JOURNAL OF VIROLOGY, Aug. 1980, p. 572-574
0022-538X/80/08-0572/03$02.00/0

Vol. 35, No. 2

Iodination of Hepatitis A Virus Reveals a Fourth Structural Polypeptide

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Hepatitis A virus present in the feces of two patients with naturally acquired hepatitis A was purified, radiolabeled with ^{125}I, and analyzed by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In addition to the three structural polypeptides previously reported, a fourth polypeptide with a molecular weight of 14,000 was detected and shown to be a component of hepatitis A virus by immune precipitation techniques. Intact virions were also shown to sediment at 160S on sucrose gradients. These findings are consistent with hepatitis A virus being an enterovirus within the family Picornaviridae.

Since the discovery of hepatitis A virus (HAV) by Feinstone et al. in 1973 (2), detailed study of its biochemical characteristics has been hampered by the absence of tissue culture systems for propagating the virus and by the difficulty in obtaining large quantities of virus from humans (6) and experimentally infected animals (7). In 1977, the detection of moderate quantities of virus in fecal specimens from three patients with naturally acquired hepatitis A provided us with an opportunity to commence biochemical studies on HAV (1). These studies, which involved the analysis of purified virus by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), revealed three major polypeptides (VP1, VP2, and VP3) with molecular weights of 34,000, 25,500, and 23,000 (1) which were similar to three of the four structural polypeptides found in members of the genus *Enterovirus* within the family Picornaviridae (1, 8).

In an attempt to detect other polypeptides which may be present in trace amounts, the sensitive technique of radiolabeling with ^{125}I was investigated.

Fecal specimens and acute and convalescent sera were collected from a number of patients with hepatitis B surface antigen-negative hepatitis and their family contacts. Extracts of the feces were tested for the presence of HAV by solid-phase radioimmunoassay (9) and immune electron microscopy (6). After preliminary screening, two specimens containing large numbers of HAV particles were chosen for further study. The two specimens were kept separate and purified by a process of differential centrifugation, solvent extraction, agarose gel filtration, and isopycnic ultracentrifugation in cesium chloride as described previously (1, 3, 5, 6).

Direct electron microscopy of the two purified fecal specimens revealed large numbers of 27-nm particles and minimal background debris. Immune electron microscopy with appropriate sera confirmed that these were preparations of HAV. In addition, no adventitious agents were detected by electron microscopy or cell culture.

After purification, 30-μl samples of purified HAV (25 μl of HAV in 0.25 M phosphate buffer [pH 7.4] plus 5 μl of 1 M phosphate buffer [pH 7.4]) were mixed with 200 μCi of $Na^{125}I$ (New England Nuclear Corp., Boston, Mass.) previously diluted in 0.05 mM NaI. Next, 10 μl of chloramine T (3.5 mg/ml) was added to the reaction mixture for 15 min at room temperature (22°C). The reaction was stopped by the addition of 10 μl of sodium metabisulphite (4.8 mg/ml) followed by 10 μl of potassium iodide (10 mg/ml). The volume of the reaction mixture was then made up to 100 μl by the addition of 30 μl of 0.25 M phosphate buffer (pH 7.4), and free unreacted ^{125}I was removed by gel filtration in Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) with a column measuring 1.5 × 8 cm. A total of 20 1-ml fractions were collected. The excluded volume containing radiolabeled HAV was pooled and concentrated by ultracentrifugation at 100,000 × *g* for 2 h at 4°C, and the pellets were suspended in 1 ml of phosphate-buffered saline (pH 7.4). Samples of this material were layered on top of 16-ml 10 to 50% (wt/vol in phosphate-buffered saline [pH 7.4]) linear sucrose gradients in SW41 cellulose nitrate tubes. The gradients were centrifuged for 3 h at 25,000 rpm at 4°C in a Beckman model L5-65B ultracentrifuge. A total of 17 1-ml fractions were collected from the bottom of each tube, and the presence of HAV was determined by solid-phase radioimmunoassay. Fractions containing HAV were centrifuged at 100,000 × *g* for 2 h at 4°C, and the pellets were suspended in 2% SDS for electrophoresis and in 0.5 ml of 0.25 M phosphate buffer (pH 7.4) for rate zonal

ultracentrifugation to determine the sedimentation coefficient of the intact virion.

Samples of iodinated HAV in 2% SDS were processed for discontinuous SDS-PAGE as described previously (1) in a slab gel system by the method of Laemmli (4). After electrophoresis, gels were fixed in 5% glacial acetic acid for 30 min and then dried under vacuum onto a piece of Whatman 3 MM filter paper. Autoradiographs from the dried gels were obtained by exposing the gels to X-ray film (X-Omat/S Medical X-Ray Film; Kodak, Melbourne, Australia) in the dark. The molecular weights of virion polypeptides were determined by comparing their electrophoretic mobilities to those of the following iodinated internal markers: RNase, lysozyme, chymotrypsinogen, carbonic anhydrase, ovalbumin, and bovine serum albumin (protein calibration kit from Polysciences Warrington, Pa.).

