J Clin Pathol 1986;**39**:808–809

Technical method

Rapid method for the concentration of hepatitis B virus particles using Beckman airfuge

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Many experimental manipulations of hepatitis B virus (HBV) particles and clinical assays on intact particles, such as measurement of the endogenous DNA polymerase (DNAP) activity[1 2] require a preliminary concentration step. This is usually achieved by the centrifugation of serum samples using the conventional preparative ultracentrifuge. This ultracentrifugation step can be so time consuming that it restricts the number of samples that can be analysed, unless a specially manufactured ultracentrifuge rotor, with a large number of sockets is available. We investigated the use of the Beckman airfuge as a rapid and simple alternative method for this concentrating step, which may be more suitable for routine use in clinical pathology.

Material and methods

PATIENT SAMPLES

Sera were obtained from patients positive for the markers HBsAg and HBeAg, HBV-DNA (by DNA dot hybridisation), and endogenous DNAP activity. Negative serum samples were obtained from patients lacking these four markers. The serial dilution used and recovery experiments made were obtained from one patient throughout the study.

CONVENTIONAL ULTRACENTRIFUGATION

Each serum sample (200 ul) was layered over 5 ml of 30% sucrose (w/v) containing 0·01M Tris-hydrochloride (pH 7·5), 0·15M sodium chloride, 0·005M edetic acid, and 1%–2% 2-mercaptoethanol in 5 ml polycarbonate tubes and particles were pelleted at 40 000 rpm for four hours at 4°C in an MSE SW45 rotor.

Accepted for publication 30 January 1986

BECKMAN AIRFUGE CENTRIFUGATION

The serum samples (up to 175 ul) were centrifuged in cellulose propionate airfuge tubes for various lengths of time in an 18° A-100 rotor at 30 psi (nominally 100 000 rpm at 20°C). Immediately after centrifugation supernatants were removed with an automatic pipette.

ENDOGENOUS DNA POLYMERASE

After centrifugation the pellets were resuspended in 50 ul of 1·5% triton X-100, 0·1% 2-mercaptoethanol, 0·01M Tris-hydrochloric acid (pH 7·5), and 0·15M sodium chloride, and allowed to stand overnight at 4°C. To each sample was added 25 ul of 0·16M Tris-hydrochloric acid (pH 7·5), 0·08M magnesium acetate, 1·2M potassium chloride, 0·001M each of dATP, dGTP and dTTP, 2·5 ul of (α-^{32}P) dCTP (7000 Curi/mmol, 20 UCI/ul; New England Nuclear). The reaction was incubated at 37°C for four hours. DNAP activity was assessed by measuring the trichloracetic acid-precipitable radioactivity by scintillation counting.

HBV DNA DOT HYBRIDISATION

This was assayed as previously described.[3]

RESTRICTION ENZYME ANALYSIS

Hepatitis B virus (HBV) particles were pelleted from sera from patients known to be positive for HBV-DNA and DNAP. The DNAP reaction was carried out and then the sample was treated with proteinase K (Boehringer) (50 ug/ml for two hours at 37°C). The DNA was extracted by phenol and chloroform and precipitated with ethanol. The pellet was resuspended in the appropriate restriction enzyme buffers and cut with various restriction enzymes. The resulting fragments were resolved by electrophoresis in 0·8% agarose, transferred to nitrocellulose paper by Southern blotting,[4] and the fragments visualised by probing with nick translated ^{32}P labelled HBV probe followed by autoradiography.

ELECTRON MICROSCOPY

After the Beckman airfuge centrifugation step the pellet was resuspended in distilled water (16 hours at 4°C) and negatively stained with 2% phosphotungstic acid. The preparation was examined in a high resolution transmission electron microscope.

Results

Initially, we investigated the minimum time required for the Beckman airfuge to concentrate HBV into a pellet. Centrifugation of a positive serum sample for

Table *Detection of HBV in various fractions of serum samples, as assayed by DNA dot hybridisation*

Specimen	*Total counts*	*Recovery (%)*
Original specimen	2492	100
Pellet 1	1705	68
Pellet 2	37	<2
Pellet 3	29	<2
Pellet 4	28	<2
Supernatant 4	0	

Four serial centrifugations were performed in the airfuge. Aliquots of the original specimen, each of the four pellets, and the final supernatant were assayed by dot hybridisation. Portions of the nitrocellulose containing the dots were cut out and quantitated by liquid scintillation counting. A control negative serum sample was used to correct for background.

less than 20 minutes failed to concentrate HBV particles as a pellet (when assayed by HBV-DNA hybridisation). After 30 to 40 minutes, however, the virus particles were retained as a pellet when the supernatant was removed. Centrifugation for periods in excess of this time produced negligible improvement in yield and we therefore used 40 minutes of centrifugation as the time for subsequent experiments.

We confirmed that the pellets contained intact HBV virions by examining them under the electron microscope, and intact double shelled Dane particles were seen. We also extracted HBV-DNA from four pellets and subjected them to restriction enzyme analysis. In each case this produced a restriction map consistent with that expected from a full genome length of HBV (data not shown).

We compared the DNAP activity of six positive and six negative serum samples, using the conventional ultracentrifugation method, with those using the Beckman airfuge method, and the results were entirely concordant.

The recovery of viral particles was measured using the airfuge procedure. A positive serum sample was centrifuged and the supernatant removed. This supernatant was subjected to three further serial centrifugations, and the four pellets were kept separate. Aliquots of the original serum, resuspensions of the four pellets, and the final supernatant were then assayed by HBV-DNA hybridisation (Table). We estimated that a 68% recovery of HBV was obtained in the first pellet and only small amounts of HBV (less than 5%) present in the original sample were recovered in subsequent pellets.

Discussion

We developed a simple protocol for the preliminary step of concentrating HBV particles by using the Beckman airfuge as an alternative to the conventional ultracentrifuge. The airfuge procedure can be carried out in 40 minutes, in contrast to a conventional ultracentrifugation protocol, which may require specially designed ultracentrifuge rotors and a run time in excess of four hours. The method uses a small initial volume (175 ul) and gives a high yield of particles, comparable with that expected from sucrose cushion ultracentrifugation.

The HBV particles obtained by this procedure seem to be morphologically intact and retain DNA polymerase activity. While there is the possible disadvantage that the HBV particles obtained by the airfuge method are contaminated by residual serum from the original sample, the pellet is not contaminated with sucrose, as would happen with conventional ultracentrifugation. In practice this does not seem to be a problem, and we found the method yields HBV particles whose DNA can be probed, cut with restriction enzymes, ligated, and cloned.

The development of this simple protocol for the Beckman airfuge has enabled us to perform assays for HBV endogenous DNAP activity as a routine clinical service with a turnaround of one night.

We thank B Bennetts and M Hobbs for helpful encouragement and advice. This work was supported by the Leo Leukemia and Cancer Research Trust (who provided the Beckman airfuge) and by grants from the National Health and Medical Research Council of Australia.

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