Studies on Mammary Tumor Inducing Virus in Mice (Bittner Virus).* (25181)

L. Dmochowski, C. E. Grey, L. O. Pearson, D. N. Ward, R. B. Hurlbert, A. C. Griffin and A. L. Bresson

Section of Virology and Electron Microscopy, and Dept. of Biochemistry, University of Texas M. D. Anderson Hospital and Tumor Inst., Depts. of Microbiology and Biochemistry, Baylor University College of Medicine, Houston, Texas

Biological, biophysical and biochemical methods have been employed in studies on properties of mammary tumor inducing Bittner virus(1-3). These studies demonstrated non-lipid fraction of fresh milk of agent-harboring mice to be best source of virus, and enzymes, such as trypsin, chymotrypsin, prostate phosphatase, and snake venom diesterase, and chelating agents, have little influence on virus present in milk(4). Electron microscope studies of sections of mammary tumors from virus-carrying mice revealed virus-like particles in these tumors(5-6), but not in lactating breast tissue of virus-carrying mice(4-7), known to be good source of virus(8). Attempts to correlate electron microscope observations with bioassays of these tumors for tumor-inducing activity failed to produce conclusive evidence that the observed particles are the Bittner virus(9). Experiments were therefore undertaken to study the morphological, biophysical and biochemical properties of the agent present in milk of mice.

Material and methods. Milk from mice of virus-carrying A, genetically identical virus-free Af, and RIII virus-harboring strains was used as source of the agent. Bioassays were carried out in 1 to 4-week-old $(C_{57} \times Af)F_1$ female mice to determine tumor-inducing activity following each treatment of milk. For electrophoretic fractionation, milk was defatted by centrifugation at 600 x g for 20 minutes, the remaining fraction decaseinated with chymotrypsin (0.2 mg/ml) for 5 minutes at 38°C. Coagulated milk was centrifuged at 600 x g for 20 minutes and supernatant at 105,000 x g for 2 hours at 4°C. Part of resulting pellet resuspended in citrate buffer of ionic strength 0.01 at pH 6.0 to the original volume of milk sample was used for bioassays, the rest for zone electrophoresis at 4°C in a trough with powdered cellulose in citrate buffer with current of 450 V and 15 m.a. After 20 hours, cellulose block was cut in ½ inch segments, buffer removed from each segment by filtration through separate filters with Whatman No. 1 paper and analyzed for phosphorus(10) and protein(11). High-speed centrifugal pellets of defatted and decaseinated milk were tested for presence of RNA and DNA. Ribonucleic acid was determined in hot perchloric acid extracts of pellets by modified orcinol method(12) and by ultraviolet absorption readings in Beckman DK2 recording spectrophotometer. For DNA determinations, Burton-Dische reaction(13) was used directly on the pellet. Ribonuclease digestion of pellet material was carried out at 38°C for 30 minutes with 10 μg of ribonuclease/ml of pellet resuspended in physiological saline, citrate buffer or distilled water. Material treated was then centrifuged at 105,000 x g for 2 hours at 4°C, and the supernatant and sediment tested for acid-soluble and insoluble RNA and tumor-inducing activity. High-speed centrifugal pellet material was treated with 75% cold ethanol, precipitate spun at 600 x g for 20 minutes at 4°C, resuspended to the original volume of defatted, decaseinated milk and tested for tumor-inducing activity. Phenolic deproteinization of defatted milk or of high-speed centrifugal pellets from such milk was carried out according to Gierer and Schramm technic(14), modified by Kirby (15). Biphthalate or salicylate buffer (0.15 M pH 7.3) was used as diluent. Fluorocarbon deproteinization technic(16-19), using 1, 1,2-trichloro-1, 2,2-trifluoroethane (Genetron 113), was also employed in attempt at extraction of the virus from high-speed centrifugal pellets of defatted and decaseinated milk.

* This work supported by Research Grants from Nat. Cancer Inst., N.I.H., U.S.P.H.S., and Am. Cancer Soc.

