Biophysical Properties of Australia Antigen

J. L. GERIN, R. H. PURCELL, M.D. HOGGAN, P. V. HOLLAND, AND R. M. CHANOCK

Rockville Laboratory¹ of the Molecular Anatomy Program, Oak Ridge National Laboratory,² Rockville, Maryland 20862, Laboratories of Infectious Diseases and Viral Diseases, National Institute of Allergy and Infectious Diseases, and the Blood Bank Department, Clinical Center, National Institutes of Health, Bethesda, Maryland 20014

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Biophysical studies with Australia complement-fixing (CF) antigen showed it to be a particle with a buoyant density of 1.20 g/cm³ in CsCl, a sedimentation coefficient of 110, and an average diameter of 25 nm. The CF antigen was not inactivated by ether, 1% deoxycholate, 1% Tween 80 or overnight heating at 56 C. The antigen was unstable when treated with 1% sodium dodecyl sulfate. A procedure is described for the isolation and partial purification of Australia antigen from serum by using isopycnic banding and rate separation techniques. Treatment of the 1.20 g/cm³ Australia antigen with 1% Tween 80 yielded a minor peak of CF activity with a buoyant density of 1.39 g/cm³ in CsCl.

In 1965, Blumberg, Alter, and Visnich (2) described a "new" precipitating antigen in the serum of an Australian aborigine. Subsequent study revealed that the antigen ("Australia antigen") was associated with several diseases of man but most notably with serum hepatitis.

There is growing evidence (3, 4, 6-8) that Australia antigen (Au) is a virus or a virus-associated protein which may be etiologically associated with serum hepatitis and perhaps also with infectious hepatitis.

Recently, a complement-fixation (CF) test was developed (9) which offers a more sensitive method for the detection of Au than the commonly-used agar-gel diffusion assay. In this paper, the complement-fixation technique was used to quantitate the Au during purification procedures and to characterize some of its biophysical properties.

MATERIALS AND METHODS

Source of Au. The serum of a young adult with chronic anicteric serum hepatitis was used as the source of antigen. The serum was stored frozen at -70 C until used in the biophysical studies to be described.

CF test. The CF test was described in a previous communication (9). Serum containing antibody to Au was obtained from a multiply transfused patient with

hepatitis and was heated at 56 C for 30 min to destroy endogenous complement; partially purified Au was tested without prior heat inactivation. The CF test was performed by the microtiter method as modified by Sever (10); 4 to 8 units of antibody and 1.7 exact units of complement were used in the test; antigen-antibody-complement mixtures were incubated overnight at 4 C before addition of sensitized sheep erythrocytes. The test was performed by using Cooke microtiter loops (0.025 ml) and Linbro disposable U-bottom plates and 0.025-ml droppers.

Density gradients. Au was isopycnically banded by using discontinuous density gradients of cesium chloride, potassium tartrate, or sucrose as described below. Rate separations were made on linear preformed sucrose gradients. All gradient experiments were performed in the Spinco L2-65B preparative ultracentrifuge by using the Spinco SW 40 rotor. A Spinco $\omega^2 t$ integrator was used in all centrifuge studies.

Density determination. The density of individual fractions was determined by direct weighing in tared 100- μ liter micropipettes.

Optical density (OD) measurements. The OD of individual fractions at 280 nm was measured by using 0.3-ml microcuvettes in the Gilford model 2400 spectrophotometer and was corrected for solvent absorption.

Detergent treatment. Au antigen partially purified by isopycnic banding was treated with 1% (w/v) sodium deoxycholate (DOC), 1% (v/v) Tween 80, or 1% (w/v) of recrystallized sodium dodecyl sulfate (SDS) for 1 hr at room temperature with mild agitation.

Acid stability studies. Isopycnically banded Au was incubated for 3 hr at room temperature at pH 2.7 and then adjusted to pH 7.2. The acid-treated and the control samples at pH 7.2 were then assayed for CF activity.

