A prospective study of acute viral hepatitis with particular reference to hepatitis A *

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In order to investigate the relationship of hepatitis A antigen to viral hepatitis, a prospective study was carried out on 97 patients admitted to Fairfield Hospital, Melbourne, with suspected viral hepatitis, and 3 of their family contacts. Evidence of infection with hepatitis A virus was obtained by detecting hepatitis A antigen in stools, and/or antibody to it in sera, by immune electron microscopy. Infection with hepatitis B virus was determined by testing for hepatitis B surface antigen and antibody in serum, by solid phase radioimmunoassay. Sixteen patients were found to have diseases other than viral hepatitis and 2 patients (child contacts) suffered no illness. There was clinical and/or biochemical evidence compatible with viral hepatitis in 82 patients, of whom 35 were confirmed as having hepatitis A and 31 as having hepatitis B infections. In the remaining 16 patients there was no evidence of infection with either hepatitis A or B virus. It is possible that some of these patients may have been infected with viral agents as yet unidentified.

In 1973 Feinstone et al. (*1*), using the technique of immune electron microscopy (IEM), detected 27-nm virus-like particles in the stools of patients in the acute phase of MS-1 type hepatitis (*2*). Morphologically and serologically identical particles were visualized by Locarnini et al. (*3*) in the stools of patients with sporadic non-B hepatitis in Melbourne and by Gravelle et al. (*4*) in specimens from a common source epidemic in Phoenix, AZ, USA. Provost et al. (*5*) recovered a morphologically similar agent from patients with non-B hepatitis in Costa Rica and showed it to be indistinguishable from the particle identified in MS-1 type hepatitis.

The accumulated evidence now suggests that a specific 27-nm particle is the etiological agent of hepatitis A. This particle has been provisionally designated hepatitis A antigen (HAAg), and antibody to it, anti-HA (*6*).

Other antigens have been found in the faeces of patients with hepatitis A (*7–11*) but their significance as specific markers for this disease and their relationship to HAAg is unclear.

A prospective study of patients admitted to Fairfield Hospital with the presumptive diagnosis of viral hepatitis was undertaken in order to determine the specificity of tests for HAAg and anti-HA and the prevalence of different types of hepatitis in Melbourne.

METHODS

Patients

The study group comprised 100 patients admitted to Fairfield Hospital, Melbourne, during the period February–April 1975. The diagnosis on admission was viral hepatitis in 97 patients. The remaining 3 patients, all children, were admitted with a parent for social reasons.

Specimens

Faecal specimens were obtained from all patients as soon as possible after admission to hospital and stored at −50°C until examination. Further samples were obtained from some patients.

Sera were collected on admission and at weekly intervals whilst patients were in hospital and stored at −20°C until tested. Additional specimens were obtained from some patients 3–6 months later.

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Table 1. Correlation of clinical grouping with HAAg in stool and anti-HA response in the 100 patients studied.

Clinical group	No. of patients	Antibody to HAAg in paired sera [a]			
		No. of paired sera tested	Rising titre [b]	Stationary titre	Not detected
Hepatitis A					
(i) HAAg detected	18	16	11	5	0
(ii) HAAg not detected	17	17	17	0	0
Hepatitis B	31	2	0	1	1
Hepatitis of undetermined etiology	16	16	0	9 [c]	7
Miscellaneous	18	12	0	6 [c]	6
Total	100	63	28	21	14

[a] The rating for antibody was: 0 = no antibody; 1+ = large complexes of particles with light antibody coating; 2+ = moderate complexes with heavier antibody; 3+ = smaller complexes heavily coated with antibody; 4+ = small complexes so heavily coated with antibody that particle morphology was obscured.

[b] A 1+ difference in antibody rating was considered to be significant.

[c] Two patients in each group had falling anti-HA titres.

Test methods

Standard biochemical tests of sera for bilirubin, aspartate-amino-transferase (AST), alkaline phosphatase, fructose monophosphate, thymol turbidity, and prothrombin activity were used to assess liver function. Sera were tested for hepatitis B surface antigen (HB_sAg) and antibody (anti-HB_s) by solid phase radioimmunoassay (Ausria II and Ausab [a]). Positive results obtained by radioimmunoassay were confirmed by specific neutralization tests. Tests for antibodies to cytomegalovirus (CMV), herpes simplex virus, Epstein Barr virus (EBV), *Mycoplasma pneumoniae*, *Coxiella burnetii*, *Toxoplasma gondii*, *Leptospira interrogans*, *Treponema pallidum*, and *Brucella abortus* were performed by standard techniques. Faecal extracts were prepared as described previously (*3*) and aliquots inoculated into primary monkey kidney and three additional cell lines for the isolation of enteric viruses. Isolates were identified by standard techniques.

