Recently data were reported suggesting that these substances inhibit virus growth by blocking penetration of the cells by the virus (11). In contrast to the ammonium ions and other amines, which are active only against viruses in tissue culture, the piperazinecarboxylate and 1-adamantanamine are active against influenza A viruses in mice. This activity may be related to their ability to reach the site of infection in the body.

The antiviral action of both the piperazinecarboxylate or 1-adamantanamine can be reversed, at least during the early stages of infection, by simply washing the cells free of the compounds. This result suggests that the interaction between the compound and the virus and the cells is probably ionic in nature.

Summary. Graded concentrations of 2-diethylaminoethyl 4-methylpiperazine-1-carboxylate produced graded inhibition of influenza virus growth in Rhesus monkey kidney cultures as measured by hemagglutination and infectivity titration. The piperazinecarboxylate was effective against strains of influenza A, A1, A2, and the Great Lakes strain of influenza B, but it was not effective against the Lee strain in influenza B. The compound was most effective when added to the culture at the time of virus inoculum. When the multiplicity of infection was relatively low, the piperazinecarboxylate inhibited viral growth even when added after infection. The compound did not inactivate the virus on contact and it did not interfere with the adsorption of the virus to the cells. It appears to act by interfering with the penetration of the host cells by the virus. The compound has a mode of action and an antiviral spectrum similar to that reported for 1-adamantanamine.

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Studies of Murine Leukemia Viruses I. Detection of Moloney and Rauscher Leukemia Viruses by Indirect Immunofluorescence.* (30701)

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The investigation of murine leukemia viruses has been hampered by dependence on time-consuming, cumbersome mouse inoculation methods for their detection and assay. Although the immunofluorescence method has been applied to detection of Friend leukemia virus (FLV) and Rauscher leukemia virus (RLV), results have been inconsistent. Some investigators have reported nuclear and cytoplasmic fluorescence in Friend leukemic cells (1) while others have described fluorescence confined to the cytoplasm in these cells and in FLV-infected mouse embryo cells(2,3). Both nuclear and cytoplasmic fluorescence have been observed in a wide variety of cells from RLV-infected animals but nuclear fluorescence only in megakaryocytes(4), where high concentrations of virus particles are

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visible in the cytoplasm by electron microscopy(5). Recently a tissue culture and complement fixation assay method for murine leukemia viruses has been described(6) in which common CF antigens were detected among cells infected with several murine leukemia viruses.

The present study was undertaken preliminary to the development of an *in vitro* quantitative immunofluorescent assay method for murine leukemia viruses. Antisera against Moloney leukemia virus (MLV) and RLV were produced in rabbits and used in the indirect immunofluorescence technique to detect infection with these viruses in cells from animals with virus-induced leukemia and in virus-infected cell cultures. Unlike Fink and Malmgren(4) we observed specific fluorescence only in the cytoplasm of RLV-infected cells, particularly of megakaryocytes. MLV-infected cells showed similar cytoplasmic fluorescence but in contrast to Hartley *et al* (6) we found no evidence of antigenic cross reactivity between MLV and RLV.

Material and methods. Virus. The MLV and RLV concentrates used in this study were prepared at The John L. Smith Laboratory, Maywood, N. J., and provided by Dr. John Moloney and Dr. Frank Rauscher, Nat. Cancer Inst. Each ml contained the virus concentrated from 10 ml of blood of infected mice by the method of Moloney(7). The concentrates were kept frozen at —70°C until used.

Immunization procedure. Full grown female New Zealand rabbits were injected initially with 4 ml of an emulsion of equal parts of virus concentrate and Freund's complete adjuvant, 1 ml intramuscularly into each hip, and 0.5 ml subcutaneously into each foot pad. One ml of undiluted virus concentrate was administered intravenously 4 weeks later and 1 ml intraperitoneally each week for 3 weeks thereafter. Subsequently the animals received repeated injections of 1 ml of virus concentrate intraperitoneally every 2 weeks. Later, the volume of maintenance injections was reduced to 0.1 ml of the virus concentrate. Animals were bled prior to immunization, 7 to 10 days after the initial series of injections, and monthly afterwards. Sera from the animals receiving each virus were pooled and heated at 56°C for 30 minutes.

