Aetiological Agent of Enterically Transmitted Non-A, Non-B Hepatitis

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SUMMARY

Virus-like particles (VLPs) with a mean diameter of 32 nm were recovered from the
stools of three acute phase cases of enterically transmitted non-A, non-B hepatitis (ET-
NANBH) occurring in the Soviet Union, North Africa and North America. VLPs
from two of these cases were studied in detail and were shown to react specifically with
antibody in acute phase sera obtained from other cases of ET-NANBH in Asia, the
Soviet Union, North Africa and North America. Partially purified VLPs were found to
sediment at 183S in sucrose gradients and to cross-react with antibody in acute phase
sera from geographically isolated cases of ET-NANBH. The latter virus preparations
were also used to document the seroconversion of experimentally ET-NANBH-
infected cynomolgus macaques to 32 nm VLPs. Our findings indicate that one virus or
class of viruses is responsible for the majority of ET-NANBH.

Enterically transmitted non-A, non-B hepatitis (ET-NANBH) has been documented to occur
in epidemic or sporadic fashion in India (Wong et al., 1980; Tandon et al., 1982; Khuroo, 1980;
Khuroo et al., 1983), Nepal (Kane et al., 1984), Burma (Myint et al., 1985), Pakistan (De Cock
et al., 1987) and the Soviet Union (Balayan et al., 1983). Outbreaks of ET-NANBH have also been
reported in Algeria (Belabbes et al., 1985), Ivory Coast (Sarthou et al., 1986), Somalia (I.
Weisfuse, unpublished data) and, more recently, Mexico (H. Stetler, unpublished data).
ET-NANBH is associated with a high mortality rate, approaching 20%, in infected pregnant
women (Tandon et al., 1982; Khuroo et al., 1983; Kane et al., 1984; Myint et al., 1985;
Shrestha & Malla, 1975) and is one of the leading causes of acute viral hepatitis in young to middle-aged
adults in developing countries (Kane et al., 1984; Myint et al., 1985).

Although several investigators have reported the finding of 27 to 34 nm virus-like particles
(VLPs) in stool specimens of acutely infected cases (Balayan et al., 1983; Kane et al., 1984;
Sreenivasan et al., 1984; Zairov et al., 1986; De Cock et al., 1987; Bradley et al., 1987), definitive
studies linking these VLPs to ET-NANBH have been prevented by the absence of a suitable
primate model and by the lack of reagent materials, including partially purified virus and
disease-specific antibody. We have recently developed a reliable experimental model of ET-
NANBH in cynomolgus macaques (Balayan et al., 1983; Andjaparidze et al., 1986; Bradley et
al., 1987) and have recovered sufficient virus from stools of patients during outbreaks occurring
in the U.S.S.R. (Tashkent; 1981 to 1982), Somalia (Tug Wajale refugee camp; 1986) and Mexico
(Telixtac; 1986) to permit the serological and biophysical characterization of geographically
isolated strains.

Key words: hepatitis, non-A, non-B[enteric transmission/aetiological agent]
Approximately 2200 stool specimens obtained from ET-NANBH cases in geographically distinct regions of the world were screened by immune electron microscopy (IEM) for the presence of disease-associated VLPs. Only two specimens, Tashkent no. 1435 and Telixtac no. VLPs 14, were found to be in sufficient quantity and to contain adequate numbers of 27 to 34 nm (approximate mean diameter, 32 nm; referred to hereafter as 32 nm VLPs) to facilitate the comparative studies we describe here. IEM for VLPs in stool preparations was performed as previously described (Gravelle et al., 1975). Briefly, control and case sera were diluted 1:20 in phosphate-buffered saline (PBS) pH 7.6 prior to use; crude stool suspensions were diluted to 10% (w/v) in PBS pH 7.6, clarified at 2500 g for 45 min at 5 °C, then filtered through 1.2 μm and 0.45 μm membrane filters (or centrifuged at 12000 g for 20 min). Virus from the gradient fraction pools was further concentrated by pelleting through a sucrose cushion (see below) or used in its partially pure state. Diluted antiserum (0.1 ml) was mixed with 0.2 ml stool filtrate and incubated overnight (18 h) at 4 to 8 °C. After incubation 0.7 ml of PBS was added, the sample was vortexed briefly and then centrifuged at 35000 g for 2 h at 4 °C to pellet immune aggregates. Pellets were resuspended on one to four drops of distilled water and one drop (20 μl) of each sample was then applied to a Formvar- or parlodion-coated carbon-stabilized 400-mesh copper grid, allowed to react 3 to 5 min, blotted, air-dried, and stained with one drop of 1 to 2% (w/v) phosphotungstic acid (PTA), pH 6-8, before viewing. For IEM of 32 nm VLPs partially purified from the Tashkent no. 1435 stool, 5 μl of virus concentrate was mixed with 5 μl of diluted antibody (1:30) and incubated for 30 min at room temperature; a grid was placed on the drop containing the virus–antibody mixture for 10 min, removed, blotted dry, and stained with PTA. All specimens were coded and examined. Length and breadth measurements of 135 disease-associated VLPs were independently made by two observers using selected (high-resolution) electron micrographs. Measurements of individual particle diameters were averaged; the mean diameters of VLPs recovered from stools of four geographically isolated cases of ET-NANBH were statistically analysed by a one-way analysis of variance to reveal any possible differences in particle size.

