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Evaluation of Irradiation-Plus-Urethan-induced Murine Leukemia Virus "Release" Using a New Method for Quantitation of Oncornaviruses in Tissues[1]

Robert C. Brown,[2] J. A. Kostyu, and Alexandra Kilgore

Department of Pathology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514 [R. C. B., J. A. K.], and Department of Pathology, Rutherford Hospital, Inc., Rutherfordton, North Carolina 28139 [R. C. B., A. K.]

SUMMARY

The quantity of C-type RNA tumor viruses in homogenate-sonicates of thymus-bone marrow tissues of C57BL/6J and RFM/Un mice 10 days after irradiation (X-rays or γ-rays)-plus-urethan treatments is no greater than that in thymus-bone marrow homogenates from nontreated control mice. These results indicate that the leukemogenic activity, shown to be present in such thymus-bone marrow homogenates at this time after irradiation-plus-urethan treatment, is not due to change in quantity of C-type viruses as has been proposed.

Virus quantity in tissues was evaluated by a new procedure that includes use of a microchamber with the sides situated on rotor radii so as to produce a uniform virus-containing sediment of tissue homogenate-sonicate that is evaluated by electron microscopic examination of thin sections cut perpendicular to the membrane surface. Samples containing as little as 10^5 to 10^6 viruses can be relatively easily counted. Semipurified or purified viruses can also be counted after mixing with a tissue homogenate-bovine serum albumin diluent.

INTRODUCTION

Since the discovery of membrane-bound RNA viruses (C-type particles) in certain tumors and their proposed causative role in murine leukemia, as determined by leukemia production by cell-free preparations rich in these viruses, it has been proposed that the mechanism of irradiation leukemogenesis in the mouse and other animals known to harbor such viruses may be "indirect," being dependent in some way on the presence of these viruses. Haran-Ghera (7) reported that X-ray-plus-urethan treatment (4 weekly doses) of C57BL/6 mice produced leukemogenic activity in homogenates of their thymus-bone marrow tissues. This leukemogenic activity was measured by tumor production following injection of the homogenate into thymus grafts beneath the renal capsule of irradiated and thymectomized isologous hosts. It was proposed that this "leukemogenic activity," which was greatest 10 days after the last X-ray dose, was due to "virus release." In order to determine whether virus release was related to change in virus quantity, we performed electron microscopic examinations of sections of thymus-bone marrow tissue homogenates from X-ray-plus-urethran-treated and from control RFM/Un and C57BL/6 mice. Our results showed that C-type particles were present in homogenates of both treated and control mice and indicated the need for developing a method for quantitating relatively small numbers of virus in tissue homogenates to determine whether irradiation had produced a change in the amount of virus (1).

Herein is presented a new method for quantitating murine leukemia viruses and other oncornaviruses in tissues and results utilizing this method to determine whether virus release associated with leukemogenic activity produced by X-ray-plus-urethan treatment is related to change in virus quantity. This new virus quantitation method is sufficiently sensitive to allow relatively easy evaluation of C-type RNA viruses even in normal murine tissues and is useful in counting virus in tissue samples containing as little as 10^5 to 10^6 viruses.

MATERIALS AND METHODS

Animals and Virus Preparations. C57BL/6J and AKR mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. RFM/Un and BALB/c mice were grown and maintained at the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.; the former were maintained in a specific-pathogen-free environment. Mice were housed 6 to 8 per cage on wood shavings or wood particles (Ab-Sorb-Dri, Garfield, N. J.) and were fed Purina laboratory chow and acidified water (0.023% HCl) *ad libitum*. All mice were male.

Concentrated semipurified cell-culture-propagated Rauscher virus was obtained from Electro-Nucleonics Laboratories, Inc., Bethesda, Md. Animal-passaged Rauscher virus was harvested from spleens of newborn BALB/c mice

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[2] To whom requests for reprints should be addressed, at Department of Pathology, Rutherford Hospital, Rutherfordton, N. C. 28139.

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that had been given injections 2 to 3 weeks previously of animal-passaged Rauscher virus that originally had been propagated in JLV-9 BALB/c cell line.

