

The present invention relates to the isolation and characterisation of a viral agent which plays a role both in epidemic and sporadic or posttransfusional hepatitis non-A, non-B.

5 Viral hepatitis can be caused by the hepatitis A virus (HAV), by the hepatitis B virus (HBV) and by an unknown number of hepatitis non-A, non-B (NANB) viruses which have not yet been identified. In the developed countries in which the prophylaxis of HBV is effective,
10 the NANBs are currently the most important cause of infectious hepatitis leading to hospitalisation. This disease is thought to involve at least three viruses: two are transmitted by contaminated blood and contaminated blood products; for the third, which is responsible for
15 epidemic hepatitis NANB, the inventor has been able to establish that it is also responsible for 50 to 60 % of the hepatitis cases requiring hospitalisation, which are reported in western countries and its mode of transmission is unknown. In the developing countries, NANB
20 also appears to be associated with large scale epidemics linked to a faecal-oral mode of transmission for which water is the vehicle. Currently, five cases of this type of epidemics have been reported since 1980: in India, Siberia, Nepal, Algeria and recently the Ivory Coast. In
25 all of these cases, a high mortality rate was observed among pregnant women.

Numerous research studies have been carried out in order to isolate a causative agent of hepatitis NANB and to obtain antigenic preparations which allow its
30 diagnosis: there may be mentioned for example European Patent Application 68 485 in the name of EISAI CO LTD, PCT Application 820 330 and European Application 61 974 in the name of C. TREPO, PCT Application 82 02774 in the name of BAXTER TRAVENOL LABORATORIES.

35 Our current knowledge regarding the viruses which are responsible for hepatitis NANB is discouragingly vague and is limited to clinical and epidemiological data. Filterable agents have indeed been isolated by inoculating blood or blood products obtained from

subjects suffering from posttransfusional hepatitis NANB to chimpanzees and infectious blood products are available. In the case of epidemic hepatitis NANB, very few experimental infections have been carried out: the
5 infection was found to be transmissible to Marmosets (*Saguinus mystax*), to Macac monkeys (*Macacus cynomolgus*) and to a human volunteer and, recently, to African green monkeys (*Cercopithecus aethops* and *Erythrocebus patas*), but no particular infectious preparation was proposed.

10 In the case of posttransfusional hepatitis, in spite of the plethora of reports on NANB antigen-antibody systems, ultrastructural modifications and particles analogous to viruses, which are all potentially promising, no causative agent and no system or virus which
15 meets the serological criteria accepted for a specific causative association was identified prior to the work which resulted in the present invention. In the case of epidemic hepatitis, particles analogous to viruses, spherical in shape and 27 nm in diameter have been
20 visualised in very rare faeces and the immunological characterisation was poorly performed by agglutination under an electron microscope. Viral replication has never been obtained in cell cultures for any NANB virus at all.

SARTHOU et al. [Ann. inst. Virol. 137 (2) 225-252
25 (1986)] describe the experimental infection of monkeys with faecal extracts from patients suffering from hepatitis NANB; the infected monkeys produce a class IgM antibody which recognises a specific antigen in the stools of patients suffering from hepatitis NANB.

30 The subject of French Patent Application No. 86 05437, in the name of INSTITUT PASTEUR, and which mentions Mr PILLOT as inventor, is an appropriate reagent which permits the detection of epidemic viral hepatitis NANB and a diagnostic process using this reagent; the
35 reagent conforming to this Patent Application consists of anti-NANB IgMs isolated from the sera of monkeys artificially infected with faecal extracts from patients known to be suffering from epidemic hepatitis NANB, which anti-NANB IgMs are advantageously attached to solid supports

such as especially PVC plates.

The pursuit of its research studies based on the invention which is the subject of the abovementioned Patent Application made it possible for the Inventor to achieve the following objectives:

- 1) he infected monkeys and he used their antibodies (Ac), as well as the Ac of convalescent humans, to develop an ELISA test for the specific detection of antigens (Ag) associated with the virus and for the detection of anti-viral Ac;
- 2) the Inventor succeeded in replicating the virus in a continuous liver cell line with a good production of antigenic material;
- 3) he succeeded in visualising viral particles in purified preparations of faecal samples and in infected liver cell lines;
- 4) he was able to establish the involvement of the same causative agent in both epidemic and sporadic viral hepatitis NANB;
- 5) by examining the role of the epidemic NANB agent in sporadic hepatitis, the Inventor was able to verify that the said agent can be disseminated not only by water, but can also be transmitted by routes other than water and especially by the blood transfusion route.

The objective of the present invention is therefore the identification and the characterisation of a new viral agent, as well as an NANB antigen (Ag) associated with this new viral agent and a method for the diagnosis of epidemic, sporadic as well as posttransfusional hepatitis NANB, by detection of the Ag associated with the NANB virus, using the antibodies (Ac) which are the subject of the abovementioned INSTITUT PASTEUR Patent Application or by investigation of the patient's Ac, using the virus or its fractions adsorbed onto a suitable support.

The objective of the present invention is also to provide immunological diagnostic reagents which can be used in the method for the diagnosis of hepatitis NANB

conforming to the present invention as well as a ready-for-use kit for carrying out the said diagnostic method.

