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Characterization and Classification of Virus Particles Associated with Hepatitis A

III. Structural Proteins

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Hepatitis A virus was purified from fecal samples collected at various times in the incubation period of patients with naturally acquired hepatitis A. The proteins of particles banding at around 1.34 g/ml in CsCl and sedimenting at about 160S were radioiodinated in vitro and separated by electrophoresis on polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate and 8 M urea. Under these conditions, the capsid proteins resolved into four polypeptides with molecular weights of approximately 31,000, 24,500, 21,000, and 9,000, respectively. A fifth protein of about 40,000 daltons in size and assumed to be equivalent to the precursor polypeptide VP0 of the picornaviruses was present in particles sedimenting at only 150 to 155S and banding at around 1.33 g/ml in CsCl. The physicochemical characteristics of these particles are consistent with those of the provirion structures of picornaviruses. In several of the fecal samples, these particles represented a considerable fraction of all particles present. The significance of this finding with respect to the antigenicity of hepatitis A antigen extracted from stool specimens is discussed.

Hepatitis A virus (HAV) has been identified repeatedly in samples of feces, bile, and liver collected from patients in an epidemic of hepatitis or from volunteers, chimpanzees, and marmosets in controlled experimental studies. The virus particles measured 27 nm in diameter, banded at around 1.34 g/ml in CsCl, and sedimented at about 160S (3,7, 16-18). Furthermore, there is evidence to suggest that the genome of HAV consists of single-stranded RNA (8, 13, 17, 19). These physicochemical characteristics of the purified particles strongly support the classification of HAV with the picornaviruses.

Intact picornavirus particles contain four well-defined proteins. The available data on the polypeptide spectrum of HAV, however, are only partially in agreement with this requirement. Three polypeptides in the size range of picornavirus structural proteins VP1 to VP3 were regularly detected (5, 8), but only very recent experiments revealed trace amounts of a fourth, minor polypeptide migrating in the appropriate position of VP4 (6). On the other hand, Feinstone et al. (8) reported the presence of an additional virus-associated protein with a molecular weight of 59,000. The size of this protein is much larger than that of the precursor polypeptide VP0 (38 to 44 kilodaltons [kD]) which is present in immature picornavirus capsids.

Here we provide evidence that HAV particles collected at various times in the incubation period of patients with naturally acquired hepatitis A show a protein spectrum compatible with that of known picornaviruses. Our results also suggest that variations in the protein pattern can be related to the relative concentrations of immature, provirion-like structures and mature virions in the HAV samples under investigation.

MATERIALS AND METHODS

Origin and purification of viruses. The fecal specimens used as a source of HAV were collected from an individual involved in an epidemic of hepatitis A in Germany (sample no. 1) (9) and from patient no. 2, who contracted the infection during holidays in the Mediterranean area. A complete series of fecal samples from patient no. 2 was available from days −9 to 4 (day 0 was the onset of disease) (8a). Samples no. 2 11/5, no. 2 11/6, and no. 2 11/9, collected 5, 4, and 1 day before the onset of disease, respectively, were used as a source of HAV particles. Stool of patient no. 1 was obtained on the 6th day before the onset of jaundice. The serological and physicochemical characteristics of particles from the latter material, as well as the method used for purification, have been described in detail previously (18, 19).

All steps in the purification and concentration of

HAV were monitored by assaying aliquots of samples or gradient fractions for the presence of hepatitis A antigen by means of a solid-phase radioimmunoassay (9, 18). Poliovirus type 2 antigen could not be detected by this radioimmunoassay. Therefore, the virus was radiolabeled as described previously (18) and then could be used as an internal density and sedimentation marker in the analysis of HAV sedimentation and buoyancy characteristics.

Radioiodination of viral proteins. For labeling of virion proteins with the ^{125}I-Bolton-Hunter reagent (1), virus particles suspended in 0.1 M sodium borate (pH 8.4)–1 mM EDTA were disrupted in the presence of 1% sodium dodecyl sulfate (SDS) by boiling for 1 min. Samples of 50 to 100 μl of the SDS-complexed polypeptides were then iodinated with 100 to 200 μCi of the ^{125}I-labeled Bolton-Hunter reagent in a siliconized glass tube at 0°C for 90 min under standard conditions (1). Labeled proteins were separated from low-molecular-weight components by gel filtration on a Sephadex G-10 column equilibrated with elution buffer (50 mM Tris-hydrochloride [pH 6.8], 0.2% gelatin) and prerun with 0.5 ml of a 10% solution of bovine serum albumin.

