Canine Systemic Lupus Erythematosus

TRANSMISSION OF SEROLOGIC ABNORMALITIES BY CELL-FREE FILTRATES

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ABSTRACT The presence of viruses was sought in a colony of dogs bred from parents with systemic lupus erythematosus (SLE). Cell-free filtrates prepared from the spleens of these animals were injected into newborn dogs, mice, and rats. The canine recipients developed antinuclear antibody (ANA) and positive lupus erythematosus (LE) cell tests; ANA and, in some cases, antinative DNA antibodies were produced by the murine recipients; no abnormalities were detected in the rats. Serial passage of spleen cells or cell-free filtrates of spleen tissue in syngeneic mice reduced the time required for appearance of ANA from 9 to 4 mo. Some murine recipients of the canine filtrate developed malignant lymphomas. Murine leukemia viruses were identified in these tumors by electron microscopic, virologic, and serologic technics. These neoplasms, but not other tumors known to contain murine leukemia viruses, were associated with the production of ANA. Puppies inoculated with the canine filtrate-induced mouse lymphoma developed ANA and positive LE cell tests within 4 mo.

The results were interpreted to indicate the presence in canine SLE of a virus capable of: (*a*) inducing the serologic abnormalities of SLE in normal dogs and mice; (*b*) activating latent murine leukemia viruses; and (*c*) spreading by both horizonal and vertical routes.

INTRODUCTION

In a preceding paper we described a colony of dogs that was developed to study systemic lupus erythematosus (SLE)[1] in this species (1). The clinical and laboratory features of the canine disorder are strikingly similar to human SLE and include autoimmune hemolytic anemia, thrombocytopenic purpura, glomerulonephritis, polyarthritis, thyroiditis, positive LE cell tests, and antibodies to nuclear antigens, including native DNA (2–4). Although the offspring of the original members of the colony have not yet developed clinical signs of SLE—perhaps because they are still too young—many of them have positive LE cell tests and antinuclear antibody (ANA) in their serum. These serologic markers have been used to test the hypothesis that SLE is a genetically determined disease (1). Results in the inbred progeny, as well as those of outcross and backcross breeding experiments, failed to uphold this idea. Instead, the data suggested the possibility of an infectious agent which was transmitted from one generation to the next. We therefore designed a new series of experiments with the aim of testing this alternative hypothesis. This paper presents the results, which indicate the presence of a filterable infectious agent in the tissues of members of the SLE colony.

METHODS

Animals

Dogs. SLE colony dogs are members of a closed inbred colony derived from affected parents as described in the original report of this series (1). Normal dogs utilized in

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[1] *Abbreviations used in this paper:* ANA, antinuclear antibody; ATS, antimouse thymocyte serum; D-DNA, denatured DNA; FITC, fluorescein isothiocyanate; MST, median survival time; MSV, murine sarcoma virus; N-DNA, native DNA; PBS, phosphate-buffered saline; RER, rough endoplasmic reticulum; SLE, systemic lupus erythematosus; XC, mixed-culture cytopathogenicity.

these experiments were either purebred beagle dogs or newborn mongrel puppies born of serologically negative dams.

Rats. Wistar/Furth (W/Fu) rats were injected intraperitoneally (i.p.) on the day of birth with 0.5 ml of cell-free filtrate.

Mice. Recipient mice utilized in these experiments were BALB/c × A/Jax (CAF_1). Newborn recipient mice received 0.2–0.3 ml of cells or cell-free filtrate i.p.

Random bred normal dogs, cats, rats, rabbits, guinea pigs, and mice were purchased from commercial sources.

Spleen cell suspensions. Spleens were removed from intact, anesthetized donors. The tissue was minced in chilled phosphate-buffered saline (PBS) pH 7.2, 0.01 M and then passed through sterile tantalum gauze into a sterile beaker. Cell viability was determined with trypan blue and the volume of fluid was adjusted so that 0.2–0.3 ml containing the required number of cells could be injected into newborn recipients.

Cell-free filtrates were prepared by either freezing and thawing the cell suspension three times (30 min at −70 and 37°C, respectively) before passing it through a Millipore filter (Millipore Corp., Bedford, Mass.), or by homogenizing a chilled cell suspension in a VirTis tissue homogenizer (VirTis Co. Inc., Gardiner, N. Y.) for 30 min before Millipore filtration. Initially, filters with pore sizes of 0.220 and 0.450 μm were utilized in the preparation of cell-free filtrates. However, since little difference was noted in the results obtained with these two kinds of filters, the remaining experiments used filters with a pore size of 0.450 μm. All cell-free filtrates not used immediately after preparation were stored at −70°C.

Serology. Dogs, rats, and mice were bled at monthly intervals. The serum was analyzed for antibodies to nuclear antigens utilizing LE cell tests (2), the fluorescent ANA test, and a radioactive binding assay for native and denatured DNA.

Fluorescent test for ANA

Antibody. A group of 12 rabbits was immunized with purified Cohn fraction II IgG prepared from either dog, rat, or mouse serum (Worthington Biochemical Corp., Freehold, N. J.). After four immunizing injections (subcutaneously, in complete Freund's adjuvant) at 20-day intervals, the animals were rested 30 days and exsanguinated. Homologous whole serum was subjected to immunoelectrophoresis and reacted with the above-mentioned antisera. Those antisera producing a single precipitin band which appeared in the migratory pattern of IgG were selected for conjugation to fluorescein isothiocyanate (FITC). Analysis of the antisera by gel diffusion demonstrated that they were species specific; e.g., the rabbit antidog IgG did not react with mouse serum, nor did the rabbit antimouse IgG react with dog serum.

Conjugation. Each antiserum was precipitated three times in 50% saturated NH_4SO_4 at 4°C, dialyzed against normal saline (pH 5.5) overnight, and adjusted to a protein concentration of 10 mg/ml. FITC, 0.1 mg/mg of protein was dissolved in carbonate-bicarbonate buffer pH 9.0, 0.025 M, and then dialyzed for 21 h at 4°C against the globulin fraction of the respective antisera. After dialysis, excess fluorescein was removed by passing the conjugate through a G-25 Sephadex column. Portions of the labeled antisera were then absorbed with an equal volume of rabbit liver powder, adjusted to a protein concentration of 1.0 mg/ml, and stored at 4°C. The fluorescein: protein ratios varied from 2.8 to 5.0.

Antigen. Fresh cryostat sections of baby rat liver provided the source of nuclei.

Staining procedure. Test sera (0.1 ml) were layered over the cryostat sections, incubated at 22°C for 30 min, washed three times in phosphate-buffered saline, pH 7.2, and stained 30 min with the appropriate conjugated antisera. After staining, the sections were washed three times in PBS, blotted dry, coverslip-mounted in Permount (Fisher Scientific Co., Pittsburgh, Pa.), and examined in dark field with a Zeiss Ortholux research microscope (Carl Zeiss, Inc., New York), exciting filter BG 12, barrier filter 5.20. The morphologic patterns of positive reactions were recorded, and fivefold dilutions of the positive sera were prepared and tested for antibody titer. Control tests included known ANA positive and negative sera and an unrelated third party (rabbit antifeline IgG) conjugate. The undiluted sera of 25 normal, 12-month old CAF_1 mice failed to yield a positive reaction when tested in the manner described.

