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Some Structural and Antigenic Properties of Intracisternal A Particles Occurring in Mouse Tumors

(complement fixation/immunodiffusion/neuroblastoma/plasma-cell tumor)

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ABSTRACT **Intracisternal A-particles were isolated from three different myeloma lines in BALB/c mice and from cultured neuroblastoma cells of A/J origin. All preparations contained a major structural protein with an apparent molecular weight near 70,000 as estimated by electrophoretic mobility in sodium dodecyl sulfate-containing polyacrylamide gels. Solubilization of this component by sodium dodecyl sulfate was dependent on prior or concomitant treatment with sulfhydryl compounds. The size distribution of A-particle proteins was markedly different from that observed for extracellular murine leukemia and mammary tumor viruses. Rabbit antiserum was developed that reacted with the major A-particle protein in both complement fixation and immunodiffusion assays. The antigen was detected in isolated neuroblastoma A-particles, in cytoplasmic membrane fractions prepared from various mouse tumors known to contain intracisternal particles, but not in preparations from normal mouse cells, in samples of leukemia and mammary tumor virus, or in JLS-V9 cells infected with Rauscher leukemia virus. Conversely, isolated A-particles did not react in complement fixation or immunodiffusion assays with antisera against leukemia virus antigens.**

Intracisternal A-particles (1) have been observed by electron microscopy in a wide variety of transplantable murine tumors (in ref. 2, see also refs. 3, 4) and recently in apparently normal cells of certain strains of mice (5). The particles are doughnut-shaped structures between 70 and 100 nm in diameter, consisting of two concentric shells enclosing a relatively electronlucent center. Characteristically, the particles form by budding at the endoplasmic reticulum (ER) membranes; when fully formed, they are localized exclusively within the cisternae. In appearance they somewhat resemble immature forms of certain oncogenic RNA viruses. Although no biological activity has yet been shown to be associated with them, these particles are of interest because of their extensive association with tumor cells and the possibility that they reflect the expression of a viral genome within affected cells.

Earlier, we described the extraction of intracisternal A-particles from a transplantable plasma-cell tumor of BALB/c mice (2). The isolated particles, which retained their characteristic morphology, consisted of 80% protein, 14% phospholipid, and 5–6% RNA (carbohydrates and other types of lipid were not studied). No DNA was detected. The phospholipid was contained largely in the detergent-sensitive outer shell, derived from the ER membrane.

In the present study, we have further analyzed A-particles isolated from several different plasma-cell tumors and from cultured neuroblastoma cells. A major protein component of the particle has been identified and antigenic determinants present on this protein have been detected in various other mouse tumors. The structural and immunological relationships between intracisternal A-particles and some oncogenic RNA viruses have been examined. Certain aspects of this work have been briefly presented (6).

Abbreviations: ER, endoplasmic reticulum; MuLV, murine leukemia virus; MTV, mammary tumor virus; gs, group specific; CF, complement fixing; SDS, sodium dodecyl sulfate.

MATERIALS AND METHODS

Tumors. Plasma-cell tumors (lines MOPC-104E, MOPC-321, RPC-5, and RPC-20) in BALB/c mice and the X5563 myeloma in C3H mice were obtained from Dr. M. Potter of NCI and maintained by subcutaneous transplantation. Mice bearing subcutaneous transplants of the squamous cell carcinoma D were kindly provided by Dr. V. Suntzeff, Washington University School of Medicine, St. Louis, Mo.

Tissue Culture Cells. Neuroblastoma cultures derived from tumor C1300 (7) were provided by Dr. R. V. Gilden, Flow Laboratories, Bethesda, Md.; the NCTC lines 4700 and 4953 (8) by Dr. K. K. Sanford, NCI; and the L1210 cells by Dr. K. W. Kohn, NCI.

Fractionation Procedures. Intracisternal A-particles were isolated from plasma-cell tumors as described (2). Briefly, cytoplasmic extracts were centrifuged at 10,000 $\times$ *g* for 10 min to yield pellets of mixed mitochondria and microsomes (membrane fraction). The A-particles were liberated from microsomal vesicles by shearing the resuspended membrane fractions in the presence of Triton X-100, or more recently, by sonic vibration in the absence of detergents (3 min at 10 kc and 0–5°C in a model DF 101 Raytheon magnetostrictive oscillator). The liberated particles were purified by two cycles of sedimentation in sucrose–potassium citrate solutions (pH 7.2) and finally banded in linear sucrose gradients containing 50 mM potassium citrate or 10 mM sodium phosphate (pH 7.2). Particles released by treatment with Triton and shearing were recovered at an average density of 1.22 g/cm^3; particles released by sonication were recovered at a density of 1.19 g/cm^3.