Table 1 summarizes the results of our IEM studies of 32 nm VLPs recovered from Soviet (Tashkent no. 1435) and Mexican (Telixtac no. 14) case stools. IEM examination of the Tashkent no. 1435 10% (w/v) (crude) stool suspension using PBS pH 7-6, normal human serum (NHS) and one serum positive for antibody to hepatitis A virus (anti-HAV) as negative controls revealed no 32 nm VLPs coated with antibody. In contrast, acute phase sera from well documented cases originating in the U.S.S.R., Pakistan, Nepal, Burma, Sudan, Somalia and Mexico were found to contain antibody against these VLPs. A human volunteer (HV) who had ingested a filtered stool pool extract (composed of 14 stool specimens obtained from nine cases during a 1981 to 1982 outbreak of ET-NANBH in Tashkent) was also shown to seroconvert to the above 32 nm VLPs. Antibody coating on the above particles was rated 2 to 4+ (on a scale of 0 to 4+) and appeared to be of the IgM class since characteristic protein halos and staple-like bridges were found associated with them (Fig. 1). The latter finding is suggestive of a disease-specific, acute phase (IgM) antibody response similar to that observed in humans and chimpanzees acutely infected with HAV (Bradley et al., 1979, 1982).

Although not shown here, convalescent ET-NANBH case sera often reacted less well than did acute phase sera with the above 32 nm VLPs (Bradley et al., 1987). This finding implies that antibody reactivity does not normally increase during the convalescent phase of ET-NANBH. As a consequence, determination of the serospecificity of VLPs for ET-NANBH by demonstrating a difference in antibody reactivity in patient sera collected during the acute and convalescent phases of disease has not been possible. However, the availability of paired pre-inoculation and acute phase sera from experimentally infected primates has enabled us to document, by IEM, development of disease-specific antibody to 32 nm VLPs recovered from two different (unrelated) ET-NANBH case stools. Primates infected with either a Burma virus isolate (Bradley et al., 1987) (CY no. 17, MY no. 43) or a human volunteer stool specimen (Balayan et al., 1983) (CY no. 1191) were found to develop acute phase, virus-specific antibody when their paired sera were reacted with the Tashkent no. 1435 10% (w/v) stool suspension (Table 1).
**Table 1. Immunoreactivity of 32 nm VLPs in samples from Soviet Asia and North America**

<table>
<thead>
<tr>
<th>Antiserum* (1:60 final dilution)</th>
<th>Tashkent 1435 (U.S.S.R.)†</th>
<th>Telixtac 14 (Mexico)§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crude, 10% (w/v) suspension</td>
<td>Partially purified</td>
</tr>
<tr>
<td>PBS pH 7.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NHS 1 (U.S.A.)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NHS 2 (U.S.A.)</td>
<td>0</td>
<td>NT</td>
</tr>
<tr>
<td>Anti-HAV (U.S.A.)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HV (U.S.S.R.) pre-inoc.</td>
<td>0</td>
<td>NT</td>
</tr>
<tr>
<td>HV (U.S.S.R.) acute</td>
<td>176</td>
<td>58</td>
</tr>
<tr>
<td>Pak 1 acute</td>
<td>236</td>
<td>42</td>
</tr>
<tr>
<td>Nepal acute pool 1</td>
<td>38 (1:5)</td>
<td>NT</td>
</tr>
<tr>
<td>Burma acute pool 1</td>
<td>35 (1:5)</td>
<td>NT</td>
</tr>
<tr>
<td>Sudan acute pool 1</td>
<td>23 (1:5)</td>
<td>NT</td>
</tr>
<tr>
<td>Somalia acute pool 1</td>
<td>45 (1:5)</td>
<td>NT</td>
</tr>
<tr>
<td>Mexico acute pool 2</td>
<td>96 (1:2)</td>
<td>63</td>
</tr>
<tr>
<td>Mexico acute 4</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>CY 1191 (P1) pre-inoc.</td>
<td>0</td>
<td>NT</td>
</tr>
<tr>
<td>CY 1191 (P1) acute</td>
<td>158</td>
<td>2-3+</td>
</tr>
<tr>
<td>CY 17 (P1) pre-inoc.</td>
<td>0</td>
<td>NT</td>
</tr>
<tr>
<td>CY 17 (P1) boosted</td>
<td>163</td>
<td>2+</td>
</tr>
<tr>
<td>CY 29 (P2) pre-inoc.</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>CY 29 (P2) acute</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>CY 74 (P3) pre-inoc.</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>CY 74 (P3) acute</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>MY 43 (P1) pre-inoc.</td>
<td>0 (1:5)</td>
<td>0</td>
</tr>
<tr>
<td>MY 43 (P1) acute</td>
<td>49 (1:5)</td>
<td>3+</td>
</tr>
</tbody>
</table>