In routine screening of faecal specimens for HAAg by IEM, the pelleted faecal extracts were diluted 1 : 4 in phosphate-buffered saline (PBS, pH 7.4) and extracted with an equal volume of chloroform (British Drug Houses, AR) to remove faecal lipids and débris. A 1.0-ml aliquot of this extract was allowed to react with 0.1 ml of a 1 : 20 dilution of a human convalescent hepatitis A serum rated 4+ for specific anti-HA (*3*). After incubation at 37°C for 1 h, then overnight at 4°C, the reaction mixture was centrifuged at 37 000 *g* for 90 min and the resultant pellet resuspended in 1 drop of PBS. The deposit was then negatively stained with 4% (weight for volume) phosphotungstic acid (pH 7.4) and examined immediately in a Philips EM 300 electron microscope at a plate magnification of approximately 44 000.

All specimens were examined under code. Faecal extracts containing 27-nm particles complexed by antibody were then re-examined with a second human convalescent hepatitis A serum rated 3+ for specific anti-HA. A stool was regarded as positive for HAAg only when it contained 27-nm particles which were complexed by both of the human convalescent sera. In some cases specimens were further examined by using pre- and post-infection sera from a chimpanzee successfully infected with a HAAg-rich stool filtrate (*12*).

The same IEM method was used to detect and measure specific anti-HA in sera. In order to deposit particulate material, sera were diluted 1 : 10 with PBS and centrifuged at 10 000 *g* for 30 min. Supernatants were collected and 0.1 ml mixed with 1.0 ml of an HAAg-positive chloroform-extracted faecal pellet. Reactant mixtures were incubated, spun, stained, and examined as described above. The same HAAg-positive faecal pellet was used for testing all

[a] Abbott Laboratories, North Chicago, IL, USA.

sera. The amount of antibody in each serum was rated on a 0–4+ basis (see Table 1), a 1+ difference between acute and convalescent sera being considered significant (*13*, *3*). Four intact squares were examined on each 400-mesh grid and antibody ratings were determined under code from photographed complexes.

Diagnostic criteria

A diagnosis of viral hepatitis was made by specialist physicians on the basis of clinical assessment aided by liver function tests.

A patient was regarded as being infected with hepatitis A virus if HAAg was detected in the stool and/or a rising anti-HA titre was demonstrable.

A diagnosis of hepatitis B infection was made if transitory HB_s antigenaemia or a primary anti-HB_s response was detected.

RESULTS

After correlation of all the results, the patients were finally allocated to four groups: (*i*) hepatitis A, (*ii*) hepatitis B, (*iii*) viral hepatitis without evidence of infection with hepatitis A or B virus, and (*iv*) miscellaneous.

Hepatitis A (35 patients)

Hepatitis A antigen was detected in stools from 18 of these patients (51%). These particles, measuring 27 nm in diameter (see Fig. 1) were morphologically and serologically identical with particles found in stools in a previous study (*3*). Crystalline arrays of particles (*3*) were observed in 6 of the 18 positive specimens.

From the 18 patients who were shedding HAAg, 15 stool specimens had been collected within 9 days of the patient's first passing dark urine and two were collected on day 16. A positive specimen from a child with a subclinical infection was collected 10 days after admission to hospital (see Fig. 2).

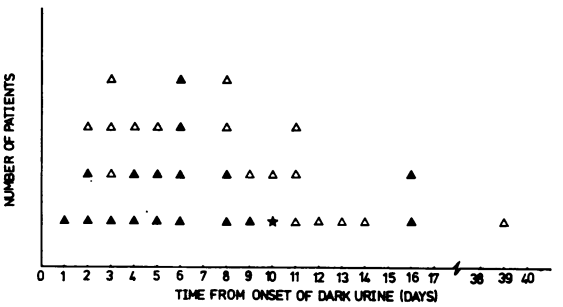

Fig. 2. The presence or absence of HAAg in the first stool specimen from 35 patients suffering hepatitis A in relation to the time from the onset of dark urine. Black triangle = HAAg-positive; white triangle = HAAg-negative; star = HAAg-positive subclinical infection.

