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USE OF THE ZONAL CENTRIFUGE TO SEPARATE PARTICLES CONTAINING TRANSPLANTATION ANTIGEN

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SUMMARY

Subcellular particles of homogenates of mouse spleen were separated at various centrifugal forces between $5000 \cdot 10^6$ and $30\,000 \cdot 10^6$ $\omega^2 t$ in the zonal centrifuge. Each fraction was analyzed for transplantation antigen, ATPase, nonspecific esterase and alkaline phosphatase. Under appropriate conditions, zonal centrifugation proved to be an efficient procedure to separate particles containing high concentrations of transplantation antigen. After a centrifugal force of $5000 \cdot 10^6$ $\omega^2 t$, the antigenic particles were located in the 10–12 % sucrose region, but they sedimented into denser sucrose particles and became more disperse when subjected to larger total centrifugal forces. The density of the particles in CsCl was about 1.2 g/cm^3. Transplantation antigen was solubilized by autolysis; the antigen was eluted with the exclusion volume during gel filtration on Sephadex G-200.

INTRODUCTION

The genetics and the biological function of the transplantation antigens (H-2 antigens) of the laboratory mouse have been extensively studied[1]. Because certain gene products induce strong rejection responses, investigators have attempted to isolate and characterize these gene products[2]. The use of many different procedures to isolate subcellular components from tissue homogenates that retain antigenic activity has resulted in conflicting reports that antigen is associated with cell membrane, lysosome, microsome, ribosome, endoplasmic reticulum, *etc.* The successful use of the zonal centrifuge for the isolation of subcellular particles and viruses[3] suggested that it might be a useful tool for the isolation of subcellular particles that contain transplantation antigens. The method would be particularly useful as the first step if the antigenic particles were recovered in a limited region of the zonal centrifuge profile and if the particles were resolved from other cellular components. Data on the physical properties of the antigenic particles during zonal centrifugation would also give some information on the location of the antigen in the intact cell. This paper describes (1) the isolation using zonal centrifugation of particles from mouse splenic homogenates that contain H-2 antigens and (2) some physical and antigenic properties of these particles.

MATERIALS AND METHODS

Tissue

Spleens were removed from C57BL/6Cum, C3H/Cum and RFM/Un male mice that were 4 months old. Each sample for the zonal centrifuge contained approx. 4 g (60 spleens) of tissue. The spleens were homogenized in 25 ml of 8.5% sucrose in a glass homogenizer, using a teflon plunger with a tolerance of 0.006 inch to preserve the cell nucleus. The homogenate was strained through eight layers of cheesecloth to remove large pieces of connective tissue.

Gradient

A sucrose gradient was established in a B-XV rotor[4]. The solution of sucrose was buffered with Miller-Golder buffer[5] (pH 7.5) having an ionic strength of 0.2. Volumes of solutions in the rotor after sample application were 300 ml of 55 % sucrose, 1200 ml of 10–30 % sucrose gradient, 25 ml of splenic homogenate and 200 ml of Miller–Golder buffer overlay. The rotor speed, continuously monitored by a Speedomax rev./min indicator[6], was increased to and maintained at 20 000 rev./min for varying intervals of time; the integral of $\omega^2 dt$ was obtained electronically. The speed was then reduced to 3000 rev./min, and the rotor was unloaded in 40-ml fractions for analysis. The absorbance of the rotor effluent was continuously monitored at 260 nm with the use of a flow cell[7] in a modified Beckman spectrophotometer. A few ml of water were added to each fraction, and the fractions were centrifuged for 1 h at 100 000 $\times$ g to sediment the particles; the pellet was washed by resuspending it in 0.85 % saline, and the sample was centrifuged again for 1 h at 100 000 $\times$ g. The washed pellet was resuspended in 0.6 ml of 0.85 % saline, and aliquots of this suspension were assayed for H-2 activity, nonspecific esterase, ATPase and acid phosphatase.