Absorption of antisera. Pooled sera were absorbed with normal Balb/c mouse plasma until no precipitation developed when the plasma and sera were tested together in the double diffusion agar technique. Approximately 0.1 ml of plasma was required for absorption of 1 ml of antiviral serum. Absorption with animal tissues was performed as described by Hiramoto *et al*(8) using homogenates of Balb/c mouse spleen, thymus and liver washed three times in Dulbecco's phosphate buffered saline (pH 7.3)(PBS). One ml of the immune serum was added to 1 g of the washed spleen sediment and 0.06 g of the washed thymus sediment, mixed thoroughly and centrifuged at 17,300 *g* for 30 minutes at 5°C. The supernatant serum was removed and the procedure repeated twice. Then the serum was mixed with 1 g of washed liver sediment and treated in a similar manner. By the double diffusion agar technique this absorbed serum was shown to be free of precipitating antibody toward Balb/c mouse plasma protein and its concentrated globulin fraction. The final supernatant serum was used for immunofluorescence testing of imprints and sections of mouse tissues. For testing in the $JLSV_5$, $JLSV_6$, and $JLSV_8$ cell cultures (see below) an additional absorption was performed. One ml of absorbed antiserum was added to 10^6 washed $JLSV_6$ cells, shaken for 30 minutes at room temperature and centrifuged at 885 *g* for 15 minutes at 5°C. This absorption was repeated twice.

Absorption of antiviral serum with virus-infected cells was performed by shaking 1 ml of antiserum with 10^6 MLV-infected cells ($JLSV_8$) or RLV-infected cells ($JLSV_5$) for 1 hour at room temperature. The mixture was centrifuged at 885 *g* for 10 minutes at 5°C and the supernatant further centrifuged at 17,300 *g* for 10 minutes at 5°C. The final supernatant was used in the test. Virus absorption of antiviral serum was accomplished by incubating a mixture of equal parts of RLV or MLV mouse plasma concentrate diluted 10-fold in PBS and anti-RLV or anti-MLV antiserum diluted 2-fold in PBS for

1 hour at room temperature. The mixtures were centrifuged at 59,364 *g* for 2 hours at 5°C and the resulting supernatants used in the test. Absorbed immune sera were stored at −20°C.

Preparation of fluorescein isothiocyanate-goat-anti-rabbit-globulin-globulin (FITC-GRGG). This reagent was obtained from Dr. Raymond Hiramoto who used the methods of Riggs *et al*(9) and of Marshall *et al*(10) to produce a conjugate with a final protein concentration of 2.4 mg per ml and a fluorescein to protein ratio of 1.8 residues per mole. It was stored at −20°C and prior to use it was absorbed 3 times with normal Balb/c mouse liver homogenate.

Rauscher and Moloney virus leukemic tissues. Balb/c mice with Rauscher virus leukemia and Moloney virus leukemia were sacrificed and their spleens, thymuses, livers, and mesenteric lymph nodes removed. A portion of each organ was fixed and embedded for electron microscopy and another part was used to make imprints on glass slides for Wright-Giemsa and immunofluorescence staining. Imprints of bone marrow were also made for immunofluorescence testing.

Cell cultures. Continuous Balb/c spleen-thymus cell culture lines, $JLSV_5$, $JLSV_6$, and $JLSV_8$, were obtained in July, 1964, from The John L. Smith Laboratory, Maywood, N. J. The $JLSV_6$ cell lines consisted of passage 120 of spleen-thymus cells not containing RLV or MLV but demonstrating a relatively low concentration of virus particles morphologically similar to them on electron microscopy (11). The $JLSV_5$ consisted of RLV-infected spleen-thymus cells in passage 115 and the $JLSV_8$ of MLV-infected spleen-thymus cells in passage 33. These had been grown on a modified medium NCTC 109 with 25% human serum and 3% newborn calf serum but shortly after delivery in this laboratory the cell lines were placed in Eagle's minimal essential medium (MEM) with 20% fetal calf serum. These lines have been carried continuously for 50 additional passages in this laboratory in 4-oz prescription bottles and checked for Moloney and Rauscher leukemia viruses by inoculation of media in which they have been grown into susceptible Balb/c mice. The medium was modified several times to establish better growth of monolayers with less serum and finally consisted of 10% fetal calf serum in MEM with 0.05% yeast extract, 0.25% lactalbumin hydrolysate and 0.5% bacto-peptone (YELP). Penicillin, 100 units /ml, streptomycin, 50 μg/ml, and amphotericin, 1.5 μg/ml, were added and the pH adjusted to 7.4 with 10% $NaHCO_3$ solution. For immunofluorescence studies 4 ml of a cell suspension containing 2×10^5 cells per ml in the above growth medium were plated in 55 mm diameter plastic petri dishes (Falcon) with two 18 mm diameter glass cover slips in each dish and incubated at 37°C in a humidified atmosphere of 3% CO_2 in air.