TABLE I. Bioassays of Various Fractions of Virus-Carrying Strain A Milk in (C_{57} × Af)F_1 Mice.

Numerators = Mice with mammary tumors. Denominators = Mice alive at earliest tumor appearance. Duration of bioassays—18 mo.

Type of fraction tested (treatment)	Dilutions 10^{-2}	10^{-3}	10^{-4}
Defatted milk (600 × g for 20 min.)	16/20 80% 12M*	12/16 75% 12M	13/18 72% 10M
Whey (incubation with chymotrypsin)	7/19 37% 17M	12/20 60% 12M	3/15 20% 16M
Supernate (10^{-1}) (105,000 × g for 2 hr)	12/19 63% 12M		
Pellet (105,000 × g for 2 hr)	9/20 45% 11M	10/19 53% 14M	11/22 50% 10M

* Avg tumor appearance in mo.

Electron microscope studies of ultrathin sections of pellets of milk from RIII, A, and Af strain mice were carried out according to method previously described(20).

Results. Bioassays of fractions of A strain milk following different treatment, preceding electrophoretic fractionation, are shown in Table I. High-speed centrifugal pellet from defatted, decaseinated milk retained tumor-inducing activity. Activity of supernatant may be due to resuspension of some of particulate material before or during removal of the supernatant. Chemical analysis for phosphorus and protein of high-speed centrifugal pellet (Table I) from defatted and decaseinated strain A virus-carrying milk after zone electrophoresis is shown in Fig. 1. On the basis of Folin-Lowry test, all segments of the cellulose block were pooled into 3 fractions for bioassays. Most of protein and 65% of phosphorus was found in Fraction II or central segment around the origin, and only small quantities in Fractions I and III towards anode and cathode. Bioassays of electrophoretic fractions of high-speed centrifugal Strain A milk pellet (Table II) showed tumor-inducing activity only in Fraction II. The tumor-inducing particle, therefore, does not appear to have a large net charge at pH 6. Electrophoretic analysis of strain Af milk with no tumor-inducing activity gave a pattern essentially similar to that of strain A milk with high tumor-inducing activity.

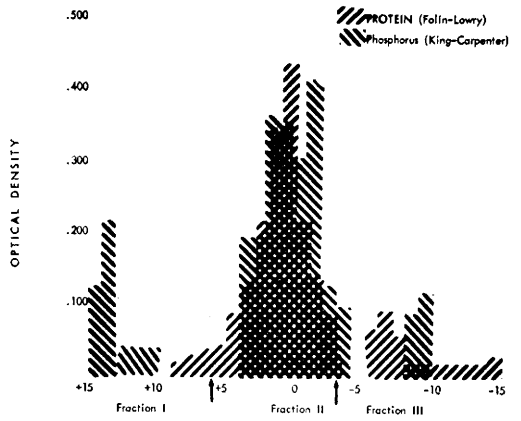

FIG. 1. Electrophoretic pattern of high-speed centrifugal pellet from defatted, decaseinated, virus-carrying strain A milk.

The amount of RNA in centrifugal pellets of defatted and decaseinated strain A virus-containing milk ranged in samples tested from 8 to 23 μg/ml of original milk. Estimations of RNA content in pellets of similarly treated virus-free Af strain milk have so far been unsuccessful because of color interference with the orcinol reaction. Similarly prepared pellets from virus-harboring A and virus-free Af milk showed no detectable DNA by method which could have shown quantities greater than 0.2 μg/ml of original milk. Absorption spectra of extracts of pellet material from both types of milk were characteristic of nucleic acids and agreed quantitatively with results of orcinol reaction. Ribonucleic acid analysis of high-speed centrifugal pellet from strain A milk before and after ribonuclease treatment (Table III) demonstrated alteration in solubility and sedimentability of RNA in the original pellet. Small fraction of RNA appeared to be resistant to ribonuclease. Bioassays on ribonuclease-treated high-speed cen-

TABLE II. Bioassays of Electrophoretic Fractions from High-Speed Centrifugal Pellet (105,000 × g—2 Hr) of Strain A Milk in (C_{57} × Af)F_1 Mice.