Neuroblastoma cells were washed at 5°C with Hank's buffered saline, allowed to swell in four volumes of cold 10 mM Tris·HCl (pH 7.4)–1.5 mM $MgCl_2$, and then disrupted with a tightly fitting Dounce homogenizer followed by vigorous expression through a 23-gauge hypodermic needle. A-particles were isolated as usual, except that the membrane fraction was sedimented initially for 30 min at 60,000 $\times$ *g*. A-particles isolated from plasma cell tumors and neuroblastoma appeared similar on electron-microscopy; particles released by sonication showed a greater association with microsomal membrane fragments.

Membrane fractions were prepared from other cultured cells or ascites tumors as from neuroblastoma cells, and resuspended in 10 mM sodium phosphate buffer (pH 7.2).

Tumor Virus. Rauscher and Moloney murine leukemia virus (MuLV) preparations were purchased from Electro-Nucleonics Laboratories, Inc., Bethesda, Md. Both viruses were grown on JLS-V9 cells (9) of BALB/c orgin. Mammary tumor virus (MTV) was prepared from milk of C3H/He mice at Meloy Laboratories, Springfield, Va., under contract no. PH-43-66-458. The viral content of each preparation was verified by electron microscopy.

Sample Preparation and Electrophoresis. Proteins were analyzed by electrophoresis in 6% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) (10). Samples in 10 mM sodium phosphate (pH 7.1) and 1% SDS were heated for 1-2 min at 100°C both in the presence and absence of a disulfide-bond reducing agent, 0.1 M 2-mercaptoethanol or 1 mM dithiothreitol. Samples were applied to the gels at once, or, with comparable results, after having first been alkylated with iodoacetamide and dialyzed against 0.1% SDS in 10 mM phosphate buffer (pH 7.1) (10). Gels (0.5 × 5.5 cm) were first electrophoresed for 90 min in the running buffer, 0.1 M sodium phosphate (pH 7.1), containing 0.1% SDS. Then samples, containing 10-20 μg of protein, were electrophoresed at 3 mA per gel for 4 hr at room temperature. Gels were fixed in trichloroacetic acid and stained with Coomassie Blue. The molecular weights of A-particle and viral components were estimated by comparing their electrophoretic mobilities in SDS-containing gels with those of known marker proteins (11). Proteins were quantitated by a Folin procedure (12).

Complement-fixation (CF) Assays. A-particle antigen was measured by microtiter assay (13). Two New Zealand rabbits were injected twice with MOPC-104E A-particles that were washed with 1% sodium deoxycholate (2): initially via footpad with complete Freund's adjuvant (70 μg of protein), and 5 weeks later intravenously in saline (90 μg of protein). About 16 months later, each rabbit was inoculated subcutaneously with a homogenized gel cut containing the main protein component (see *Results*) that was separated electrophoretically from about 100 μg of total A-particle protein (RPC-20 origin). Sera were obtained 10 days after the last inoculation. Both rabbits yielded antisera of similar specificity. The antiserum with the higher titer (98/11) reacted at dilutions up to 1:1280 in CF test against preparations containing A-particle components. Both antiserum 98/11 and the corresponding preimmunization serum (98/0) reacted with sonicates or membrane fractions of normal BALB/c cells at dilution ≤ 1:48. The antiserum was routinely used at a dilution of 1:300 for detection of A-particle protein in CF test. Tissue fractions were first treated for 1 hr at 25°C with 0.1% SDS and 1 mM dithiothreitol to unmask the antigen.

Microtiter assays for MuLV antigen(s) were performed (14) with a pooled rat antiserum, MSV-20, broadly reactive toward the major group specific antigen, *gs*-1 (15); assays were kindly performed under the direction of Mr. H. C. Turner, Viral Carcinogenesis Branch, NCI.

Immunodiffusion Assays. For A-particle antigens, double diffusion was performed in 0.75% agarose gels containing 10 mM sodium phosphate (pH 7.4), 0.1 M NaCl, 0.1% SDS, and 20 mM iodoacetamide. Immunodiffusion assays for the *gs*-1 and *gs*-3 antigens of MuLV and related C-type viruses (16–81) were performed by Dr. R. V. Gilden.