Suspensions of Balb/c mouse fetal cells (MFC) were prepared by trypsinization of 12- to 17-day-old fetuses, and grown in YELP medium with 20% fetal calf serum on plastic petri dishes containing cover slips. For maintenance the serum concentration was reduced to 2%. For inoculation, medium was removed, 0.2 ml of a 10^{-1} dilution of MLV- or RLV-infected plasma concentrate in PBS was inoculated onto the monolayers and the cultures incubated in maintenance medium.

At regular intervals cover slips were removed for immunofluorescence and Wright-Giemsa staining and the remaining cells scraped off the dish for electron microscopy.

Cytotoxicity tests. Monolayers of $JLSV_5$, $JLSV_6$, and $JLSV_8$ cells were incubated in 20% rabbit anti-RLV serum in MEM, in 20% rabbit anti-MLV serum in MEM or in 20% normal rabbit serum in MEM for 8 hours, and examined for rounding and necrosis. In addition, the monolayers were stained with trypan blue and the relative proportions of stained and unstained cells were estimated.

Neutralization tests. Serial 2-fold dilutions of antisera in PBS were mixed with equal volumes of 10-fold dilutions of virus concentrate in PBS and incubated 1 hour at room temperature. For RLV, 0.1 ml of each mixture was injected intraperitoneally into 24- to 28-day-old Balb/c mice; for MLV, 0.1 ml of each mixture was injected subcutaneously into 1- to 3-day-old Balb/c mice. Sixteen ran-

domized mice of mixed sex were used for each dilution and for the controls, which consisted of equal volumes of normal rabbit serum and 3 serial 10-fold dilutions of virus concentrate. Animals were examined twice weekly for splenomegaly. Both splenomegaly and death with autopsy evidence of leukemia were used as end points of assay. At day 116 following inoculation all RLV-infected animals remaining alive and their controls were sacrificed and their total body and spleen weights measured. When the ratio of spleen weight to body weight multiplied by 100 equaled or exceeded 5, splenomegaly was recorded. At day 155 the same procedure was carried out for surviving MLV-infected animals and their controls.

Preparation of cells and tissues for electron microscopy. Tissue culture cells were gently scraped from the petri dish using a rubber policeman, sedimented from the media by low speed centrifugation, fixed in 5% glutaraldehyde, washed with PBS and cut into 1 mm^3 sections under a drop of 1% osmium tetroxide in White's saline(12). Leukemic spleen and thymus were cut into small sections, fixed in glutaraldehyde and washed with PBS.

Cell and tissue blocks were post-fixed in 1% osmium tetroxide in White's saline, washed in PBS, dehydrated in ethanol and propylene oxide, embedded in Maraglas® (13), sectioned on a Porter-Blum ultramicrotome, and stained with uranyl acetate and lead citrate.

Indirect immunofluorescence test. The cell monolayers on cover slips or the imprints on glass slides were washed twice with PBS, air dried, fixed with cold acetone and washed again with PBS. The specimens were covered with the rabbit antiviral serum for 1 hour in a humid chamber and rinsed thoroughly with PBS. FITC-GRGG was added to the specimens and they were placed again in a humid chamber for an hour, rinsed in PBS, allowed to dry in air, and mounted in a 9:1 glycerin-PBS solution. The preparations were examined for characteristic apple-green fluorescence in a Leitz ultraviolet microscope with the exciter filter BG 12 and a barrier F blue absorption filter system. In cell monolayer preparations the percentage of fluorescent cells was determined after counting 200 cells on each of 3 cover slips.

TABLE I. Neutralization of RLV with Rabbit Anti-RLV Serum.

Final concentration		Splenomegaly	Death with leukemia
Virus	Serum	Positive/ Total	Positive/ Total
$10^{-1.3}$	1: 2 Normal	16/16	14/16
$10^{-3.3}$	1: 2 "	2/15	1/15
$10^{-5.3}$	1: 2 "	0/10	0/10
$10^{-1.3}$	1: 2 Anti-RLV	0/11	0/11
$10^{-1.3}$	1: 8 " "	0/16	0/16
$10^{-1.3}$	1:16 " "	1/16	1/16
$10^{-1.3}$	1:64 " "	7/15	3/15