* NHS, normal human serum. Anti-HAV, antibody to hepatitis A virus. HV, human volunteer from Soviet Union (Balayan et al., 1983). Pak 1, acute phase serum from case of ET-NANBH imported to U.S.A. from Karachi, Pakistan (De Cock et al., 1987). Nepal, Burma acute serum pools: pools of acute phase sera from four cases of ET-NANBH from each epidemic (Kane et al., 1984; Myint et al., 1985) (collected between 1 and 12 days after onset of symptoms; also same for Sudan acute phase serum pool 1 and Somalia serum pool 1). Mexico serum pool 2 is composed of two acute phase sera collected from ET-NANBH cases during an outbreak in Huitzililla, Mexico 4 and 5 days, after the onset of icterus. Mexico 4 is an acute phase serum from one of two cases contributing to the Mexico serum pool 2. After collection of serum (pre-inoc.), CY 1191 was infected with an acute phase stool from HV (Balayan et al., 1983) with ET-NANBH. CY 17, 29 and 74 represent the first, second and third primate passages (P) of the Burma ET-NANBH virus (Bradley et al., 1987) MY 43 was infected with an aliquot of the same Burma virus preparation used to infect first passage macaques. Acute phase sera from all primates were collected within a 2 week period encompassing the major peak of alanine aminotransferase/serum isocitrate dehydrogenase activity.

† The Tashkent 1435 (U.S.S.R.) stool was collected from a case of ET-NANBH within 4 days after the onset of illness (Balayan et al., 1983), this stool was used as a source of reagent virus for IEM studies as either a crude suspension (10% w/w) or as a partially purified virus preparation (see Fig. 2). Thirty-two nm VLPs coated with antibody were counted in a total of three grid squares (sq). Antibody coating (Ab) was rated on a scale of 0 to 4+. Some virus preparations were diluted in PBS as noted. NT, Not tested.

‡ Partially purified 32 nm VLPs were prepared from the Tashkent 1435 stool as described in the legend to Fig. 2 and used as described above.

§ IEM for 32 nm VLPs in the Telixtac 14 virus preparation (see Fig. 3) was performed as described above, except that the total number of antibody-coated VLPs in three grid squares were counted.

|| Three sources of antisera were tested for antibody using partially purified virus that had been stored for 1 week at 4 to 8°C.

A smaller, but representative, number of control, case and serial passage primate sera were further tested by IEM for antibody to 32 nm VLPs that had been partially purified from either the Tashkent no. 1435 or Telixtac no. 14 stool suspensions (Table 1). Sucrose gradient fractions encompassing the major virus peaks (see Fig. 2, 3) were individually pooled and used either undiluted or after pelleting the virus, for the detection of virus-specific antibody in the above sera. Neither virus preparation reacted with antibody in the control sera, nor was antibody
Fig. 1. Electron micrographs of 32 nm diameter VLPs recovered from the stools of cases of ET-NANBH occurring in the Soviet Union, Somalia and Mexico. Particles are heavily coated by the antibody contained in the patient or primate acute phase serum which was used for immune aggregation. IEM for VLPs was performed as described in the footnotes to Table 1. (a) Tashkent no. 1435 virus aggregated with antibody from acute phase serum from U.S.S.R. human volunteer. (b, c) Telixtac no. 14 virus (partially purified, 18S peak fraction from rate zonal gradient) aggregated with antibody from acute phase ET-NANBH case serum (Mexico acute no. 4). (d) Telixtac no. 14 virus
Fig. 2. Rate zonal banding of HAV and ET-NANBH-associated 32 nm VLPs in linear sucrose gradients (10 to 40% w/w; △) preformed and prepared in PBS pH 7.5. One ml of purified HAV (strain HAS-15) (Bradley et al., 1984) and 1.0 ml of a 10% (w/v) suspension of the Tashkent no. 1435 stool were layered onto separate gradients and centrifuged in a SW41Ti rotor at 35000 r.p.m. for 3-0 h at 5°C. A total of 21 0-6 ml fractions of each gradient were collected into siliconized tubes containing 0.5% (w/v) (final concentration) bovine serum albumin. IEM for VLPs in gradient fractions of the Tashkent no. 1435 preparation (a) was performed using 1:60 final dilutions of acute phase sera from well documented cases of ET-NANBH originating in Pakistan (○) (De Cock et al., 1987) or Mexico (●) (H. Stetler, unpublished data). The number of antibody-coated 32 nm VLPs in five grid squares was counted; more than 90% of the virus counted was contained in immune aggregates of three to 25 particles each. EIA for HAV antigen (represented as • in b) in gradient fractions was performed as previously described (Wheeler et al., 1986). IEM for 27 to 30 nm HAV particles in each fraction (○) was done by conventional techniques (Gravelle et al., 1975). Sedimentation coefficients were computed by standard methods. Gradient fractions (no. 13 and no. 14) containing the major peak of ET-NANBH-associated VLPs were pooled, diluted to 4.5 ml in PBS, underlayed with 0.5 ml of 30% (w/w) sucrose in PBS and centrifuged in a SW50 rotor for 90 min at 15°C to pellet the virus. The virus-containing fraction was resuspended in 100 μl PBS for use in further IEM assays (See Table 1).