Numerators = Mice with mammary tumors. Denominators = Mice alive at earliest tumor appearance. Duration of bioassays—18 mo.

Type of fraction tested		Dilutions 10^{-2}	10^{-3}	10^{-4}
I	Anode side	0/19	0/18	0/19
II	Origin area	20/20 100% 13M	8/17 47% 12M	1/18 6% 18M
III	Cathode side	0/15	0/20	0/20

trifugal pellets from defatted or defatted and decaseinated strain A milk (Table IV), although not complete, show at least some tumor-inducing activity remains following this, and after cold ethanol treatment.

TABLE III. Ribonucleic Acid Analyses of High-Speed Centrifugal Pellet (105,000 × g—2 Hr) of Strain A Milk before and after Ribonuclease Treatment.

Material (fraction)	Amt of ribonucleic acid/10 ml: Acid soluble	Acid insoluble	Total (μg)
Original pellet (I)	103	131	234
Pellet after ribonuclease treatment (II)	20	10	30
Supernate from (II) (105,000 × g)	25	154	179

Aqueous phase from phenolic deproteinization of strain A milk has, so far, shown no tumor-inducing activity (after 14 months of bioassay duration) and control material considerable activity (86% tumor incidence in 9 months). The aqueous layers contain nitrogen in quantities of 80-140 μg/ml of original milk. This represents 1-2% nitrogen of original defatted milk. Bioassays of aqueous phase from fluorocarbon deproteinization of milk, because of short duration, do not permit any conclusion.

High-speed centrifugal pellets of virus-harboring RIII and A strain and of Af virus-

TABLE IV. Bioassays of High-Speed Centrifugal Pellets of Strain A Milk following Ribonuclease or 75% Ethanol Treatment in (C_{57} × Af)F_1 Mice.

Numerators = Mice with mammary tumors. Denominators = Mice alive at earliest tumor appearance.

Duration of exp. (mo)	Material tested	Dilutions: 3.2×10^{-1}	2×10^{-1}	10^{-1}	10^{-3}
11	Milk defatted & decaseinated			2/15 13% 10M	
	Pellet incubated		6/23 27% 9M		
	Pellet treated with ribonuclease		3/22 14% 6M		
	Pellet treated with ethanol	5/25 20% 10M			
10	Milk defatted & decaseinated			0/27	
	Pellet treated with ribonuclease			9/42 21% 7M	
9	Milk defatted				1/16 6% 9M
	Pellet untreated				3/17 18% 7M
	Pellet treated with ribonuclease			2/19 11% 6M	2/17 12% 6M
	Supernate from treated pellet			2/19 11% 6M	0/19