Serological analysis

Antisera used were C3H anti-C57BL/6, C57BL/6 anti-C3H and C3H anti-RFM. The hemagglutination inhibition assay is based on the principle that particles containing antigen will react with isoantibody and inhibit it. The absorbed antibody is used to agglutinate erythrocytes for which it is specific; an inability to agglutinate red cells after absorption indicates that the particles contain specific antigen. Conversely, persistence of agglutination after absorption indicates that the particles contain little or no specific antigen. To assure maximum sensitivity, the antisera were diluted in 0.85 % saline to a titer four tubes below the peak titer. Samples (10 μl) of the suspended pellet from each fraction were added to 0.4 ml of the diluted antiserum; the mixture was incubated for 40 min at room temperature and 20 min at 37° and then was centrifuged at 1200 $\times$ g for 20 min. An aliquot of the absorbed serum was adjusted with 4 % dextran to 2 % dextran, and a total of 4 to 6 two-fold dilutions were made with 2 % dextran. An equal volume of specific erythrocytes suspended in 50 % human serum was added. The extent of agglutination was read after incubation at 37° for 90 min.

Immunization

Pellets from the three major regions of a zonal centrifuge preparation of C57BL/6 spleens were injected into B10.D2 mice. Each mouse received three intraperitoneal

injections at weekly intervals, and the serum was collected 8 and 9 days after the third injection.

Autolysis

A modification of procedures described by NATHENSON AND DAVIES[8] was used to solubilize a portion of the particulate fraction that contained H-2 active material. The fractions containing antigenic particles, Tubes 7–15 from four zonal centrifuge separations at $5000 \cdot 10^6\ \omega^2 t$, of C3H splenic homogenates were suspended in 40 ml of 0.05 M Tris–HCl buffer (pH 7.4). The suspensions were allowed to stand for 8 h at 5°, and the particles were removed by centrifugation at 100 000 × *g* for 1 h. The particles were resuspended in Tris–HCl buffer for 24 h and were again made into pellets. The supernatant fractions were pooled, dialyzed overnight against 0.002 M Tris (pH 7.4) and lyophilized.

Sephadex chromatography

Sephadex G-200 (column 1.9 cm × 100 cm) equilibrated with 0.05 M Tris–HCl buffer (pH 7.4) was used. Elution was performed with the same buffer.

Electrophoresis

Samples of soluble antigenic material recovered from the Sephadex column were electrophoresed on cellulose polyacetate (Gelman Instrument Company, Ann Arbor). Soluble material was applied to strips (2.5 cm × 17 cm) which were wetted with Tris–barbital–sodium barbital buffer (pH 8.8). A constant current of 300 V was applied to the electrophoresis chambers for 20 min.

Enzyme analysis

ATPase. The substrate was prepared as described by SCHWARTZ[9] and the inorganic phosphate was determined according to the procedure of ERNSTER *et al.*[10]. To 0.9 ml of the substrate, 0.1 ml of suspended pellet was added. The reaction time was 15 min at 37°.

Esterase. The Gomori procedure for esterase determination has been described[11]. 10 μl of suspended pellet and 5 ml of substrate were incubated at 30° for 30 min before adding Fast Red ITR salt for color development.

Acid phosphatase. Procedures for the acid phosphatase reaction were similar to those for esterase. As substrate, 0.02 % sodium α-naphthyl phosphate in 0.02 M sodium acetate buffer (pH 5.0) was used; 10 μl of suspended pellet for every 5 ml of substrate were incubated for 30 min at 37° before the color developer was added.

RESULTS

Spleen homogenates were subjected to forces of approx. 5000, 10 000, 20 000 and $30\,000 \cdot 10^6\ \omega^2 t$, where ω is the angular velocity in radians/sec and t is the time in sec. Absorption curves of the effluent from each of such preparations are shown in Fig. 1. Fig. 1a shows a selected profile of the rotor effluent of a splenic homogenate after zonal centrifugation with a total force of $5000 \cdot 10^6\ \omega^2 t$. The soluble peak extends from Fractions 6–10; microsomes are in Fractions 11–25; and lysosomes, mitochondria, cell membrane and a few remaining whole cells are layered at the interface of the gradient