The subject of the present invention is a purified viral agent associated with sporadic, epidemic and posttransfusional viral hepatitis NANB, characterised in that it can be obtained from the faeces of patients suffering from hepatitis NANB and in that it is in the form of two main types of spherical viral particles associated with the same viral antigen:

10 a) spherical particles of about 75 nm in diameter whose density in a CsCl gradient is between 1.28 and 1.32 g/cm³ and which sediment in sucrose gradient fractions containing between 40 and 41.5% (w/v) of sucrose,

b) spherical particles from 25 to 30 nm in diameter whose density in a CsCl gradient is between 1.12 and 1.19 g/cm³ and which sediment in sucrose gradient fractions containing between 26 and 28% (w/v) of sucrose.

The subject of the present invention is furthermore a process for isolating a viral agent which plays a role in epidemic, sporadic and posttransfusional viral hepatitis NANB, which is characterised in that a biological extract containing the said viral agent is centrifuged in a CsCl gradient or in a sucrose gradient, and in that the said agent is collected in the CsCl gradient fractions whose density is between 1.29 and 1.32 g/cm³ or in the sucrose gradient fractions containing between 40 and 41.5% (w/v) of sucrose, in the form of particles of about 75 nm in diameter, and/or in the CsCl gradient fractions whose density is between 1.12 and 1.19 g/cm³, or in the sucrose gradient fractions containing between 26 and 28% (w/v) of sucrose, in the form of particles from 25 to 30 nm in diameter.

According to one embodiment of the process for isolating the abovementioned viral agent, the centrifugation treatment advantageously consists of a centrifugation operation at about 5000-8000 g for about 10 minutes.

According to another embodiment of the process for isolating the abovementioned viral agent, prior to

the abovementioned centrifugation and filtration operations, the faecal extract is subjected to a treatment by sonication or to a chemical dissociation treatment.

5 The subject of the present invention is also another process for producing the abovementioned viral agent, which is characterised in that liver cells or continuous liver cell lines are inoculated with a faecal extract from a patient suffering from hepatitis NANB, or with a liver extract from a monkey (preferably Saimiri)
10 inoculated with a faecal extract from patients suffering from hepatitis NANB, and cultured until the culture becomes confluent, from which a subculture is prepared, which is centrifuged after several passages in order to recover the desired viral agent.

15 According to an advantageous embodiment of this process, the centrifugation pellet is taken up in an appropriate buffer so as to be subjected to a sonication treatment which releases the NANB Ag into the supernatant.

20 Strains of the viral agent conforming to the invention, isolated as described in the preceding text and below, and characterised as indicated later, were deposited with the COLLECTION NATIONALE DE CULTURE DE MICROORGANISMES held by INSTITUT PASTEUR, on the date of
25 30 October 1986, namely:

- a strain of viral agent isolated from the faeces of a patient suffering from sporadic hepatitis NANB, called "Clamart strain", which was given the deposit number: I-617,
- 30 - a strain of viral agent isolated from the faeces of a patient suffering from epidemic hepatitis NANB, called "Ivorian strain", which was given the deposit number: I-616.

The subject of the invention is also a process
35 for the detection of the antibodies present in the blood of the patient or of the convalescent or in the products used in blood transfusion or in the fractions derived from blood, which have been in contact with the virus which is the causative agent of hepatitis non-A, non-B,

as defined according to the present invention, including the strains I-617 and I-616 and those having an immunological cross-reactivity with the two reference strains, according to this process, the serum of the patient or of
5 the convalescent patient is brought into contact with the purified viral preparation (or with a fraction thereof) as recovered under the conditions mentioned above, after centrifugation and isolation of the band corresponding to a density of 1.30.

10 The said viral preparation is attached for example to the walls of a microplate according to known techniques.

The Ag/Ac complex is visualised by a preparation of anti-human immunoglobulins labelled with a suitable
15 marker, for example an enzyme.

An analogous system can be used to characterise an antibody activity in IgMs of a patient for the purpose of a very early serological diagnosis of the disease by using the anti-human IgMs, or better still by capturing
20 the IgMs of a serum by reacting them with the virus or its fractions and by characterising the Ag attached to the patient's IgM.

The process for the in vitro diagnosis of the presence of the hepatitis NANB virus conforming to the
25 present invention therefore consists in bringing a serum or another biological medium of a patient for whom it is desired to establish the diagnosis, into contact with at least one of the proteins or glycoproteins of the NANB virus and with a viral lysate or with a viral extract,
30 and then in detecting the immunological reaction by the ELISA method or by another immunoenzymatic method or an immunofluorescence method which can measure, directly or indirectly, the immunofluorescence or the immunoenzymatic reaction.

35 Accordingly, the present invention also encompasses labelled viral extracts, whether they are labelled with an enzyme, by fluorescence or whether they are labelled with a radioisotope.

The immunodetection methods comprise, as known:

- the deposition of quantities of a specific extract or of the viral proteins conforming to the invention, into or onto a support such as the wells or a microtitre plate;

5 - the introduction of increasing dilutions of the serum to be diagnosed onto the said support, such as the said wells;

- the incubation of the support such as the abovementioned microplate;

10 - the thorough washing of the support such as the abovementioned microplate by means of a suitable buffer;

- the introduction of labelled, specific anti-human immunoglobulin antibodies into or onto the support such as the wells of the abovementioned microplate, the labelling being carried out using an enzyme chosen from
15 those capable of hydrolysing a substrate in such a manner that the absorption of radiation by the latter is modified at least in a specific band of wavelengths, and

- the detection, preferably in a comparative
20 manner, compared with a control, of the quantity of substrate hydrolysed, both by measuring the hazardous biological product and by the effective presence of the disease.