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Heat stability studies. Isopycnicly banded Au was heated at 56 C overnight in a water bath. This sample and a 4 C control were then tested for CF activity.

Ether stability studies. Isopycnicly banded Au was treated with diethyl ether (20%, v/v) overnight at 4 C. In another experiment, Au was incubated with 20% (v/v) ether at room temperature. Samples of the material incubated at room temperature were taken at 15, 30, and 60 min and at 2, 3, and 4 hr. Ether was removed by evaporation. All samples and comparable controls incubated without ether were assayed for CF activity.

Electron microscopy. For electron microscopic examination, Au preparations were placed on carbon-coated Formvar films on 300-mesh copper grids. After 1 to 4 min, the grids were washed twice with distilled water and then stained for 0.5 to 1.5 min with 1% uranyl acetate at pH 5. Some preparations were fixed with osmium tetroxide before staining. Excess stain was removed by touching with filter paper. After the preparations were allowed to air dry, they were examined in a Siemens Elmiskop 1A at 80 kv with an instrumental magnification of 10,000 to 40,000. Purified preparations of type 1 adenovirus-associated virus (AAV-1) were prepared in a similar manner and served as controls for the electron microscope procedures.

RESULTS

Initial biophysical characterization of Au antigen. Biophysical studies were first performed with serum containing Au. The Au CF antigen banded with a buoyant density of 1.20 g/cm³ in cesium chloride (Fig. 1A); a visible band was observed at this density. When the fraction containing the peak CF activity of the isopycnic step

was rebanded on a shallower CsCl gradient (Fig. 1B), the CF activity was recovered in a sharp distribution at a density of 1.20 g/cm³. The Au CF antigen migrated in sucrose as a zone of homogeneous particles with a sedimentation coefficient of about 100S (Fig. 1C).

An attempt was made to pellet the Au antigen from 100 ml of serum through a 2-ml 30% (w/w) sucrose cushion. A visible pellet was recovered; however, subsequent analysis revealed that all of the Au CF activity remained in the supernatant fluid. The 30% sucrose cushion appeared to exclude the Au antigen. The supernatant clarified by the direct pelleting step was used for the subsequent studies.

Preparation of partially purified antigen. The following procedure was used to prepare concentrated and partially purified antigen. Five ml of serum clarified as described above was layered onto a 5-ml three-step CsCl density gradient ranging from 1.1 to 1.6 g/cm³ and overlaid with 3 ml of light mineral oil. Six tubes were centrifuged at a time in the L2-65B centrifuge by using the SW 40 rotor at 5 C for $40 \times 10^{10} \omega^2 t$ (8 hr at $152,000 \times g$). Three discrete bands were seen, and these were carefully extracted. The CF activity was recovered in the middle band (no. 2, Fig. 2) at a density of 1.20 g/cm³. With the use of this procedure, 90 ml of clarified serum was banded in CsCl, and the 1.20 g/cm³ bands were combined. This material was then rebanded on 10-ml 1.1 to 1.4 g/cm³ CsCl density gradients. A single sharp band was

FIG. 1. Biophysical studies with serum containing Au. (A) Isopycnic banding of Au in cesium chloride density gradient. A 0.5-ml sample of serum was layered on a 4.5-ml discontinuous 1.1 to 1.6 g/cm³ CsCl gradient and run at $152,000 \times g$ for 4 hr at 5 C ($20.8 \times 10^{10} \omega^2 t$). Fractions of 0.5 ml were collected from the bottom of the nitrocellulose centrifuge tube. (B) Second isopycnic banding of Au antigen in a 9.5-ml discontinuous 1.1 to 1.4 g/cm³ CsCl density gradient with a 0.5-ml starting sample ($117,000 \times g$ for 20 hr at 5 C ($65.5 \times 10^{10} \omega^2 t$)). (C) Rate separation of Au antigen on a preformed 6-ml 10 to 30% (w/w) sucrose gradient with 0.2 ml of serum as starting sample, $117,000 \times g$ for 2.5 hr at 5 C ($9.2 \times 10^{10} \omega^2 t$). CF titer is the reciprocal of the antigen dilution.