and the cushion in Fractions 33–42. The small peak in Fraction 11–12 (Fig. 1a) was not observed in all zonal preparations. Since antigen was not localized there, its significance was not investigated. The double peak in the dense sucrose fractions also did not always develop, so it was ignored since it did not seem significant for our purposes. At $10000 \cdot 10^6\ \omega^2 t$ (Fig. 1b) little difference in the two main peaks were seen when compared with $5000 \cdot 10^6\ \omega^2 t$. At $20000 \times 10^6\ \omega^2 t$ particles previously associated with the soluble fraction began to sediment into the denser sucrose fraction. At $30000 \cdot 10^6\ \omega^2 t$ the soluble peak had become broader and much of the material had

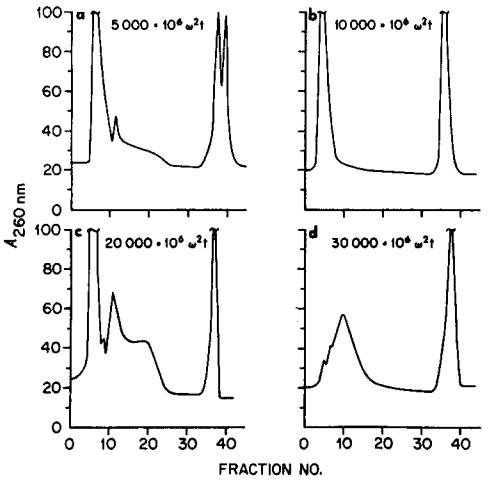

Fig. 1. Effect of varying the centrifugal force on the separation of subcellular particles of C57BL/6 splenic homogenates. The relative absorbance of the effluent is plotted against each 40-ml fraction. a. Profile after $5000 \cdot 10^6\ \omega^2 t$. b. Profile after $10000 \cdot 10^6\ \omega^2 t$. c. Profile after $20000 \cdot 10^6\ \omega^2 t$. d. Profile after $30000 \cdot 10^6\ \omega^2 t$.

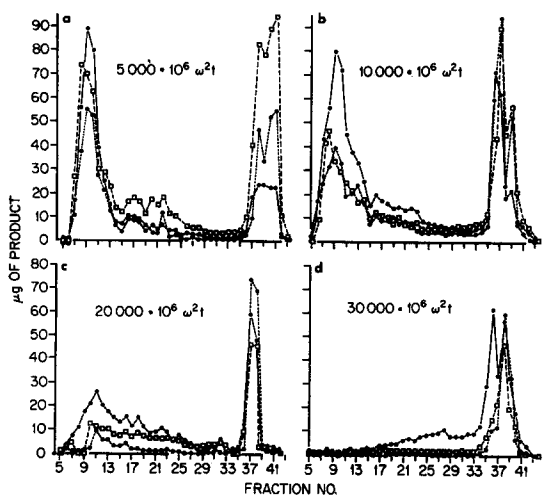

Fig. 2. Distribution of enzymic activity in the zonal centrifuge effluent after varying centrifugal forces. The relative activity of ATPase (●—●), acid phosphatase (□---□) and nonspecific esterase (○···○) is plotted for each 40-ml fraction. Total centrifugal forces were the same as in Fig. 1.

sedimented to the interface of the sucrose gradient and the 55 % sucrose cushion.

Enzyme analyses (Fig. 2) were performed to show the distribution of three enzymes for the zonal centrifuge preparations in Fig. 1. Two major peaks for all three enzymes were found at 5000 and $10\,000 \cdot 10^6\ \omega^2 t$. At $20\,000 \cdot 10^6\ \omega^2 t$, the first peak had fewer particles with these enzymes, and they sedimented even farther at $30\,000 \cdot 10^6\ \omega^2 t$. In the first peak, the highest activity for ATPase and esterase always occurred in the same fraction, but the highest activity for acid phosphatase activity always occurred in the preceding fraction. In the second peak, the esterase and acid phosphatase activity curves coincided, but the ATPase activity did not coincide perfectly with the highest activity for the other enzymes.