Thymus-Bone Marrow Homogenate-Sonicate Preparation. Thymus-bone marrow homogenates for evaluation of virus release by irradiation-urethan treatments were prepared essentially as described by Haran-Ghera (7). RFM/Un mice (4 to 6 weeks old) received 4 weekly doses of 170 rads of X-rays given at 80 to 90 rads/min as measured by a Victoreen dosimeter and produced by a General Electric Maxitron 300 X-ray machine at 250 kV, 25 ma, with inherent 1-mm aluminum plus 3-mm added aluminum filtration. C57BL/6J mice (4 to 6 weeks old) received 4 weekly doses of 200 rads of γ-rays given at 160 rads/min as measured by a Baldwin-Farmer Dosimeter (Nuclear Enterprises, Ltd., Beenham, Reading, England) and produced by a Theratron 80 ^{60}Co teletherapy unit (Atomic Energy of Canada Ltd., Ottawa, Canada). The γ-ray beam was passed through a 0.625-cm-thick leucite sheet located just above the mice. With both kinds of irradiation treatments the mice were rotated on a horizontal leucite platform in individual ventilated chambers (leucite type for X-rays and polycarbonate tubes for γ-rays) at 6 rpm. Three days after each irradiation dose, urethan (1 mg/g body weight) was injected i.p. in a 3.3% aqueous solution.

Ten days after the last irradiation dose, thymus-bone marrow tissue homogenates were prepared as follows. In Step 1, a 16-2/3% (w/v) thymus homogenate (pooled thymus glands from 12 to 70 mice to give 20 to 200 mg) was prepared in NaCl (8.0 g), KCl (0.2 g), Na_2HPO_4 (0.2 g), and distilled H_2O to make 1000 ml (adjusted to pH 7.2, 300 milliosmoles/liter) using a 2-ml TenBroeck glass tissue grinder (Kontes Glass Co., Vineland, N. J.; clearance, 0.004 to 0.006 inch). The tissue homogenate was kept at 4–10°, except as noted, during the entire procedure. In Step 2, 2 parts of thymus homogenate were combined with 1 part of NaCl (8.0 g), KCl (0.2 g), Na_2HPO_4 (0.2 g), and distilled H_2O to make 1000 ml (adjusted to pH 7.2, 300 milliosmoles/liter) containing 30 to 40 $\times$ 10^7 bone marrow cells/ml, and 2 ml of this solution were ultrasonically disrupted in 10-ml "Oak Ridge" type polycarbonate tubes for four 30-sec periods with intervening 1-min periods. Ultrasonication was in a water-cooled well cup (7–13°) of a Sonifier cell disrupter (Model 185; Heat Systems Ultrasonics, Inc., Plainview, N. Y.) at a frequency of 20 kHz and 150 watts. In Step 3, centrifugation at 1000 $\times$ g in a Sorvall RC2-B centrifuge (rotor SS-34) was performed to remove large debris. In Step 4, the supernatant was placed in a 12- x 50-mm Petri dish (Falcon Plastics, Oxnard, Calif.) and was irradiated with 15,000 rads X-ray (450 rads/min) or 10,000 rads γ-ray (450 rads/min). In Step 5, the virus quantitation procedure was then performed as indicated below.

Preparation of Other Tissue Homogenate-Sonicates for Virus Quantitation. Tissues, 20 to 200 mg/ml in NaCl (8.0 g), KCl (0.2 g), Na_2HPO_4 (0.2 g), and distilled H_2O to make 1000 ml (adjusted to pH 7.2, 300 milliosmoles/liter, were homogenized, ultrasonically disrupted, and centrifuged as described in Steps 1 to 4 above. The supernatant was evaluated for virus quantity as described below.

