol., 12:161-178 (1983), Bradley, et al., Proc. Nat'l. Acad. Sci., 84:6277-6281, (1987), Akatsuka, T., et al., J. Med. Virol, 20:43-56 (1986), Seto, B., et al., U.S. Patent Application Number 07/234,641 (available from U.S. Department of Commerce National Technical Information Service, Springfield, Virginia, No. 89138168), Takahashi, K., et al., European Patent Application No. 0 293 274, published November 30, 1988, and Seelig, R., et al., in PCT Application PCT/EP88/00123. The recent report of isolated cDNA sequences encoding antigens which react immunologically with antibodies present in infected patients has provided one diagnostic assay which may use recombinantly produced antigens. See, Houghton et al., European Patent Application publication number 0 318 216 and the related papers Kuo et al., Science, 244:359-361 (1989) and Choo et al., Science, 244:362-364 (1989).

The infectious agent itself has not been successfully isolated and characterized. See, for example, Fowler, et al., J. Med. Virol., 12:205-213 (1983) and Weiner, et al., J. Med. Virol., 21:239-247 (1987). Mori et al., Lancet, 318 (Aug. 9, 1980), Seto et al., Lancet 941-943 (October 27, 1984), and Bradley et al., J. Med. Virol, 3:253-269 (1979). Bradley et al., Gastroenterology, 88:773 (1985) reported sizing of HCV viral particles with polycarbonate filters. A variation of this technique using a graded series of polycarbonate filters to purify human rotavirus from seeded chimpanzee

blood plasma is more fully described by Miller et al., Proceedings of the 46th Annual Meeting of the Electron Microscopy Society of America, pages 76-77 (August,1988) and Miller et al., Proceedings of the 12th International Congress for Electron Microscopy, pages 308-309 (August, 1990) .

Clearly, there exists a need to isolate and fully characterize the infectious agent associated with HCV. The successful isolation of the infectious agent will provide for further development of additional diagnostic and prognostic assays.

SUMMARY OF THE INVENTION

The present invention relates to an antibody capable of selectively binding with one or more HCV antigen which are associated with the whole virus particle and which produce an immune response in a patient. Preferably, HCV antigens associated with the virus particle are isolated from a mammal selected from the group consisting of chimpanzees and humans.

Both monoclonal and polyclonal antibodies are provided by this invention. Both of these types of antibodies may be prepared according to processes known in the art for generating hybridoma cells from immuno-challenged mammals. Specifically, these antibodies may be isolated from cell lines selected from the group consisting of the hybridoma cell lines H18 C27 and H18 C68 which were deposited on August 15, 1990 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, 20852 under ATCC Nos. HB 10529 and HB 10530, respectively.

This invention also provides a process for isolating an infectious HCV particle in a plasma sample taken from an infected patient which includes clarifying the plasma sample by centrifugation; filtering the clarified plasma through a series of filters having sequentially smaller pore sizes, wherein the pore size of the filters is 3.0, 1.0, 0.8, 0.6, 0.4, and 0.2 microns, respectively; pelleting the filtered plasma through a 10% sucrose solution; and resuspending the pelleted material.

The present process provides resuspended material having infectivity titers up to and about 10^7 CID/ml even though this material contains less than about 1%, and preferably less than 0.1%, of the total pelletable protein of the original plasma sample.

The antibodies of the present invention are useful in an immunological assay for detecting an antigen specifically associated with HCV.

Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description which includes illustrative examples of the practice of this invention.

DETAILED DESCRIPTION

The present invention provides a method to isolate and visualize the agent(s) known to cause human and chimpanzee HCV. Past efforts to immune aggregate a putative virus, raise hyperimmune sera, or clone this illusive agent have been thwarted by the lack of availability of adequately purified virus from blood plasma. Little was known about the physical properties of this infectious agent except that it is probably a

chloroform and B-propiolactone sensitive virus capable of passing through about a 100 nm filter. Attempts by others to determine the bouyant density of the virus have yielded ambiguous results and, until now, there has not been a reliable means for purifying and, at the same time, concentrating the virus. The present invention describes a scheme for purifying and enriching whole, infectious blood plasma and using the isolate to generate antibodies.

Methods for purifying virus particles from blood plasma or cell extracts have traditionally been based on the bouyant density of the virus to be isolated. Both differential centrifugation or density gradient methods, such as isopycnic or rate zonal banding, collect all classes of cellular debris having densities similar to the virus in a given centrifugation run. Such debris varies considerably in overall size and the presence of the debris generally makes it difficult to detect or identify virus particles using electron microscopy.

