Heavy-density Particles of Australia Antigen Detected in a Human Plasma Pool

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SHIRACHI, R., SUKENO, N., UMENAI, T. and ISHIDA, N. *Heavy-density Particles of Australia Antigen Detected in a Human Plasma Pool*. Tohoku J. exp. Med., 1972, 107 (4), 359-365 — Biophysical study of Australia antigen (Au) was made with 5 specimens including 3 plasmas and 2 plasma pools which had been known to be Au carriers. A fraction which could be detected both by complement fixation test and by Ouchterlony double diffusion test was obtained with all of the specimens and this could be banded at a buoyant density of 1.21-1.23 g/cm³ in CsCl. In addition, another fraction with a buoyant density of 1.28 g/cm³ was recovered from one of the plasma pools. This fraction was detected, however, only by complement fixation test. Electron microscopic study of the latter fraction revealed small particles about 3-4 m μ in diameter, suggesting subunits of known Au antigen particles. ——— hepatitis; Au antigen

In 1968, Bayer, Blumberg and Werner described 20 m μ particles in Australia (Au) antigen-containing serum fraction from sucrose gradients. The particles were virus-like and aggregated by the same sera that precipitated the antigen. On the basis of such observations, many investigators have been convinced that the antigen is intimately associated with a virus-like structure that is responsible for B type hepatitis (Prince 1968, Blumberg *et al.* 1968). When studies were undertaken to determine the densities of the Au antigen and the particle morphology with various plasma specimens collected throughout Japan, one plasma pool obtained from Tokyo University through the courtesy of Dr. Okochi contained two antigen fractions of different densities. This report describes characteristics of these two antigens, which bear on the subunit structure of the known Au particles.

MATERIALS AND METHODS

Source of Au antigen

Five plasma specimens were purified under the same condition. Three plasmas (B, H, C) collected through Japan Red Cross Blood Center of Sendai were from healthy carriers of Au antigen. Two plasma pools (K, P) were obtained from Tokyo University. The pool K was made from two individuals (5210 and 1008), and P from three (2129, 2290 and 0128). They were stored frozen at -20°C until use.

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Complement fixation (CF) test

The CF antigen titers were measured in microtiter plates, using 2 CF antibody units and 2 exact units of complement (Sever, 1962). Serum containing antibody to Au was obtained from a healthy high school boy (Chubachi) and was heated at 56°C for 30 min. This serum has been used as a reference antibody for these 2 years. Gradient fractions of Au antigen were tested with prior heat inactivation.

Ouchterlony double diffusion (AGD) and immunoelectrosyneresis (IES) tests

The micro-Ouchterlony technique was carried out with the use of 1% agar-agar (Wako Pure Chemicals) and 0.01 M veronal buffer (pH 8.6) system. Au antiserum was added in the center well of seven hole micro-Ouchterlony pattern (LKB) on slide and samples containing Au antigen were added in the peripheral wells. The slides were kept for 48 hrs at room temperature in a moist chamber. The IES test of sample (5 μ l) was performed on slides layered with 1.0% agar solution in veronal buffer of pH 8.6, with an electric strength of 15 V/cm for 40 min at room temperature.

Reagents

Cesium chloride (Merck AG) was used without further purification. Sucrose solutions were prepared by dissolving ribonuclease free sucrose (Nakarai Chem.) in 0.01 M phosphate buffer (pH 7.4) with gentle mixing.

Pretreatment of serum before 2-step purification

Two tenth ml of 1 M CaCl_2 solution was added to 20 ml of plasma or plasma pool for the removal of fibrinogen. This was left at room temperature for 3 hrs and stood at 4°C overnight. After light centrifugation, fibrin clot was removed. For further purification, defibrinated plasmas were diluted 3 times with 0.01 M phosphate buffered saline of pH 7.2 (PBS).