Virus Quantitation Procedure. The virus-containing homogenate-sonicate supernatant to be evaluated for virus quantity was centrifuged onto a 0.6-mm-thick agar membrane (14) in a microchamber in a Sorvall SU rotor at 4° for 1 hr at 23,500 $\times$ g. The microchamber is like the chambers originally designed for this rotor by Sharp *et al.* (13, 14) except for size; it contains only 0.1 ml volume and has an agar membrane surface of 3 x 3 mm, or 9 sq mm; like the original larger chambers its sides lie on rotor radii. After centrifugation, the agar membrane containing the virus sediment on its surface was carefully removed with a metal spatula and placed onto a filter paper in a Petri dish. The filter paper was then saturated with cold phosphate-buffered 2.5% glutaraldehyde (pH 7.2, 300 milliosmoles/liter) and fixation at 4° was allowed for 12 to 72 hr, after which osmium tetroxide (2% in phosphate buffer, pH 7.2) vapor postfixation was performed for 30 min. At this time a uniform gray appearance of the sediment-agar membrane, when viewed from the sediment surface, indicated uniform sediment thickness. After ethanol and propylene oxide processing the sediment-containing membrane was bisected and each half was embedded in Epon 812 within a flat mold. Cross-sections (1 μm thick) of the sediment-agar membrane stained with crystal violet were examined by light microscopy to assure uniform sediment thickness. Silver-gold thin sections (about 0.090 μm (11) stained with uranyl acetate and lead citrate were mounted on carbon-coated Formvar membranes on round-hole copper grids and examined in a JEM-100B or Hitachi HU-11C electron microscope. Virus was counted at $\times$20,000 with added magnification of $\times$7 oculars, and any questionable virus forms were photographed at $\times$50,000 and examined.

The thickness of the sediment layer on the agar varies with the concentration of tissue used in preparations of the homogenate-sonicate. Sediment layers up to 0.07 mm thick (approximate thickness of sediment from homogenate-sonicate prepared from 200 mg tissue per ml buffer) can be evaluated easily.

Quantitation of Semipurified or Purified Virus Preparations. Quantitation of semipurified Rauscher virus preparations was performed after diluting the sample in a solution of tissue homogenate-sonicate consisting of 25 mg murine thymus tissue per ml 5% BSA[3] (Ortho Diagnostics, Raritan, N. J.) in NaCl (8.0 g), KCl (0.2 g), Na_2HPO_4 (0.2 g), and distilled H_2O to make 1000 ml (adjusted to pH 7.2, 300 milliosmoles/liter). Other parts of the virus quantitation were as described above. The thymus homogenate-sonicate BSA diluent assures adherence of the sedimented virus to the agar surface.

Calculation of Virus Quantity. Effective section thickness, which is dependent on virus size and the proportion of virus that must be in a thin-section for virus recognition, must be considered in calculating virus quantity (9). If one considers that the average murine leukemia virus diameter is 0.100 μm, that visualization of at least one-third of a virus is necessary for its recognition in thin sections, and that silver-gold sections are 0.090 μm thick (11), then effective section thickness is 0.123 μm or 0.000123 mm (9).

[3] The abbreviations used are: BSA, bovine serum albumin; CF, complement fixation.

The formula for calculating the number of viruses in the 0.1 ml of homogenate-sonicate placed in the microchamber, or its equivalent the total centrifuged sediment in the microchamber, is:

{Total area of sediment (9 mm^2)/[effective section thickness (mm) × length of sediment counted (mm)]} × no. of viruses in the length of sediment counted

Calculations of virus per volume or weight of original sample can then be calculated as desired considering sample dilution.

Virus in the tissue sample, even though freed from the tissue by homogenate-sonicate treatments, was present in the heavy debris that was removed by low-speed centrifugation. Examination of debris removed by low-speed centrifugation of tissue homogenate-sonicates showed mostly heavy nuclear fragments, small amounts of fragmented cytoplasmic material, and virus particles most of which were freed from cytoplasmic material. The amount of virus in the debris was similar to that in an equal volume of homogenate-sonicate supernatant and was considered as part of the total homogenate-sonicate volume for calculating virus quantity in the original homogenate-sonicate sample or tissue.

CF. Ultrasonicated homogenates, ether-treated to disrupt the viral membrane, were assayed for virus antigen by the method of Hartley *et al.* (8). The rat anti-murine sarcoma virus serum (Lot 9S-162; Huntington Research Center, Inc., Baltimore, Md.) used has a CF titer of 1:64 against an ether-treated, ultrasonically disrupted, semipurified Rauscher virus preparation (Catalog No. 1012; Electronucleonics) when diluted to 4 antibody units and less than 1:2 titer at the same dilution against ether-treated ultrasonicated thymus homogenates from untreated 4- to 6-week-old C57BL/6J mice. This rat anti-murine sarcoma virus had been shown to react to group-specific murine leukemia virus antigen (Maloney and Gross) and to a lesser extent against virus surface antigen.