Sizing of virus particles by ultrafiltration has been used as an aid in virus classification. As previously discussed, Bradley et al. report the use of polycarbonate filters of defined pore size to gain some information about the size of the HCV particle. This invention provides an HCV purification scheme based on the sequential passage of blood plasma through a series of polycarbonate filters of decreasing pore size.

To isolate the HCV particles, polycarbonate filters are preferred because, unlike nitrocellulose, polyester or other types of filters which are made of a relatively thick meshwork of interwoven fibers, polycarbonate filters are thin and contain precisely

sized, straight-bore holes. These features limit the loss of particles caused by non-specific absorption or entrapment in the filter matrix associated with nitrocellulose, polyester or other types of filters.

In initial experiments, the ability of a graded series of filters to sequentially reduce the sizes of particles present in whole blood plasma was examined. Nuclepore filters 25 mm in diameter were mounted in Swinlok holders which were, in turn, coupled to 10 cc hypodermic syringes filled with plasma. This set up, however, allowed only a small amount of plasma to successfully pass through a series of filters before the filters plugged. One solution, dilution of the plasma was not pursued because this would only serve to increase the overall amount of handling of plasma and lead to excessive ultracentrifugation steps to concentrate final viral isolate. Ultimately, a small press was used to facilitate the filtration steps.

Table 1 illustrates a preferred scheme to isolate HCV particles. Whole blood plasma was first clarified by low speed centrifugation and then sequentially pressure filtered through 3.0, 1.0, 0.8, 0.6, 0.4 and 0.2 micron Nuclepore filters. The 0.2 micron filtrate was then layered on top of buffered 10% sucrose and pelleted for 4 hours at 105,000 x g. Following pelleting thru 10% sucrose, fine protein was removed. Pellets were resuspended in phosphate buffered saline to yield a 100X stock concentrate and then further diluted as desired. The portion of the final concentrate to be used for infectivity studies was diluted in buffer containing 2% BSA as a cryroprotectant.

TABLE 1

Step #1	Whole Undiluted Plasma (Clarify at 2000 rpm, 20 min)
	↓
Step #2	3.0 Micron Filter
	↓
Step #3	1.0 Micron Filter
	↓
Step #4	0.8 Micron Filter
	↓
Step #5	0.6 Micron Filter
	↓
Step #6	0.4 Micron Filter
	↓
Step #7	0.2 Micron Filter
	↓
Step #8	10 % Sucrose Pellet (105,000 x g for 4 hrs)
	↓
Step #9	Resuspend to 100 x

The partially purified plasma passed easily through the filters and at each step of the purification process 100 microliter aliquots of plasma were prepared for electron microscopic viewing using a method developed by Miller (Miller, M.F., Electron Microscopy in Biology, Vol.2, J.D. Griffith, ed., John Wiley and Sons, Inc., New York, N.Y., 1982, pp. 305-339) which employs the commercially available Beckman Airfuge ultracentrifuge and Em-90 electron microscopy particle counting rotor. Briefly, 100 microliters of starting plasma and plasma derived from various stages of the polycarbonate filter purification process is sedimented at 100,000 x g in the EM 90 rotor onto 5mm^2 Millipore filter supports. Supports with sedimented particulates are fixed with 3% glutaraldehyde followed by osmium tetroxide, dehydrated and embedded in epoxy resin. Embedded filter supports are then thin-sectioned and stained with uranyl acetate followed by lead citrate.

At low magnification, the appearance of whole, unfiltered chimpanzee blood plasma prepared for EM viewing showed large quantities of fine proteinaceous material, clumps of fibrin and a variety of sizes of particulates. At higher magnification, the appearance of a 0.2 micron filtrate of chimp plasma showed that all particulates larger than 0.2 micron were removed by filtration and that the fine protein was removed following the 10% sucrose pelleting step. These results clearly demonstrated the efficacy of the purification scheme for selectively eliminating larger particulates and removing fine protein.

To evaluate virus recovery using this filtration procedure, whole chimp plasma was seeded with approximately 10^{10} particles per ml of highly purified

human rotavirus. Rotavirus, although non-blood born, is about 65-70 nm in diameter and is relatively easy to recognize and count in thin sections of impure samples. Seeded plasma samples derived from various stages of purification were processed for EM viewing as described above and the virus particles were counted. Samples were also assayed for total pelletable protein using a sensitive spectrophotometric protein assay. It was found that only a fraction of a log of virus was lost during purification but there was an overall 250-fold reduction of total pelletable protein.