For iodination of proteins by the chloramine-T method, virus particles were dialyzed against 50 mM sodium phosphate (pH 7.5) before disruption by boiling. Iodination under the conditions given by Carthew and Martin (4) was allowed to take place at room temperature for 1 min, and labeled proteins were separated from unreacted iodide by gel filtration as described above.

The ^{125}I-labeled Bolton-Hunter reagent (specific activity, 2,000 Ci/mmol) and [^{125}I]iodide (carrier-free) were obtained from the Radiochemical Centre, Amersham, England.

Gel electrophoresis. Samples of labeled HAV proteins were prepared in the appropriate sample buffers of the gel systems described below and, in general, were heated to 90°C for 15 min before loading onto the gels. Three types of gel and buffer system were used for electrophoresis: (i) SDS-polyacrylamide gels in the discontinuous buffer system described by Laemmli (12); (ii) SDS-polyacrylamide gels in the same discontinuous buffer system supplemented with either 1 or 4 M urea by the method of Bowen et al. (2); and (iii) a modification of the continuous phosphate buffer system containing 8 M urea as described by Summers et al. (21). In the latter system, both the gel and the running buffer contained 0.1 M sodium phosphate (pH 8.0)–0.1% SDS–8 M urea. Protein samples were prepared in the same buffer plus 1% β-mercaptoethanol, 2% SDS, 8 M urea, 20% sucrose, and 0.002% bromophenol blue. Electrophoresis in discontinuous gels was carried out in slab gels (120 by 130 by 1.5 mm) at 80 V per gel, whereas electrophoresis in continuous phosphate-urea gels was performed in glass tubes (5 by 130 mm) at a constant current of 7 mA per tube. Proteins were fixed and stained in 1.5% Coomassie blue–1% amido black–45% ethanol–9% acetic acid and destained in 15% ethanol–7.5% acetic acid. Gels were finally sliced into 1-mm sections, and the radioactivity was determined without further treatment of the slices in a Packard PRIAS Auto-Gamma counter.

RESULTS

Electrophoresis of the proteins of mature poliovirus particles disrupted by boiling and radioiodinated in vitro revealed the well-known pattern (14) of the four structural polypeptides VP1 to VP4, as well as traces of the precursor polypeptide VP0. When polypeptides of HAV particles were analyzed under identical conditions, only particles purified from stool no. 1 gave rise to a polypeptide spectrum that was more or less consistent with that of the marker picornavirus (Fig. 1). It consisted of four polypeptides tentatively labeled VP1 to VP4 and about 35, 31, 26, and 7 kD in size. Moreover, two additional polypeptides with apparent molecular weights of 45,000 and 48,000 could be distinguished. The latter polypeptides were also present in all HAV preparations purified from fecal specimens of patient no. 2. In general, they constituted the majority of labeled proteins in these samples, whereas the polypeptides migrating in the positions of poliovirus polypeptides VP1 and VP4 could barely be detected or were not present at all.

A variation in several experimental parameters (e.g., the technique used for radioiodination, treatment of samples before electrophoresis, origin and concentration of acrylamide, and addition of 1 and 4 M urea to the discontinuous gel system) had no significant effect on the qualitative and quantitative aspects of the polypeptide pattern of individual samples. However, electrophoresis in a continuous phosphate buffer system containing 0.1% SDS and 8 M urea resulted in the following drastic changes (Fig. 2). Besides a high-molecular-weight peak, three major proteins and a fourth minor protein could be detected regularly in all HAV samples. All of these polypeptides appeared to be smaller than the respective structural proteins of poliovirus (Table 1). The only high-molecular-weight polypeptide resolved in the presence of 8 M urea had an apparent molecular weight of about 40,000. This is clearly within the size range of the precursor polypeptide VP0 of well-characterized picornaviruses.