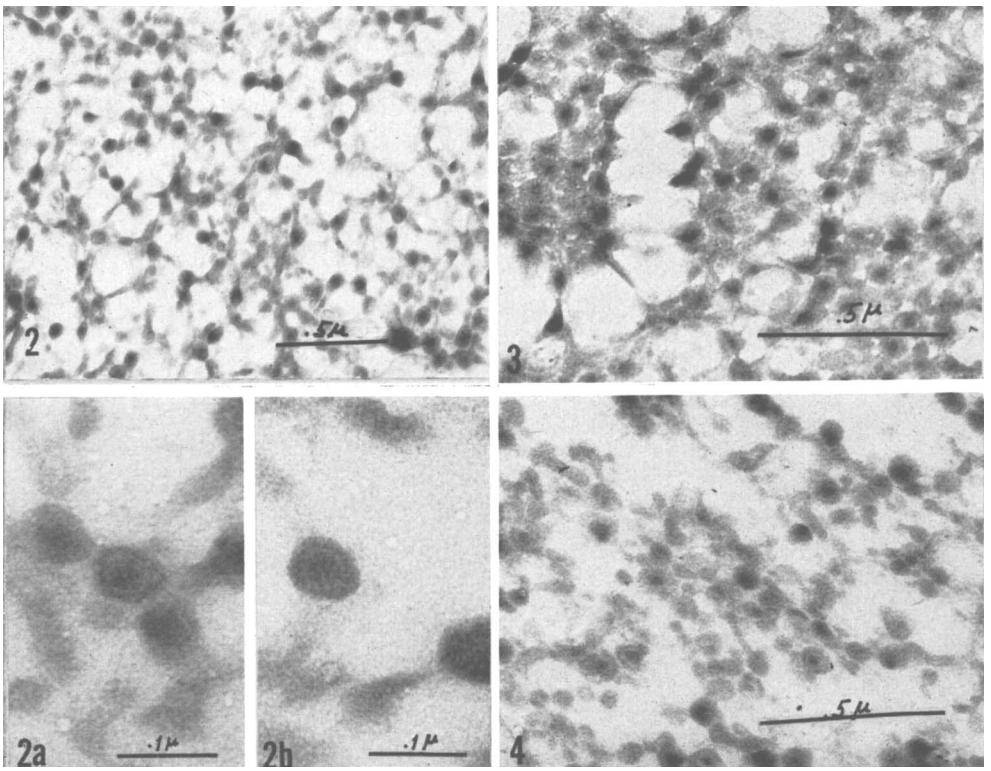

FIG. 2. Electron micrograph of section of upper layer of high-speed (105,000 × g—2 hr) centrifugal pellet from defatted, decaseinated virus-carrying RIII strain milk. Particles with characteristic structure. × 30,000.

FIG. 2a & 2b. Selected fields of Fig. 2 at higher magnification. × 130,000.

FIG. 3. Electron micrograph of section of upper layer of high-speed centrifugal pellet from defatted, decaseinated virus-carrying A strain milk.

FIG. 4. Selected field of upper layer of high-speed centrifugal pellet from similarly treated virus-free Af strain milk. × 50,000.

free strain defatted and decaseinated milk have similar macroscopic appearance. They are composed of an upper pale layer and lower more dense brownish zone. Electron microscope studies of sections of upper layer of pellets have revealed characteristic particles of 700 Å average diameter, composed of inner dense core, and outer pale zone, surrounded by 2 limiting membranes. They have been found in every section of upper layer of high-speed centrifugal pellets from RIII and A strain milk (Fig. 2, 2a, 2b, and 3). Similar particles have only occasionally been found in sections of upper layer of pellets from Af strain milk (Fig. 4). Similar study of lower zone of pellets from milk of all 3 strains of mice has shown only amorphous material. High-speed centrifugal pellets from aqueous phase of fluorocarbon-treated pellet material from milk of the 3 strains have macroscopic appearance similar to that of pellets before treatment but contain a much smaller lower layer. In the electron microscope apparently unaltered particles are present in the upper layer (Fig. 5). Denatured protein at the interphase between the aqueous and fluorocarbon layers has failed to reveal any characteristic particles.

Discussion. Bioassays of different fractions from virus-containing strain A milk, following defatting, decaseination, high-speed centrifugation, have demonstrated high-speed centrifugal pellets of treated milk to be suitable starting material for biophysical and biochemical study of the agent. Electrophoretic fractionation has led to localization of tumor-inducing activity in the electrophoretic pattern. Contamination of other fragments has not been observed in the present electrophore-

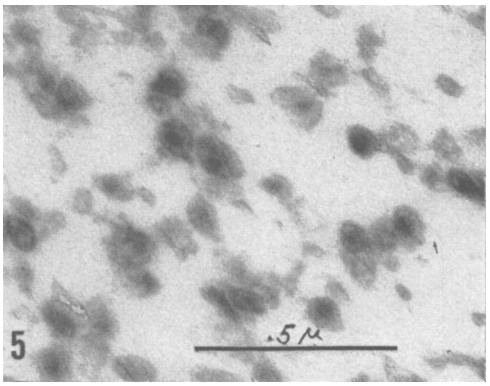

FIG. 5. Electron micrograph of section of upper layer of high-speed (105,000 × g—2 hr) centrifugal pellet from aqueous layer of Genetron-treated high-speed centrifugal pellet from defatted, decaseinated RIII strain milk. × 52,000.

sis experiments. Previous attempts by column electrophoresis showed tumor-inducing activity in varying amounts in all fractions(4), probably from cross-contamination during elution. The present studies indicate more extensive electrophoresis studies of the virus are feasible.