The filtration procedure may be used to provide concentrated plasma needed to isolate the infectious agent associated with HCV. One method to isolate HCV, immune aggregation electron microscopy, may be performed by mixing appropriate concentrations of the plasma concentrate and a suitable antibody and visualizing resultant immune aggregates using a transmission electron microscope. The antibody is generally pre-centrifuged at about 40,000 RPM for 10 minutes to remove undesirable background particulates found in most sera. When working with unknown antibody titers it may be necessary to experimentally determine optimum dilutions using a systematic trial and error approach. Too dilute or concentrated an antibody solution will result in incomplete or no aggregation.

The preferred aggregation procedure to study a variety of hepatitis viruses includes: a) mixing 20 microliters of well dispersed plasma concentrate with 50 microliters of PBS, pH 7.2, and 20 microliters of antibody, b) incubating the mixture for one hour at room temperature and then overnight at 4°C, c) pelleting the immune aggregates for 30 minutes at 20 psi in a Beckman

Airfuge Ultracentrifuge, d) discarding the supernatant and resupsending the pellet in 20 microliters of water, e) applying 5 microliter droplets of suspension to Formvar coated EM specimen grids, f) blotting away the droplet with absorbent paper, and g) negatively staining adherent particulates with 2% phosphotungstic acid. Specimen grids are then carefully surveyed for immune aggregates with a transmission electron microscope at magnifications ranging from 10 to 45,000 x.

The monoclonal antibody or other monoclonal antibodies derived using the described isolated HCV preparations may be used in diagnostic tests for antigens of HCV separately, in combination with each other or in combination with human, chimpanzee, rabbit or other mammalian polyclonal antibodies.

Several alternative assay configurations may be used with the antibodies of the invention. For example, the antibody may be bound to a suitable solid support, contacted with a sample or aliquot containing HCV, and then contacted with a second labeled antibody which may be developed to provide a detectable signal. An assay which uses antibody of the present invention which has been labeled with a signal generating moiety is an alternative procedure. In addition, the labeled antibody may be used in a competitive antibody assay. In this type of assay, the labeled antibody is incubated with the sample or aliquot in the presence of a solid support which is coated with an antigen containing the immunodominant epitope of the antigen contained in the sample. Antibodies to HCV present in the sample will compete for binding with the labeled antibody and with the bound antibody. Therefore, a sample containing an antibody to HCV will produce a detectably lower signal

compared to the signal produced in an assay which has no added labeled antibody.

The purified virus prepared by the process of this invention may also be used to characterize unknown HCV antigens. The purified virus used to generate an antibody may be labeled with radioactive iodine using both Chloramine T and Bolton Hunter reaction conditions according to established procedures before and after solubilization of the virus with detergents. The antibodies may be used in an immunoprecipitation experiment where the antibody is allowed to react in solution with the solubilized labeled virus. After this incubation period, several micrograms of fixed Staphalococcus aureus (Staph A) cells are incubated with the antigen-antibody mixture. Immunoglobulin receptors on these cells will bind the antigen-antibody complexes as well as free immunoglubulins. The bound cells are pelleted by centrifugation and washed repeatedly to remove unadsorbed labeled antigen. The Staph A cells with the adsorbed complexes are then dissociated by heating to 100°C in a solution containing 2-mercaptoethanol, detergents and buffer and then electrophoresed on polyacrylamide slab gels. After electrophoresis the gels are fixed, treated with chemicals known to enhance detection of radioactivity, dried and exposed to x-ray film at -70°C for an extended period of days, weeks or even months. Upon development of the x-ray film bands will appear on the film at positions where the radiolabeled antigens migrated in the gel based on their molecular size. By comparing the positions of these bands to those of radioactive standards of known molecular weight, the molecular size of the precipitated viral antigen can be determined. The information may be valuable in the determination of the

size of the actual gene products of HCV which are unknown at this time.

Variations of this technique may also be utilized to identify labeled viral antigens produced in cell lines capable of supporting viral replication. In essence, this technique can be used to screen various cell lines for the presence of viral replication and therefore is useful as a tool for establishing a tissue culture system for hepatitis C diagnosis.

It is expected that monoclonal antibodies according to the invention will be useful in assays for detecting HCV infection and infectious agents in bodily fluids, tissues, experimental reagents and fluids.

The monoclonal antibodies of the invention may also be used to isolate antigens specifically associated with HCV by affinity chromatography. Such immunopurified antigen may be used to immunize non-primate species such as goats and rabbits, in order to obtain antibodies having multiple epitope specificities. Alternatively, the isolated antigen may be used to immunize mice or rats allowing for preparation of hybridoma cell lines capable of producing additional monoclonal antibodies.