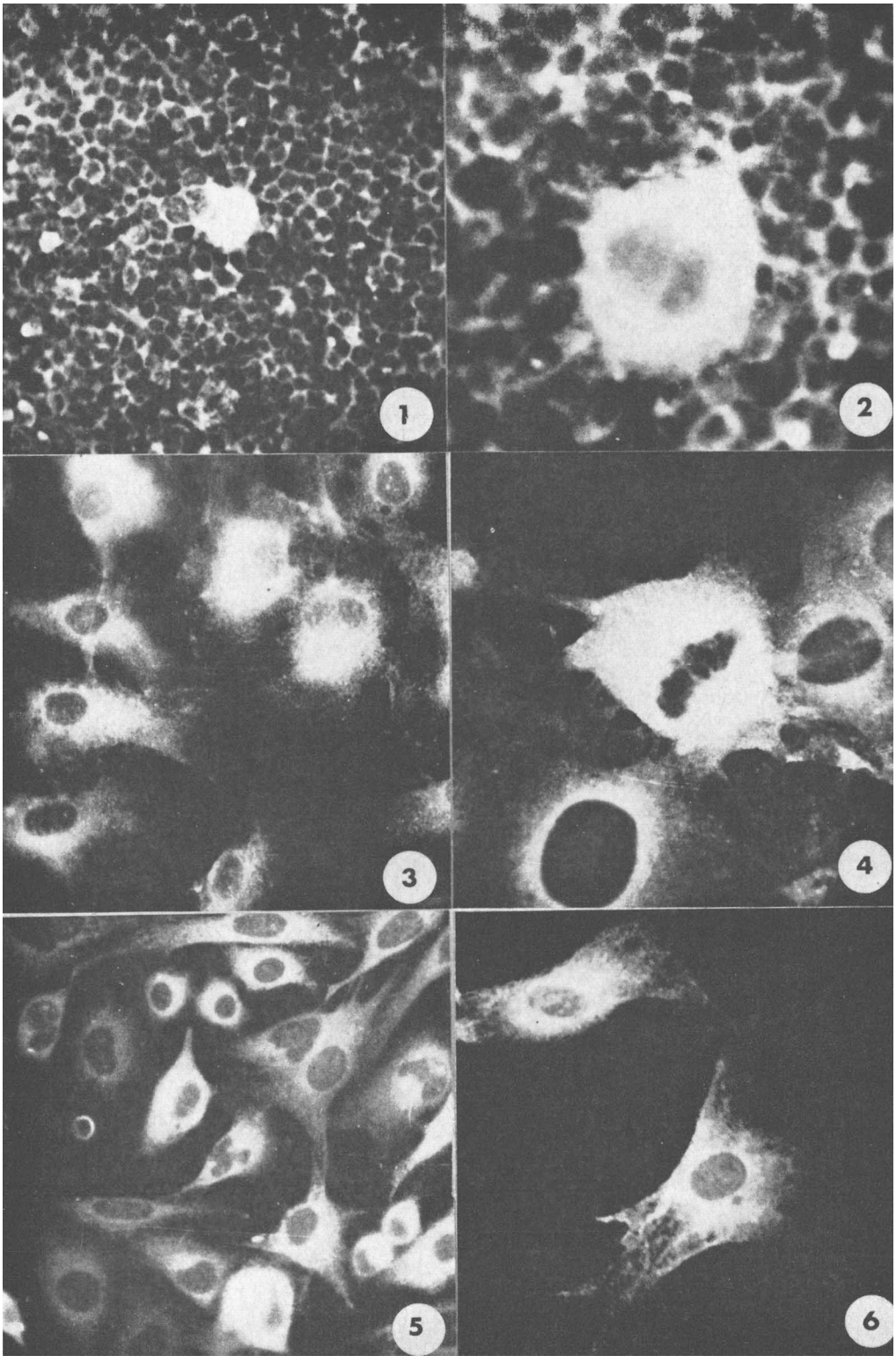
Balb/c mice, 24 to 28 day-old, were inoculated intraperitoneally with 0.1 ml of virus-serum mixture and observed for splenomegaly and death with leukemia. On day 116 after inoculation survivors were sacrificed and their spleen and body weights measured.

Results. Neutralization of RLV and MLV by homologous antisera. Results of a neutralization test with RLV and anti-RLV serum are summarized in Table I. At a dilution of 1:16 the antiserum was almost completely effective in neutralizing the amount of virus which produced splenomegaly in all of 16 mice within 23 days after inoculation and death in 14 out of the 16 mice within 79 days after inoculation. Table II shows the neutralizing effect of the anti-MLV serum on MLV. At a dilution of 1:8 it neutralized a dose of virus which produced splenomegaly in 15 out of 16 mice and death with leukemia in 14 of the 16 mice within 155 days.

TABLE II. Neutralization of MLV with Rabbit Anti-MLV Serum.