Studies on presence of RNA and DNA in high-speed centrifugal pellets from virus-carrying, defatted and decaseinated A strain milk were possible because of removal of a substance (possibly lactose) which interfered with previous attempts at colorimetric determination of RNA in mouse milk. It should be pointed out that the interfering substance is not always removed by high-speed centrifugation. For this reason, RNA determinations in pellets of similarly treated Af strain milk have so far not been successful. The absence of DNA in pellets of virus-carrying milk, at least in quantities greater than 0.2 μg/ml, is of interest. It should be pointed out that Bittner virus is of cytoplasmic origin, as shown in fractionation studies of tumor cells by differential centrifugation(21) and as indicated by electron microscopy(4-7,22-23). Presence of tumor-inducing activity following ribonuclease treatment of high-speed centrifugal pellets from defatted and decaseinated milk, and treatment with cold ethanol may prove helpful in isolation of the virus from mouse milk.

Attempts to demonstrate virus-like particles in lactating breast tissue of virus-carrying mice similar to those in cytoplasm of mammary tumor cells have been unsuccessful(4-7). Observation of characteristic particles in high-speed centrifugal pellets from virus-carrying milk which have shown considerable tumor-inducing activity is therefore of interest. However, the particles are smaller (700 Å) than those in tumor cells (900 Å). It is not known whether the difference in size, assuming the particles are the same, is the result of treatment of milk and subsequent preparation of pellet material for electron microscopy. Fluorocarbon deproteinization of pellets from virus-carrying milk does not appear to affect the appearance of particles to any appreciable extent, although they decrease in number. Presence of a small number of similar particles in pellets from Af milk, with no tumor-inducing activity, requires exploration. Particles of similar size and appearance to those in virus-carrying mammary tumors have also been observed in some tumors from agent-free mice(7,9). The incidence of mammary tumors in strain Af mice of our colony is about 3% at an age between 400-600 days. In the dairy colony of this strain, only one mammary tumor has, so far, been observed. It is not known whether the smaller number of particles observed in Af milk compared with that in RIII and A strain milk is connected with difference in tumor incidence of these strains (70% at 389 days average age in RIII and 66% at 382 days in A strain).

Further studies based on biochemical fractionation combined with electron microscope examination of sections and bioassays of various milk fractions may finally demonstrate the relationship of these and other particles (24-25) to the tumor-inducing activity of mouse milk. It has been pointed out(4) that shadow-cast preparations of mouse milk do not differentiate virus from calcium caseinate and lipoprotein particles present in milk.

Summary. 1). Zone electrophoresis of high-speed centrifugal pellets from virus-containing defatted and decaseinated strain A milk has led to good recovery of mammary tumor-inducing activity and to its localization in electrophoretic pattern. Nucleic acid determinations have shown presence of considerable quantities of RNA in pellets from similarly

treated A strain milk and absence of DNA. High-speed centrifugal pellets from defatted and decaseinated strain A milk treated with either ribonuclease or cold 75% ethanol retained tumor-inducing activity. 2) Characteristic particles of 700 Å average diameter, have been observed in sections of high-speed centrifugal pellets of agent-harboring RIII and A strain milk which have shown high tumor-inducing activity. They have also been found following treatment of pellets with fluorocarbon. Similar particles have been observed in considerably smaller number in pellets of Af strain milk without tumor-inducing activity. The relationship of these particles to the origin of mammary tumors of mice requires further study.