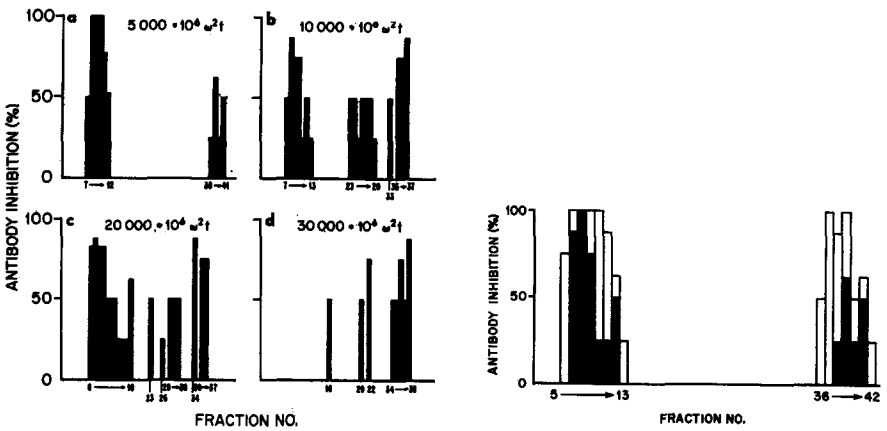

Fig. 3. Distribution of splenic particles that demonstrate hemagglutination inhibition after varying centrifugal forces. The extent of hemagglutination inhibition is plotted for each 40-ml fraction from the zonal centrifuge. Total centrifugal forces were the same as in Fig. 1.

Fig. 4. Effect of a 10-fold increase in the quantity of splenic particles for the detection of H-2 antigen by the hemagglutination inhibition assay. Solid and open bars indicate the degree of hemagglutination inhibition expressed by 10- and 100-μl suspensions, respectively, of splenic particles from each 40-ml fraction.

Aliquots of the suspensions of particles from each fraction were mixed with antiserum. The distributions of H-2 antigen are shown in Fig. 3 for four conditions of zonal centrifugation. At a force of $5000 \cdot 10^6\ \omega^2 t$, Fractions 8–10 completely absorbed the antibody activity from the antiserum used. Fractions 7, 11 and 12 removed lesser amounts of antibody. Smaller concentrations of antigen were found in Fractions 38–41 in which whole cells were identified by microscopy; the antibody inhibition was attributed primarily to the presence of antigen on whole cells. Although the antigen was localized in regions that had the highest enzymic activity, the antigen and enzyme peaks did not completely coincide.

At the higher centrifugal forces, a greater distribution of antigenic particles occurred in the sucrose gradient. At $10\,000 \cdot 10^6\ \omega^2 t$, the first peak removed antibody activity; some antibody absorption occurred from Fractions 23–29, and a slightly increased degree of antibody inhibition was found in the last peak (Fig. 3b). At $20\,000 \cdot 10^6\ \omega^2 t$, the antigen was detected throughout the gradient; at $30\,000 \cdot 10^6\ \omega^2 t$,

antigen was found primarily in the last peak. Comparative analyses made with 10- and 100-μl suspensions from each fraction reveal a similar distribution of antigen (Fig. 4).

Fractions that inhibit antibody also have high enzymic activity and undoubtedly contain proteolytic enzymes. Thus, it was necessary to establish that the reduced hemagglutination after incubation with splenic particles resulted from antibody inhibition by antigen and not from nonspecific degradation of antibody by proteolytic enzymes. For this purpose, 100 μl of splenic preparation from C57BL/6 mice were incubated with a C3H anti-RFM serum, a situation in which antibody inactivation might be caused by proteolysis but not by specific antibody absorption. Because such serum remained active against RFM red cells, the hemagglutionation inhibition test appeared to measure specific antigen–antibody interaction and not the proteolytic inactivation of antibody.

Whole spleens were frozen at –30° for 1 month to analyze the zonal centrifuge profile of frozen spleens. After centrifugation with a total force of $5000 \cdot 10^6\ \omega^2 t$, the principal antigen peak was located in Fractions 7–13, as with fresh spleen. The distribution of enzymic activity was also similar to that obtained with fresh spleen.

