Isolation and purification of a non-A, non-B hepatitis-associated microtubular aggregates protein

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Blood-borne type non-A, non-B (NANB) hepatitis-associated microtubular aggregates protein was isolated and partially sequenced. The microtubular aggregates were isolated from the hepatocytes of NANB-infected chimpanzees and were found to have a buoyant density in sucrose solution of 1.21 to 1.23 g/ml. A single protein, recognized by our anti-microtubular aggregates monoclonal antibodies, was found to have an Mr of 44000 (p44). This p44 protein was not found in uninfected chimpanzees. We determined a partial amino acid sequence for p44, and showed that it has no homology to any known proteins.

Introduction

Non-A, non-B (NANB) hepatitis is a term for a viral hepatitis not due to hepatitis A virus (HAV), hepatitis B virus (HBV), or other known hepatotropic viruses, including hepatitis delta virus (HDV), cytomegalovirus, Epstein–Barr virus and human parvovirus (B19). There are at least two types of NANB hepatitis, namely water- and blood-borne NANB hepatitis (Alter et al., 1982; Sherlock, 1986).

Water-borne NANB hepatitis is endemic to the Indian subcontinent and clinically resembles hepatitis A (Khuroo, 1980; Bradley et al., 1987; Bradley & Maynard, 1986). The RNA of the causative agent has recently been cloned and shown to contain a positive-stranded RNA of at least 10000 nucleotides, a feature which is also characteristic of the togaviridae or flaviridae (Choo et al., 1989). It is now tentatively designated the hepatitis C virus.

The second type of virus, the so-called non-tubule-forming agent, is not chloroform-sensitive and does not induce tubule formation when injected into chimpanzees. The causative agent is still unidentified.

In a search for antibodies associated with post-transfusion hepatitis, we established monoclonal antibody-producing cell lines derived from chimpanzee (Shimizu et al., 1985), human (Shimizu et al., 1986) and murine lymphocytes (Maeda et al., 1989). Immunoelectron microscopy revealed that these monoclonal antibodies recognized microtubular aggregates that appeared in the cytoplasm of NANB-infected and HDV-infected chimpanzee hepatocytes, but not in normal hepatocytes or those infected by HAV or HBV (Krawczynski et al., 1985; Shimizu et al., 1985, 1986, 1987). Western blot analysis also confirmed that the antigen purified from the microtubular aggregates (p44) exists in the hepatocytes of a chimpanzee with delta hepatitis (Shimizu et al., 1987).

These findings raised the possibility that the antigen associated with the microtubular aggregates may be a host protein specifically induced during NANB or HDV
infection. In this report we describe the purification of the microtubular aggregates protein from an experimentally infected chimpanzee liver, and a partial amino acid sequence of the antigen.

Methods

Source of microtubular aggregates. The liver from chimpanzee no. 41, which developed chronic NANB hepatitis after inoculation with infectious serum, was used as the starting material for purification of the microtubular aggregates (Shimizu et al., 1986). The liver from a healthy uninfected chimpanzee (no. 57) was used as a control.

Antigen assay. The p44 antigen was measured using a sandwich radioimmunoassay (RIA) system with our monoclonal antibodies (Maeda et al., 1989). Each well of 96-well microtitre plates (Falcon 3912, Becton Dickinson Labware) was coated with mouse monoclonal antibody, M17, in phosphate-buffered saline (PBS) (50 µl/well) at 4 °C overnight. After blocking with PBS containing 1% gelatin (Nakarai Tesque), 50 µl of the 125I-labelled M17 antibody (50 000 c.p.m.) was added to each well. After a 2 h incubation at room temperature, the plates were washed and 50 µl of the 125I-labelled M17 antibody (50 000 c.p.m.) was added to each well. The plates were then further incubated for 2 h at room temperature. The antibodies were radioiodinated by the Chloramine-T method. After washing, individual wells were cut out and the bound radioactivities were measured.