1. Dmochowski, L., *Advances in Cancer Research,* Academic Press, N. Y., 1953 v1, 104.
2. ———, *Cancer,* Butterworth & Co., England, 1957, v1, 214.
3. ———, *Proc. IInd International Symposium on Mammary Cancer,* Perugia, 1958, 655.
4. ———, *Acta Union Intern. Contre le Cancer,* 1956, v12, 582.
5. ———, *J. Nat. Cancer Inst.,* 1954, v15, 785.
6. Bernhard, W., Bauer, M., Guerin, M., Oberling, Ch., *Bull du Cancer,* 1955, v42, 163.
7. ———, Guerin, M., Oberling, Ch., *Acta Union Intern. Contre le Cancer,* 1956, v12, 544.
8. Bittner, J. J., *Harvey Lectures,* 1946, v42, 221.
9. Dmochowski, L., Grey, C. E., *Ann. N. Y. Acad. Sci.,* 1957, v68, 559.
10. King, E. J., *Biochem. J.,* 1932, v26, 292.
11. Lowry, O. H., Rosebrough, N. T., Farr, A. L., Randall, R. J., *J. Biol. Chem.,* 1951, v193, 265.
12. Hurlbert, R. B., Schmitz, H., Brumm, A. F., Potter, R. V., *ibid.,* 1954, v209, 23.
13. Burton, K., *Biochem. J.,* 1956, v62, 315.
14. Gierer, A., Schramm, G., *Nature,* 1956, v177, 702.
15. Kirby, K. S., *Biochem. J.,* 1957, v66, 495.
16. Gessler, A. E., *Trans. N. Y., Acad. Sci.,* 1956, v18, 707.
17. Gessler, A. E., Bender, C. E., Parkinson, M. C., *ibid.,* 1956, v18, 701.
18. Epstein, M. A., *Brit. J. Exp. Path.,* 1958, v39, 436.
19. ———, *Brit. J. Cancer,* 1958, v12, 248.
20. Dmochowski, L., Grey, C. E., *Blood,* 1958, v13, 1017.
21. Dmochowski, L., Haagensen, C. D., *Acta Union Intern. Contre le Cancer,* 1955, v11, 646.
22. Bang, F. B., Vellisto, I., Libert, R., *Bull. Johns Hopkins Hosp.,* 1956, v98, 255.
23. Bang, F. B., Andervont, H. B., Vellisto, I., *ibid.,* 1956, v98, 287.
24. Graff, S., Moore, D. H., Stanley, W. M., Randall, H. T., Haagensen, C. D., *Cancer,* 1949, v2, 755.
25. Moore, D. H., Lasfargues, E. Y., Murray, M. R., Haagensen, C. D., Pollard, E. C., *J. Biophys. Biochem. Cytol.,* 1959, v5, 85.

Received June 15, 1959. P.S.E.B.M., 1959, v102.

Use of Contrasting Fluorescent Dye as Counterstain in Fixed Tissue Preparations. (25182)

CHAUNCEY W. SMITH, JOHN D. MARSHALL, JR., AND WARREN C. EVELAND
(Introduced by F. D. Maurer)
Bacteriology and Immunology Branch, Armed Forces Inst. of Pathology, Washington, D.C.

One of the major problems in fluorescent antibody studies is the nonspecific uptake of fluorescein dye in formalin fixed tissues which often makes differentiation between specific and nonspecific fluorescence difficult. It was observed that a conjugated bovine antiserum using Lissamine rhodamine RB 200 (LR), according to Chadwick *et al.*(1), not only stained the specific organism but imparted a reddish-orange background to the tissue as well. An investigation was made as to the possibility of using LR-conjugated normal serum as a nonspecific counterstain to eliminate the nonspecific fluorescence of the fluorescein dye.

Materials and methods. Normal bovine, ovine and rabbit whole sera and bovine serum albumin were conjugated with Lissamine rhodamine RB 200* according to Chadwick

* Lissamine rhodamine RB 200 was kindly supplied by Arnold, Hoffman, and Co., Providence, R. I.