DNA binding assay. ^{14}C-labeled *Escherichia coli* was prepared by growing a thymidylate synthetase-deficient mutant of *E. coli* in 1 mCi/ml (0.03 mol/ml) of ^{14}C-labeled thymine, extracting the bacterial DNA by the method of Marmur (9), and adjusting the DNA to a concentration of 10 mg/ml in PBS before testing. The final product failed to react immunologically with rabbit antisingle-stranded DNA, but was reactive with antibodies to native DNA in the serum of a human patient with SLE.[2] To test for DNA binding, 0.05 ml [^{14}C]DNA was added to a mixture of 0.94 ml of 0.06 M phosphate buffer, pH 8.0 and 0.01 ml heat-inactivated test serum and incubated for 30 min at 45°C. After incubation, the reaction mixture was filtered through a wet 2.4 cm GF/A Whatman glass fiber filter. The filter was supported on a stainless steel precipitating apparatus and filtration carried out by suction applied through the side arm of a 500 ml Erlenmeyer flask. The filter was washed three times with an equal volume of buffer, removed from the apparatus and allowed to air dry before being placed on planchets for counting in a Nuclear-Chicago gaseous scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.). 1,000 counts were obtained for each plate to calculate the percentage of total radioactivity retained by the filter. The machine background, in counts per minute (cpm), has been substracted from all values. The final value is expressed as percent binding. Controls include normal serum and known, standardized antisera to native DNA (N-DNA) or denatured DNA (D-DNA).

With this method, the normal ranges of N-DNA binding in 13 normal, 12-mo old CAF_1 mice were: mean 6.69%; SE 1.22%; SD 4.40%; and normal range 0–16%.

Antithymocyte serum. Rabbit antimouse thymocyte serum (ATS) was purchased from Microbiological Associates, Inc., Bethesda, Md. Potency of the ATS was tested in our laboratory as follows: 21 CAF_1 ($H\text{-}2^d \times H\text{-}2^a$) mice received six injections of ATS over a 2 wk period (total dose 2.5 ml). 1 day after the first injection of ATS the mice were grafted with C57Bl/6($H\text{-}2^b$) skin. The median survival time (MST) of the grafts was 30.0±3.0 days. The experiment was repeated in 30 CAF_1 mice that had been thymectomized 2 wk before receiving the first injection of ATS. The MST of the C57Bl/6 grafts was 41.0±6.6 days. The MST of C57Bl/6 grafts on 10 control CAF_1 mice was 13.0±1.0 days. The differences in the survival

[2] Stollar, D. Personal communication.

times between the ATS-treated mice and the control mice were significant ($P < 0.05$).

Tissue culture studies. Tissue suspensions were tested for murine leukemia viruses by a mixed-culture cytopathogenicity (XC) assay described by Klement, Rowe, Hartley, and Pugh (5), and by a focus assay on mouse cells transformed by a murine sarcoma virus (MSV), described by Bassin, Tuttle, and Fischinger (6).

In the XC assay, 60-mm Falcon plastic petri dishes (Falcon Plastics, Los Angeles, Calif.) were seeded with 350,000 cells from 14 to 17-day CD-1 Swiss mouse embryos. On the next day, these cultures were inoculated in duplicate with 20% tissue suspensions of a stock pool of Rauscher leukemia virus (lot no. R-2-5300). Cultures were maintained at 37°C in Eagle's medium with 10% fetal calf serum, 2 M glutamine, 250 U/ml penicillin, and 250 μg/ml streptomycin. 14 days after the cultures were inoculated, longitudinal strips were scraped out of the cell sheet with a capillary pipette and 1.0×10^6 XC cells, derived from a rat tumor induced with Rous sarcoma virus, were added. The cultures were then incubated for an additional 5 days, fixed in methanol, and stained with Wright's mixture. The dishes were examined under a light microscope at a magnification of × 200 and multinucleate cells (more than three nuclei per cell) were counted per 100 fields/dish. Several dishes treated as described above, but containing neither tissue suspensions nor Rauscher virus served as negative controls in each assay. Because rare multinucleate cells were seen in control cultures, as well as in cultures of XC cells grown alone, the criterion for caling a given dilution positive for virus was a number of multinucleate cells four times the mean control value per 100 fields.

In the focus assay, 10^5 mouse cells transformed by MSV but not releasing infectious virus (sarcoma-positive, leukemia-negative cells provided by Dr. R. Bassin) were plated per 60 mm Falcon dish and were infected the following day. Lytic lesions, easily seen by light microscopy, appear 4–5 days after inoculation. Positive and negative controls, as in the XC assay, were included in each test.

Tissue suspensions were also cultured on confluent and subconfluent monolayers of both CD-1 Swiss mouse embryo cells and dog fetal cells for evidence of viral cytopathogenic effects. The mouse embryo dishes were maintained for up to 2 mo on medium described above and the dog fetal cells were maintained on McCoy's medium with 10% inactivated fetal calf serum, 250 U/ml penicillin and 250 μg/ml streptomycin. Hemadsorption assays were performed on representative dishes at biweekly intervals using human O erythrocytes, according to standard techniques (7).

Postmortem examination of dogs and mice. Complete postmortem examination of all dogs and mice utilized in these experiments was conducted, excluding examination of the central nervous system in mice. Histologic examination of representative tissues from all major organ systems was performed. Tissue prepared for electron microscopic examination was processed as previously described (8).

RESULTS

Development of ANA in recipients of cell-free filtrates

Mice. Cell-free filtrates prepared from the spleens of five first generation SLE colony dogs (B-95, B-97, B-44, C-4, and B-10)[3] were injected into newborn CAF_1 mice (Table I). Recipients of filtrates prepared from four of these dogs developed ANA by 12 mo of age in 59/119 cases (49.2%). The filtrate from donor B-10 was ineffective. The mean age of the recipients at the time ANA was first detectable was 9 mo. Filtrates prepared with 0.220 or 0.45-um. filtrates were equally effective. A diffuse nuclear-staining pattern characterized the positive ANA tests of the donor animals. ANA with a homogeneous-staining pattern was found in 42 mice; the ANA of one mouse produced a rim pattern; and in 16 recipients of the filtrate the ANA was characterized by a mixture of homogeneous and rim patterns (Fig. 1). In no instance was a speckled pattern found. Serial testing of seropositive recipients at monthly intervals demonstrated rising titers of ANA in a majority of the mice during the course of the experiment (Fig. 2). Patterns of the staining of the ANA tests did not change as the titers rose. None of the 85 uninjected, control mice produced ANA during the 12 mo period of observation.

TABLE I

Induction of ANA in Primary Recipients of SLE Dog Colony Spleen Cell-Free Filtrates

Donor dog	Filter size	
	0.220 μm	0.450 μm
B-95	17/30	21/46
B-97	4/7	2/4
B-44	2/2	6/11
C-4	4/7	3/5
B-10	—	0/7

Denominator, number of mice tested; numerator, number of mice with positive ANA test in any titer (undiluted to 1:625). None of 85 age-matched, control mice had detectable ANA in undiluted serum.

In addition to ANA, 12/26 recipients of colony dog cell-free filtrate also developed antibody to N-DNA. Binding of DNA by the serum of noninjected control mice did not exceed the binding levels of normal 12-mo old CAF_1 mice. (Fig. 3).

Cell-free filtrates were also prepared from the spleens of two normal beagle dogs that were housed in the same quarters as the SLE colony. These beagles had been in close physical contact with dogs of the SLE colony for 2 yr. ANA developed in 8/21 CAF_1 recipients of filtrates obtained from both beagles. Because of this finding, filtrates were prepared from the spleens of three dogs that had never been in contact with the colony. These filtrates failed to induce ANA in any of the 23 CAF_1 recipients (Table II). As a further control, fil-

[3] Dogs of the B line were derived from two affected parents. Only the dam had SLE in the C line.