The subject of the present invention is also a
25 ready-for-use kit - or set - for implementing the diagnostic process (detection of the antibody or of the NANB virus) conforming to the invention, defined above, which kit is characterised in that it comprises:

- an extract or a purified fraction of the viral
30 agent conforming to the invention, which extract or fraction is labelled, for example by a radioisotope, an enzyme or by immunofluorescence;

- anti-human immunoglobulins or a protein A optionally attached to a water-insoluble support such as
35 agarose or latex spheres which are magnetic or otherwise;

- buffers and, if necessary, substrates for visualising the markers.

The characterisation of some of the antigens which enter into the composition of the viral agent

isolated in conformity with the present invention was carried out, in the first instance, on the Clamart strain, the procedure being carried out as described below.

5 The Clamart strain was isolated from stools from a patient suffering from sporadic hepatitis NANB.

 The supernatant of the said stools was centrifuged in a cesium chloride gradient from which the band containing the viral antigen purified as defined above
10 was recovered and its density determined as being 1.30.

 The epitopes recognised in the diagnostic test [sic] defined above are carried by these antigens which can thereby be used in the said test.

 The detection of antibodies by means of this
15 method of detection can be carried out in the blood of patients or convalescents, in the products used in blood transfusions and in the fractions derived from the blood of individuals who have been exposed to the causative agent of hepatitis NANB as defined above, including the
20 strains I-617 and I-616 and those having an immunological cross-reactivity with these two reference strains, especially the strain isolated from patients suffering from posttransfusional hepatitis NANB, whose immunological properties are identical to those of the strain
25 I-617, and which is identified as "H-TEST" viral strain deposited on 5 February 1987 under the No. 87.02.05.02 with the ECACC Collection (Great Britain).

 Although the clinical and epidemiological data suggested that the epidemic form of the hepatitis NANB
30 presented by patients suffering from epidemic hepatitis NANB is caused by a virus different from that which is responsible for the posttransfusional hepatitis NANB transmitted by parenteral injection, in conformity with the present invention, the Inventor was able to isolate
35 from patients suffering from posttransfusional hepatitis NANB a new NANB virus strain whose immunological properties are practically identical to those of the strains deposited on 30 October 1986 under the Nos. I-616 and I-617 with the CNCM of Institut Pasteur. Very sensitive

serological tests for the presence of the HAV and HBV viruses made it possible to exclude these two viruses as aethiological agents and similarly to exclude other hepatotropic viruses such as Cytomegalovirus, Epstein-
5 Barr virus and yellow fever virus.

The subject of the present invention is furthermore a new purified viral agent which plays a role in the sporadic, epidemic and posttransfusional forms of hepatitis NANB. This virus differs from the HAV and HBV viruses
10 in relation to the antigenic homology of its proteins as genetic material.

The compositions prepared in conformity with the present invention (purified viral antigens, recombinant proteins obtained by expressing the NANB virus in prokar-
15 yotic or eukaryotic cells or synthetic peptides deduced from the genome sequence) can be used for the diagnosis of hepatitis NANB or for appropriate vaccination to induce the formation of a protective immune response after administration to the host.

The present invention encompasses all the compositions of this type which contain an antigen exhibiting immunological properties equivalent to those of the Clamart (CNCM I-617) or Ivory Coast (CNCM I-616) NANB virus. Indeed, two proteins or antigens are con-
20 sidered equivalent within the framework of the present invention when they are capable of being recognised by the same antibodies. The products expressed by corresponding sequences of the coding genetic material of the corresponding genetic sequences are accordingly included
25 within the scope of such equivalent antigens.

The present invention also encompasses the sera which may be produced from animals by inoculating them with the NANB virus by means of the abovementioned compositions. In particular, the present invention
35 encompasses the polyclonal antibodies specifically directed against each of the NANB virus antigens. It also encompasses the monoclonal antibodies capable of being obtained by appropriate methods and directed more specifically against the NANB virus antigens.

In conformity with the present invention, the use of the virus of its lysate or of a fraction of this lysate may be envisaged for the preparation of monoclonal antibodies and the viral lysate or the fraction thereof
5 or the proteins purified from this virus, from the lysate or from its fractions may be used for the detection of antibodies in the serum of patients using conventional tests of the RIPA, ELISA or Western blot (immunoblotting) type.

10 These polyclonal or monoclonal antibodies can be used in a large number of applications among which there may be mentioned the neutralisation of the corresponding antigen or of the total virus. They can be used to detect viral antigens in biological preparations or to purify
15 the corresponding antigens.

The present invention also encompasses any equivalent hepatitis NANB virus which exhibits the same immunological characteristics. The work carried out by the Inventor has demonstrated the immunological relation-
20 ship between the antigenic materials isolated from faecal extracts of patients suffering from epidemic, sporadic and posttransfusional hepatitis NANB respectively.