An isopycnic banding was performed to determine the density of antigenic particles in the first peak of the zonal centrifuge profile. After zonal centrifugation at a force of $5000 \cdot 10^6\ \omega^2 t$, fractions 6–13 were pelleted at 100000 $\times$ *g* for 1 h. The pellet from each of these fractions was resuspended in 15 ml of saline; 10 ml of CsCl saturated at 5° (density 1.81 g/cm^3) was layered at the bottom of the tube, and a plastic bead of density 1.27 g/cm^3 was introduced into each tube as a density marker. After centrifugation at 100000 $\times$ *g* for 1 h, the position of the particles was observed using light scattering. One band, with an estimated density of 1.20 g/cm^3, was observed in each tube just above the bead. The particles in the band were recovered, washed in saline and tested for their ability to inhibit antibody. Antibody inhibition was observed with the particulate preparations in Fractions 7–10; thus, the band was judged to contain particulate bound antigen.

If the particle represents "complete antigen" (*i.e.*, retains the total biological function), it should induce as well as inhibit antibody. Antibody specific for C57BL/6 erythrocytes was induced in B10. D2 mice that received injections of C57BL/6 splenic pellets from the region in which hemagglutination inhibition was demonstrated; no antibody was observed in mice that received injections of pellets from the microsomal region, and a very weak antibody titer was found in mice that received injections of pellets from the lysosomal and mitochondrial region. This antiserum was tested against a panel of red cells to study its antibody specificity. The expected antibody specificities include anti-D^b, -E and -V. The titer against C57BL/6 erythrocytes was greater than 1/128. The titers to A, A.SW, C3H and C57BR erythrocytes ranged from 1/128 to 1/500. The serum was negative for B10.D2, BALB/c, YBR and DBA/2 erythrocytes; it was also negative for erythrocytes from A.CA and RFM mice. The reactivity to C57BL/6 erythrocytes may be due to anti-D^b, -E and/or -V. A, A.SW, C3H and 101 erythrocytes have antigen E, but not D^b and V, so this serum must have anti-E activity. This conclusion is supported by the inability of the serum to agglutinate A.CA and RFM erythrocytes, which also lack antigen E. Absorption of this serum on splenic cells from C3H mice to remove anti-E still gave hemagglutination of C57BL/6 erythrocytes, suggesting that the serum also contained anti-D^b and/or V activity.

Zonal centrifugation of homogenates of spleens from C57BL/6, C3H and RFM mice showed that the H-2 antigens are located on particles similar for each strain. The antisera used identify antigens A, C and E for C3H and antigens G and I for RFM splenic material. Because antigen localization was identical for all strains and antigens tested, these antigens may be located on similar subcellular structures.

The H-2 active pellets from four zonal centrifuge separations at $5000 \cdot 10^6\ \omega^2 t$ of C3H splenic homogenates were partially solubilized by autolysis. Hemagglutination inhibition analyses of the lyophilized soluble fraction indicated that about 15 % of the particulate antigen had become soluble during autolysis. Gel filtration on Sephadex G-200 showed that the fraction with H-2 activity was in the exclusion volume, suggesting a molecular weight greater than 100 000 (Fig. 5). Electrophoresis of samples on cellulose acetate after gel filtration showed that the antigen-active fraction contained three major and two or more minor electrophoretically distinct components.

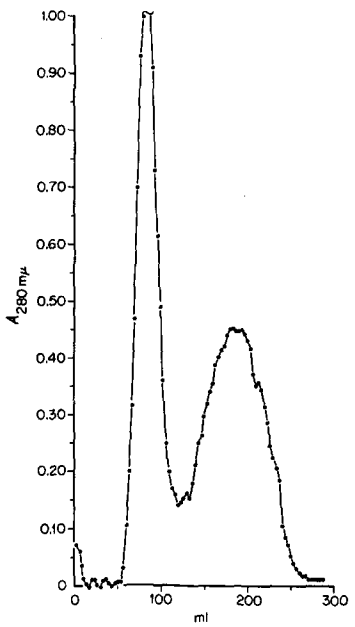

Fig. 5. Gel filtration of the soluble phase of C3H splenic particles that contain H-2 antigen. The absorbance of the effluent from Sephadex G-200 is plotted against the volume of effluent.