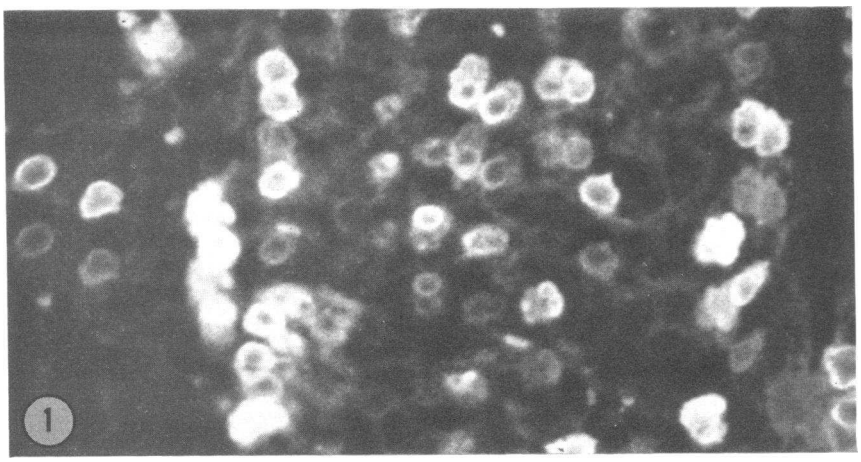

FIGURE 1 Peripheral- and diffuse-staining pattern of positive fluorescent test for ANA in murine recipients of seropositive SLE dog spleen cell-free filtrate. Cryostat section of baby rat liver. × 400.

trates prepared from the spleens of normal cats, rabbits, guinea pigs, rats, and mice were also injected into newborn CAF_1 mice. None of the 134 recipients developed ANA within the 1 yr period of testing (Table III).

Rats. Filtrates prepared from four SLE colony dogs (B-95, B-44, B-97, C-4) as well as from one normal cohabitant dog (S-24) were injected into newborn Wistar/Furth (W/Fu) rats. Neither these recipients nor 24 uninjected control rats developed ANA during the 12 mo period of the experiment.

Dogs. A cell-free filtrate made from the spleen of SLE colony dog B-95 was injected i.p. into four normal newborn beagle puppies. All recipients developed ANA with 12 mo. The antibody persisted until the animals were sacrificed at 18 mo of age. Titers of the antibody ranged from 1:5 to 1:125, and the staining patterns were diffuse (1), rim (1), and both diffuse and rim (2). Each of the four dogs also developed positive LE cell tests following the appearance of ANA.

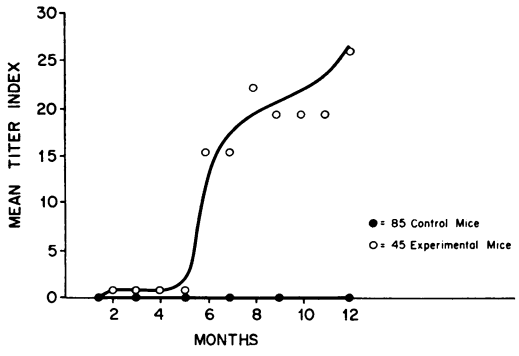

FIGURE 2 Rising titers of ANA in seropositive CAF_1 mice that were injected at birth with SLE colony dog cell-free filtrate.

Three additional newborn puppies of the same litter were injected with a filtrate prepared from a normal dog obtained outside our breeding colony and each of these animals also developed ANA and positive LE cell tests between 12 and 18 mo of age. All seven puppies used in this experiment were housed together as a family unit during the course of the experiment and were in close proximity to the SLE colony at the Animal Research Center. These results led to additional transmission studies in which 19 normal newborn puppies were injected with cell-free filtrates from 3 normal dogs. All animals used in this experiment, including donors and recipients, were never in contact with the SLE colony and were housed at the New England Medical Center Hospital, which is 30 mi from the Animal Research Center. None of these animals developed ANA or LE cell tests.

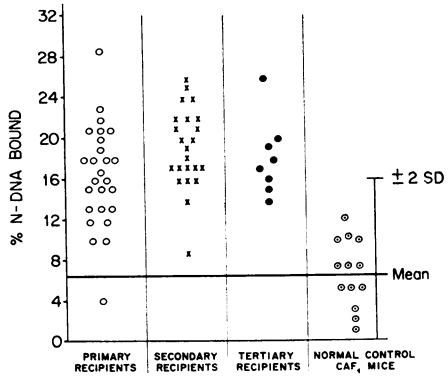

FIGURE 3 Development of anti-N-DNA antibody in primary, secondary, and tertiary CAF_1 recipients. "Mean" refers to percent of N-DNA bound by the serum of 13 normal 12-mo old CAF_1 mice.

TABLE II

Induction of ANA by Spleen Cell-Free Filtrates

Donors	Recipients		
	CAF_1 mice	W/Fu rats	Normal puppies
SLE colony dogs	59/119	0/32	4/4
Cohabitant normal dogs	8/21	0/36	0/7
Normal mongrel dogs	0/23	—	—
Uninjected control mice	0/85	0/33	0/12

Denominator, number of mice tested; numerator, number of mice with positive ANA test in any titer.

TABLE III

Induction of ANA in CAF_1 Mice by Spleen Cell-Free Filtrates

Source of filtrate	No. of positive recipients
SLE colony dogs	59/119
Normal dogs	0/23
Normal cat	0/17
Normal rabbit	0/23
Normal guinea pig	0/20
Normal white rat	0/21
Normal CAF_1 mice	0/30

Induction of ANA in CAF_1 mice by cell-free filtrates of spleen from various species. Three normal donors of each species were used. Individual filtrates were prepared in each case. At least six newborn mice were used for each filtrate. One recipient of the cat filtrate died before testing.

To test further the mode of transmission of the agent responsible for the development of ANA and LE cells in the SLE colony, a pregnant F_2 dam was shipped to the Germ-Free Unit at the Ohio State University School of Veterinary Medicine. Four F_3 cesarean-derived and formula-fed puppies are being raised in isolator units within that facility. These dogs never had contact with the dam after delivery. Although they are now 14-mo old and have undergone serial testing at monthly intervals, they remain negative for both LE cells and ANA.

The probability of a litter of four conventionally delivered and reared SLE colony dogs having negative LE cell tests and lacking ANA is 0.06 in the third generation of the A breeding line (1).

Serial passage of spleen cells from seropositive mice. The high incidence of ANA in CAF_1 mice given cell-free filtrates prepared from tissues of SLE colony dogs led to the following experiment. Recipients of filtrate from four SLE colony dogs were splenectomized after they had developed ANA, and 5×10^6 viable spleen cells were injected into syngeneic mice. Serial analyses for ANA in these recipients were then conducted at monthly intervals for 1 yr. During this time, 37 of the 41 recipients (90.2%) of syngeneic spleen cells developed ANA (Table IV). Moreover, 16/26 of these secondary recipients, as well as 4/8 tertiary recipients also developed antibodies against N-DNA (Fig. 3).

In an attempt to decrease the latent period required for ANA production, the experiment was redesigned as follows. Seropositive recipients were splenectomized at the time ANA was first detected and 5×10^6 of their spleen cells were injected into newborn syngeneic mice. These mice were bled at monthly intervals and their sera were assayed for antibodies to nuclear antigens. Detection of ANA was followed immediately by splenctomy and passage of the spleen cells into another group of newborn mice. In this fashion, the percentage of seropositive mice increased in the group of secondary recipients (Table IV) and the mean age at which ANA was first detected was reduced from 9 to 4 mo (Fig. 4). As a rule, the staining pattern of the ANA produced by the original donor was maintained through the several passages. None of nine newborn control CAF_1 mice injected with normal adult CAF_1 spleen cells developed positive tests for ANA.