RESULTS

Electrophoretic Studies. A-particles treated with 1% SDS at pH 7.2 in the absence of reducing agent were incompletely dissociated, and most of the protein was retained at the upper gel surface upon electrophoresis. Solubilization was obtained when particles were treated simultaneously with SDS and 2-mercaptoethanol (Fig. 1) or first reduced and alkylated in the absence of SDS and subsequently exposed to the detergent.

Purified A-particles from various sources all exhibited a major electrophoretic component, appearance of which in the gel was rigorously dependent upon prior sulfhydryl treatment (Fig. 1). This component had an apparent molecular weight near 70,000 on the basis of its relative mobility over a range of gel concentrations between 5 and 10%. Little or none of it entered the gels in systems without SDS, such as 8 M urea at pH 9 or 5 M urea in 35% acetic acid, even when the particles had been previously reduced with 2-mercaptoethanol and solubilized with SDS. Deoxycholate and Triton X-100 were ineffective in solubilizing the major protein.

Other electrophoretic components varied in proportion among preparations from different sources and, to a lesser extent, according to whether the particles had been liberated by detergent treatment or sonic vibration (Fig. 1). A more detailed structural analysis (Wivel *et al.*, manuscript in preparation) has shown that some of these minor components are derived from the outer membranes, while the major protein is localized in the detergent-resistant inner particle shells (2). The latter may be recovered by centrifugation (e.g., 60 min at 105,000 × *g*) from particle suspensions treated with SDS [0.05-1% (pH 7.1); 20°C] in the absence of reducing agent.

The A-particle electrophoretic patterns differed markedly from those presented by MuLV (Fig. 2). A-particles contained little or no material in the molecular weight range of the main viral bands, about 30,000 for MuLV and 55,000 for MTV

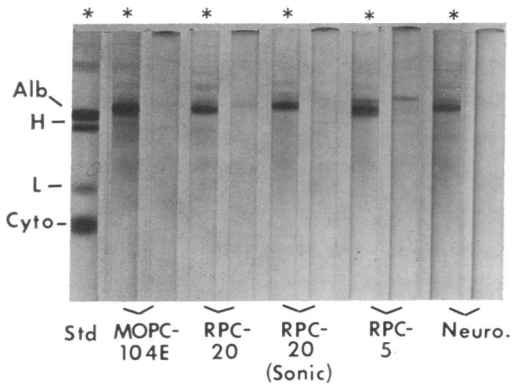

FIG. 1. Protein electrophoretic pattern of intracisternal A-particles isolated from mouse plasma-cell tumors and neuroblastoma. Electrophoresis was performed in 6% polyacrylamide gels in the presence of 0.1% SDS, samples having first been heated at 100°C for 1 min in 1% SDS, either with* or without 0.1 M mercaptoethanol. The gels were stained with Coomassie Blue. *Std*, a standard mixture of proteins, human serum albumin (*Alb*, 69,000 daltons), heavy (*H*, 55,000) and light (*L*, 23,000) chains of mouse myeloma IgG, and bovine cytochrome *c* (*cyto*, 12,500). Other details are given in *text*.

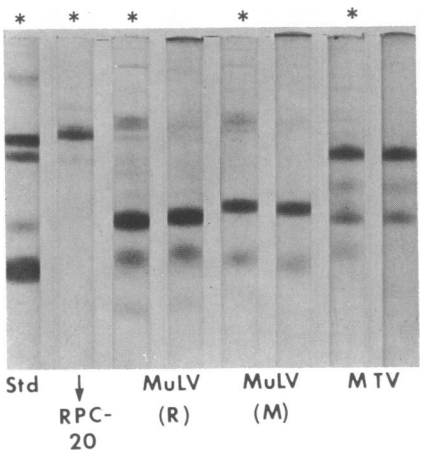

FIG. 2. Protein electrophoretic patterns given by intracisternal A-particles (*RPC-20*), Rauscher (*R*) and Moloney (*M*) types of murine leukemia virus (*MuLV*), and a sample of mouse mammary tumor virus (*MTV*). Other details as in Fig. 1.

Also, most of the MuLV and MTV proteins were solubilized by treatment with SDS in the absence of 2-mercaptoethanol, in contrast to the A-particle components. However, both leukemia virus preparations exhibited a triad of minor bands in the molecular weight range of 70,000–100,000; the appearance of the triad in the gel depended on prior disulfide reduction (Fig. 2). Application of periodic acid–Schiff stain to gels of reduced Rauscher virus produced two positively staining bands (see ref. 19), one of which corresponded to the more slowly moving component of this triad. No periodic-acid–Schiff-positive components were seen in gels containing A-particle material derived from RPC-tumors.

