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NOTES

Separation of B and C Type Virions by Centrifugation in Gentle Density Gradients

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Murine type B particles were separated from type C (Rauscher leukemia virus) by means of gentle (low-increment rate) density gradients. The best separation was obtained when the density ranged from 1.13 to 1.20 g/cm^3 when sucrose was used and from 1.12 to 1.28 g/cm^3 with CsCl. The buoyant densities of the B and C particle bands in sucrose were 1.18 and 1.16 g/cm^3, respectively. The CsCl gradient gave a better separation with the B particles banding at a density of 1.20 g/cm^3 and with the C particle density little different from its value in sucrose.

During our recent attempts to find oncornavirus-like particles in human milks, we observed two morphologically distinct types of particles in a single band obtained after density gradient centrifugation in sucrose (7, 11). One type resembled the B particle of mouse mammary tumor virus, and the other resembled the C particle of leukemia and sarcomas in several species. Isolated particles from human milk are claimed to contain both 70*S* RNA and reverse transcriptase (13), but it was not known whether these products came from the B-type or C-type particles. Furthermore, besides B particles, milks of high-breast-cancer-strain mice contain a small amount of C particles (11), and even mouse mammary tumor cells cultivated in tissue culture produce both B and C particles (2, 4, 5). Thus, it was of interest to devise methods by which these two kinds of particles could be separated. We, therefore, attempted to separate B and C particles from an artificial mixture of these two types of particles derived from murine sources by density gradient centrifugation.

The B particles used in these experiments were purified from milk of RIII mice (10), and the C particles, the Rauscher strain of murine leukemia virus produced in tissue culture, were obtained from Pfizer, Inc., Maywood, N.J. The number of each type of highly purified particles in suspension was estimated by particle counting in the electron microscope by using known amounts of latex particles (5). About 10^{12} particles each of B and C type were mixed together thoroughly in 0.5 ml of Tris-NaCl-EDTA buffer (TNE) and layered on preformed gradients of sucrose or cesium chloride (CsCl) in 5-ml ultracentrifuge tubes. The linear gradients were made by a mechanical device (12). A wide range of density differences were used, and all gradient centrifugations were carried out for 2 h at 32,000 rpm in the Spinco SW 39. In most cases two distinct bands were visible in the gradient tubes (Fig. 1 and 2). The bands were carefully removed by skimming from the meniscus, diluted separately with buffer, and centrifuged for 1 h at 32,000 rpm. The respective viral pellets were allowed to soak in 0.1 ml of TNE overnight and were examined in the electron microscope by using negative staining techniques. Micrographs of randomly selected fields were taken, and the number of type B or C particles were counted on the basis of their surface morphology (Fig. 3).

The best separation of the two types of particles was obtained when the density ranged from ρ = 1.135 to 1.20 g/cm^3 in the case of sucrose and from 1.12 to 1.285 g/cm^3 when CsCl (gentle gradients) was used (Table 1). In sucrose gradient (Fig. 1a), the C particles banded at ρ = 1.155 g/cm^3 and were about 93% free of B particles, but the B particle band (ρ = 1.173 g/cm^3) contained about 25% of the C particles. This indicated that the C particles were more highly dispersed than the B particles, but that the two types have distinct enough sedimentation rates and densities to cause most of the particles to separate. An excellent separation of the particles was obtained in CsCl when C particles were banded at a density of 1.165

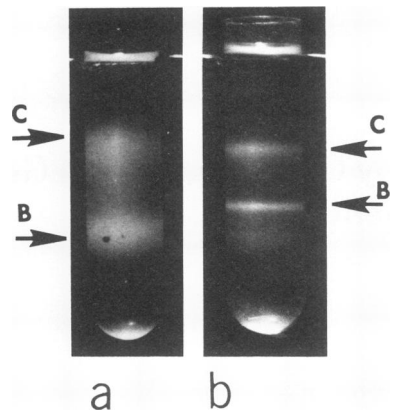

FIG. 1. *Photographs of centrifuge tubes. (a) Centrifugation of a mixture of B and C particles (≃10^{12} particles each) in sucrose gradient (ρ = 1.135 to 1.20 g/cm³) for 2 h at 32,000 rpm in the SW 39 rotor. The upper band (ρ = 1.155 g/cm³) contained about 93% C particles and only 7% B particles, whereas the lower band (ρ = 1.173 g/cm³) contained 75% B particles and 25% C particles. (b) CsCl gradient (ρ = 1.12 to 1.285 g/cm³), centrifuged under identical conditions. The upper band (ρ = 1.165 g/cm³) contained 96% C, and the lower band (ρ = 1.205 g/cm³) contained 97% B particles (see Table 1).*

g/cm³, whereas B particles banded at a density of 1.205 g/cm³ (Fig. 1b and 2). The C particle band contained about 5% B particles, while the B particle band had 8% C particles. Neither B nor C particles reached equilibrium in CsCl gradient after 2 h of centrifugation, but prolonged centrifugation did not improve the separation.