Serial passage of cell-free filtrates of spleen cells from seropositive mice. The induction of ANA production

TABLE IV

Induction of ANA by Serial Passage of Viable Spleen Cells from Seropositive Mice to Syngeneic Newborn Mice

Colony dog —Filtrate→ ANA + primary CAF_1 recipient —Spleen Cells→		
	ANA + primary CAF_1 recipient	ANA + secondary CAF_1 recipient
B-95	38/76	24/27
C-4	7/12	4/4
B-44	8/13	5/5
B-97	6/11	4/5

Denominator, number of mice tested; numerator, number of mice with ANA in any titer. Note that 50% of primary recipients of B-95 filtrate were positive, whereas 82% of secondary recipients were positive. A similar trend is seen with the other filtrates.

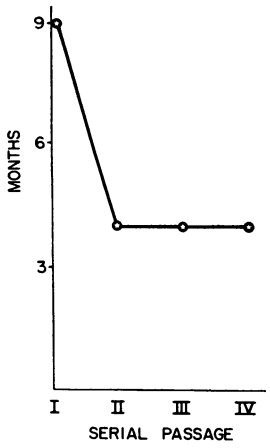

FIGURE 4 Transfer of spleen cells from seropositive CAF_1 to syngeneic newborn mice at the time the ANA test was first found to be positive in the donors reduced the average time required to develop ANA in the recipients from 9 to 4 mo postinjection.

in recipients of serially passaged murine spleen cells raised the question whether viable spleen cells were necessary to transmit the serologic abnormality. To test this hypothesis, the spleen was removed from a seropositive recipient of filtrate prepared from colony dog B-95. A cell-free filtrate was prepared from this spleen and injected into newborn syngeneic mice. 6 of 14 recipients of this filtrate developed ANA before reaching the age of 12 mo.

Development of lymphomas. During the course of these experiments 7/119 mice (5.9%) that had received cell-free filtrates from the SLE colony, or that were injected with viable murine spleen cells from recipients of the canine filtrate, developed lymphomas. All tumors developed in mice less than 1 yr of age. We (30) previously found the incidence of spontaneous

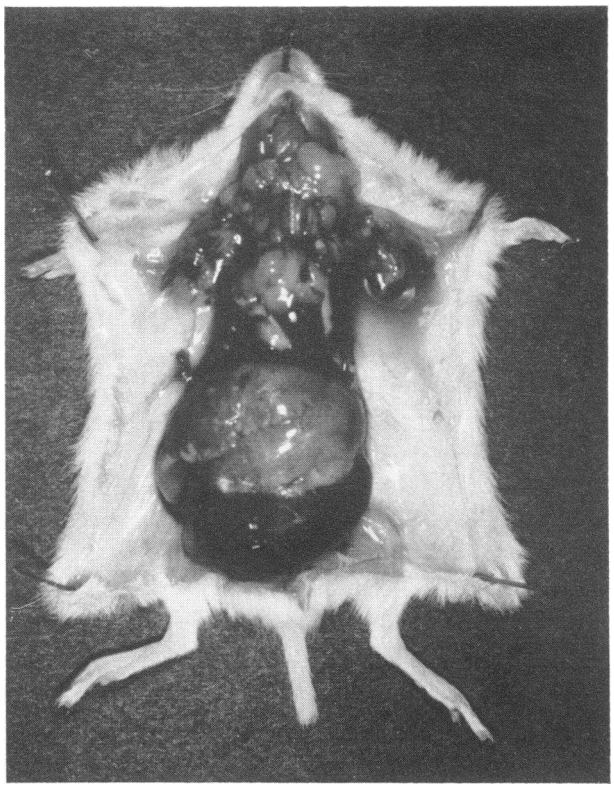

FIGURE 5 SLE dog-induced murine lymphoma. Splenomegaly, hepatomegaly, generalized lymphadenopathy and an enlarged thymus characterize the gross features of the tumor.

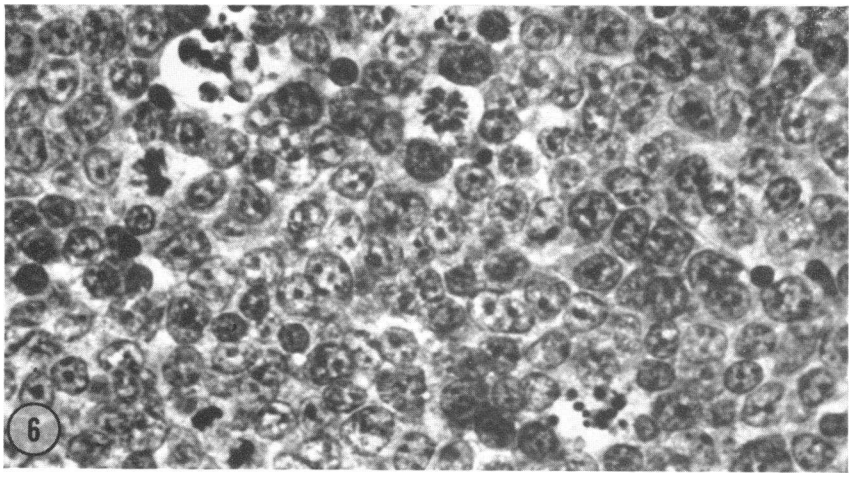

FIGURE 6 SLE dog-induced reticulum cell tumor. Infiltration of the reticuloendothelial system by sheets of uniform, large, immature reticulum cells was accompanied by frequent mitotic figures and macrophages containing ingested nuclear debris. (Hematoxylin-eosin stain, × 400.)

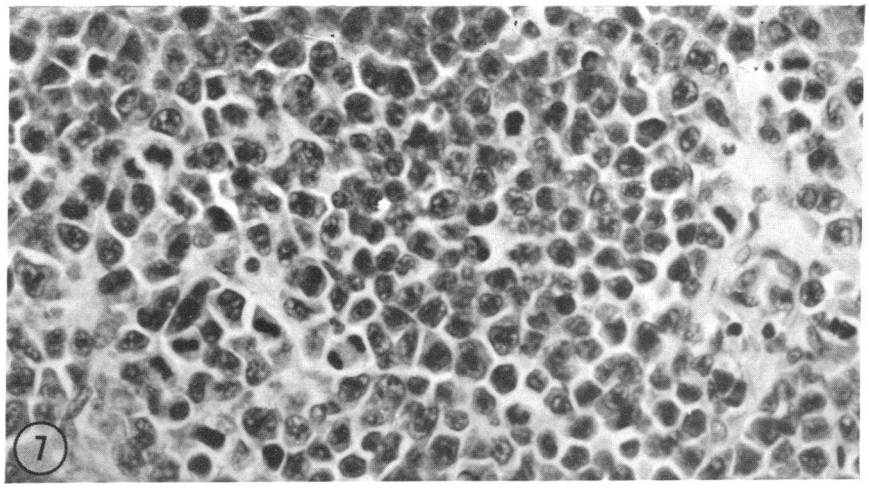

FIGURE 7 SLE dog-induced murine lymphoblastic lymphoma. Uniform small, round, discrete cells with many mitotic figures are present. (Hematoxylin-eosin stain, × 400.)

lymphomas in 324 control CAF_1 mice to be 0 at 6 mo of age (58 mice); 0 at 12 mo of age (50 mice); 12% at 18 mo of age (68 mice); and 10.2% at 24 mo of age (65 mice); 45 CAF_1 mice injected with 150×10^6 syngeneic spleen cells failed to develop neoplasms by 1 yr; 38 CAF_1 mice received 150×10^6 BALB/c spleen cells that had been subjected to three cycles of freezing and thawing and no tumors were found in that group by 1 yr. The tumors found in the recipients of the canine filtrate affected all reticuloendothelial organs and characterically produced splenomegaly, lymphadenopathy, hepatomegaly, and an enlarged thymus (Fig. 5).