Antibodies generated according to the present inventions will allow the characterization of antigens specifically associated with HCV infection and infectious agents. In this regard, standard methods such as analytical and preparative gel electrophoresis may be employed to determine the molecular weight of antigens reactive with the antibodies of this invention. Reactive isolates so obtained may be subjected to further characterization through use of various selective agents

including reducing agents, proteases, lipases, glycosidases, and the like. Antibodies which react with HCV viral antigens may be utilized in a variety of immunoassays including competitive and non-competitive immunoassays, radioimmunoassays, ELISA, EIA and the like.

The following examples illustrate the practice of this invention and are included only for the illustrative purposes. These examples are not intended to limit the scope of the invention or the scope of the appended claims.

Example 1 describes a process to purify HCV from an infected chimpanzee, Example 2 describes the production of a monoclonal antibody, and Example 3 describes a diagnostic test.

EXAMPLE 1
Purification of Hepatitis C Virus
Using Polycarbonate Filters

Chimpanzee blood plasma having an infectivity titer of 10^5 CID/ml (chimp infectious doses/milliliter) of HCV was purified as follows. Raw plasma was first clarified by low speed centrifugation for 20 minutes at 1000Xg in a tabletop centrifuge to remove large cellular debris and then sequentially pressure filtered through polycarbonate membrane filters having pore sizes of 3.0, 1.0, 0.8, 0.6, 0.4 and 0.2 micron diameter. Particulates remaining in the 0.2 um filtrate were then pelleted by ultracentrifugation through phosphate buffered 10% sucrose for 4 hours at 105,000 X g. This step removed fine proteins and served to concentrate the virus. Pelleted virus was resuspended in phosphate buffered

saline to yield a final 100X concentrate. The portion of the concentrate to be used for infectivity studies was diluted with buffer containing 0.2% Bovine Serum Albumin as a cryoprotectant.

Electron micrographs showing sedimented and thin-sectioned particulates derived from the purified chimpanzee plasma were compared to the similarly prepared raw starting plasma. Using a sensitive spectrophotometric assay it was found that total pelletable protein was decreased more than 250 fold. When purified virus product was inoculated into chimpanzees it was found to be infectious (100% of titer recovered as evidenced by the characteristic elevation of serum enzymes and appearance of hepatocyte ultrastructural alterations). The purified product was used to generate monoclonal antibodies.

EXAMPLE 2

Production of a Monoclonal Antibody

Six female Balb c/J mice were selected on the basis of low reactivity of their sera by immunofluorescent microscopy on liver sections from hepatitis C infected chimpanzees. These selected mice were used for inoculation with purified HCV. Pre-inoculation bleeds were collected from all mice prior to inoculation with virus. Purified virus obtained by the procedure described in Example 1 was used as the inoculum. Two mice each were inoculated according to the following regimen: intraperitoneally (IP) with 0.1 ml of the purified virus stock, 0.2 ml of a 1/10 dilution of the virus stock in normal saline, or 0.2 ml of a 1/100 dilution of the virus stock in normal saline. Blood samples were collected by eye bleeds at day 21. On day

35, all mice were boosted (IP) with the same inocula and dose as their primary immunization regimen. Blood samples were again collected by eye bleeds on day 49. On day 129, all mice were again boosted, (IP) as before. Blood samples were collected by eye bleeds on day 141. On day 199, one mouse from each dosage category was inoculated intravenously (IV) with 0.1 ml of a 1/10 dilution of the purified virus stock. Three days later, the IV boosted mice were sacrificed and their spleen lymphocyte cells were isolated and fused to SP 2/0 mouse myeloma cells following the procedure of Kohler and Milstein (Kohler G., and Milstein C., Nature, 256:495-497, 1975). On day 241 the remaining 3 mice were boosted IV as previously described, and sacrificed three days later and their spleen lymphocytes were isolated and fused to SP 2/0 cells.

Supernates from hybridoma cells resulting from the fusions of mouse lymphocytes with SP 2/0 cells were screened by an enzyme immunoassay (EIA) using synthetic peptides representing sequences from the Chiron C-100 clone (described in European Patent Application publication number 0 318 216 published May, 31, 1989) as the antigen coated onto a solid polystyrene bead. A hybridoma cell line producing a positive signal in the peptide EIA was cloned by limiting dilution. Monoclonal cell lines producing IgM class antibodies specific for an epitope contained within the Chiron C-100 sequence were isolated. Two of these cell lines, H18 C27 and H18 C68, were expanded in cell culture and frozen under liquid nitrogen and are now on deposit with the ATCC under accession nos. HB 10529 and HB 10530, respectively.