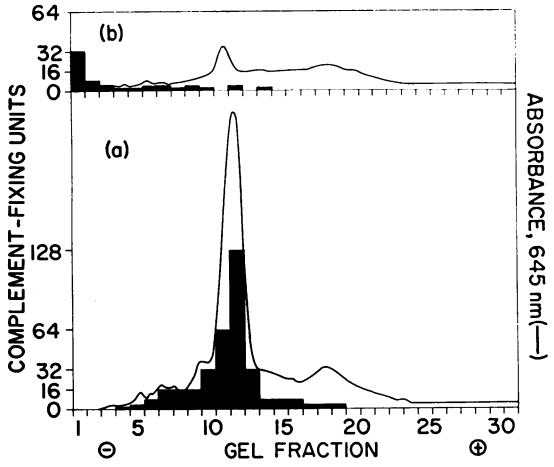

FIG. 3. Complement-fixing antigen in electrophoretically separated A-particle fractions. A-particles were isolated from RPC-20 tumors and aliquots containing 100 μg of protein were electrophoresed on 5% polyacrylamide gels for 3 hr in the presence of 0.1% SDS, as described in the text. In (*a*), the sample was heated at 100°C for 1 min in 1.0% SDS and 0.1 M 2-mercaptoethanol before electrophoresis; in (*b*), 2-mercaptoethanol was omitted. After electrophoresis, the gels were sliced at calibrated intervals and eluates of the slices were assayed by CF with antiserum 98/11. Smaller aliquots of reduced and unreduced A-particles (about 10 μg protein) were also electrophoresed, the gels were stained with Coomassie Blue and scanned at 654 nm in a Gilford spectrophotometer, equipped with a gel-scanning device. Data obtained by the two types of analysis were then combined on a single graph (see *text* for additional details).

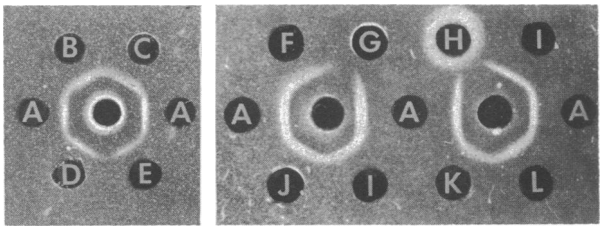

FIG. 4. Immunodiffusion reactions for A-particle antigen(s). Unmarked wells contained rabbit antiserum 98/11 prepared against A-particle material (see *text*). Well *H* was filled with preimmunization serum 98/0, from the same rabbit. Other wells were filled as follows: *A*, isolated A-particles from MOPC-104E myeloma; *B*, cytoplasmic extract; *C*, membrane fraction; *D*, cut from electrophoretic gel in region of main A-particle component; *E*, SDS-washed inner A-particle shell (fractions B-E prepared from RPC-20 myeloma). In the right hand grouping, wells contained membrane fractions from *F*, neuroblastoma; *G*, normal BALB/c liver; *I*, RPC-20 myeloma; *J*, carcinoma D; *K*, X5563 myeloma; *L*, NCTC-4953.

Immunologic Studies. Antiserum 98/11, diluted 1:300, was titrated by CF test against the electrophoretically separated A-particle components (Fig. 3*a*). The major antigen activity was localized in the position of the main protein band. Weak reactions observed in gel fractions 5–9 could have resulted from trailing and/or aggregates of the main component. Also, the antiserum may have reacted with more than one specificity, even at 1:300 dilution. Little reaction was observed when A-particles were electrophoresed in SDS without prior reduction (Fig. 3*b*).

CF antigen titers observed with purified particles and crude fractions were increased as much as 8-fold upon pretreatment of the samples with SDS and sulfhydryl compounds. The conditions described in *Methods* were found sufficient to unmask antigen, while avoiding SDS-induced lysis in the CF assay. Cells and tissues were compared on the basis of the CF titer of their cytoplasmic membrane fractions: such fractions were easily prepared in concentrated form (equivalent to 1 or 2 g of tissue per ml) and rarely showed anticomplementary activity. Also, addition of SDS to homogenates or sonicates produced DNA gels that interfered in CF assay.

Antiserum 98/11 also reacted with A-particles on immunodiffusion. The reaction was strictly dependent upon prior reduction of the samples in SDS and upon inclusion of some SDS (0.01–0.1%) in the gel itself. Conditions for pretreatment of the samples were apparently not critical: 0.1% SDS–1 mM dithiothreitol at 25°C (as for CF), or 1% SDS–10 mM 2-mercaptoethanol at 100°C gave similar results.