Three histologically distinct, transplantable lymphoid tumors were induced by the canine cell-free filtrate. The first type was an atypical reticulum cell tumor (Fig. 6), the second was a lymphoblastic lymphoma (Fig. 7), and the third encompassed cell types ranging from undifferentiated reticulum cells to plasmablasts (Fig. 8).

The gross appearance of the latter neoplasm differed greatly from the previous two tumors in that multilobulated grey-pink tumor masses were located unattached in the abdominal cavity, as well as along the mesentery and omentum (Fig. 9). Serosanguinous ascitic fluid accompanied the presence of the tumor and both the serum and the ascitic fluid contained a monoclonal protein spike. Characterization of the monoclonal protein produced by this tumor is now in progress.

By electron microscopy, cell types varied from one tumor to another, some neoplasms being primarily lymphocytic (Fig. 10*a*), whereas others contained chiefly

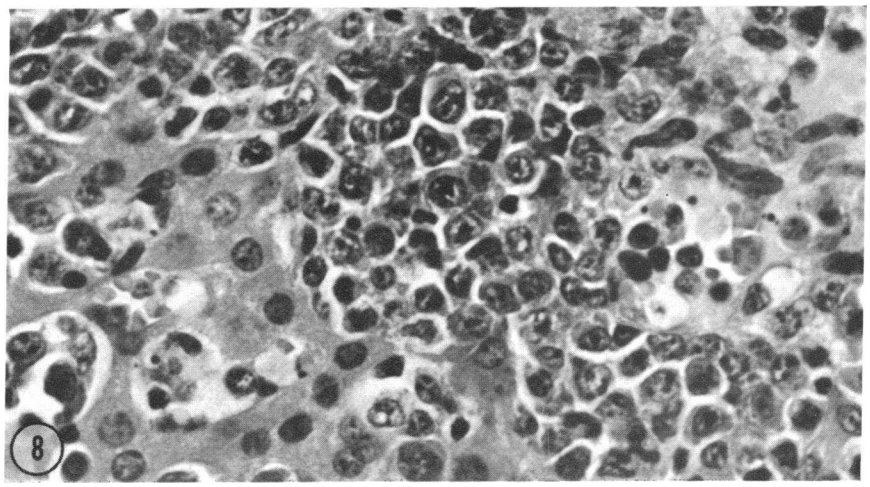

FIGURE 8 SLE dog-induced plasmacytoma. Infiltration of liver by large, immature cells, many of which are plasmablasts. A monoclonal protein spike is present in animals affected with this type of tumor. (Hematoxylin-eosin stain, × 400.)

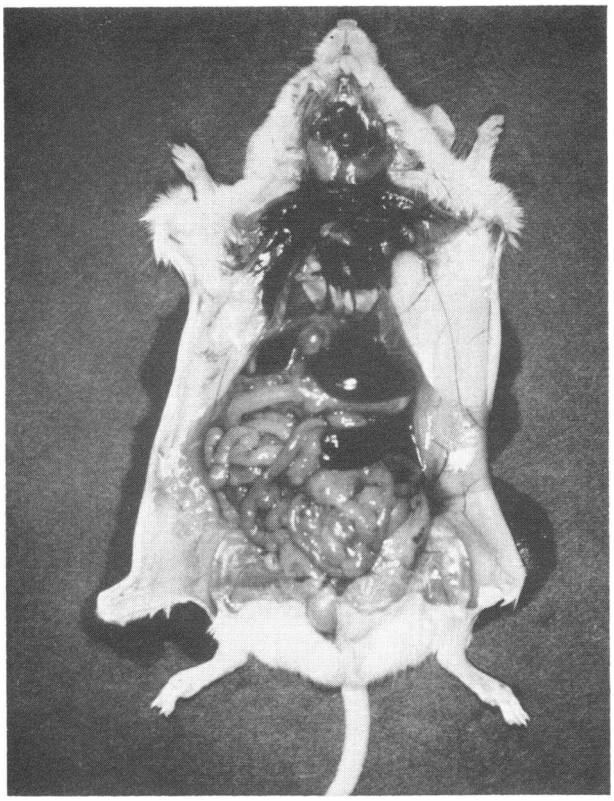

FIGURE 9 SLE dog-induced plasmacytoma. Multilobulated grey-pink masses in the mesentery, omentum, and unattached in the peritoneal cavity characterize the gross appearance of this tumor.

reticulum cells and plasmacytic cells (Fig. 10*b*). In addition to the monocytic type of reticulum cell (Fig. 11*a*), a second type was characterized by the presence in its cytoplasm of numerous electron-opaque globular inclusions of varying size. Several cytoplasmic vacuoles, which seemed to result from extrusion of the contents of the globular formations, were also a striking feature of these cells (Fig. 11*b*). A third type of reticulum cell contained abundant rough endoplasmic reticulum (RER) and only a few phagosomes (Fig. 11*c*). Finally, plasmablasts and plasmacytes, which were often difficult to differentiate from this latter cell (Fig. 10*d*), were seen. These cells were prominent in tumors associated with the production of a monoclonal protein.

Numerous viral particles were seen in all three types of tumors and in all types of cells comprising the tumors, except mature lymphocytes and reticulum cells. Single doughnut-shaped A particles (10), about 70–75 nm in diameter, were observed in the RER cisternae of both plasmablasts and plasmacytes (Fig. 12 *a*). "A-type" particles were also present in clusters in the cytoplasm of hemocytoblasts, plasmablasts, and plasmacytes (Fig. 12*b*); their size was similar to that of the intracisternal particles, but they often exhibited a thicker inner membrane and a moderately electron-dense core (Fig. 12*b*).

Particles of the immature C type (10), about 100–110 nm in diameter with an outer membrane, a thin intermediate shell and a thicker, dense, spherical inner nucleocapsid with an electron-lucent core were also present. These particles were seen budding from the cell membrane of hemocytoblasts (Fig. 12 *c*), lymphoblasts (Fig. 12 *d*), plasmablasts, and plasmacytes. They were also observed in extracellular spaces.

No other type of virus, nor cytopathic lesions evocative of the presence of a second virus, were found.

Passage of 3×10^6 viable tumor cells into newborn syngeneic mice consistently transferred all three types of neoplasms, resulting in death of the recipient within 4 wk. Occasional recipients surviving beyond 1 mo of age developed ANA in conjunction with the tumor. Passage of tumor cells into other inbred strains of mice resulted in the uniform development of ANA, but not tumors. Cell-free filtrates prepared from the reticulum cell and lymphoblastic tumors regularly induced the development of ANA, and occasionally produced lymphoid tumors in syngeneic recipients (Table V).

Injection of 10×10^6 viable tumor cells into newborn W/Fu rats resulted in the development of transplantable malignant lymphoma in 25/25 recipients. The tumor was characterized by gross enlargement of the liver, spleen, lymph nodes, and thymus. Histologically, all organs within the reticuloendothelial system were diffusely infiltrated with lymphoblasts. None of the rats developed ANA.

Eight seropositive CAF_1 recipients of the cell-free filtrates from SLE colony dogs and four normal control mice were given six injections of ATS over a 2 wk period (total dose 2.5 ml). Five of the eight experimental mice developed a lymphoma after ATS treatment. In three of these mice the tumor appeared within 1 mo after the animal had received ATS. None of the control animals developed a tumor.