RESULTS

Acceptable uniformity of virus quantity was found in multiple counts of the sediment layers. Counting of 50 to 84 viruses in each of 10 different areas in the central part of a sediment layer of a semipurified virus preparation showed a mean of 1.75 ± 0.22 (S.D.) × 10^7 viruses/sediment layer. Counting of 100 to 120 viruses in each of 10 different areas of another sediment layer, including areas near the edges, showed virus counts to be within 2 S.D. of a mean count of 7.12 × 10^7 viruses/sediment layer; the coefficient of variation was 22% for these counts. Chart 1 shows the good consistency of virus counts that can be achieved by this virus quantitation method using dilutions of a semipurified virus preparation. Virus samples (0.1 ml) with less than 10^5 to 10^6 are tedious to count without use of virus concentration procedures because of the length of time necessary to examine a portion of sediment containing a significant number of virus particles. For example, a sample (0.1 ml) with 7.1 × 10^5 C-type viruses requires counting a length of 0.3 mm of sediment to visualize 3 viruses.

Virus counts and CF titers of 4 semipurified cell-culture-propagated Rauscher virus preparations and 1 preparation of pooled splenic tissue from neonatal BALB/c mice given injections of animal-passaged Rauscher virus are shown in Table 1. The quantity of viruses found in semipurified cell-culture-propagated preparations was within the limits of that indicated to be present by Electro-Nucleonics Laboratories, Inc., using an indirect negative stain method for virus counting (10). The titer of virus antigen in the splenic tissue as determined by CF was much higher per virus quantity than that in the semipurified cell-culture-

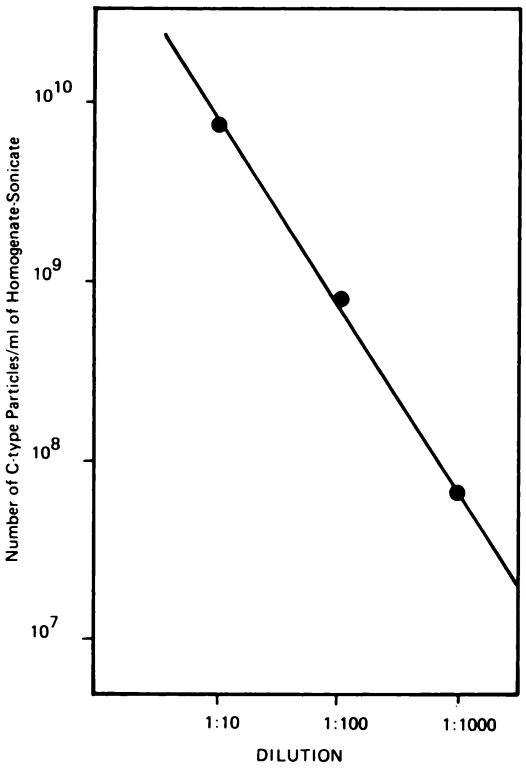

Chart 1. Correlation of virus concentration with dilutions of a homogenate-sonicate of semipurified cell-culture-propagated Rauscher virus. Thymus homogenate-sonicate BSA solution is used as the diluent.

Table 1

Quantity of C-type virus and virus antigen assayed by CF in semipurified cell-culture-propagated rauscher virus preparations and in splenic tissue from mice injected with animal-passaged rauscher virus

Material assayed	No. of C-type virus/ml homogenate or g tissue	CF titer[a]
Rauscher virus, semipurified cell culture propagated		
Stated 10^{11}-10^{12} virus/ml		
Lot 117-19-4	1.2 × 10^{11}	1:64
Lot 117-30-4	1.6 × 10^{11}	ND[b]
Stated 10^{10}-10^{11} viruses/ml		
Lot 146-9-3	8.4 × 10^{10}	1:40
Lot 104-49-9	7.1 × 10^{10}	ND
Rauscher virus, animal-passaged, BALB/C Spleen	5.3 × 10^{10}	1:640

[a] Maximum dilution giving a 3+ reaction using 4 units of rat anti-murine sarcoma virus serum.

[b] ND, not done.

propagated Rauscher virus preparations. The normal thymus homogenate in BSA necessary as a diluent for the semipurified virus sample in order to allow consistent adherence of the sedimented virus to the agar membrane contained 5.1×10^6 virus/ml, an insignificant amount of virus compared to that present per ml of semipurified virus preparation. This quantity of virus in the diluent was subtracted from the total virus count to give the count present in the semipurified virus preparation.