As shown in Fig. 4, the antiserum gave a single precipitin line with isolated plasma-cell tumor A-particles, the SDS-treated inner particle shells, and an acrylamide gel cut containing the major electrophoretic component. Lines of identity were also obtained with cytoplasmic extract and membranes, indicating that the antiserum was specifically detecting the major A-particle protein in these crude fractions.

CF titers of 8 or greater were found for membrane fractions from a number of mouse tumors known by electron microscopy to contain intracisternal A-particles (Table 1). In each

TABLE 1. *Survey of intracisternal A-particle antigen(s) in the membrane fractions from normal and neoplastic cells*

Cell type	Mouse strain	A-particle antigen(s) in membrane fraction: CF units* (per g of tissue)	CF units* (per mg protein)	Immunodiffusion†	Intracisternal A-particles by electron microscopy‡
Normal					
Mixed (liver, kidney, spleen)	BALB/c	<1	<0.06	−	Extremely rare (5)
Mixed (liver, kidney, spleen)	A/J	<1	<0.06	−	N.E.
Mixed (liver, kidney, spleen)	C3H/He	<1	<0.05	−	Extremely rare (5)
Mixed (liver, kidney, spleen)	DBA/2	<1	<0.06	−	N.E.
Mixed (liver, kidney, spleen)	C57L	<1	<0.06	−	N.E.
3T3 (fibroblast TC)	BALB/c	<2	<1	−§	Absent
JLS-V9 (marrow TC)¶	BALB/c	<1	<0.2	−	Absent
Embryo (primary TC)	Swiss	<2	<0.7	−	Absent
Transformed					
MOPC-104E (myeloma)	BALB/c	128	7	+	Numerous
RPC-20 (myeloma)	BALB/c	160	8	+	Numerous
MOPC-321	BALB/c	36	2	+	Rare
Neuroblastoma TC	A/J	53	19	+	Numerous‖
Carcinoma D (squamous)	C57L	≥50	≥20	+	Numerous
X5563 (myeloma)	C3H/He	256	9	+	Numerous
NCTC 4700 (fibroblast)	C3H/He	32	2	+	Numerous (8)
NCTC 4953 (fibroblast)	C3H/He	16	1.5	+	Numerous (8)
L1210 TC (lymphoma)	DBA	16	1	+	Occasional‖
Ehrlich	C3H/He	8	1	+	Present (in ref. 2)
70429 (reticulum cell)	C3H/He	2	0.25	−	Rare (in ref. 2)
129(P) (hepatoma)	C3H/He	2	0.3	−	Extremely rare

* Reciprocal of CF titer for 25 μl of membrane fraction divided by the wet weight of tissue represented in 1 ml of fraction or by the mg of membrane protein per ml.

† Reaction of identity with A-particles isolated from BALB/c myelomas did (+) or did not (−) occur; negative reactions for antigen wells filled three times.

‡ Observed in cells actually used for the present study, or previously reported as indicated by the references. N.E., not examined.

§ A weak reaction was given that did not fuse with A-particle line and was also developed by preimmunization serum.

¶ Whole cell sonicate tested.

‖ Electron micrographs kindly provided with cells (see *Materials and Methods*).

such instance, immunodiffusion gave a precipitin line that fused with that given by a standard myeloma A-particle preparation (Table 1; examples in Fig. 4). The antigen-positive tumors represented various cell types and were derived from five different mouse strains. Minimal CF titers and negative immunodiffusion results were found with two tumors of C3H origin. Normal tissues of adult mice and cultured cells of BALB/c and Swiss mice were negative by both assays.

Representative CF titers obtained with antiserum 98/11 for A-particles that were gradient-isolated are shown in Table 2. Preparations of MuLV and MTV were not reactive with this antiserum, nor was A-particle antigen detected in JLS-V9 cells infected with Rauscher virus. Conversely, A-particles isolated from MOPC-104E and RPC-20 tumors have consistently failed to react in CF test with antisera broadly reactive for the murine leukemia-sarcoma group of viruses. Negative results (not illustrated) were also obtained in immunodiffusion tests with antisera against both the *gs*-1 and *gs*-3 leukemia virus antigens.