Viable cells or a cell-free filtrate of the lymphoma developing in murine recipients of the canine filtrate were injected into four newborn normal beagle dogs, which were geographically isolated from the SLE colony. All four animals developed positive LE cell tests by 4 mo of age and three of the four also have ANA (Table VI). These animals are now 10-mo old and, to date, no neoplasm has appeared. Tumor cells and cell-free filtrates from two transplantable lymphoid tumors of mice (MOPC 315 [11]) and GVH (12) that were not induced by the canine filtrate, but which are known to contain murine leukemia virus, failed to elicit ANA production after administration to normal newborn puppies.

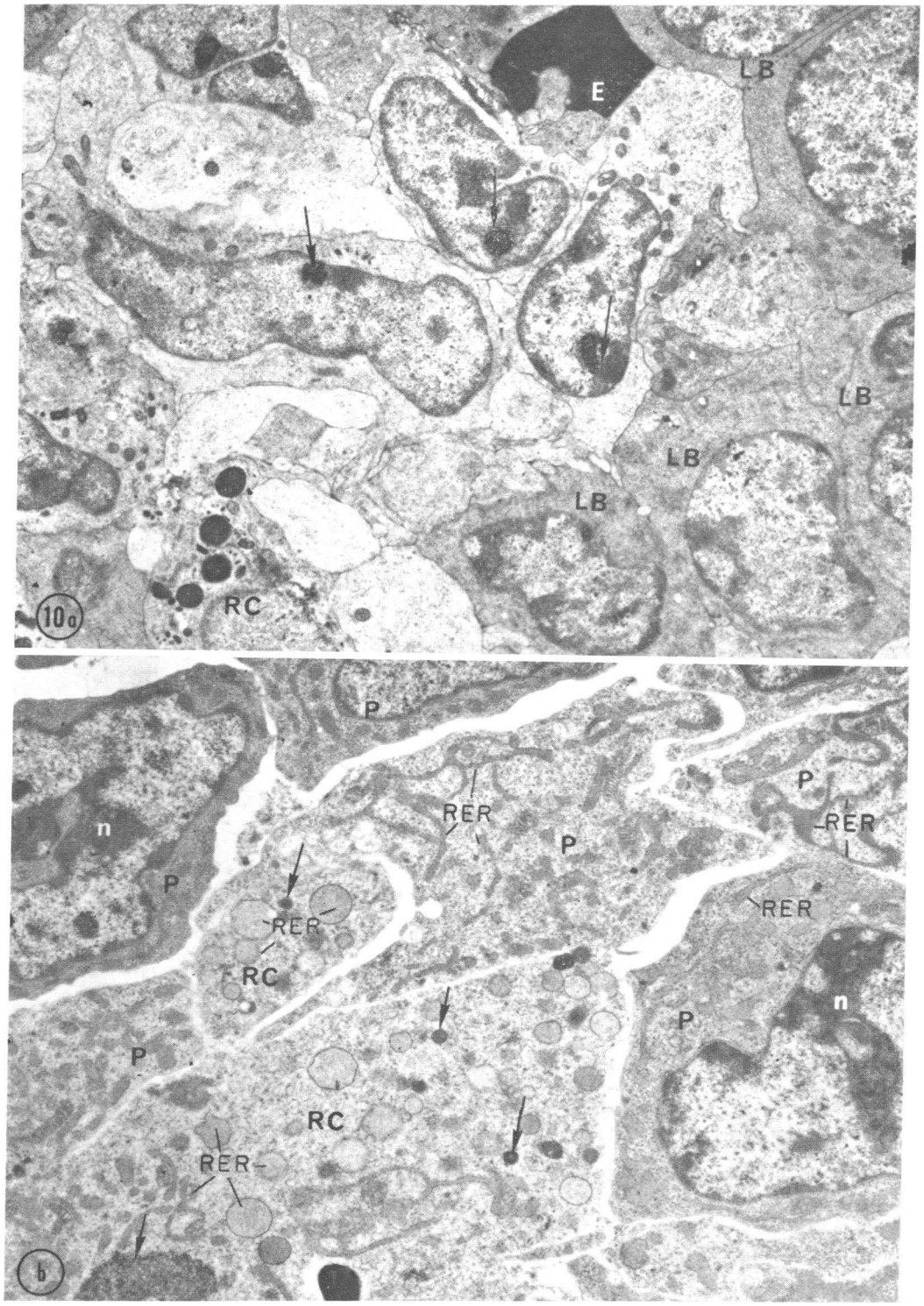

FIGURE 10 (*a*) Primarily lymphocytic type of tumor. Numerous lymphocytes with dense nucleoli (arrows) and lymphoblasts (LB) with darker cytoplasm are seen. A reticulum cell (RC) with large, round inclusions is visible at bottom left. Erythrocyte (E) is present on top right of the field. × 3,200. (*b*) Reticular plasmacytic type of tumor. Plasmablasts (P) with large nuclei and prominent nucleoli (n) are observed; their dense cytoplasm contains abundant, elongated RER. Parts of two reticulum cells (RC) are also seen. They can be distinguished from plasmablasts by the presence of phagosomes (arrows) in their cytoplasm; the ground substance of their cytoplasm is electron-lucent and their RER cisternae are round and less electron-dense than those of plasmablasts. × 3,800.