Polypeptide VP0 of picornaviruses is present in significant quantities only in "empty" capsids or in immature virus particles. These types of particle differ from the mature virion in their buoyancy and sedimentation behavior (14). As buoyant density centrifugation of aliquots of the HAV samples in shallow CsCl gradients indicated (Fig. 3), only particles purified from stool no. 1 and known to be almost devoid of the VP0-like polypeptide had a buoyancy behavior comparable to that of poliovirus; yet peak concen-

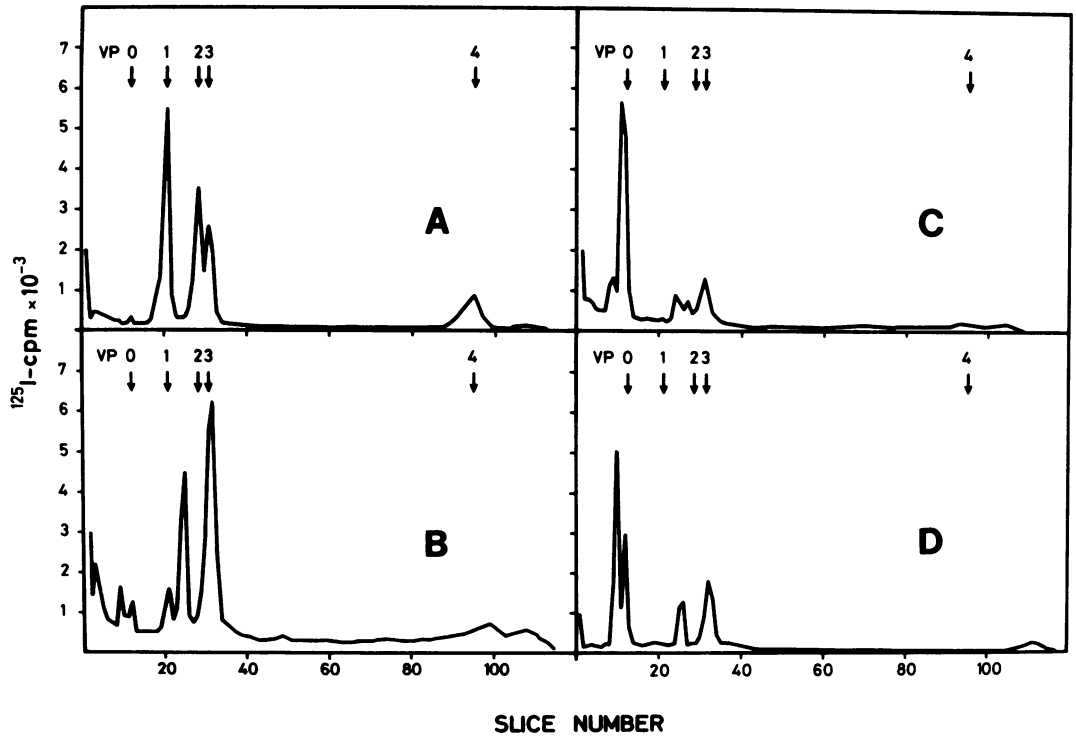

FIG. 1. *SDS-polyacrylamide gel electrophoresis of the capsid proteins of poliovirus type 2 (A) and of HAV particles extracted from stool samples no. 1 (B), no. 2 11/5 (C), and no. 2 11/6 (D). The purified viruses were disrupted in the presence of 1% SDS by heating to 100°C for 1 min before radioiodination with the Bolton-Hunter reagent. Labeled proteins were again heated to 100°C for 2 min (1% SDS, 2.5% β-mercaptoethanol), and polypeptides were separated by electrophoresis in a discontinuous 15% SDS-polyacrylamide slab gel by the method of Laemmli (12). Arrows indicate the positions of polypeptides VP0 to VP4 of poliovirus.*

trations of hepatitis A antigen accumulated at a slightly lower density (1.336 g/ml) than did the marker picornavirus (1.34 g/ml). In contrast, virus samples extracted from the fecal specimens of patient no. 2 and containing the VP0-like protein in considerable quantity regularly banded at densities of below 1.336 g/ml. In some of these samples, there was also evidence that suggested the presence of more than one particle species (e.g., no. 2 11/6 in Fig. 3). Sedimentation studies yielded consistent results (data not shown in detail). Particles extracted from sample no. 1 sedimented at 157S, whereas 150 to 154S was recorded for HAV contained in the stools of patient no. 2.