Table 2 shows virus quantities found in thymus tissue of "normal" newborn and adult AKR mice and splenic tissue from 2- and 19-month-old RFM/Un mice. There is no significant difference in virus quantity in the thymus of newborn and adult AKR mice. Assay for virus antigen by CF was negative. There appears to be an age-related increase in C-type particles in splenic tissue of RFM/Un mice. The spleens of these mice contained no tumors by histological examination.

Table 3 shows a comparison of virus counts in thymus-bone marrow tissue homogenates from X-ray- or γ-ray-plus-urethan treated mice (10 days following the last irradiation dose) and control (nontreated) similarly aged mice. As can be seen, thymus-bone marrow homogenates from treated mice contain no greater quantity of C-type particles than tissue homogenates from control mice. The 1 X-ray (only)-treated group of RFM/Un mice showed a slightly greater, but probably insignificant, amount of C-type particles in thymus bone-marrow homogenates than that in the control homogenates. Assay of virus antigen by CF was consistently negative in the C57BL/6J homogenates. The relative proportion of mature and immature C-type particles (5) was similar in bone-marrow tissue homogenates from irradiation-plus-urethan and control groups of both C57BL/6J and RFM/Un mice. Fig. 1 illustrates examples of C-type particles counted in homogenate samples.

DISCUSSION

Our new method of quantitation of C-type RNA viruses in tissue homogenate-sonicates, with which one can easily

Table 2

Quantity of C-type virus and virus antigen assayed by CF in murine tissues

Tissue assayed	No. of C-type virus/g tissue ($\times 10^8$)	CF titer[a]
AKR thymus		
Adult		
Pool 1	23	0
Pool 2	16	0
Newborn	11	0
RFM/Un spleen		
2 mo. old		
Animal 1	5	ND[b]
Animal 2	1	ND
19 mo. old		
Animal 1	21	ND
Animal 2	36	ND
Animal 3	37	ND

[a] Maximum dilution giving a 3+ reaction using 4 units of rat anti-murine sarcoma virus serum.

[b] ND, not done.

Table 3

Quantity of C-type virus and virus antigen assayed by CF in homogenates of thymus-bone marrow tissues following irradiation-urethan treatments

Mouse strain and type of treatment	No. of C-type virus/ml homogenate ($\times 10^7$)	CF titer[a]
C57BL/6J		
γ-ray-plus-urethan treated		
Group 1	0.4	0
Group 2	0.8	0
Group 3	0.6	0
control (untreated)		
Group 1	2.3	0
Group 2	1.1	0
Group 3	6.6	0
RFM/Un		
x-ray-plus-urethan treated		
Group 1	4.0	ND[b]
Group 2	2.7	ND
x-ray treated		
Group 1	10.0	ND
control (untreated)		
Group 1	3.0	ND
Group 2	6.0	ND

[a] Maximum dilution giving a 3+ reaction using 4 units of rat anti-murine sarcoma virus serum.

[b] ND, not done.