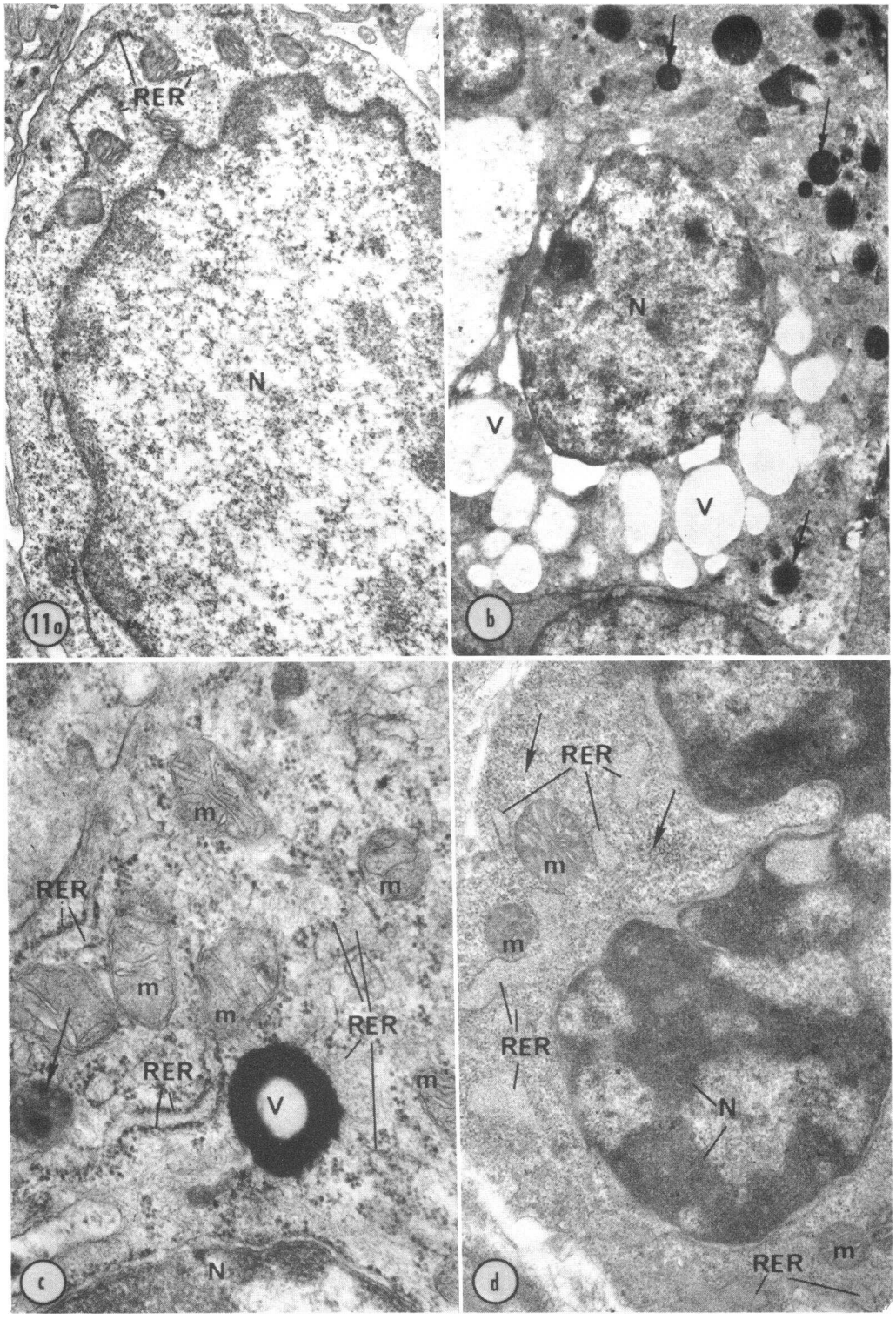

FIGURE 11 (*a*) Monocyte. Its nucleus (N) has a finely scattered chromatin and slightly indented contour. A few RER strands are present in its cytoplasm. × 8,400. (*b*) A reticulum cell with globular inclusions (arrows) and numerous similarly shaped, oval vacuoles (v) is

TABLE V

Passage of Dog-Induced Murine Lymphoma in CAF$_1$ and BALB/c Mice*

Passage no.	Tumor filtrate		Tumor cells	
	ANA+	Tumor+	ANA+	Tumor+
1	5/6	0/6	—/—	4/4
2	8/12	2/12	—/—	12/13
3	5/12	0/9	—/—	11/12
4	2/2	0/2	—/—	3/3
5	2/4	0/3	—/—	14/14
6	4/8	1/5	—/—	10/10
7	—/—	—/—	—/—	9/9
8	—/—	—/—	2/2*	0/2*
9	—/—	—/—	2/2	0/2
10	—/—	—/—	2/2	0/2

Numerator, number positive; denominator, number of recipients, —, not tested. Newborn BALB/c recipients were used for the eighth passage and lymphomas did not develop. Nevertheless, transfer of viable spleen cells from two of these BALB/c recipients 3 wk later led to the formation of ANA in CAF recipients (ninth passage) by 4 mo of age. Each of the two remaining eighth passage BALB/c recipients developed positive ANA tests at 11 mo of age.

Tissue culture studies. Leukemia virus assays of spleen cell suspensions were positive in all four tumor-bearing CAF$_1$ mice tested. Each of these mice also had C- and A-type particles demonstrable in their tumor cells by electron microscopy and ANA in their sera (Table VII). An additional eight mice inoculated with splenic filtrates of SLE colony dogs were sacrificed before the development of any tumors; four of these mice were leukemia virus positive. Thus, 8 of 12 mice inoculated with SLE colony material became leukemia virus positive; in contrast, only 4 of 26 uninoculated control CAF$_1$ mice were positive for leukemia viruses using the same assays. Tests on tumor cells for group specific murine leukemia virus antigens (gs-1), kindly performed by Dr. P. S. Sarma, were also positive.

No direct cytopathogenic effects were observed on fetal dog cells or mouse embryo cells when cultured with the above described mouse spleen preparations or with bone marrow or heparinized blood suspensions from serologically positive SLE colony dogs. These cultures were maintained for periods of up to 2 mo without significant morphological alterations or the development of positive hemadsorption of human O erythrocytes.

DISCUSSION

The results of these experiments indicate that the serologic hallmarks of SLE—ANA, LE cells, and antibodies to N-DNA—can be induced in appropriate recipients by cell-free filtrates of tissues from dogs with ANA and positive LE cell tests. The data do not show, nor is it claimed, that the *disease* SLE can be transferred by cell-free filtrates. Only further experiments can support or refute that idea.

Transmission studies of the type described here are fraught with difficulties, and any result, whether positive or negative, requires the most cautious and skeptical interpretation. For example, it is well known that some strains of mice spontaneously produce ANA, especially in old age. We chose to work with CAF$_1$ mice because they have been studied extensively in our laboratory for several years. We have never found a positive ANA test in a CAF$_1$ mouse less than 1 yr of age. Thus, the findings of positive ANA tests within a year in 49.2% of CAF$_1$ recipients of the canine filtrate is, we believe, of great significance.

TABLE VI

Specific Association between Dog-Induced Lymphoma and ANA

	Donor dog	Recipient	
		CAF$_1$ mice	Normal puppies
Dog-induced tumor	SP 49	9/9	3/4
MuLV + control tumors	MOPC	0/21	0/3
	GVH	0/8	0/5
	K 36	0/5	—

MOPC, GVH, and K 36 are transplantable murine lymphomas that arose independent of the present study and contain readily detectable murine leukemia viruses.

TABLE VII

Correlation of Murine Leukemia Virus with Dog-Induced Murine Lymphomas

Mouse tumor	ANA	EM virus particles	Leukemia virus titer
			*TCID$_{50}$/ml**
SP 37 no. 3	+	+	5×10^4
SP 49 A	+	+	5×10^3
SP 69 no. 1	+	+	5×10^3
SP 37 no. 1	+	+	5×10^2

Each tumor is associated with the formation of ANA by its host and each contains leukemia viruses, detectable by electron microscopy and the XC assay.

* $TCID_{50}$ = 50% tissue culture infectious doses as determined by focus assay; all were also positive by XC assay.

shown. × 5,000. (*c*) Part of a reticular cell with abundant RER. Numerous mitochondria (m), a phagosome (arrow), and a vacuole (v) with a thick electron-opaque rim are present in its cytoplasm. Its nucleus (N) is visible at the bottom of the picture. × 12,000. (*d*) A plasmacyte is shown: note its patchy nuclear chromatin (N), its cytoplasm studded with ribosomes (arrows), its numerous RER cisternae and their moderately electron-opaque content. Note the absence of phagosomes. The golgi apparatus is not seen in this plane of section. × 9,000.