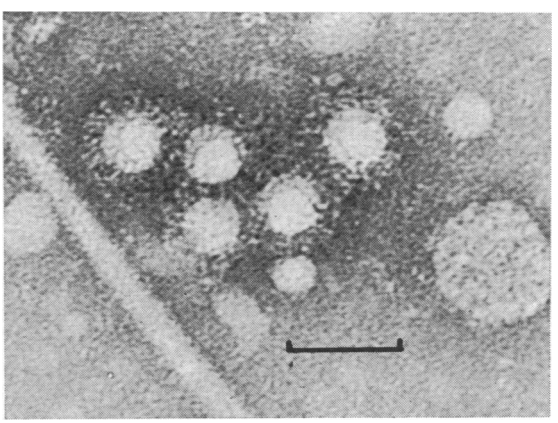

Fig. 1. An immune complex of hepatitis A antigen. The five particles are heavily coated with antibody, and core-like structures can be observed in four. The bar represents 60 nm.

The shedding of HAAg in the stool was followed up in 3 patients. From one patient 5 consecutive stool specimens were collected at 8, 10, 12, 19, and 20 days after the first appearance of dark urine. Only the first specimen was positive for HAAg (approximately 250 HAAg particles per grid square). From the second patient 2 specimens were collected at 4 and 8 days after the first appearance of dark urine. These specimens contained approximately 250 and 50 HAAg particles per grid square respectively. The third patient had 3 specimens collected 9, 13, and 14 days after the first appearance of dark urine. Only the first specimen, containing approximately 200 HAAg particles per grid, was positive.

The kinetics of the anti-HA response was studied in serial serum specimens from 5 patients who shed HAAg in the stools. Antibody levels in the 26 sera from these patients are shown in Fig. 3. The results indicate that over the first 8 or 9 weeks of illness at

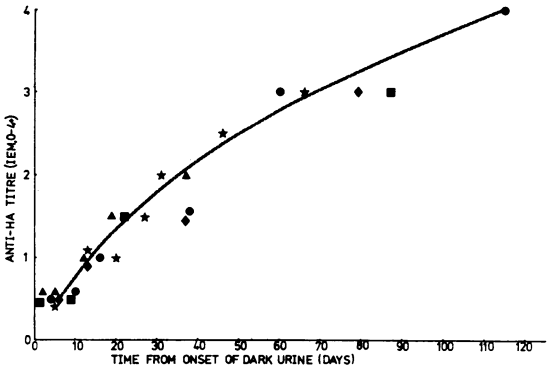

Fig. 3. Anti-HA levels in serial serum specimens from 5 patients who were shedding HAAg. The ratings (0–4+ scale) are shown in relation to the first appearance of dark urine.

least 3–4 weeks must elapse before a 1+ or greater difference in antibody levels can be detected by IEM in paired sera.

Tests on pairs of acute and convalescent sera from 16 of the 18 patients shedding HAAg in stools indicated that 11 had rising and 5 had stationary anti-HA levels (see Table 1). However, in 4 of these 5 patients the time interval between obtaining acute and convalescent sera was considerably less than 4 weeks. Seventeen patients, in whom HAAg was not detected, had rising anti-HA titres. It is interesting to note that in 26 of the 28 patients who had rising anti-HA titres, detectable levels of anti-HA were present on admission to hospital. IgM-like structures (*14, 3*) were often visualized in these acute sera but were not seen in convalescent-phase sera.

Two family groups were included in the 35 patients who suffered hepatitis A infection. In the first, the index case was the mother, her two children being admitted to hospital for social reasons. One of these children was shedding HAAg in stool, and represents the only subclinical infection detected in this study. In the second group, a child was the index case and 2 of her siblings were admitted to hospital with her. Two of the 3 children had elevated AST values and HAAg was detected in their stools. The remaining child, who was not ill, had no evidence of hepatitis A infection. Five weeks later their mother was admitted with hepatitis; HAAg was visualized in her stools and a rise in anti-HA titre was demonstrated.