EXAMPLE 3

A Diagnostic Test

The monoclonal antibody prepared according to the procedure of example 2 is used as an antigen capture reagent bound to a solid phase polystyrene bead support. An aliquot of bodily fluid such as serum, plasma or cerebral spinal fluid from a patient is incubated with the antibody bound to the bead. During the incubation, an antigen or whole virus binds to the solid phase. After incubation, unbound antigens are washed away from the solid support. In the second step of the assay, the same antibody, or another monoclonal antibody binding to a different epitope of an HCV antigen, or a polyclonal antibody, coupled to horseradish peroxidase is incubated with the bead. This labeled antibody will bind to antigen trapped on the solid phase forming an antibody-antigen-antibody sandwich. Excess peroxidase labeled antibody is washed away and the bead is exposed to a suitable enzyme substrate such as OPD (orthophenylene diamine). The resulting colored product is detected spectrophotometrically in amounts directly proportional to the amount of antigen which was present in the original aliquot.

While the present invention has been illustrated in terms of specific methods and compositions, it is understood that variations and modifications will occur to those skilled in the art upon consideration of the present invention.

WHAT IS CLAIMED IS:

1. An antibody capable of selectively binding with one or more hepatitis C virus antigens which may be associated with the whole virus particle and which produces an immune response in a mammal selected from the group consisting of chimpanzees and humans.

2. The antibody of claim 1 wherein said antibody is a monoclonal antibody or a polyclonal antibody.

3. The antibody of claim 1 produced by a hybridoma cell.

4. The antibody of claim 3 produced by the cell lines selected from the group consisting of ATCC Nos. HB 10529 and HB 10530.

5. A cell line capable of producing an antibody which selectively binds to an antigen associated with a hepatitis C virus selected from the group consisting of ATCC Nos. HB 10529 and HB 10530.

6. A process for isolating an infectious hepatitis C virus from a plasma sample taken from an infected patient comprising the steps of:

a) clarifying the plasma sample by centrifugation;

b) filtering the clarified plasma through the following series of polycarbonate filters having sequentially smaller pore sizes, wherein the sequential pore size of the filters is 3.0, 1.0, 0.8, 0.6, 0.4, and 0.2 microns, respectively;

c) pelleting the filtered plasma through a 10% sucrose solution; and

d) resuspending the pelleted material.

7. The process of claim 6 wherein the infectivity titer of the resuspended material is greater than about 10^5 CID/ml, and wherein the resuspended material comprises less than about 1% of the total protein of the plasma sample.

8. In a process for producing an antibody generated by an immune response to a hepatitis C virus isolated from a plasma sample taken from an infected patient, the improvement comprising:

generating an antibody to purified plasma prepared by the process of claim 6 wherein the purified plasma has an infectivity titer greater tan about 10^5 CID/ml.

9. In an immunological assay for detecting an antigen specifically associated with hepatitis C virus, wherein the assay is based on a selective immunological reaction with an antibody specific for the antigen, the improvement comprising:

employing an antibody of claim 1 wherein the antibody is a monoclonal antibody.

10. The assay of claim 9 wherein the monoclonal antibody is produced by the cell lines selected from the group consisting of ATCC Nos. HB 10529 and HB 10530.

11. A process for isolating an infectious hepatitis C virus from a plasma sample taken from an infected patient comprising the steps of:

a) clarifying the plasma sample by centrifugation;

b) filtering the clarified plasma through a series of filters having sequentially smaller pore size;

c) pelleting the filtered plasma through a 10% sucrose solution; and

d) resuspending the pelleted material.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/07571

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

IPC(5) : C07K 15/00; C12N 5/00,7/02,15/00; C12Q 1/70

US Cl.: 530/387; 435/240.26, 239,5

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
USCl.	530/387; 435/240.26, 239, 172.2,5

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

APS DIALOG

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	EP,A, 0,318,216 (Houghton et al.) 31 May 1989, see page 47, lines 40-52 and Claim 10.	1-3,9 4-8,10,11
Y	G. W. Bailey, "Proceedings of the 46th Annual Meeting of the Electron Microscopy Society of America," published 1988 by San Francisco Press (San Francisco), see pages 76-77.	6-8,11

* 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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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
03 February 1992	10 MAR 1992
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Donna C. Wortman tf

Form PCT/ISA/210 (second sheet) (Rev.11-87)