	FRACTION			
	1	2	3

Volume	0.6 ml.	0.6 ml.	0.5 ml.	
Concentration by Volume	8.3 x	8.3 x	10 x	
CF TITER (AC)	0	≥ 65,536 (4)	4 (8)	
Concentration of CF Titer	NONE	≥ 8 x	NONE	
Percent Recovery of CF Activity	0	≥ 100%	0	

FIG. 2. Concentration and initial isopycnic banding of Au antigen. A 5-ml sample of serum was layered onto a 5-ml 1.1 to 1.7 g/cm³ three-step discontinuous CsCl density gradient with an oil overlay and centrifuged for 8 hr at 152,000 ($39.6 \times 10^{10} \omega^2 r$) at 5° C. Three discrete bands were visible and extracted. All of the starting CF activity was recovered in the no. 2 band. CF titer is the reciprocal of the antigen dilution. AC indicates anti-complementary activity.

observed at a density of 1.20 g/cm³ in each tube; these bands were extracted, combined, dispensed in 0.1-ml amounts, and stored at -150 or 4° C. This preparation, designated Au (Cs2) comprised the starting material for subsequent experiments. The reciprocal of CF titer of the Au (Cs2) pool was 65,536, and this preparation remained stable at both -150 and 4° C for several months.

Electron microscopy (Fig. 3A) of the negatively stained Au (Cs2) preparation revealed a high concentration of particles ranging in size from 20 to 30 nm with an average diameter of 25 nm.

Rate separation of Au antigen in a sucrose gradient. A diluted sample of Au (Cs2) was centrifuged on a 5 to 20% (w/w) sucrose gradient. The sample also contained purified and tritium-labeled type 1 adeno-associated virus (AAV-1; $S_{20,w} = 104$; reference 5) as an internal sedimentation marker. The Au CF antigen migrated slightly ahead of the AAV marker and behaved as a particle with a sedimentation coefficient of 110S (Fig. 4). The CF antigen was associated with a sharp peak of optical density, and approximately two-thirds of the total 280-nm ultraviolet (UV)-absorbing material in the gradient was removed from the peak CF activity by this rate separation step.

A considerable loss of CF activity was observed after rate separation of Au (Cs2). Examination of the fraction containing the activity revealed clumps of aggregated particles (Fig. 3B). These particles had a morphology characteristic of small icosahedral viruses.

Buoyant density of Au in various gradient materials. The solvation properties of different gradient materials affect the banding characteristics of virus particles. Hence, it was not surprising to observe a marked difference in the buoyant

density of Au in CsCl [1.20 g/cm³ (Fig. 1B)], potassium tartrate [1.15 g/cm³ (Fig. 5)] and sucrose [1.16 g/cm³ (Fig. 6, Au control)].

Effect of detergents on Au antigen. Certain properties of Australia antigen, such as its low density and affinity for Sudan Black, led Alter and Blumberg (1) to suggest that the antigen contained a lipid component. We tested the effect of a 1-hr pretreatment with SDS, DOC, or Tween 80 on the buoyant density of Australia antigen. When Au not treated with detergent was banded in a sucrose density gradient (Fig. 6, Au control), two UV-absorbing peaks were observed. The major peak (fraction 4) banded at a density of 1.16 g/cm³ and contained the CF activity; no CF activity was associated with the lighter peak (fraction 2). When Au antigen was pretreated with 1% SDS for 1 hr and then banded in sucrose, a shift of UV-absorbing material from the 1.16 g/cm³ peak to the lighter band occurred and was accompanied by the loss of all CF activity. Treatment of Au with either DOC or Tween 80 resulted in a marked shift in the buoyant density of the antigen (Fig. 7). After DOC treatment both the UV-absorbing material and the CF activity banded at a higher density (1.23 g/cm³) than the untreated control (1.20 g/cm³). Examination of the 1.23 g/cm³ fraction by electron microscopy revealed no gross alteration of the particles. In contrast to the effect of DOC, Tween 80 pretreatment of Au caused a shift in density to the lighter region of the CsCl gradient (1.18 g/cm³). The CF activity banded with the major optical peak, but an exception to this will be described below.