Attempts were also made to separate B and C particles from a crude mixture of the two in the presence of cell debris by using the best conditions of gradients described above. For this set of studies the viral suspensions used for mixing were prepared as follows. (i) Tissue culture fluid containing C particles was centrifuged for 5 min at 5,000 rpm in the SW 25.1 rotor. The pellet containing most of the cell debris was discarded, and the supernatant was centrifuged for 1 h at 21,000 rpm in the same rotor. The resuspended pellet was used as the source of C particles. (ii) For B particles, 4 ml of RIII mouse milk was diluted with 16 ml 0.15 M EDTA and 10 ml of phosphate-buffered saline, pH 7.4, and centrifuged for 5 min at 5,000 rpm in the Spinco SW 25.1 rotor. After discarding the cream fraction and the pellet, the skim milk was centrifuged for 1 h at 21,000 rpm. The supernatant was decanted and 0.5 ml of TNE was added to the pellet, which was allowed to stand overnight at 4 C. This viral suspension and that of C particles were diluted appropriately to get a particle count of about 10^{12} particles/ml. After mixing equal volumes (0.25 ml) of the two viral preparations, they were subjected to centrifugation in a gentle gradient of sucrose or CsCl.

In sucrose, a broad band (ρ = 1.15 to 1.18 g/cm³), which sometimes could be identified as two, was obtained. Particle counts on the material contained in the area around ρ = 1.16 g/cm³ and ρ = 1.17 g/cm³ in the diffuse bands showed that the separation of B and C particles was poor (Table 1). However, if these two fractions were recentrifuged separately in similar gradients, both B and C particles could be isolated effectively (Table 1). Separation of particles in CsCl gradient was much better than sucrose gradient, and, although slightly diffuse, in most cases two distinguishable bands were obtained. A second cycle of centrifugation of the respective bands in CsCl gradient was necessary for a good separation of the particles.

From these results it is obvious that for purification of either B or C particles, CsCl gradients are better than sucrose gradients, although the latter have usually been employed in purifying oncornaviruses. It is known that B particles derived from milk or cell cultures band at 1.170 to 1.180 g/cm³ in sucrose (6; Sarkar,

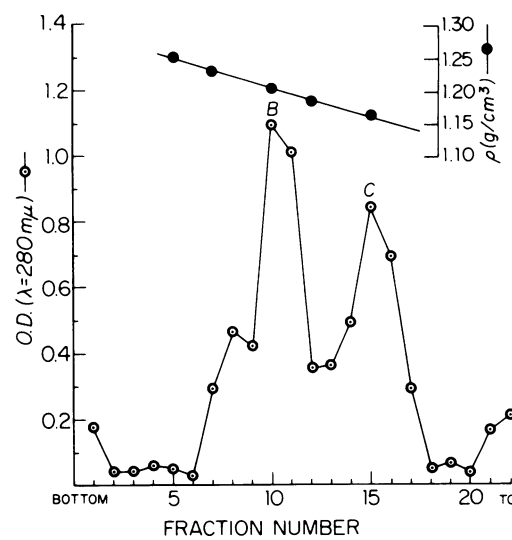

FIG. 2. *Distribution of B and C particles in CsCl gradient as measured by optical density at the wave length 280 nm. The pooled fractions 9 to 12 contained mostly B particles, whereas a pool of the fractions 14 to 17 contained mostly C particles. B particles most often form another diffuse band following the major band (cf. Fig. 2 with 1b). After 2 h of centrifugation, 0.2-ml fractions were collected by puncturing the bottom of the tubes. The density of each fraction was determined by weighing.*

FIG. 3. *A survey picture of the particles contained in the upper band in sucrose gradient. This band contained mostly C particles and only a small amount of intact B particles (solid arrow); however, the majority of these B particles were in the form of fragments (open arrows), which are easily identified due to the spikes. The inset shows a higher magnification of B and C particles. ×60,000; inset ×150,000.*

TABLE 1. *Separation of B and C particles*

Virus	Gradient material	Percentage of particles in bands[a]			
		Upper band[b]		Lower band[c]	
		B	C	B	C
Mixture of B and C, both purified in sucrose gradient	Sucrose	7	93	75	25
	CsCl	5	95	92	8
Mixture of B and C, both crude preparations	Sucrose	35 (B-1)[d]	65 (B-1)	50 (B-2)	50 (B-2)
	CsCl	15 (B-3)	85 (B-3)	75 (B-4)	25 (B-4)
Bands:					
B-1	Sucrose	4	96	85	15
B-2		6	94	80	20
B-3	CsCl	6	94	95	5
B-4		4	96	97	3

[a] Each value is a mean of at least three separate experiments from individual bands, and the number of total particles counted ranged from 500 to 1,000.

[b] The mean density of the upper band was 1.155 g/cm^3 in sucrose and 1.165 g/cm^3 in CsCl.

[c] The mean density of the lower band was 1.173 g/cm^3 in sucrose and 1.205 g/cm^3 in CsCl.

[d] Bands designated as B-1 and B-3 (upper area of the gradient) and B-2 and B-4 (lower area) were centrifuged on gentle gradients to obtain better separation as is demonstrated in lower part of the table.

unpublished data), whereas C particles from a variety of sources band at 1.15 to 1.16 g/cm^3 (1, 3, 6, 9, 14). Whether CsCl gradients will affect the biological activity of C particles is not known, but we established earlier that CsCl has practically no detrimental effect on the infectivity of murine mammary tumor virus (8). Furthermore, CsCl gradient should be an obvious choice when one deals with mouse mammary tumor virus or attempts to isolate murine mammary tumor virus-like particles from human milks, whereas in both cases contamination with C-type particles is very likely. We recommend, however, two cycles of centrifugation. From our electron microscope data, we are hopeful that such a purification procedure will also be acceptable to those who are interested in biochemical and immunological analysis of the viral isolates.

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