One patient shedding HAAg in stool during the acuted phase of his illness was HB_sAg-positive on admission to hospital. This patient was still HB_sAg-positive more than 3 months later. Unfortunately his acute phase serum was exhausted but high levels of anti-HA were detected in two convalescent sera. Further evidence that the particles detected in his stool were HAAg was obtained by showing that they were complexed by post-infection but not pre-infection chimpanzee sera (*12*). It seems probable that this illness represented an acute attack of hepatitis A in a hepatitis B carrier.

Evidence of past infection with hepatitis B was obtained in 3 of the 35 patients who suffered hepatitis A infection. Stationary anti-HB_s levels were detected in sera from these three patients.

Hepatitis B (31 patients)

The diagnosis of hepatitis B was made in 29 patients by detection of HB_sAg in acute phase sera and in 2 patients by the detection of a primary antibody response.

Hepatitis A antigen was not detected in the stools from any of these patients. Transitory HB_s antigenaemia in 29 patients was regarded as proof of hepatitis B infection and no tests for anti-HA were carried out. Tests for anti-HA were performed on the remaining two patients, because the diagnosis of hepatitis B was made on the basis of a primary anti-HB_s response; in one patient anti-HA was not detected, and the other had a stationary level.

Hepatitis of undetermined etiology (16 patients)

Sixteen patients suffered an illness which, on clinical and biochemical criteria, was regarded as viral hepatitis but could not be identified as either hepatitis A or B by the available laboratory techniques. Tests for HAAg were negative in all patients. Seven patients failed to develop anti-HA, while 7 had stationary and 2 had falling anti-HA titres. Tests for HB_sAg and anti-HB_s were negative in 15 patients but late convalescent sera, suitable for detection of anti-HB_s, were available in only 7 patients. The other patient was HB_sAg-positive on admission and remained so for the entire period of follow-up.

In 9 patients, therefore, hepatitis B infection could not be excluded owing to the absence of late convalescent sera, but in the other 7 patients a diagnosis of hepatitis B seemed most unlikely. Of these 7 patients, 3 failed to develop anti-HA and 4 had stationary levels consistent with previous hepatitis A

infection. Examination of paired sera from these 7 patients also failed to reveal evidence of recent infection with CMV, herpes simplex virus, EBV, *Mycoplasma pneumoniae*, *Coxiella burnetii*, *Toxoplasma gondii*, *Leptospira interrogans*, *Treponema pallidum*, or *Brucella abortus*.

Miscellaneous (18 patients)

The final diagnosis for patients in this group included alcoholic liver disease, obstructive jaundice, halothane-induced hepatitis, acute cholecystitis, cholelithiasis, and septicaemia. Two children, both hepatitis A contacts, suffered no illness.

Hepatitis A antigen was not detected in stools and rising anti-HA titres were not observed in any of the paired sera tested. Two patients demonstrated a slight fall in anti-HA titre. Neither HB_sAg nor anti-HB_s was detected in sera from patients in this group.

Other viruses and virus-like particles

Adenoviruses were visualized in 3 specimens, 2 of which also contained HAAg. Adenovirus type 2, the only virus cultivated from stools in this survey, was isolated from another patient, whose stool also contained HAAg.

Particles 27 nm in diameter and morphologically similar to HAAg were visualized in specimens from 5 patients. These particles were complexed by the first but not the second human convalescent hepatitis A serum. Two of these 5 patients suffered from hepatitis A (diagnosed on the basis of rising anti-HA titres), 1 had hepatitis B, and the remaining 2 had miscellaneous illnesses.

Other 27-nm particles, which did not react serologically with either of the two convalescent hepatitis A sera, were seen in a further 14 specimens. Four of these came from patients with hepatitis A, 5 from patients with hepatitis B, 1 from the miscellaneous group, and 4 from patients with hepatitis of undetermined etiology.

Particles 22–24 nm in diameter, sometimes complexed by antibody, were visualized in an additional 9 specimens. Four of these patients suffered from hepatitis A, 2 had hepatitis B, 1 did not suffer from viral hepatitis, and 2 had hepatitis of undetermined etiology. Hepatitis A antigen was also detected in specimens from 2 of the 4 patients with hepatitis A.

DISCUSSION

The presence of HAAg in stools, and/or rising antibody titres to this antigen, appear to be specific markers of infection with the hepatitis A virus. These markers were detected only in patients with HB_sAg-negative hepatitis, both sporadic cases and family outbreaks, apart from one subclinical infection and one episode of acute hepatitis occurring in a hepatitis B carrier. Hepatitis A antigen and rising anti-HA levels were not detected in 31 patients with hepatitis B, nor in 18 patients with diseases other than viral hepatitis.