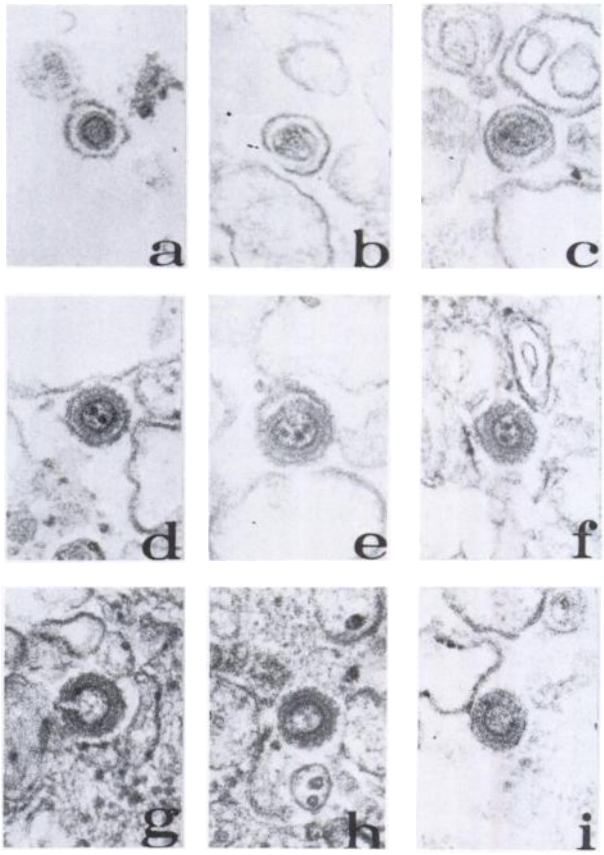

Fig. 1. Electron micrographs of typical virus forms counted in thin sections of murine tissue homogenate-sonicates; *b* and *c* are mature C-type particles; *a* and *d* to *i* are immature C-type particles. Uranyl acetate and lead citrate, × 85,000.

count samples containing as little as 10^5 to 10^6 viruses, has a number of advantages over currently used methods for quantitation of murine RNA tumor viruses. Use of the microchamber (0.1-ml volume) allows analysis of small samples. Use of a centrifuge chamber with sides that are radii of the centrifuge rotor provides uniform numbers of virus in all full-thickness volumes of sediment. Electron microscopic examination of thin sections of sedimented virus-containing material allows positive identification of C-type particles. This method can be used for counting any virus that can be recognized in thin section by electron microscopy.

Oncornaviruses in tissues cannot be quantitated with suitable accuracy by current methods that use negative stains or metal shadowings of virus on membrane surfaces (2, 10, 12) because of poor virus recognition and failure to distinguish virus from similar-shaped and -sized cellular debris that cannot be completely separated from virus. Methods that use electron microscopic examination of thin sections of tissue (3, 4, 15, 16) are impractical unless relatively large numbers of virus are present; nonrandom distribution of virus in tissues can lead to erroneous results. Published methods using thin sections of sedimented virus-containing pellets (6, 9) have used improperly shaped centrifuge tubes or chambers that cause irregular distribution of virus in the sediment and nonuniform thickness of the sedimented virus-containing material; this makes quantitation uncertain since it is impractical to examine more than a small portion of the total virus-containing sediment.

Results of quantitation of C-type particles in tissue by electron microscopic examination of thin sections of tissue homogenate-sonicates clearly show that irradiation-plus-urethan treatment did not increase virus quantity in thymus-bone marrow tissues. This suggests that the leukemogenic activity found in such thymus-bone marrow homogenates from X-ray-plus-urethan-treated mice by Haran-Ghera (7) reflects change in some parameter other than virus quantity caused by virus release or virus proliferation. Possible change in the biological quality of virus or a change in quality and/or quantity of certain cellular substances in such homogenates are possible considerations to explain the leukemogenic activity. These counted homogenate-sonicates and additional counted homogenate-sonicates are currently being bioassayed for leukemogenic activity by the method of Haran-Ghera (7). Measurement of virus group antigen by CF testing did not show positive reaction with thymus-bone marrow homogenates from either treated or control mice. Thus our virus quantitation method appears to be considerably more sensitive than the CF tests used for assaying of murine leukemia viruses. Preliminary results of tests for virus group antigen (gs-1) by radioimmune assay, which is much more sensitive than assay by CF, did not show evidence of increased virus antigen in thymus-bone marrow of treated mice compared to that of controls. This indicates that neither intact virus nor virus antigens unassembled into virus are increased by irradiation-plus-urethan treatments.

The considerably higher assay of virus by CF, in relation to virus quantity in the spleen tissue of mice given injections of animal-passaged Rauscher virus, compared to that in the semipurified tissue culture-propagated Rauscher virus preparations may be due to CF measurement of virus antigen unassembled into virions but present within cell nucleoplasm or cytoplasm. The possibility that animal-passaged Rauscher virus contains a greater quantity of CF-reacting antigen than tissue-cultured propagated Rauscher virus cannot be excluded as the cause of this. The apparent greater quantity of C-type particles in nonneoplastic spleens of old RFM/Un mice compared to that in spleens of young adult mice suggests again that factors other than virus quantity are important in leukemogenesis, since irradiation-plus-urethan treatments of these older mice do not increase leukemia incidence nearly to the extent of that caused by these treatments in young adult mice. Examination of other organs of varied aged mice for virus particles and quantity of virus antigen is in progress.

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