That is how the Clamart type strain (deposited under the No. I-617 with CNCM and also deposited with the
25 ECACC Collection under the No. 87.02.05.02) was isolated from the stools of a patient who had an acute hepatitis NANB duly characterised as transfusional hepatitis NANB. The stools contained the antigen conforming to the invention, which is also characterised, in conformity
30 with the invention, as epidemic (Ivory Coast) and sporadic (France) hepatitides.

One antigen associated with the hepatitis NANB virus was characterised for 15 days during the acute phase of the disease. Other similar cases were observed
35 with characterisation of the same antigen in their stools and isolation of a virus exhibiting immunological characteristics identical to the viruses I-616 and I-617.

In addition to the preceding arrangements, the invention also comprises other arrangements which will

emerge from the description below.

The invention will be more clearly understood with the aid of the additional description below which refers to exemplary embodiments of the process which is
5 the subject of the present invention.

It should be clearly understood, however, that these exemplary embodiments are given solely as illustration of the subject of the invention and do not constitute in any manner a limitation thereof.

10 During a first stage, two *Cercopithecus aethiops* monkeys and one *Erythrocebus patas* monkey were inoculated orally with 2 ml of a mixture of a 10 % aqueous extract of faeces from Ivorian patients presumed to be suffering from epidemic hepatitis NANB, which were collected during
15 the first few days following the appearance of the symptoms. The mixture was previously centrifuged and filtered through 0.22 μ m Millipore® filters to remove bacteria and parasites. The serum samples and the faecal samples from these monkeys were collected, on the one
20 hand, before administration of the faecal extract and, on the other hand, on the twenty-seventh day after the inoculation. The liver functions of these monkeys were not studied; these animals did not have apparent clinical manifestations. One *Saimiri* monkey was also inoculated
25 under the same conditions with 0.5 ml of a faecal extract obtained from an Ivorian patient different from those used for the African green monkeys. A second *Saimiri* monkey which had been inoculated three months earlier with a faecal hepatitis A virus preparation and sero-
30 converted by means of anti-HAV IgM antibodies on day 29, was inoculated intravenously with 0.5 ml of the same filtered faecal sample. The two *Saimiri* monkeys were bled twice weekly in order to carry out biochemical and serological examinations and their faeces were examined
35 daily. The two *Saimiri* monkeys died on day 28 without exhibiting specific symptoms. None of the monkeys exhibited convincing biochemical proof of hepatic dysfunction and the post-mortem histological examination carried out showed a congestion of the liver without

lesion of the parenchyma. The sera from these five monkeys were fractionated in 0.1M Tris-NaCl buffer pH 8, over "Sephacryl" S 300" and the fractions containing IgMs and IgGs were collected. They were used as receptor antibodies in an immunoenzymatic test. IgM was chosen for its high capture capacity and in order to avoid false positive reactions due to a rheumatoid factor or a rheumatoid factor analogue which has been recognised to stimulate the NANB antigen.

Indeed, IgG may also be used although its background is slightly higher than that of IgM and it has a lower specificity for certain preparations.

To reveal the binding of the faecal antigen onto the antibody in a solid phase, the IgGs obtained from sera of convalescents were fractionated by chromatography on DEAE-triacryl, after $(\text{NH}_4)_2\text{SO}_4$ precipitation. The IgGs were conjugated with β -galactosidase or with peroxidase. Labelled IgGs were used for the detection of the antigen: one IgG preparation obtained from an Ivorian patient convalescing from an epidemic NANB hepatitis; one IgG preparation collected from an Algerian patient who recovered during the Medea epidemic in 1981-1982; finally the IgG from a Cercopithecus monkey inoculated experimentally. The IgG preparations from the three sources gave equivalent results. For each monkey, preinoculation IgM in a solid phase was used as control for postinoculation IgM specificity. Non-immune labelled human IgG was used as control for Ag labelling. The faeces of inoculated monkeys were examined to establish the presence of an Ag presumed to be NANB in the ELISA test. An antigenic activity was detected in the faeces of both Saimiri monkeys (cf. Table 1). The application of a sonication treatment made it possible to increase the quantity of antigen detected and increased the sensitivity of the method. The Ag appeared from the fourth day after the inoculation and persisted up to the thirteenth day.

Table 1 shows the NANB Ag detected in the faeces of monkeys experimentally infected with faecal extracts obtained from patients suffering from epidemic NANBH

[sic].

The detection was carried out as follows, using the ELISA technique: NUNC[®] immunoplates were coated with 100 μ l of 50 μ g/ml of IgM fraction, collected 27 days after inoculation, in phosphate buffered saline (PBS), for one hour at 37°C and overnight at 4°C. After several washes, the plates were covered with 200 μ l of PBS containing 1% of bovine serum albumin and 0.1% of Tween[®] 20, for three hours at 37°C. After washing, 100 μ l of the faecal extracts (1/10 V/V), filtered over an element with a porosity of 0.22 μ m, were incubated over the IgMs of the coating, at 37°C for one hour and then overnight at 4°C. After several washes, 100 μ l of immune monkey IgG, labelled with peroxidase at 5 mcg/ml in 1% of BSA + 1% of Tween[®] 20 in Tris sodium buffer, pH 7.6 (TNB) containing 0.1 % NaN₃, were used for the detection of Ag. After incubating for one hour at 37°C and several washes, the reaction was visualised by means of 100 μ l of ortho-phenylenediamine in 0.005 M citric buffer pH 5.6 with 1 μ l/ml H₂O₂. The results obtained are expressed by the ratio between the studied sample and the mean of five negative samples (P/N). The background of the negative samples was of the order of 0.04-0.06 in optical density (OD) at 492 nm. It is higher if NaN₃ is omitted, but in this case, smaller quantities of reagent should be used in the reactions. The samples whose P/N ratio 2.1 [sic], were considered as positive. The faeces (1/10 weight/weight) which were examined were of two types: those which were subjected to a sonication treatment and those which were not subjected to such a treatment; the sonication treatment applied lasts for three minutes at 4°C, with an output intensity of 4 with the BRENSON apparatus for volumes of 2 to 3 ml before centrifugation and filtration through an element with a porosity of 0.22 μ m. The positive results are underlined in Table 1. The faeces of a Saimiri monkey which received a faecal extract from a patient suffering from a non-infectious liver disease were used as control and constantly gave negative results.