detected in pre-inoculation sera from experimentally infected primates. Both virus preparations, however, were observed to react with antibody in acute phase sera from well documented cases of ET-NANBH occurring in the U.S.S.R., Mexico and Pakistan, as well as in acute phase or boosted sera of experimentally infected primates (Table 1). In addition, acute phase sera from Nepal (Kane et al., 1984) and Burma (Myint et al., 1985) were found to contain antibody to 32 nm VLPs in the Telixtac no. 14 virus preparation. The latter VLPs were also aggregated by antibody in the acute phase sera of three cynomolgus macaques (Table 1, no. 17, no. 29, no. 74) representing the first, second and third primate passage, respectively, of a Burmese isolate of ET-NANBH in this species (Bradley et al., 1987).
It is of interest to note that 32 nm VLPs contained in both the Tashkent no. 1435 and Telixtac no. 14 stool suspensions sedimented significantly faster in rate zonal (sucrose) gradients that did HAV run in parallel gradients. The computed sedimentation coefficient of the ET-NANBH-associated virus was approximately 183S, in contrast to 157S for that of HAV (Fig. 2, 3). A similar sedimentation coefficient was found for 32 nm VLPs isolated from an acute phase stool of a case of ET-NANBH in Tug Wajale, Somalia (data not shown). VLPs recovered from the 183S peak fractions often appeared to have a somewhat fuzzy or ragged capsid profile. Size measurements of antibody-aggregated VLPs recovered from stools of ET-NANBH cases in Somalia, the Soviet Union, Mexico and Burma (Somalia no. 30, Tashkent no. 1435, Telixtac no. 14 and FVH-I 11) revealed a mean particle diameter of 32.2 nm (Fig. 4). Approximately 90% of the particles measured were between 27 and 34 nm in diameter. No statistically significant difference in particle size was found for any of the four geographically distinct virus isolates.

Our studies suggest that the majority of ET-NANBH occurring in geographically isolated regions of the world, including Soviet Asia, Asia, North Africa and North America is caused by one virus or a serologically related class of viruses. Thirty-two nm VLPs visualized in stools obtained from ET-NANBH cases in Mexico and the Soviet Union were clearly shown to cross-react with antibody in acute phase sera from other well defined cases originating in Burma, Nepal, Pakistan, U.S.S.R., Sudan, Somalia and Mexico. The demonstrated seroconversion of
experimentally infected primates to 32 nm VLPs partially purified from the stools of two unrelated cases of ET-NANBH further supports our contention that these VLPs are aetiologically linked to disease. Sedimentation analysis of 32 nm VLPs recovered from the acute phase stools of three geographically isolated cases also revealed that the putative agent of ET-NANBH has a higher sedimentation coefficient than that of HAV, a member of the picornavirus family. The latter finding is consistent with our observation that ET-NANBH-associated VLPs appear to be slightly larger in diameter than HAV, a 27 to 30 nm particle. Although the mean diameter and sedimentation coefficient of the ET-NANBH-associated VLPs are similar to those of two Norwalk-like agents responsible for acute, non-bacterial gastroenteritis (Greenberg et al., 1981; Madore et al., 1986), limited serological studies of some of the ET-NANBH case stools described here have revealed no immunological relationship (data not shown). Studies are currently in progress on the molecular characterization of the genome and capsid polypeptide(s) of the ET-NANBH-associated 32 nm VLPs.

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