First-step rate zonal centrifugation on sucrose gradient

All centrifugation steps were performed in a Hitachi model 65P centrifuge. 20 ml of diluted serum were layered on a preformed 10 ml discontinuous 40 and 60% (w/w) sucrose gradient and centrifuged at $68,000 \times g$ for 15 hrs at 5°C. Fractions of 0.5 ml were collected from the bottom of the centrifuge tube. CF titer in each fraction shown in Fig. 1 is the reciprocal of the antigen dilution.

Second-step isopycnic banding in CsCl density gradient

A 3.5 ml sample was layered on a 1.5 ml discontinuous 1.1 to 1.5 g/cm³ CsCl gradient and run at $68,000 \times g$ for 20 hrs at 5°C.

Density determination

The density of individual fractions was determined from curves relating refractive index to density. Refractive indexes were read at 20°C with an Abbe 3 L type refractometer.

Electron microscopy

Preparations of purified Au antigen were examined in a Hitachi HU-11 B electron microscope at an instrumental magnification of $40,000 \times$. The preparation was placed on a carbon-coated collodion-covered grid and stained with 2% phosphotungstic acid at pH 7.2.

RESULTS

Preparation of partially purified Au antigen

Five Au positive specimens obtained from different sources were subjected to the same two-step purification procedure to prepare concentrated and partially purified antigen.

First step purification was a centrifugation at $68,000\times g$ for 16 hrs on a discontinuous gradient of sucrose (7 ml of 40% and 3 ml of 60%). Almost all of the antigenicity was concentrated in 40% sucrose fraction as illustrated in Fig. 1. A visible turbid band was observed at this fraction.

The recovery rate was almost 100% with all of the 5 specimens. The second step isopycnic banding made on preformed discontinuous CsCl gradients ($\rho=1.1$, 1.3 and 1.5) at $68,000\times g$ for 20 hrs resulted in the high condensation of antigen in $\rho=1.3$ fraction (Fig. 1). Again the same recovery rate of almost 80% was obtained with 5 specimens.

Fig. 1. Centrifugation profile of Au antigen in two step purification process.

Three times diluted, defibrinated plasma specimens were used as the starting material. First sucrose cushion centrifugation was at $68,000\times g$ for 16 hrs. Second step centrifugation was made on preformed discontinuous CsCl gradients at $68,000\times g$ for 20 hrs. All fractions were collected by the bottom-drip method and tested for antigenicity by IES and CFT.

Isopycnic banding of Au antigen in CsCl density gradients

Partially purified Au antigens obtained from 5 different sources described above were finally isopycnically banded by using continuous density gradients of CsCl, at $158,000\times g$ for 48 hrs. For this 0.5 ml sample containing 81,920 CF titer/ml was mixed with 4.5 ml of CsCl in water with final density of 1.26 g/cm^3 . Gradient fractions of Au antigen were tested with CF, AGD and IES tests. Before tests, all fractions were dialyzed against PBS for 24 hrs for 5 times.

Obtained results with plasma H was illustrated in Fig. 2-a and with the plasma pool P, in Fig. 2-b. With the rest of the sera B and C and the plasma pool K, almost identical result as that of H, shown in Fig. 2-a, was obtained.

In Fig. 2-a, Au was sedimented as a discrete zone of CF activity at a density of $1.21\text{--}1.23\text{ g/cm}^3$. In AGD or IES test, almost parallel results were obtained

Fig. 2. Isopycnic banding of Au in continuous CsCl density gradient.

A 0.5 ml sample of partially purified Au antigen was layered on a 4.5 ml continuous 1.26 g/cm³ CsCl gradient and run at 158,000×g for 48 hrs at 5 C. Fractions of 0.2 ml were collected from the bottom of the centrifuge tube.

as those of CF test. In two out of the four specimens mentioned above, a shoulder was found at a heavier fraction, which was not clearly resolved from the main peak, whereas with the plasma pool P, two discrete peaks appeared as shown in Fig. 2-b. The CF activity distribution at a density of 1.21 g/cm³ was not different from that obtained with the rest four specimens, and the AGD and IES activity paralleled to the CF titer distribution, whereas another high titer peak in CF test was recovered in a sharp zone at a density of 1.28 g/cm³. Nevertheless, any activity was not found in AGD and IES with this fraction.