Dienstag et al., in a study of experimentally infected human volunteers (*6*) and chimpanzees (*12*) found that shedding of HAAg was complete at or before peak aminotransferase levels were reached. Epidemiological and experimental evidence (*2*), however, suggests that the virus is probably shed for a longer period. In this study of naturally acquired disease, HAAg was detected in stools from 12 of 18 patients with hepatitis A when specimens were obtained within one week of the first appearance of dark urine, and in 6 out of 17 patients when specimens were obtained in the second and third week of the illness (see Fig. 2).

Contrary to some reports (*15*, *16*), the HAAg particles visualized in this study were of uniform size. The significance of core-like structures observed in some particles is unclear and these may represent staining artefacts. Morphologically, HAAg resembles members of the parvovirus and picornavirus groups. The mean buoyant density of HAAg in stools (*3*) is similar to that of parvoviruses, but its diameter (27 nm) is greater than that generally accepted for parvoviruses (18–22 nm) and falls within the picornavirus range (25–32 nm). Other workers (*5*, *17*) have reported buoyant densities for HAAg at variance with that described above; these discrepancies are difficult to explain.

Electron microscopic examination of faeces has resulted in the detection of a wide variety of virus-like structures (*11*, *18*, *19*). Viruses which are sensitive to lipid solvents (*20*) would not have been visualized in the present study because chloroform extraction was used in preparing the specimens. Virus-like particles which were serologically unrelated to HAAg were detected in stools from 28 of the 100 patients. These particles, some 22–24 nm and others 27 nm in diameter, were not detected in cell culture and may represent new groups of " orphan " viruses as well as unclassified gastroenteritis viruses (*13*). Since antibody to a variety of these particles may be present in human sera, immune complexing of 27-nm particles by a single convalescent human hepatitis A serum does not constitute valid evidence

for the presence of HAAg. The findings should be confirmed with other convalescent hepatitis A sera or, preferably, by using pre- and post-infection hepatitis A sera of human or animal origin.

The anti-HA response, as determined by IEM, indicates the early appearance of specific antibody. Most of the acute phase sera that were collected within one week of the first appearance of dark urine contained detectable levels of anti-HA. After the acute phase of infection the antibody appears to rise steadily, reaching a maximum level in about 14–16 weeks, and apparently remains detectable for several years. Methods for quantifying anti-HA by complement fixation and immune adherence (*21*, *22*) have been described recently but are not widely available. These techniques are more sensitive than IEM, but the kinetics of the antibody response appear to differ in that antibody detectable by immune adherence does not usually appear until 3–4 weeks after the onset of illness, and then rises rapidly (*23*).

Confirmation of a diagnosis of hepatitis B is usually made by the demonstration of HB_sAg in acute phase sera. However, if antigenaemia is over before the time of testing, the diagnosis can be made only by demonstrating the development of specific antibody. In the current study of 31 patients with hepatitis B, two (6.3%) were diagnosed on the basis of primary antibody response alone. This phenomenon has been described in experimental infections in man (*24*) and in chimpanzees (*25*) and in post-transfusion hepatitis (*26*).

Currently over 30% of patients with viral hepatitis admitted to Fairfield Hospital show evidence of infection with hepatitis B virus, but the proportion of the population with antibody to this virus is low because the disease has been common in Melbourne only since 1971 (*27*). In this study evidence of past infection with hepatitis B virus was found in only three patients.

Evidence has been produced (*26*, *28*, *29*, *30*) for the existence of transfusion-associated hepatitis not caused by hepatitis A, hepatitis B, CMV, or EBV. In the present study a possible diagnosis of non-A, non-B hepatitis was considered in 7 of 82 patients (8.5%) on the basis of failure to detect HAAg or HB_sAg in acute phase specimens of stools or serum and the absence of rising titres of anti-HA or anti-HB_s in convalescent sera collected at least 3 months after the onset of illness. It is possible that this group may contain patients with hepatitis A in whom a diagnosis was not made because of the limitations of current techniques, and patients with hepatitis B in whom anti-HB_s might have developed very late. It is tempting, however, to speculate that as yet unidentified viruses may be involved in the etiology of naturally occurring hepatitis as well as in post-transfusion hepatitis.