Analysis of iodinated HAV by SDS-PAGE revealed six polypeptides. For determining if these were all structural polypeptides of HAV, a series of immune precipitation experiments (1) was carried out (Fig. 1). Tracks A, B, and C of Fig. 1 show the disrupted products of reactions between iodinated HAV and phosphate-buffered saline and pre- and postinfection immunoglobulin G obtained from a patient with serologically confirmed hepatitis A. These immune precipitation experiments demonstrated that four of the six polypeptides, VP1, VP2, VP3, and VP4, are found in the immune complexes produced by the reaction of purified HAV and postinfection immunoglobulin G alone and are therefore structural components of HAV. Track D of Fig. 1 shows the SDS-PAGE analysis of the bovine serum albumin used throughout the purification procedure to help stabilize and pellet the virus during ultracentrifugation and also to minimize adsorption to various surfaces during purification. It appears that although p66 and p68 are not structural proteins, they are firmly bound to the surface of the virion. Their molecular weights are identical to those of bovine serum albumin. It therefore seems probable that they represent heterologous bovine serum albumin which becomes attached to the virus and iodinated with it.

For determining the sedimentation coefficient of iodinated HAV, poliovirus type 1 (Brunhilde strain, obtained from Virgo Reagents, Electronucleonics Laboratories, Inc.) was iodinated and used as a marker. Samples (0.5 ml) of iodinated poliovirus type 1 and iodinated HAV, together with non-iodinated samples of these viruses, were layered on top of parallel 16-ml 10 to 50% (wt/vol in phosphate-buffered saline [pH 7.4]) linear sucrose gradients in SW41 cellulose nitrate tubes. The gradients were centrifuged for 3 h at 25,000 rpm at 4°C in a Beckman model L5-65B ultracentrifuge. A total of 17 1-ml fractions were collected from the bottom of each tube. The presence of HAV was detected by solid-phase radioimmunoassay, and the presence of poliovirus was detected by electron microscopy (Fig. 2).

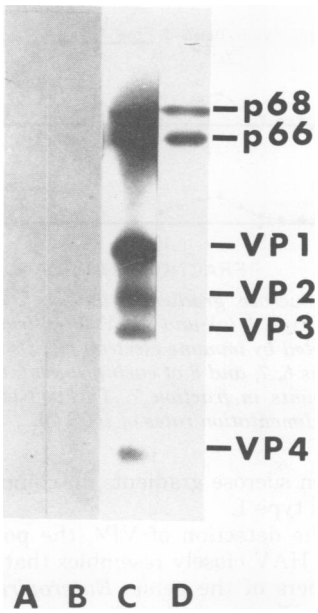

FIG. 1. *Autoradiograph showing the SDS-PAGE analysis of immune precipitates produced by the reaction of HAV which had been iodinated for a prolonged period of time (15 min) and phosphate-buffered saline (track A). Track B, Serum collected from a patient with hepatitis A before the onset of illness; Track C, convalescent serum from the same patient. Track D shows the SDS-PAGE analysis of bovine serum albumin used throughout the experiment. Under these conditions, VP1, VP2, VP3, and VP4 were detected only in the reaction product of HAV and serum containing specific antibody. In comparison to iodinated molecular weight markers, the polypeptides seen in track C had the following molecular weights: VP1, 33,000; VP2, 26,500; VP3, 22,500; p66, 66,000; and p68, 68,000.*

Iodination of both poliovirus and HAV did not affect the sedimentation of these viruses through sucrose, and HAV was found to have a sedimentation rate similar to that of poliovirus (160S) (8).

In summary, the sensitive technique of radioiodination was successfully used to detect a fourth previously unrecognized polypeptide with a molecular weight of 14,000, in addition to the polypeptides previously reported (1). HAV was also shown to have a sedimentation coefficient

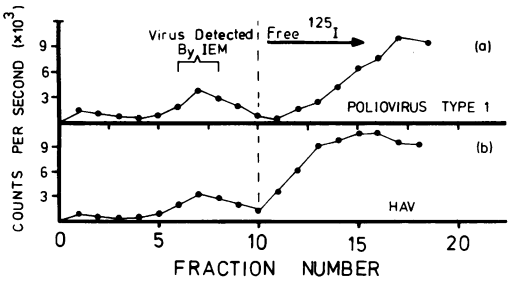

FIG. 2. *Sucrose gradient studies of ^{125}I-labeled poliovirus type 1 (a) and HAV (b). Virus particles were detected by immune electron microscopy (IEM) in fractions 6, 7, and 8 of each gradient, with maximum amounts in fraction 7. The two viruses had similar sedimentation rates of 160S (8).*

of 160S on sucrose gradients, in comparison to poliovirus type 1.

With the detection of VP4, the polypeptide profile of HAV closely resembles that reported for members of the genus *Enterovirus* within the family Picornaviridae (8).

This work was supported by grants from the Wenkart Foundation and the National Health and Medical Research Council of Australia.

We are grateful for the cooperation of the medical and nursing staff of Fairfield Hospital in the collection of specimens; E. G. Westaway, P. D. Cooper, R. Pringle, and D. Guthrie for helpful discussions; A. Stratton for technical assistance; and Loris Brenton for typing the manuscript. This study was greatly assisted by international direct calling facilities provided by the Australian Overseas Telecommunications Commission.

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