TABLE 1

Number of days after inoculation	P/N without sonication treatment		P/N with son- ication trea- tment	
	Monkey No.1 (intravenous inoculation)	Monkey No. 2 (oral inocu- lation)	Monkey No.1	Monkey No. 2
0	1.15	1.14	1.18	1.07
4	<u>2.47</u>	1.60	<u>5.10</u>	0.79
7	<u>6.48</u>	<u>3.28</u>	<u>8.78</u>	<u>5.52</u>
9	3.64	<u>2.24</u>	<u>4.72</u>	<u>5.82</u>
11	1.12	1.40	1.95	5.20
13	1.30	1.46	<u>2.40</u>	0.80
15	1.51	1.68	0.93	0.90
19	1.53	1.51	1.20	0.80

A second stage consisted in studying faeces from patients suffering from a presumed acute hepatitis NANB, both in its epidemic form (Ivorian patients) and in its sporadic form (French patients). The diagnosis of hepatitis NANB was established in conformity with the conventional immunological criteria for the three types of infections caused by hepatitis viruses (A,B, delta): presence or absence of anti-HAV IgM and IgG for the infection caused by the A virus; HBsAg, anti-HBc and anti-HBc IgM, anti-HBs, for the infection caused by HBV; the delta and anti-delta Ag for the infection caused by HDV, where appropriate. For the Ivorian patients, yellow fever markers were investigated. All the patients were also investigated for the infection caused by Cytomegalovirus by immunocapture of IgM antibodies, and for the infection caused by Epstein-Barr virus by detection of anti-VCA IgM in the immunofluorescent spots. The Ag associated with NANB was detected both in the epidemic cases and in the sporadic cases (cf. Table 2). No Ag was detected in 90 control extracts from European patients having other gastrointestinal disorders. In most cases, these results were confirmed several times using various

IgMs of IgGs in a solid phase and using various labelled IgGs. The same positive samples were studied using preimmune IgM covering one plate and no antigen was detected. It follows that the Ag detected appears to be
5 closely linked to the NANB virus.

Table 2 below illustrates the detection of NANB Ag in the faeces of patients suffering from epidemic (Ivory Coast) and sporadic (France) hepatitis NANB. 90
10 faeces from patients suspected of gastrointestinal infections were used as controls. All the samples presumed to be NANB and 17 of the 90 controls were subjected to sonication treatment. Human antibody (Ac) labelled with peroxidase or β -galactosidase was used as Ag marker.

TABLE 2

	Faecal samples from Ivory Coast: epidemic cases	Faecal samples from France: sporadic cases	Controls from France
15 NANB AG positive	17	7	0
NANB AG negative	44	10	90

To verify that the detected Ag is indeed closely
20 linked to the NANB virus, during a third stage, continuous cell lines of hepatocytic origin, PLC/PRF 5 and HEP G2, and normal human fibroblastic cells MRC 5, were inoculated in DULBECCO's medium containing 10 % foetal calf serum with a faecal extract from a patient suffering
25 from hepatitis NANB. After the culture had become confluent, a subculture was prepared after addition of EDTA-trypsin. In some cases, toxic substances present in the faeces cause the premature death of the cells during the first week, while other faeces which are non-toxic for
30 the cell lines bring about the death of the cells after several passages. This specific lethal effect was observed only with the cell line PLC/PRF 5 but was not

observed with the cell line HEP G2 and with the cells MRC 5. After treating the cells by sonication and filtration over a porosity of 0.22 μ m, NANB Ag was detected in the supernatant of infected PLC/PRF 5 but not in the supernatant of HEP G2 which were infected under the same conditions.

For liver extracts of inoculated monkeys, no cell toxicity was observed after inoculation and the PLC/PRF 5 cells exhibited an antigenic positivity in the same manner as in the case of faecal samples (cf. Table 3).

Table 3 illustrates the detection of NANB Ag in the hepatoma cell line inoculated with a faecal extract from an Ivorian patient suffering from epidemic hepatitis NANB and with a liver extract from an infected Saimiri monkey (No. 1).

The liver extract was prepared in PBS (10% weight/weight) and homogenised by means of "Ultraturax". After centrifugation, the pellet was resuspended in 4 times its volume of TNB and subjected to a sonication treatment in the presence of 0.005 mg/ml of phenylmethylsulphonide chloride (PMSF) [sic].

A liver extract from a Saimiri monkey inoculated with a faecal extract from patients suffering from a non-infectious liver disease was used as control.