Final concentration		Splenomegaly	Death with leukemia
Virus	Serum	Positive/ Total	Positive/ Total
$10^{-1.3}$	1: 2 Normal	15/16	14/16
$10^{-2.3}$	1: 2 "	4/14	4/14
$10^{-3.3}$	1: 2 "	0/15	0/15
$10^{-1.3}$	1: 2 Anti-MLV	0/16	0/16
$10^{-1.3}$	1: 8 " "	0/16	0/16
$10^{-1.3}$	1:16 " "	4/14	1/14
$10^{-1.3}$	1:64 " "	9/16	8/16

Balb/c mice, 1 to 3 day-old, were injected subcutaneously with 0.1 ml of virus-serum mixture and observed for splenomegaly and death with leukemia. After 155 days the surviving animals were sacrificed and their spleen and body weights measured.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Cytotoxicity of RLV and MLV antisera. The absorbed antiviral sera demonstrated a low level of cytotoxicity against monolayers of all 3 JLS cell lines. At 20% concentration in MEM they produced rounding, necrosis and permeability to trypan blue stain of the cells after incubation at 37°C for 8 hours. Absorption of the antisera with $JLSV_6$ cells eliminated this effect but did not interfere with the activity of the antisera in the immunofluorescence test, indicating that the cytotoxicity was distinct from antiviral antibodies.

Localization of RLV and MLV antigen by indirect immunofluorescence. Testing of RLV- and MLV-infected cells by the indirect immunofluorescence method clearly indicated that viral antigen was confined to the cytoplasm. When imprints of spleen, liver and bone marrow from Rauscher virus leukemic animals were treated with anti-RLV serum and FITC-GRGG specific cytoplasmic fluorescence was observed, especially in the splenic megakaryocytes (Fig. 1). There was no nuclear fluorescence. Similar results were obtained when imprints of spleen, thymus, lymph nodes, bone marrow and liver of animals with Moloney virus leukemia were treated with anti-MLV serum and FITC-GRGG (Fig. 2).

RLV-infected spleen-thymus cell monolayers ($JLSV_5$) treated with anti-RLV serum and FITC-GRGG showed specific granular cytoplasmic fluorescence in 62% of the cells (Fig. 3). The fluorescence was particularly brilliant in dividing cells where it appeared homogeneous and intense about the mitotic apparatus during all stages of cell division (Fig. 4). MLV-infected spleen-thymus cell monolayers ($JLSV_8$) treated with anti-MLV serum and FITC-GRGG showed similar cytoplasmic fluorescence in 40% of the cells (Fig. 5).

TABLE III. Indirect Immunofluorescence of $JLSV_5$ Cells (RLV-infected spleen-thymus cell line).

Rabbit sera	% fluorescent cells*
Anti-RLV	62
Anti-MLV	0
Normal	0
Anti-RLV absorbed with RLV-infected cells	0.5
Anti-RLV absorbed with MLV infected cells	58
Anti RLV absorbed with RLV	8

* Based on counting 200 acetone-fixed cells in each of 3 cover slip preparations.

Specificity of the indirect immunofluorescence test for RLV and MLV. The results of control studies indicated that this indirect immunofluorescence method is specific for viral antigen and that RLV and MLV are antigenically distinct by this test. The control experiments were carried out primarily with monolayers of the continuous Balb/c mouse spleen-thymus cell lines, $JLSV_5$, $JLSV_6$, and $JLSV_8$, and are summarized in Tables III, IV, and V.

In monolayers of spleen-thymus cells not infected with MLV or RLV ($JLSV_6$), cytoplasmic fluorescence occurred in 3% of cells treated with anti-RLV serum and FITC-GRGG (Table V). This might indicate an

FIG. 1. Rauscher leukemic spleen imprint, treated with rabbit anti-RLV serum and FITC-GRGG. Cytoplasmic fluorescence is especially prominent in megakaryocyte. U-V photomicrograph, × 320.

FIG. 2. Moloney leukemic spleen imprint, treated with rabbit anti-MLV serum and FITC-GRGG. Cytoplasmic fluorescence in megakaryocyte is homogeneous. U-V photomicrograph, × 675.

FIG. 3. RLV-infected spleen thymus cell ($JLSV_5$) monolayer treated with rabbit anti-RLV serum and FITC-GRGG. Granular cytoplasmic fluorescence is generally more prominent in perinuclear areas. There is no specific fluorescence in the nuclei. U-V photomicrograph, × 320.

FIG. 4. Dividing cell in RLV-infected spleen-thymus cell monolayer ($JLSV_5$) treated with rabbit anti-RLV serum and FITC-GRGG. Fluorescence is particularly intense about the mitotic apparatus. U-V photomicrograph, × 675.