Stability of Au antigen. An eightfold or greater loss of CF titer when compared with a control preparation was used as the criterion of antigen stability. The CF antigen was not inactivated by overnight incubation at 56° C, exposure to ether overnight at 4° C or for 4 hr at room temperature, or 3 hr of incubation at pH 2.7 at room temperature. Exposure of Au (Cs2) to ether caused a shift in the buoyant density of Au in CsCl to 1.22 g/cm³ similar to that observed with DOC treatment.

The partially purified Au (Cs2) preparations lost CF activity when diluted in phosphate-buffered saline, pH 7.6. Such loss of activity probably resulted from aggregation, a phenomenon frequently encountered with purified viruses.

High-density Au CF antigen. In the course of these studies, another population of CF activity was often observed in the high-density (1.35 to 1.40 g/cm³) region of CsCl gradients. This CF antigen was most evident after Au preparations at 1.20 g/cm³ were either frozen and thawed or

FIG. 3. (A) Electron micrograph of a partially purified preparation [Au (Cs2)] of Australia antigen negatively stained with uranyl acetate. $\times 100,000$. (B) Electron micrograph of Australia antigen after rate separation of a partially purified preparation [Au(Cs2)]. Fixed with osmium tetroxide and negatively stained with uranyl acetate. $\times 100,000$.

treated with Tween 80. The distribution of Au CF activity in CsCl after Tween 80 treatment of Au (Cs2; Fig. 8) revealed that, whereas most of the CF activity corresponded with the UV peak at 1.18 g/cm^3 (Fig. 7), a significant proportion of activity banded at a density of 1.39 g/cm^3 in CsCl. This phenomenon was not observed after treatment of Au (Cs2) with either SDS or DOC. The data suggested that the high-density CF antigen was derived from the 1.20 g/cm^3 band as a result of the mild detergent treatment.

DISCUSSION

Alter and Blumberg (1), using an agar-gel diffusion assay, found that Au antigen banded in the density range of 1.063 to 1.30 g/cm^3 in KBr. We found that Australia antigen banded sharply with a buoyant density of 1.20 g/cm^3 in cesium chloride and 1.16 and 1.15 g/cm^3 in sucrose and potassium tartrate gradients, respectively. Rate separation on a sucrose gradient with AAV as an internal sedimentation marker showed that Au

FIG. 4. Rate separation of Au in a sucrose gradient. An Au antigen preparation double banded in CsCl was diluted with phosphate buffer (0.01 M, pH 7.4) after the addition of a small quantity of purified and tritium-labeled adeno-associated virus (AAV) as an internal sedimentation marker. Sample (0.5 ml) was layered onto a linear preformed 5 to 20% (w/w) sucrose density gradient and centrifuged for 3 hr at $188,600 \times g$ ($11.2 \times 10^{10} \omega^2 t$) at 10 C. Fractions of 0.75 ml were collected and assayed for CF activity and radioactivity. Arrow shows the position of the AAV sedimentation marker in the recovered fractions. CF titer is the reciprocal of the Au dilution.