After three passages, the inoculated cell cultures were centrifuged. The pellet was taken up in TNB and after addition of PMSF, the cells were subjected to a sonication treatment. The figures indicate the P/N ratio.

TABLE 3

Hepatoma cell line	Cells inoculated with faecal extract from an Ivorian patient	Cells inoculated with liver extract from an infected Saimiri monkey	Cells inoculated with liver extract from a control Saimiri monkey	Non-inoculated cells
PLC/PRF 5	8.19 *	4.39	2	1.01
HEP G2	1.62	1.14	2	1.07

* After dilution to 1/100 P/N: 6.29.

It seems to emerge from preliminary immunofluorescence labelling trials that only one cell out of about 50 is infected under the experimental conditions.

The object of a fourth stage was to purify the virus-associated antigen obtained from faecal samples of various origins. Three antigen distribution patterns were observed and are represented in the appended Figure 1 which shows that in freshly used samples, the antigenic activity appears in the form of a band at a density of 1.29-1.32 g/cm³. Some of the faecal samples stored at + 4°C for a few months, or fresh samples which had been subjected to a sonication treatment, had an antigenic activity only at a density of 1.12-1.19 g/cm³, while others had an activity at both densities.

Figure 1 represents the antigen distribution obtained in the CsCl gradient after isopycnic ultracentrifugation by carrying out the procedure as follows:

3 ml of faecal samples (10 % in RPMI) were fractionated, after filtration through a filtering element with a porosity of 0.22 µm, in a CsCl gradient with a density of between 1.1 and 1.6 g/ml. The isopycnic centrifugation (48 hours) at 78,000 g was carried out in 12-ml tubes (1,2,3,2, 0.5, 0.5 ml of CsCl with a density of 1.1, 1.2, 1.3, 1.4, 1.5 and 1.6 respectively). 0.6-ml fractions were collected and tested to determine the presence of NANB antigen after two dilutions.

In Figure 1:

A designates the fresh faeces

B and C the faeces preserved or treated by sonication.

5 Antigenic material of low density which appears
under these conditions is presumed to result from the
effect of decomposition by enzymes contained in the
faeces or by a physical treatment. The CsCl fractions
with positive antigenicity were subjected to an ultracen-
10 trifugation in a sucrose gradient ranging from 10 to 60 %
weight/volume and the reactive fractions were studied
under an ultramicroscope. The NANB antigen was found at
40-41.5 % sucrose and predominantly at 26-28 % sucrose.
Seven preparations, obtained from 9 samples with positive
15 NANB antigenicity, contained viral particles: one
obtained from an inoculated Saimiri monkey (the second
monkey was not examined), two obtained from two of the
three patients suffering from acute epidemic hepatitis
NANB (Ivory Coast), four obtained from four of the five
20 patients suffering from sporadic hepatitis NANB (France).
Two types of spherical viral particles were observed:
large-sized particles, of the order of 75 nm in diameter,
which possess a double membrane structure, and smaller
sized homogeneous particles, of about 25-30 nm in diame-
25 ter, without any defined inner structure. A tubular
structure 15 nm in diameter (or in width) and whose
length can be as much as 1 μ m, more or less attached to
the particles, was observed and is represented in the
appended Figure 2. Some images create the impression that
30 the tubules start from the envelope of the large-sized
viral particles. The most characteristic particles were
observed in fresh samples which had not been subjected to
the sonication treatment. Large-sized spherical particles
could be observed for most of the fractions with a
35 density of between 1.29 and 1.32 g/cm³ and in the frac-
tion with 40-41.5 % sucrose for the sucrose gradient,
while only smaller sized particles could be observed in
the CsCl fractions with a density of between 1.12 and
1.19 g/cm³ and in the sucrose gradient fraction

containing 26-28 % sucrose. It has not yet been possible to establish whether the smallest particles, with lower density, represent a released form of the original virus. Six faecal samples with negative antigenicity were also
5 studied simultaneously, in the same manner, in order to be used as controls. No structure having the appearance of particles was found in any of these preparations.

A final stage made it possible to show that the detection of NANB Ag in the faeces demonstrates that the
10 epidemic NANB virus frequently plays a role in sporadic hepatitis NANB. Virus-associated NANB Ag was detected in seven faecal samples among 17 examined patients suffering from acute sporadic hepatitis NANB (cf. Figure 2). Serological examinations confirmed these results: of
15 eight patients for whom sera were available, seven of them had a serum with the anti-NANB antibody.

Figure 2 illustrates the characterisation of viral particles under an electron microscope in the faeces of monkeys infected with NANB and of patients
20 suffering from hepatitis NANB.

After isopycnic ultracentrifugation as described in relation to Figure 1, the positive fractions were mixed, then dialysed against TNB, and they were then subjected to zonal ultracentrifugation in a sucrose
25 gradient (10-60% weight/weight) at 78,000 g, for 5 hours. The positive fractions were mixed, dialysed, concentrated and examined under an electron microscope. A drop of viral preparation was spread on a 200-mesh carbon-coated grid and after absorption onto filter paper, a drop of
30 2 % uranyl acetate in distilled water was added. After two minutes, the grid was wiped with filter paper and studied under a JEM 100 C x II microscope.