FIG. 5. MLV-infected spleen-thymus cell ($JLSV_8$) monolayer treated with rabbit anti-MLV serum and FITC-GRGG. About one-half of the cells demonstrate cytoplasmic fluorescence. U-V photomicrograph, × 320.

FIG. 6. MLV-infected mouse fetal cell monolayer, 28 days after inoculation, stained with rabbit anti-MLV serum and FITC-GRGG, showing cytoplasmic fluorescence. Nuclei are free of specific fluorescence. U-V photomicrograph, × 320.

TABLE IV. Indirect Immunofluorescence of $JLSV_8$ Cells (MLV-infected spleen-thymus cell line).

Rabbit sera	% fluorescent cells*
Anti-MLV	40
Anti-RLV	0
Normal	0
Anti-MLV absorbed with MLV-infected cells	0
Anti-MLV absorbed with RLV-infected cells	42
Anti-MLV absorbed with MLV	.6

* Based on counting 200 acetone-fixed cells in each of 3 cover slip preparations.

TABLE V. Indirect Immunofluorescence of $JLSV_8$ Cells (Spleen-thymus cell lines not infected with MLV or RLV).

Rabbit sera	% fluorescent cells*
Anti-RLV	3
Anti-MLV	0
Normal	0
Anti-RLV absorbed with RLV-infected cells	0

* Based on counting 200 acetone-fixed cells in each of 3 cover slip preparations.

antigenic relation between RLV and the virus particles observed by electron microscopy in association with these cells by others(11) and by ourselves since it was eliminated when anti-RLV serum absorbed with RLV-infected cells was used in the test. Absorption of antiviral sera with corresponding viral preparations considerably reduced but did not eliminate their activity in the tests (Tables III, IV). The amounts of virus used in absorption of the sera were based on results of the *in vivo* neutralization tests and evidently were not adequate for complete removal of virus antibody.

Several other controls were used to confirm the specificity of the virus-antibody reactions. Spleen imprints from normal Balb/c mice did not fluoresce with the antiviral sera, imprints from Rauscher virus leukemic mice did not fluoresce with anti-MLV and normal sera, and those from Moloney virus leukemic mice failed to fluoresce with anti-RLV and normal sera. Finally, virus-infected cell monolayers treated with their corresponding antisera and GRGG not conjugated with fluorescein isothiocyanate followed by FITC-GRGG did not fluoresce while those treated with the antisera and normal goat globulin followed by FITC-GRGG demonstrated the usual percentage of fluorescent cells. This indicated that fluorescence was produced by the combination of FITC-GRGG with rabbit globulin.

Detection of RLV and MLV multiplication in cell cultures by indirect immunofluorescence. Examination of MLV- and RLV-infected monolayers of MFC at regular intervals by immunofluorescence revealed that increasing concentration of viral antigen and presumably virus multiplication in the monolayers can be determined by this method. When RLV plasma concentrate, diluted 10-fold in PBS, was inoculated into MFC monolayers and the monolayers tested for indirect immunofluorescence using anti-RLV serum, specific cytoplasmic fluorescence appeared in 3.5% of cells 5 days after inoculation and increased until 75% of cells fluoresced at 25 days. Similar observations were made in MLV-infected monolayers treated with anti-MLV serum except that initial fluorescence did not occur until 11 days after inoculation. Fluorescence was present in 66% of cells at day 28 (Fig. 6). The fluorescence was observed first in the perinuclear areas of infected cells and tended to be less prominent with increasing distance from the nucleus. This may be a function of cell thickness rather than of difference in distribution of virus antigen in the cytoplasm.

Electron microscopic studies supporting the indirect immunofluorescence findings. Electron microscopic examination of the cells used in the immunofluorescence studies demonstrated the association of virus particles in the cytoplasm or at cell membranes of these cells with cytoplasmic viral antigen as shown by immunofluorescence. Megakaryocytes of Rauscher virus leukemic and Moloney virus leukemic spleen contained large concentrations of characteristic virus particles in various stages of completeness in their cytoplasm (Fig. 7), correlating with their intense cytoplasmic immunofluorescence. The leukemic cells had virus particles in various stages of development at their cell membranes but none in their cytoplasm, indicating that their cytoplasmic immunofluorescence represents virus antigen rather than intact

virus particles. These ultrastructural findings confirm previous reports(5,14).