DISCUSSION

Intracisternal A-particles prepared from several plasma-cell tumors and from neurobastoma had the following properties: (*a*) marked structural stability of the inner particle shell, resulting from intermolecular disulfide bonding as well as the associative properties of the constituent proteins (*b*) a major structural protein (or group of proteins) with apparent molecular weight near 70,000, and (*c*) a common antigenic specificity associated with the major protein. This antigenic determinant was also demonstrated in the membrane fractions of other neoplastic cells that differ in histologic type and host strain of origin, but have in common the presence of intracisternal A-particles. The antigen was not detected with our antiserum in normal adult cells, where A-particles are extremely rare (5). Our results suggest that intracisternal A-particles exhibit group specific properties in a manner analogous to that of known viruses or intracellular organelles.

Intracisternal A-particles occur sometimes in neoplastic cells that are also producing extracellular C-particles resembling leukemia virus or intracytoplasmic A-particles (1) of the type associated with mammary tumor virus infection. A possible relationship between intracisternal particles and known oncogenic RNA viruses has thus had to be considered. However, the studies of Hall and coworkers (8) and others (20, 21) have shown a complete lack of correlation between the presence of intracisternal A-particles and expression of leukemia virus antigens or infectivity. Similarly, with respect to the mammary tumor agent, it is known that plasma-cell tumors arising in BALB/c mice invariably contain intracisternal A-particles, but exhibit (in addition) intracytoplas-

TABLE 2. *Comparison of A-particle and leukemia virus antigens in various preparations*

	A-particle antigen(s)		MuLV antigens
	CF units (per mg protein)*	Immuno-diffusion†	CF units (per mg of protein)‡
MPOC-104E A-particles	100	+	<2
RCP-20 A-particles	190	+	<3
RPC-20 (sonic release)	265	+	<2‖
Neuroblastoma (sonic release)	128	+	N.T.
Rauscher MuLV	≤4§	−	512**
Moloney MuLV	≤4§	−	N.T.
MTV	<2	−	N.T.
JLS-V9-Rauscher MuLV¶	<0.25	−	≥16

* Reciprocal of CF titer of a 25-μl aliquot divided by mg of protein per ml of preparation. Rabbit antiserum 98/11, diluted 1:300, was used.

† As in Table 1.

‡ Assayed with rat antiserum MSV-20 diluted 1:20 (see *Methods*). N.T., not tested.

§ Preparations were anticomplementary to indicated titers; no evidence of positive reaction.

¶ Whole sonicate of JLS-V9 cells infected with Rauscher strain of MuLV. Actual MuLV CF tier was ≥1:128 for 25 μl of sonicate containing 7.8 mg of protein per ml.

‖ With or without Tween–ether treatment.

** Treated with Tween–ether.

mic A-particles only when the hosts have foster-nursed on MTV-carrying C3H females (22).

The present study provides no evidence of a structural or antigenic relationship between intracisternal A-particles and MuLV or MTV. The size spectrum of proteins represented in the intracisternal A-particles was clearly different from that seen by us and others (23–26) in either virus, and the A-particle antigen was not detected in the virus samples tested. In experiments to be described fully in another context, MuLV group specific antigen was demonstrable by CF assay in RPC-20 membrane fractions but failed to fractionate with the A-particles released by either the Triton-shear procedure or sonic vibration. Our results thus far do not support the suggestion that intracisternal A-particles in BALB/c myeloma cells represent intracellular or incomplete forms of MuLV (27, 28).

Although tumor induction has recently been reported with cell-free preparations from neoplastic tissues known to contain intracisternal A-particles (3, 29), it has not yet been possible to ascribe a biological activity to the A-particles themselves. To our knowledge, direct infectivity studies with A-particles isolated from myeloma lines have been uniformly negative, and attempts to rescue the Moloney sarcoma virus, mentioned earlier as in progress (2), proved unsuccessful. Recently, we have attempted to coinfect JLS-V9 cells with MuLV and sonically released A-particles from MOPC-104E. No evidence for production of A-particle antigen was obtained, although CF tests for MuLV became strongly positive in the treated cells.

Intracisternal A-particles could arise from abnormal production and/or processing of a cellular constituent, or they could reflect the expression of a viral genome. In the latter case, they might represent competent virus whose infectivity has simply not yet been demonstrated or they might in fact be biologically defective. It will be important to determine by more varied and sensitive techniques whether intracisternal A-particles indeed form a structurally and antigenically unique group, whether they represent the partial or aberrant expression of an integrated genome that can on occasion give rise to recognized functional viruses, and finally whether their presence is directly related to the neoplastic transformation.

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