The appended Figures 3 and 4 represent sections seen under an electron microscope after uranyl acetate staining of PLC/PRF 5 cells inoculated with a stool
35 extract from a patient suffering from hepatitis non-A, non-B in whose stools the Ag was detected according to the techniques described above.

The viral particles are found in the nucleus and

in the cytoplasm in the form of "crystalline lattices"; the diameter of the particle is very constant: 74 to 75 nm. Figure 3 is a view at magnification $\times 16,000$ and Figure 4 a view at magnification 54,000.

5 Since epidemics of hepatitis NANB are usually observed in developing countries, it appeared useful to examine whether the same viruses play, in these countries, a role in sporadic cases. Accordingly, 28 sera from Moroccan patients suffering from acute sporadic
10 hepatitis NANB were examined to determine whether they had antibodies to the epidemic NANB virus. 68 % of the patients were found to possess anti-epidemic NANB virus antibodies while the same antibodies were detected in only 10 % of the sera of blood donors in the same coun-
15 try.

 Table 4 below illustrates the detection of anti-
bodies specific to the NANB antigen in the sera of patients suffering from sporadic hepatitis NANB. The presence of antibodies was demonstrated by inhibition of
20 their reaction under the conditions described in Table 1 above. The reaction was carried out by incubating 50 μ l of a reference extract, obtained from the faeces of an infected monkey, for one hour at 37°C and overnight at 4°C, with 50 μ l of serum to be examined. All the other
25 stages of the reaction were carried out in the same manner as for the detection of the Ag. The P/N ratio for this reference antigen was of the order of 6-8. 24 undiluted normal human sera were tested in an inhibition reaction against the reference Ag compared with PBS; none
30 of them exhibited an inhibitory action. Subsequently, 50 μ l of a mixture of these sera were used to establish the positivity at 100% of the reaction. The samples tested were considered to be positive when the inhibition was greater than 50%.

35 * The NANB Ag was detected in the faeces of five patients suffering from acute hepatitis NANB; no antibody was found in one of them. In two other patients, the acute disease dated from 9 and 3 months respectively and no Ag was detected in the faeces. The last of the five

patients had previously had a hepatitis NANB with the detectable antibody disappearing after 3 years.

TABLE 4

Source of serum	Blood donors	Laboratory staff	Haemodialysis patients	Sporadic hepatitis NANB
COUNTRY				
MOROCCO	8/78	0/12	1/12	19/28
FRANCE	0/26			7/8

As shown in Table 4 above, haemodialysis or laboratory work involving the handling of human blood does not appear to increase the risk of infection with the NANB virus. The fact that Ac prepared from monkeys infected with an epidemic NANB virus are used for the detection of Ag linked to a sporadic NANB virus shows that they are the same virus.

The different stages of the study which have just been described made it possible to characterise a new viral agent which is involved in hepatitis NANB. This viral agent appears to be responsible for the epidemic form, for the sporadic form as well as for the posttransfusional form of hepatitis NANB.

It is different from the HAV virus:

- 1) An epidemic NANB virus infection was observed in one monkey and in patients exhibiting an anti-HAV immunity;
- 2) The usual markers for acute infection (anti-HAV IgM) were not detected;
- 3) This virus induces a specific immune response and the antibodies did not react with an HAV viral preparation; sera from convalescent hepatitis A patients gave negative results in the test for the detection of anti-epidemic NANB virus antibodies.

The epidemic NANB virus is also distinct from HBV

and therefore from HDV:

- 1) The disease developed in some patients having anti-HBsAg antibodies and it also appeared in one vaccinated European patient having a high anti-HBV immunity;
- 2) No HBsAg and HBeAg were generally detected; if HBV markers hepatitis NANB preexist (in some Ivorian patients), no anti-HBc IgM appears;
- 3) IgMs from monkeys infected with the epidemic NANB virus which are used for the detection of the viral antigen react neither with the HBV or with purified HBsAg preparations, nor with sera from patients actively replicating the HBV; anti-HBs antibodies do not interfere with the test for the detection of anti-epidemic NANB virus antigen.

It appears that the new purified virus, which is characterised as described in the preceding text, can be isolated from widely distributed sources. In addition to the cases described above, the virus-associated antigen has also been found in a Senegalese patient and antibodies have been found in the serum of a convalescent patient of an epidemic which raged in Algeria. The epidemic NANB agent is considered as a virus which is disseminated by water. However, its role in sporadic hepatitis, in particular in countries which benefit from good hygiene conditions, does not exclude other methods of transmission, and especially via the blood.

Nevertheless, the role played by the epidemic NANB virus in cases of sporadic or posttransfusional hepatitis NANB should result in a reconsideration of the term "epidemic".

Indeed, the result of the Inventor's work is that the virus identified in conformity with the present invention, called epidemic, sporadic and transfusional hepatitis NANB virus (abbreviated: "H-SET") is also responsible for the appearance of type non-A, non-B hepatitis in patients who are recipients of blood

transfusions.