In ultrathin sections of $JLSV_5$ cell monolayers there was abundant virus in different stages of development at cell membranes and between cells (Fig. 8) and mature forms of virus in vacuoles within the cytoplasm. Therefore, the immunofluorescence in their cytoplasm may represent viral antigen, mature virus in vacuoles or both. The same can be said for $JLSV_8$ cells, which showed similar ultrastructural findings but less abundant virus. The $JLSV_6$ cells also demonstrated virus particles at cell membranes and between cells, of a size similar to RLV and MLV but in much less concentration and with thicker and less regular outer envelopes. As mentioned previously, the small percentage of fluorescent cells seen in these monolayers when treated with anti-RLV serum may indicate an antigenic relation between this virus and RLV.

In MFC monolayers inoculated with MLV, typical virus particles appeared at cell membranes and between cells at 11 days. The relative numbrs of virus particles and of cells with virus at their cell membranes increased over the next 2½ weeks and correlated with the increasing percentage of immunofluorescent cells in the same monolayers (Fig. 9). Similar observations were made in RLV-infected MFC except that virus particles were seen earlier after inoculation. Although the immunofluorescence of these cells probably represents viral antigen synthesis in the cytoplasm primarily, it may also be due to the mature virus particles seen in phagocytic vacuoles in their cytoplasm. However, the clear association of initial appearance of virus with initial appearance of immunofluorescence in the infected monolayers and the correlation of concentration of virus particles and relative number of cells with virus particles at their cell membranes with percentage of immunofluorescent cells suggest that this immunofluorescence method is suitable for measurement of RLV and MLV replication in cell monolayers.

Discussion. The results of our studies with MLV and RLV are in agreement with those reported for Friend leukemia virus (FLV) by Osato *et al*(3). They used the indirect immunofluorescent technique and FLV-infected mouse embryo cell monolayer cultures along with FLV bioassay in mice to demonstrate a correlation between virus multiplication and progressive synthesis of viral antigen in the cell cultures. Fluorescence was confined to the cytoplasm of the infected cells.

However, our results for RLV differ from those of Fink and Malmgren(4) in which cells from RLV-infected mice and rats were treated with fluorescein isothiocyanate conjugated globulin derived either from sera of immunized rabbits or from sera of immunized Balb/c mice. Specific fluorescence was observed by them in the nuclei of leukemic cells and megakaryocytes of RLV-infected mice and in the cytoplasm of RLV-infected rat cells, but not in the cytoplasm of megakaryocytes, in which RLV particles are seen in abundance by electron microscopy. This disparity of our results with theirs is probably due to technical differences in preparation of the antisera.

Several studies of MLV and MLV lymphoma utilizing immunofluorescence methods have been reported by Klein and Klein(15,16, 17). They produced anti-Moloney lymphoma serum in mice by inoculation of homogenate or viable cells of a Moloney virus lymphoma and demonstrated fluorescence on the surface of living Moloney lymphoma cells with the indirect immunofluorescence method of Möller(18). From their methods and results it appears that they may be dealing with antibodies primarily directed toward cellular antigens although virus antigen on the cell surface may also be involved.

While we observed no antigenic cross reactivity between MLV and RLV, Hartley *et al*(6) detected common CF antigens among several murine leukemia viruses, including MLV and RLV, with their complement fixation and tissue culture assay method. It is possible that antigens reacting in our test are different from CF antigens or that their method is more sensitive than ours in detecting common antigens. On the other hand, their antiserum was obtained from rats bearing RLV-induced transplantable tumors and was absorbed only once with mouse liver