DISCUSSION

The experiments described in this study show that the zonal centrifuge is useful for preliminary separation of particles that contain murine transplantation antigens. The particles can be concentrated from a large mass of splenic tissue under appropriate conditions. At approx. $5000 \cdot 10^6\ \omega^2 t$, the heavier and/or larger particles sediment into regions of higher sucrose density, whereas the particles having antigenic activity are found along with soluble material at a density of 10–12 % sucrose. The soluble and particulate materials can be separated by centrifugation at 100 000 $\times\ g$ for 1 h; the

small pellet contains particles that will absorb antibody and induce hemagglutinating antibody.

High levels of enzymic activity are found in the fractions with the highest concentrations of H-2 antigen. The enzyme test is not a reliable index for antigen localization, however, because enzyme activity is also high in fractions that contain little or no H-2 antigen (Figs. 1 and 2).

The majority of the H-2 antigen is found on small subcellular particles. The density of the particles bearing H-2 antigen corresponds to that of cell membrane. If the particles are indeed fragments of the cell membrane, the antigen must be present on portions of the cell membrane that consistently become fragments of relatively small size because the particles do not sediment as quickly as one might expect for large fragments of cell membrane. Data being collected by BOYSE *et al.*[12] on antigen mapping suggest an organized architecture for the membrane. The geography of antigens on cell surfaces may dictate that the H-2 antigens reside in regions that are quite readily detached from other structural components of cell membranes.

The H-2 active material separates physically as a unit; the unit would appear to be a fragment of the cell membrane. Solubilization by mild autolysis at 5° gives a fraction of molecular weight greater than 100000 that contains all of the H-2 antigenic specificities (Fig. 5). Autolysis at 37° gives a soluble H-2 product of slightly lower molecular weight[13] and digestion by proteolytic enzymes yields yet smaller fragments in which some of the H-2 specificities have been separated[14]. The soluble antigen appears to be a glycoprotein, but the importance of the carbohydrate groups is uncertain because an alteration of the carbohydrate prosthetic groups does not always destroy the antigenic activity of the material[15]. Treatment which alters proteins, however, usually results in a loss of antigenic activity[15].

Genetic information suggests that the H-2 locus represents a linear portion of the chromosome. One cistron governs the synthesis of one polypeptide chain. If the recombinants which have been observed between the D and K regions of the H-2 locus really represent intracistronic crossing-over, the length of the DNA that controls the structure of the large H-2 molecule should be related to the frequency with which crossing-over has been observed. BOYER *et al.*[16] have estimated that the structural locus for hemoglobin represents about 0.001 map units for the polypeptide of a molecular weight of about 17000. By using this estimation, if map units in man and mouse are similar, the minimal size of the H-2 molecule must be about 61200 $\pm$ 8500; this calculation is based on the observed recombination frequency of 0.003 $\pm$ 0.0005 for the D and K regions of the H-2 locus[17]. The molecular weights of the H-2 molecules obtained by autolysis and papain digestion are greater than 100000 (Fig. 5) or approx. 100000 (ref. 13) and 70000 (ref. 14), respectively. If the molecule is a simple polypeptide, mild enzymic digestion may produce smaller sigments that can still participate in antigen–antibody interaction.

The antigen must be further purified before methods already in use to establish primary structures of proteins can be applied to elucidate the primary structure of the H-2 molecule. Knowledge of this structure will perhaps show what sequences of amino acids, perhaps associated with bound carbohydrates, do indeed represent transplantation antigens. Once this is known, it should be possible by chemical methods to modify or synthesize molecules either to produce tolerance or to enhance the antigenic potency of weakly antigenic materials such as those found in tumor tissues.

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