FIG. 5. Isopycnic banding of Au in a potassium tartrate density gradient. An Au preparation banded twice in CsCl was diluted 10-fold with phosphate buffer (0.01 M, pH 7.4). A 0.1 ml sample was layered onto a 12-ml four-step discontinuous 1.1 to 1.5 g/cm³ K₂ tartrate density gradient and centrifuged at 5 C for 13 hr at $111,700 \times g$ ($44.2 \times 10^{10} \omega^2 t$). The abscissa represents the centimeter distance from top to bottom along the 12-ml liquid gradient column. CF titer (○) is the reciprocal of the antigen dilution. OD₂₈₀ is indicated by the solid line. Density (●).

migrated as a particle with an $S_{20,w}$ of 110. Blumberg, Sutnick, and London (4) and Hirschman et al. (7) described the antigen as a particle with a diameter of 20 nm. Prince (8) stated that the closely related SH antigen was a particle with

FIG. 6. Effect of SDS on the buoyant density of Au in a sucrose density gradient. A CsCl double-banded Au preparation was diluted with 0.01 M phosphate buffer, (pH 7.4), and a sample treated with 1% (w/v) SDS. Control and treated samples (0.2 ml) were banded in separate sucrose density gradients for 18 hr at $111,700 \times g$ ($59.7 \times 10^{10} \omega^2 t$) at 5 C. The figure shows the distribution of optical density at 280 nm in the two gradients.

FIG. 7. Effect of detergent treatment on the buoyant density of Au in CsCl. A CsCl double-banded Au preparation was diluted with 0.01 M phosphate buffer, pH 7.4. Samples were treated with either 1% (w/v) DOC or 1% Tween 80 (v/v). Of the treated and untreated, 0.2-ml samples were then run on separate CsCl density gradients at 5 C for 18 hr at $111,700 \times g$ ($59.7 \times 10^{10} \omega^2 t$). The figure represents a composite of the distribution of OD in these gradients.

FIG. 8. Distribution of Au CF activity in a CsCl density gradient after Tween 80 treatment. Conditions of experiment are same as given in Fig. 7. The abscissa represents the centimeter distance from top to bottom along the 12-ml liquid gradient column. CF titer is the reciprocal of the antigen dilution.

a diameter of about 25 nm. Electron microscopy of Au in our laboratory revealed that the antigen was a particle with an average diameter of 25 nm, slightly larger than AAV (22 nm). Considerable purification of Au was obtained using the two-step ρ -S techniques.

Treatment of Au with 1% sodium DOC for 1 hr shifted its buoyant density from 1.20 to 1.23 in CsCl without major alteration of structure or CF reactivity. This suggests that DOC removed some lipid-associated material not essential to the particulate structure. In contrast, treatment of the antigen with SDS destroyed the CF reactivity and probably its particulate structure. Tween 80 treatment lowered the buoyant density of the major CF activity from 1.20 to 1.18 g/cm³ and appeared to release another population of particles with CF activity which banded at 1.39 g/cm³. A similar but less uniform distribution of CF activity was observed in the high-density region of the gradient when frozen-and-thawed preparations of Au were banded in CsCl. A number of infectious small icosahedral viruses band at this density (e.g., AAV, rhinoviruses, and foot-and-mouth disease virus), and it is tempting to speculate that the high density particulate population

of Au is a similar complex of protein and nucleic acid which has been released in some manner from low-density host or virus components. Electron microscopy has failed to reveal viruslike particles in the high-density fraction, but the concentration of Au in this fraction may be too low for such direct observation.

The isolation procedure herein described which involves the initial banding of Au from serum in CsCl may be useful for the detection of other possible serotypes of hepatitis virus if they are similar in density and are present in approximately the same concentration as Au.

It is interesting that Au shares with certain viruses which are loosely grouped together as "slow viruses" (i.e., scrapie, Aleutian disease of mink, and mink encephalopathy) certain characteristics: small size, resistance to inactivation by physical and chemical agents, and, in some cases, low density and presence of lipid. Viruses with such characteristics probably represent one or more new taxonomic groups. Additional work will be necessary to determine if Au is indeed a virus and if it is related to members of the "slow virus" group(s).

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