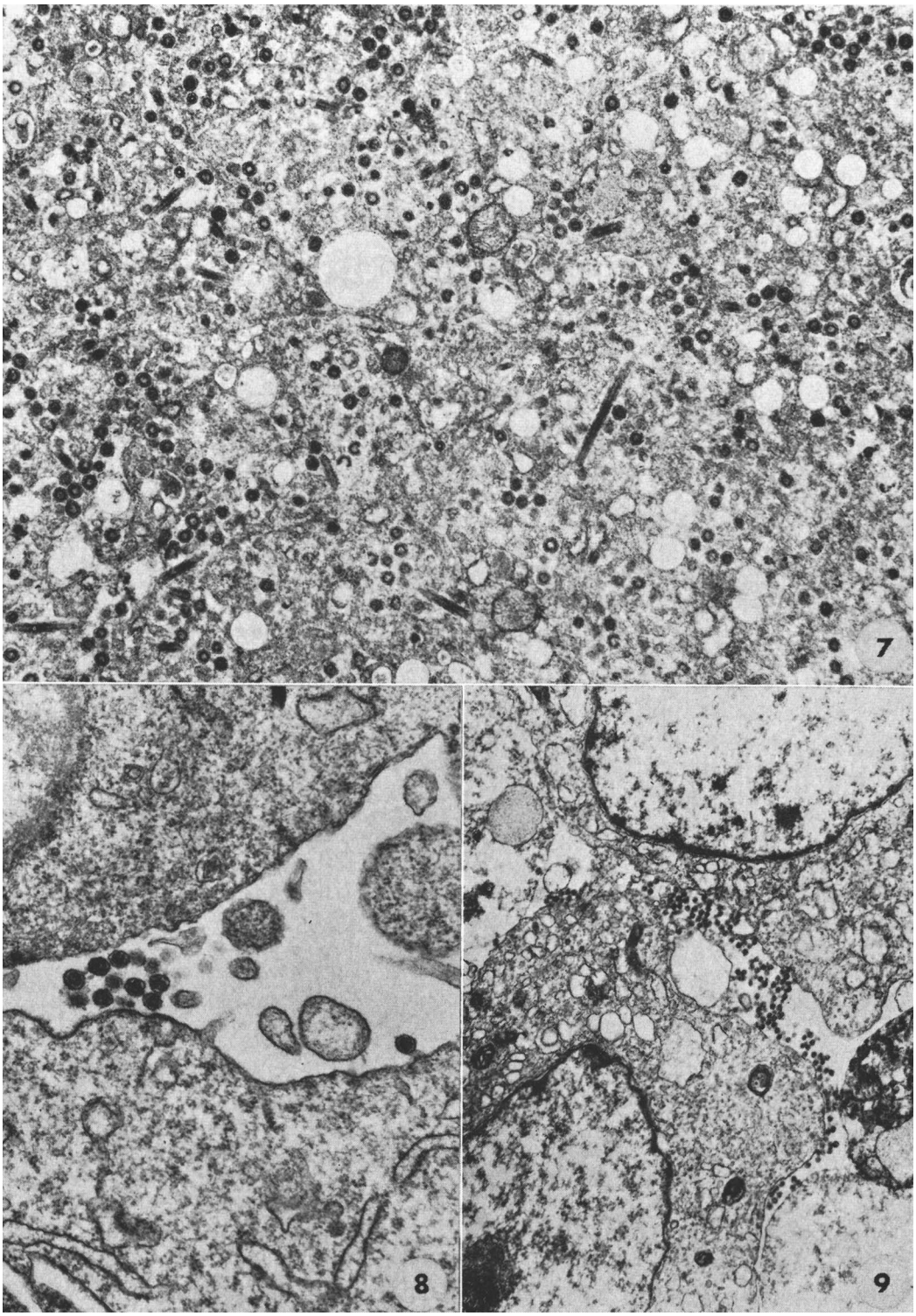
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FIG. 7. Cytoplasm of megakaryocyte, Moloney leukemic spleen, demonstrating spherical and rod forms of MLV in various stages of formation. Electron photomicrograph, lead citrate and uranyl acetate stain, × 16,000.

FIG. 8. Virus particles in MLV-infected spleen-thymus cell line (JLSV$_8$). Electron photomicrograph, lead citrate and uranyl acetate stain, × 27,200.

FIG. 9. RLV-infected mouse fetal cells 28 days after inoculation. Cells are undergoing degeneration and elaborating numerous virus particles. Non-inoculated cells show similar degeneration at this age but no virus particles. Electron photomicrograph, lead citrate and uranyl acetate stain, × 11,400.

powder so that it might have been reacting with common cellular antigens associated with leukemia virus infection as well as with virus antigens.

Preliminary experiments in this laboratory indicate that the percentage of immunofluorescent cells in RLV- or MLV-infected MFC at a given time following inoculation is related to the concentration of virus inoculum. By use of the indirect immunofluorescence technique described here a quantitative *in vitro* assay method for RLV and MLV is being developed.

Summary. Specific cytoplasmic fluorescence resulted when RLV- or MLV-infected cells were treated with their respective antiviral sera in the indirect immunofluorescence test. The fluorescence was especially intense in the cytoplasm of megakaryocytes, where abundant virus particles were seen by electron microscopy. No antigenic cross reactivity of RLV and MLV could be demonstrated by this test. Inoculation of mouse fetal cell monolayers with RLV or MLV led to the appearance of immunofluorescent cells, which increased in percentage with increasing length of incubation time. Electron microscopy of the infected cells demonstrated that the initial appearance of virus at cell membranes was related to the appearance of fluorescence and that increasing numbers of virus-forming cells and concentrations of virus particles between and within cells were associated with the increasing percentage of fluorescent cells. The indirect immunofluorescence method is useful for detection of MLV and RLV and may be applicable to the quantitative assay of these viruses in cell culture.

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