Purification of the p44 antigen. Approximately 10 g of liver tissue from each chimpanzee was finely chopped and placed in cold NTE (50 mM-Tris–HCl, 130 mM-NaCl, 5 mM-disodium EDTA pH 7-4) buffer to make a 10% (w/v) homogenate suspension using a Physcotron (Nichion Iriks Kiki Seisakusyo). After clarification of the homogenates by centrifugation at 10 000 g for 1 h at 4 °C, the supernatant was pelleted through a 20% (w/v) sucrose cushion by centrifugation at 100 000g for 4 h at 4 °C. The pellets were reuspended in 7-5 ml of NTE buffer, the sample was divided into three and each portion was layered on top of a 32-5 ml 10 to 60% (w/w) sucrose density gradient in NTE buffer and centrifuged at 77 000 g for 60 h at 4 °C. Fractions (2-5 ml) were collected by top to bottom fractionation and assayed for antigen activity by RIA. Density was measured by spectrophotometry. Protein concentrations were determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard.

Fraction 10 from the livers of both chimpanzees was further purified by reverse-phase HPLC (RP-HPLC) using a Cosmosil SC4-300 column (4-6 x 150 mm, Nakaria Tesque). The solvent system used was 0-1% trifluoroacetic acid (TFA) to 80% CH3CN in 0-1% TFA, with a linear gradient for 40 min at a flow rate of 1-0 ml/min. Elution was monitored by the absorbance at 215 nm and the chimpanzee 41-specific peak (arrow in Fig. 2a) was collected. Final purification of the p44 antigen from this fraction was achieved by cutting the 44K band from SDS-polyacrylamide gels, and then extracting the protein from the gel pieces electrophoretically using MAX-YIELD (ATTO) (Hunkapiller et al., 1983). Its purity was then checked by SDS-PAGE.

Amino acid analysis. Aliquots of the purified p44 were used for amino acid analysis. Hydrolysis was carried out in 6 M-HCl containing 0-1% dithiothreitol and 0-01% phenol at 110 °C for 24 h. The amino acid content of the hydrolysates was determined on an MCI AA-01 amino acid analyser (Mitsubishi Kasei Corporation). Aliquots of the same protein solution were used for N-terminal amino acid sequence analysis and peptide mapping. For peptide mapping, the purified p44 antigen was digested with Achromobacter protease I (1:400, mol/mol), which cleaves peptides at lysyl bonds (Masaki et al., 1981). Achromobacter protease I was obtained from Dr T. Masaki, Ibaraki University Department of Agricultural Chemistry, Ibaraki, Japan. After incubation for 6 h at 37 °C, digested peptides were separated by RP-HPLC using a Cosmosil SC4-300 column (4-6 x 150 mm). The solvent system used was 0-1% TFA to 80% CH3CN in 0-1% TFA, with a linear gradient for 50 min at a flow rate of 1-0 ml/min. Elution was monitored by the absorbance at 215 nm. Five different peaks (numbered in Fig. 3) were analysed by automatic Edman degradation on a Applied Biosystems model 470 A protein sequencer (Hewick et al., 1981).

Phenyliodohydantoin (PHT) amino acids were identified by HPLC in a TSK gel ODS-80 column (TOSO) using an acetate–SDS buffer system as previously described (Tsunasawa et al., 1985). The amino acid sequences thus obtained were submitted to the protein information analyser system, PRINAS (Mitsui Knowledge Industry), which was screened against the NBRF and PRF databases by the method of Korn et al. (1977). Western blot analysis. For protein analysis, aliquots of sucrose gradient fraction 10 from each chimpanzee or HPLC eluate were separated by 12-5% polyacrylamide gels according to the procedure of Laemmli (1970). Gels were either stained with Coomassie blue or electrotransferred onto nitrocellulose filters (BA83; Schleicher & Schuell) essentially as described previously (Towbin et al., 1979). The transferred sheets were treated for 1 h at room temperature with blocking buffer (PBS containing 10% skimmed milk) and then incubated for 2 h at room temperature with blocking buffer containing 20 µg/ml of the mouse monoclonal antibody M17. They were then washed four times with washing buffer (20 mM-Tris–HCl, 140 mM-NaCl, 2 mM-disodium EDTA, 0-05% Tween-20 pH 7-4) for 10 min each. The washed sheets were treated with a 1:500 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Cooper Biomedical) at 4 °C overnight. After washing four times with washing buffer, immunoreactivity was detected using 4-chloro-1-naphthol and hydrogen peroxide.