A comparative study comprising patients who are recipients of multiple blood transfusions without infectious hepatitis and patients suffering from hepatitis A or B has shown the absence of virus-associated antigen in their stools and the absence of viruses which can be cultivated under the conditions in which the "H-SET" virus multiplies. The "H-SET" strain, I-617, isolated from the stools of a patient suffering from transfusional hepatitis NANB, was able to be cultivated on PLC/PRP5 cells under the conditions described above. The lesions corresponding especially to numerous syncytial formations appear at J6-J7 preceding the detachment of the infected cells. An identical culture procedure is capable of being used for isolation of the virus from a blood sample. The infected cultures can be used as source of unpurified antigen for the characterisation, at their surface, of antibody molecules obtained from a subject who has contracted the infection (the virus-antibody complex being visualised by means of anti-labelled human immunoglobulins).

The Clamart strain was found to be infectious for two rhesus monkeys (inoculation by ingestion (per os) using 0.5 ml of a 10 % stool extract) with appearance of the antigen in their stools between the 20th and 24th days after the inoculation and with increase in transaminases between the 28th and the 32nd days (characteristic sign of hepatic attack).



CLAIMS for the contracting states

AT BE CH DE FR GB IT LI LU NL SE

- 1) Purified viral agent associated with sporadic, epidemic and posttransfusional viral hepatitis NANB, characterised in that it can be obtained from the faeces of patients suffering from hepatitis NANB and in that it is in the form of two main types of spherical viral particles associated with the same viral antigen:
 - a) spherical particles of about 75 nm in diameter whose density in a CsCl gradient is between 1.28 and 1.32 g/cm³ and which sediment in sucrose gradient fractions containing between 40 and 41.5% (w/v) of sucrose,
 - b) spherical particles from 25 to 30 nm in diameter whose density in a CsCl gradient is between 1.12 and 1.19 g/cm³ and which sediment in sucrose gradient fractions containing between 26 and 28% (w/v) of sucrose.
- 2) Strain of a purified viral agent according to Claim 1, called "Clamart strain", deposited with the Collection nationale de Cultures de Microorganismes on 30 October 1986 under the number I-617.
- 3) Strain of a purified viral agent according to Claim 1, called "Ivorian strain", deposited with the Collection Nationale de Cultures de Microorganismes on 30 October 1986 under the number I-616.
- 4) Strain of a purified viral agent according to Claim 1, called "H-SET strain", deposited with the ECACC Collection (Great Britain) on 5 February 1987 under the number 87.02.05.02.
- 5) Process for isolating a viral agent according to any one of Claims 1 to 4, characterised in that a biological extract containing the said viral agent is centrifuged in a CsCl gradient or in a sucrose gradient, and in that the said agent is collected in the CsCl gradient fractions whose density is between 1.29 and 1.32 g/cm³ or in the sucrose gradient fractions containing between 40 and 41.5% (w/v) of sucrose, in the form of spherical particles of about 75 nm in diameter, and/or in the CsCl gradient fractions whose density is between 1.12 and 1.19 g/cm³, or in the sucrose gradient fractions



containing between 26 and 28% (w/v) of sucrose, in the form of spherical particles from 25 to 30 nm in diameter.

6) Process according to Claim 5, characterised in that the biological extract containing the viral agent
5 consists of faeces from patients suffering from hepatitis NANB.

7) Process according to Claim 5, characterised in that the biological extract containing the viral agent consists of a culture of hepatitis NANB virus on a
10 continuous liver cell line.

8) Process according to either of Claims 6 and 7, characterised in that the biological extract is subjected beforehand to centrifugation at 5000-8000 g, at the end of which the supernatant is filtered through a filter
15 with a porosity of 0.22 μ m.

9) Process according to Claim 8, characterised in that prior to the filtration and the centrifugation, the biological extract is subjected to a physical dissociation treatment, especially by sonication, or to a chemical dissociation treatment.
20

10) Process for culturing a viral agent according to any one of Claims 1 to 4, characterised in that liver cells or continuous liver cell lines are inoculated with a faecal extract from a patient suffering from hepatitis
25 NANB, or with a liver extract from a monkey inoculated with a faecal extract from patients suffering from hepatitis NANB, and cultured until the culture becomes confluent.

11) Application of a viral agent according to any one of Claims 1 to 4, in the in vitro diagnosis of epidemic, sporadic and posttransfusional hepatitis NANB.
30

12) Process for the detection of antibodies specific for hepatitis NANB and associated at the same time with epidemic, sporadic and posttransfusional hepatitis, in a
35 blood sample or in sample of product derived from blood, which process is characterised in that, in a first stage, the said sample is brought into contact with a reagent consisting of a viral agent according to any one of Claims 1 to 4 and, in a second stage, the formation of an



antigen-antibody complex between the said viral agent and antibodies specific for hepatitis NANB, which may be present in the said sample, is detected.

5 13) Reagents for the diagnosis of epidemic, sporadic or posttransfusional viral hepatitis NANB, characterised in that they contain a preparation of the viral antigen according to any one of Claims 1 to 4.

10 14) Therapeutic agent for the treatment of epidemic, sporadic or posttransfusional viral hepatitis NANB, characterised in that it essentially contains a preparation of a viral agent according to any one of Claims 1 to 4.

15 15) Reagents for the diagnosis of epidemic, sporadic or posttransfusional viral hepatitis NANB, characterised in that they contain antibodies directed against a preparation of the viral antigen according to any one of Claims 1 to 4.

20 16) Therapeutic agent for the treatment of epidemic, sporadic or posttransfusional viral hepatitis NANB, characterised in that it contains antibodies directed against a preparation of the viral antigen according to any one of Claims 1 to 4.