DISCUSSION

The results reported in this paper are in accordance with the observations of Coulepis et al. (5) and Feinstone et al. (8) in as far as they confirm the existence of three major protein components in HAV capsids with molecular weights similar to those of the structural polypeptides VP1 to VP3 of picornaviruses. Moreover, there is evidence to suggest that the HAV particles also contain a minor polypeptide component migrating in the position of picornavirus polypeptide VP4, as well as a high-molecular-weight protein comparable in size to the precursor polypeptide VP0. The concentration of the latter proteins, however, varied with the origin of the virus particles.

Rigorous experimental conditions proved to be necessary for resolving the polypeptide spectrum of HAV in a reliable and reproducible manner. At least one of the high-molecular-weight peaks of the spectrum displayed in discontinuous gel systems disappeared upon electrophoresis in the presence of 0.1% SDS–8 M urea, and radiolabeled material lost from that region apparently added to the concentration of polypeptides VP1 to VP4 (e.g., no. 2 11/6 in Fig. 1 and 2). Therefore, it may be concluded that the formation of aggregates of one or more structural polypeptides rather than contamination of virus samples with unrelated or virus-specific

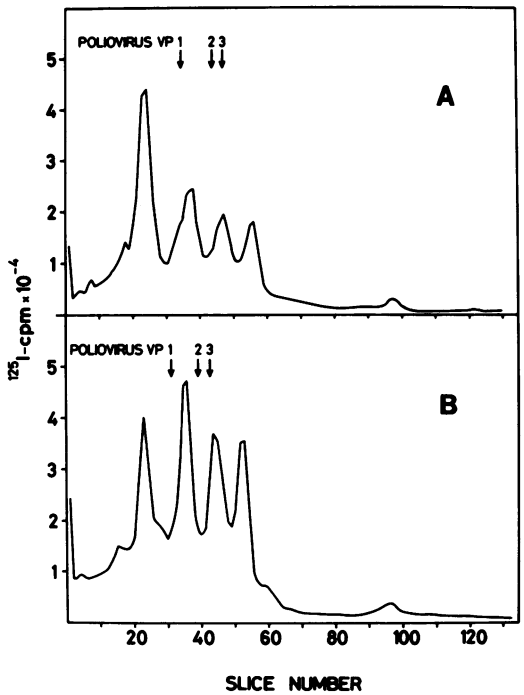

FIG. 2. *Polypeptides of HAV particles extracted from stool samples no. 2 11/6 (A) and no. 2 11/9 (B) as revealed by electrophoresis in continuous 12.5% polyacrylamide tube gels containing 8 M urea. HAV particles were disrupted as described in the legend to Fig. 1, radioiodinated by the chloramine-T procedure, and heated to 90°C in the presence of 2% SDS-1% β-mercaptoethanol-8 M urea for 15 min. Arrows indicate the positions of unlabeled poliovirus polypeptides coelectrophoresed in the same tubes and located by protein staining.*

but nonstructural proteins (e.g., the equivalents of the 52- and 57-kD proteins detected in foot-and-mouth disease [15] or Mengo virus [22] particles, respectively) might be responsible for the appearance of high-molecular-weight components in the protein pattern of HAV as occasionally revealed in Laemmli gels (8).

If we assume that HAV is a typical picornavirus, it is obvious to relate the 40-kD polypeptide resolved in SDS-urea gels to the precursor polypeptide VP0. This protein is known to be present in procapsids (empty capsids) of picornaviruses and, in the process of maturation of the virus, is cleaved into VP2 and VP4 (14). The only type of particle in which VP0, VP2, and VP4 have been found simultaneously and in various concentrations is an immediate precursor, the provirion, of mature enterovirus particles (10, 11, 20). Provirions contain a full complement of viral RNA, sediment at 130 to 150S,

TABLE 1. *Apparent molecular weight of HAV polypeptides in 10 and 12.5% polyacrylamide gels containing 0.1% SDS and 8 M urea*

Polypeptide[a]	Mol wt[b]
VP0	39,600 ± 1,000
VP1	30,800 ± 800
VP2	24,500 ± 700
VP3	20,800 ± 1,000
VP4	9,000 ± 2,000

[a] Tentative classification.