Results and Discussion

To isolate the antigen associated with the microtubular aggregates, a sandwich RIA system using one of our monoclonal antibodies, M17, was set up. A sample of liver containing microtubular aggregates was obtained from a chronically NANB hepatitis-infected chimpanzee. Fig. 1 shows typical results obtained following sucrose density gradient ultracentrifugation and RIA of liver homogenates from each of the chimpanzees. It was found that 60 h centrifugation was effective for the separation of the microtubular aggregates from the other liver proteins with a buoyant density of around 1-14 g/ml in sucrose. The microtubular aggregates formed an apparently homogeneous zone at densities between 1-21 and 1-23 g/ml. No antigen activity was detected in the gradient from the liver of the normal chimpanzee (Fig. 1b).

Aliquots of the peak fraction of antigen activity (fraction 10) were separated by SDS–PAGE, and then either stained with Coomassie blue or transferred to nitrocellulose membranes for Western blot analysis. Fig. 1(c) shows that the mouse monoclonal antibody, M17, recognized a single protein with an Mr of 44 000 (p44).
The same results were obtained using samples from the liver of a chimpanzee with acute NANB infection (data not shown).

For the next step of purification, RP-HPLC separation was carried out. Fig. 2(a) and 2(b) show typical elution profiles of fraction 10 from chimpanzees 41 (infected) and 57 (uninfected), respectively on a C₈ reverse-phase column. When the chimpanzee 41-specific peak (eluted at 39.5 min, arrow in Fig. 2a) was analysed by SDS-PAGE and Western blotting, it was found to be p44 as expected (Fig. 2c). However, this fraction still contained several other proteins (Fig. 2c, lane 1).

Final purification of the p44 antigen was achieved by cutting at 44K band from SDS-polyacrylamide gels and extracting the protein from the gel pieces electrophoretically. Its purity was then checked by SDS-PAGE. A silver-stained gel showed only a single protein (Fig. 2c, lane 3), aliquots of the same protein solution were used for amino acid analysis and N-terminal amino acid sequence analysis. Edman degradation was performed but no PTH amino acids were detected, suggesting that the N terminus of p44 was blocked.

Therefore, to obtain sequence information, purified p44 antigen was digested with *Achromobacter* protease I, which cleaves peptides at lysyl bonds. Digested peptides

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**Table 1. Amino acid sequences of cleaved p44 peptides**

<table>
<thead>
<tr>
<th>Peptide no.*</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ILQNFHFFGK</td>
</tr>
<tr>
<td>2</td>
<td>YRTYSIRDGK</td>
</tr>
<tr>
<td>3</td>
<td>VIMDLK</td>
</tr>
<tr>
<td>4</td>
<td>YNSPTNFQIDGRNRK</td>
</tr>
<tr>
<td>5</td>
<td>SFFNVSRSVFQGHV</td>
</tr>
</tbody>
</table>

* Peptide numbers correspond to the peak numbers in Fig. 3.
were separated by RP-HPLC on a C₈ reverse-phase column. Fig. 3 shows a typical elution profile for the cleaved peptides. More than 20 peptide peaks were obtained. Five different peaks (numbered in Fig. 3) were analysed by a gas-phase protein sequencer. The results obtained are summarized in Table 1. The amino acid sequences thus obtained were screened against the NBRF and PRF databases by the protein information analyser system, PRINAS. A computerized search indicated that p44 did not resemble any known proteins including prion protein and hepatitis A, B or D viral proteins.

Recently, we isolated a chimpanzee cDNA clone of the p44 gene. Determination of its amino acid sequence has allowed us to confirm the oligonucleotide sequence of the p44 gene (Takahashi et al., 1990). Studies on the expression of the p44 gene in various tissues may enable the origin and the mechanisms of microtubular aggregate biosynthesis to be determined, giving a better understanding of the pathogenesis of NANB hepatitis.

We thank Noriko Yahata, Rumiko Hashimoto, Masataka Sato and Toshiyuki Honda for technical assistance, Dr Takeharu Masaki for the supply of Achromobacter protease I and Mr Daniel Mrozek for carefully reading the manuscript.

References

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Fig. 3. Separation of Achromobacter protease I digests of p44 antigen on a C8 reverse-phase column. Numbered peaks were analysed by automatic Edman degradation on an Applied Biosystems model 470 A protein sequencer (see Table 1).


(Received 1 February 1990; Accepted 9 May 1990)