Electron micrographic study of the high-density CF antigen

High-density antigen fraction obtained from the pool P, and low-density fractions obtained from different sources were examined under electron microscope. Fig. 3 illustrates the negatively stained Au particles found in low-density fraction of specimen H. The particles revealed fairly large deviation in size but had a modal diameter of 22 mμ. Surface structures were apparent on some particles and there was a suggestive evidence of symmetric arrangements. These particles are similar in morphology to the particles so far described as Au antigen particles (Barker *et al.* 1969, Gerin *et al.* 1969). Tubular forms described by Bayer *et al.* (1968) and Almeida *et al.* (1969) were also found in this low-density fractions originated from 5 specimens, although the relative concentration to cubic particles is different from specimen to specimen. Dane's large particles (Dane *et al.* 1970, Jokelainen *et al.* 1970) were predominantly encountered at a density of 1.24 g/cm³ in 2 specimens out of 5.

In high-density antigen peak of the specimen P, no cubic, tubular and Dane's particles were found. Particles revealed in this fraction were fairly small particulates but uniform in their morphology. Average diameter was estimated to be between 3 and 4 mμ (Fig. 4). Their arrangement in negatively stained pre-

Fig. 3. Electron micrograph of low-density Au particles. Partially purified Au antigen were examined in a Hitachi HU-11B electron microscope at an instrumental magnification of 40,000 (photographic enlargement $\times 174,000$).

Fig. 4. Electron micrograph of heavy-density small particulates ($\rho=1.28$). Photographic magnification of 17,4000.

paration was fairly regular in a sense that many dimers, tetramers or polymers were found.

DISCUSSION

To obtain the partially purified Au antigens with high recovery rate, a simplified two-step purification procedure was proposed in this article. First-step sucrose

cushion centrifugation resulted in a condensation of antigen in 40% sucrose layer. By this means, Au antigens in 20 ml serum were concentrated into 5 ml solution and this was subjected to the second-step isopycnic banding in CsCl cushion. Through this second step, antigens can be concentrated into 0.5 ml with high recovery rate. Electron microscopic study of this fraction revealed the homogeneous distribution of virus-like particles of 22 m μ size. However, a trace amount of albumin could not be removed from this fraction, when examined by means of immunoelectrophoresis against rabbit anti-human serum antibody.

When these partially purified antigens are subjected to the final purification process of isopycnic banding in continuous gradient concentration of CsCl (Barker *et al.* 1969, Millman *et al.* 1970, Gerin *et al.* 1971), two peaks were encountered with the plasma pool P. The second high-density fraction could not be detected with the rest 4 specimens, and in our recent experience of 10 purification trials through the year 1971, only one out of 10 revealed such two peaks. This high-density fraction seems to be identical with the second population of CF activity described by Gerin *et al.* (1968). However, they found this activity in higher density than ours, *i.e.* 1.35 to 1.40 g/cm³. In their description, they encountered this second peak with frozen and thawed preparation as well as after the treatment of 1.20 g/cm³ fraction with Tween 80. However, their description did not extend to the electron microscopic examination of this high-density antigen. Our study clearly illustrates that the antigenicity in this second peak can be detected only by means of CF test, but not by AGD and IES tests.

Furthermore, known antigenic particles such as virus-like cubic, tubular and large particles could not be found in this fraction. Homogenous distribution of small cubic elementary particles of 3–4 m μ in diameter was encountered. When the concentration of these elementary particles was high in negatively stained preparation, they showed the tendency of lattice arrangements. Many tetramers and dimers were found. This observation is tempting to speculate that this high-density fraction is a particulate population of protein which has been released in some manner from low-density component of Au antigen. The density of 1.28 g/cm³ just fits the described density of a simple protein.

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