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RÉSUMÉ

ÉTUDE PROSPECTIVE DE L'HÉPATITE VIRALE AIGUË, ET PARTICULIÈREMENT DE L'HÉPATITE A

Cette étude de l'hépatite virale spontanée à Melbourne a été entreprise en vue de vérifier la spécificité des épreuves relatives à l'antigène de l'hépatite A (HAAg) et à son anticorps (anti-HA), de même que pour déterminer la fréquence des différents types d'hépatite.

Le groupe étudié était composé de 97 malades admis au Fairfield Hospital for Communicable Diseases parce qu'ils étaient soupçonnés d'avoir une hépatite virale; trois contacts familiaux ont également été admis à l'hôpital pour des raisons sociales.

Les échantillons de selles et les sérums étaient identifiés par des numéros de code et ont été examinés par des personnes ignorant tout des données cliniques. On a utilisé l'immuno-microscopie électronique pour détecter le HAAg dans les selles et déterminer les titres d'anti-HA dans les sérums. Le titrage radioimmunologique en phase solide a servi pour la détection de l'antigène de surface de l'hépatite B (HBsAg) et la détermination quantitative de l'anticorps correspondant (anti-HBs).

Les critères diagnostiques ci-après ont été adoptés:

a) Le diagnostic d'hépatite virale était posé par des médecins spécialistes sur la base de l'observation clinique complétée par des épreuves biochimiques de la fonction hépatique.

b) On considérait un malade comme atteint d'hépatite à virus A si l'on détectait le HAAg dans les selles et (ou) si une augmentation du titre de l'anti-HA était mise en évidence.

c) Le diagnostic d'hépatite B était posé si l'on décelait une antigénémie HBs transitoire ou une réponse primaire anti-HBs.

Après analyse de tous les résultats, on a constaté que 16 malades avaient eu diverses affections autres qu'une hépatite virale, 2 (contacts d'enfant) n'avaient aucune infection, 35 avaient une infection par le virus de l'hépatite A, 31 une infection par le virus de l'hépatite B, et 16 malades n'avaient aucun signe d'infection par le virus de l'hépatite A ou B.

La détection de HAAg et (ou) une augmentation des titres de l'anti-HA ont semblé constituer des marqueurs valables de l'infection par le virus de l'hépatite A car ces marqueurs n'ont pas été retrouvés chez les malades pour qui le diagnostic d'hépatite B a été posé ou ceux qui présentaient diverses maladies autres qu'une hépatite virale. Des arguments épidémiologiques en faveur de la spécificité du HAAg ressortent de l'examen de deux groupes familiaux qui ont été inclus parmi les sujets étudiés.

L'antigène de l'hépatite A était présent dans les selles de 18 (51%) des 35 malades atteints d'hépatite A; 12 de ces échantillons de selles avaient été recueillis dans la semaine qui a suivi l'apparition d'urines foncées, et 6 autres au cours de la deuxième ou de la troisième semaine de maladie. Le dernier échantillon positif, provenant d'un enfant présentant une infection infra-clinique, a été recueilli 10 jours après admission à l'hôpital. L'identification du HAAg dans les selles demande beaucoup de circonspection car l'immunomicroscopie électronique visualise une grande variété de structures ressemblant à des virus.

Des titres décelables d'anti-HA étaient présents dans la plupart des sérums de phase aiguë recueillis dans la semaine suivant l'apparition d'urines foncées. Les titres d'anticorps ont ensuite paru augmenter régulièrement pour atteindre un maximum au bout de 14 à 16 semaines environ.

Chez 16 sujets, la maladie a été considérée comme une hépatite virale mais n'a pu être identifiée ni comme une hépatite A, ni comme une hépatite B. Chez 9 malades, l'évaluation complète n'a pas été possible faute de spécimens cliniques suffisants, mais pour 7 on disposait de paires de sérums appropriés. Les tests auxquels ces sérums ont été soumis n'ont pas apporté de preuve d'une infection par le virus de l'herpès simplex, le cytomégalovirus le virus d'Estein-Barr et un certain nombre d'autres agents, il est donc tentant de supposer que des virus jusqu'ici non identifiés peuvent être les agents étiologiques d'hépatites spontanées aussi bien que d'hépatites post-transfusionnelles.

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