[b] Poliovirus polypeptides (VP0, 41 kD; VP1, 32 kD; VP2, 28 kD; VP3, 25 kD; VP4, 8 kD) were used as internal markers and detected by staining with Coomassie blue. Additional molecular weight markers were bovine serum albumin (68 kD), gamma globulin, H-chain (50 kD), ovalbumin (43 kD), pepsin (35 kD), chymotrypsinogen (25.7 kD), papain (23 kD), cytochrome *c* (11.7 kD), and insulin B chain (3.4 kD). Numbers represent mean values of seven determinations.

and band at lower densities in CsCl than does the mature virion. The analogies between the protein pattern and the buoyancy and sedimentation characteristics of these structures and the HAV particles under investigation are striking. Therefore, it is conceivable that the 40-kD protein indeed represents the equivalent of the precursor polypeptide VP0. At the same time it must be concluded that the majority of HAV particles present in the fecal samples collected from patient no. 2 are provirion structures. Since provirions of picornaviruses probably are not infectious (11), the concentration of physical particles allows no reliable conclusion to be drawn about the infectivity of a certain stool sample. This point must be considered when the many unsuccessful attempts to infect cell cultures with HAV-containing fecal extracts are evaluated.

Finally, it is well known that mature picornavirus capsids carry a characteristic immunological reactivity defined as D (or N) antigen. The absence or loss of VP4 from these particles is equivalent to the appearance of an entirely new set of surface determinants, the C (or H) antigen (14). The antigenicity of provirion particles has not been determined. In light of our protein data, however, it is tempting to speculate on the extent to which the use of uncharacterized fecal extracts as a source of hepatitis A antigen could affect the significance and reproducibility of the available serological tests for anti-HAV antibodies.

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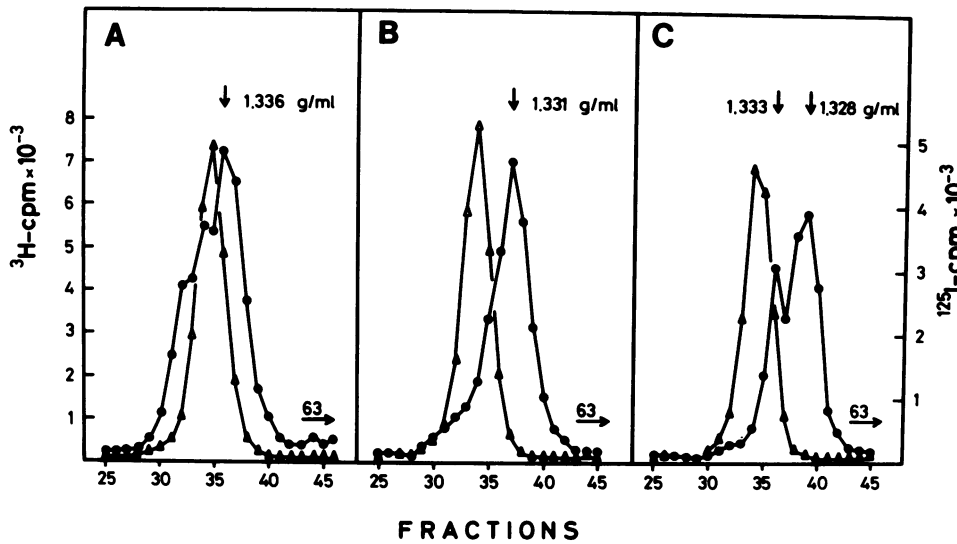

FIG. 3. *Buoyant density centrifugation of mature poliovirions (△) and HAV particles (●) purified from stool samples no. 1 (A), no. 2 11/5 (B), and no. 2 11/6 (C) in CsCl. [^{3}H]uridine-labeled poliovirus (10 μl) and unlabeled HAV particles (50 μl) were mixed with 7.9 ml of CsCl (ρ = 1.34 g/ml; made up in 50 mM Tris-hydrochloride [pH 7.4]-0.1% Sarkosyl NL97) and centrifuged to equilibrium in a Beckman 50 Ti fixed-angle rotor at 40,000 rpm at 15°C for 60 h. A total of 63 fractions were collected by bottom puncture of the tubes. Samples of each fraction were assayed for ^{3}H radioactivity by liquid scintillation counting and for the presence of hepatitis A antigen in the radioimmunoassay. Poliovirus was assumed to band exactly at a density of 1.34 g/ml.*

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