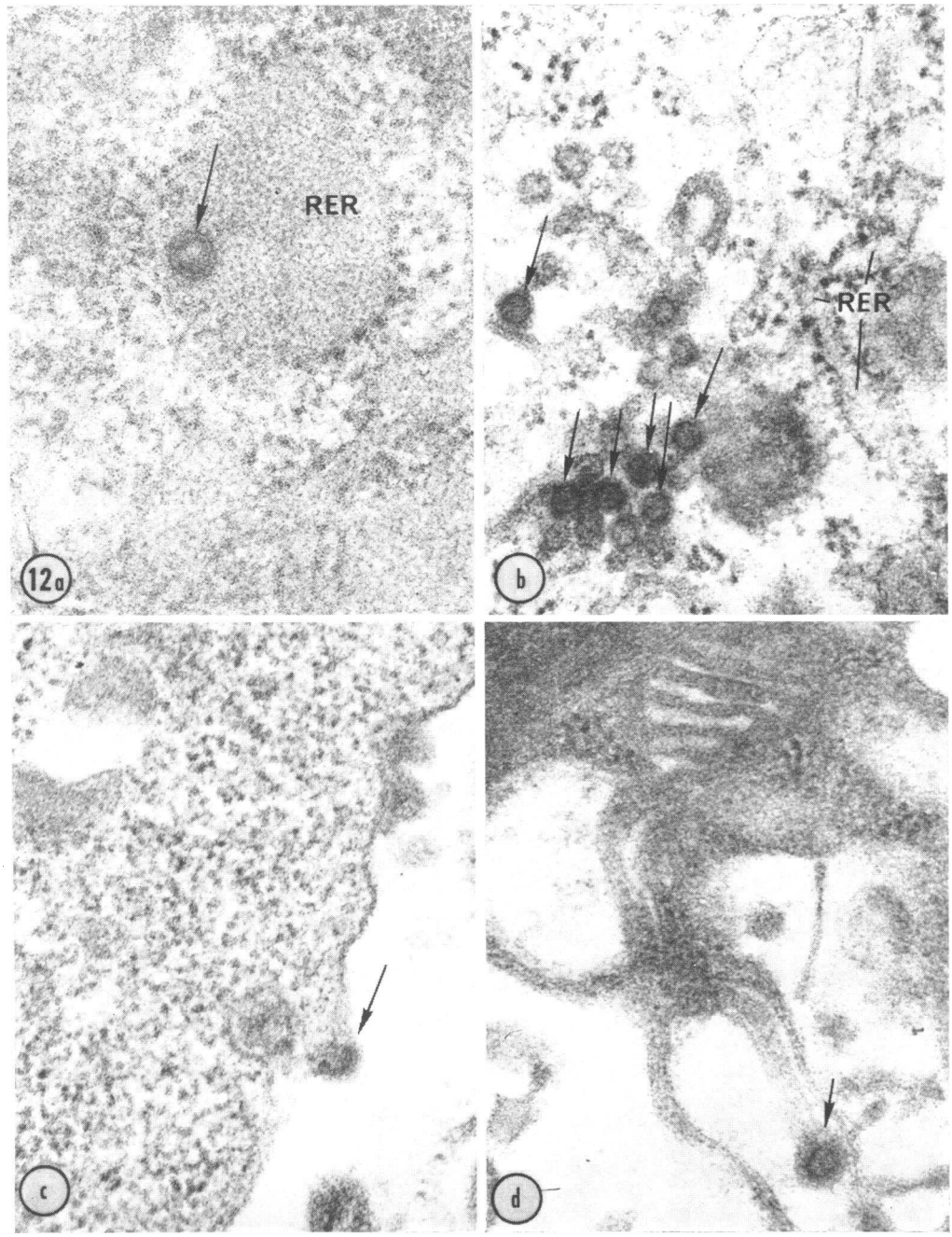

FIGURE 12 (*a*) An "A-type" viral particle (arrow) is shown in the RER cisternae of a plasmacyte. It is 70 nm in diameter and has a doughnut shape with two membranes of equal density and an electron-lucent core. × 60,000. (*b*) A cluster of "A-type" viral particles is seen in the cytoplasm of a plasmacyte. These particles are also 70 nm in diameter. Several of them (arrows) exhibit a thicker inner membrane and a moderately electron-opaque core. An RER cisternae (RER) is visible at right of the field. × 38,000. (*c*) An immature C-type viral particle (arrow) is seen budding from the membrane of a hemocytoblast. × 32,000. (*d*) An immature C-type viral particle (arrow) is shown budding from a lymphoblast. × 32,000.

It is, of course, possible to interpret this as a result of immunization by DNA or nuclear fragments present in the filtrate. We do not believe this is the case for the following reasons: (*a*) Filtrates prepared in an identical manner from spleens of normal dogs, cats, rabbits, guinea pigs, rats, and mice failed to elicit the formation of ANA in a group of 134 mice. (*b*) Murine recipients of the canine filtrate developed antibodies against N-DNA. Although antibodies against D-DNA can be produced by immunization with DNA-protein conjugates, antibodies against N-DNA have never been found in such circumstances (13). Indeed, antibodies against N-DNA occur almost exclusively in one condition, SLE, whether it affects mice, dogs, or humans (14). (*c*) The normal puppies given the canine filtrate developed, in addition to ANA, positive LE cell tests. To the best of our knowledge, the factors required for the LE cell cannot be induced by immunization of normal animals with DNA, nuclear remnants or other cellular constituents.

The rising titers of ANA over a 12 mo period in the recipients of the filtrate exclude the possibility that the ANA in mice was merely transferred from the dog through the filtrate. It is inconceivable to us that a subcellular unit—or even a small number of whole dog cells —persisted in the mouse for a year and synthesized the ANA. This unlikely possibility is excluded by the fact that the ANA was detected by an antimouse immunoglobulin reagent that did not react with canine immunoglobulins.

We thus conclude that a filterable agents or substance induced the murine recipients to produce ANA and the canine recipients to develop positive LE tests as well as ANA.

The results do not demonstrate that the causative factor in the filtrate is a replicating virus. The present experiments provide only indirect evidence favoring that view, and until the hypothetical virus is isolated and identified, we cannot accept this notion with confidence. The experiments involving serial passage of cells or or filtrate from mouse to mouse encourage the idea that a virus is involved because they suggest the serial transmission of an infectious agent, which replicates preferentially in a newborn animal, rather than a nonreplicating substance.

A possible viral etiology of SLE has been sought for many years. Although serological studies of SLE have detected antibodies to certain viruses, no single agent has been consistently incriminated in this condition (15–17). The results of other studies indicate that structures resembling viral nucleocapsids are present in vascular endothelial cells of the kidney and skin of patients with SLE (18); however, evidence that such structures are present in normal kidney tissue (19), or in kidneys from patients with a variety of other diseases (20) has also been presented. Of additional interest is the proposed etiological role of a "C"-type RNA virus in the lupus-like disorder of New Zealand black (NZB) mice (21); this agent is probably not a specific cause of the disease since other viruses, both RNA and DNA types, can provoke the development of ANA and nephritis in NZB mice (22).

Viruses could initiate the pathogenetic mechanisms of SLE by causing the release of host DNA, either through cytolytic effects, or through the action of enzymes capable of hydrolyzing DNA. Some viruses contain DNA endonucleases (23–26) and exonucleases (25); others may induce nucleases in the host cell. Whether continued liberation of intracellular contents during a chronic viral infection can result in an autoimmune response is not known at present. Most temperate viruses are known to induce new antigens in the cells they infect or transform. These antigens may be viral or altered host-cell components, either of which might result in an autoimmune host response (27–29).

The development of lymphomas in the murine recipients of the canine filtrate was unexpected. These neoplasms range in cell type from lymphoblastic lymphoma to a bizarre reticulum cell tumor, which produced a monoclonal protein. Each tumor developed before the recipient was a year old, an important point because we have never seen a malignant lymphoma in an untreated CAF_1 mouse of that age (30). Furthermore, of the few lymphomas that do develop spontaneously in much older CAF_1 mice, we have not encountered either a monoclonal gammopathy or a lymphoblastic neoplasm. We therefore believe that these tumors were triggered by the injection of the canine filtrate.

Another interesting feature of these tumors was the presence of leukemia viruses, as detected by specific in vitro assays and by electron microscopy. That the viruses in the canine filtrate-induced tumors were murine RNA tumor viruses was shown by the presence of the gs-1 antigen diagnostic of RNA tumor viruses of the mouse.

The tumors induced by the canine filtrate had a property shared by no other murine tumor we tested: The ability to induce ANA formation in both murine and canine recipients. The puppy recipients developed ANA and positive LE cell with unusual rapidity, by 4 mo of age. Often, the tumor was lethal within 2 wk after transplantation into syngeneic recipients, which had no ANA in their serum. But by using cell-free filtrates or allogeneic recipients, growth of the tumor was delayed or stopped, and sufficient time elapsed for the majority of the recipients to produce detectable amounts of ANA.

We interpret our findings as follows: The dogs of the SLE colony harbor a virus. This agent, which passes

through the pores of a 0.22 μm filter, can infect normal dogs and mice. In dogs it triggers ANA production and the development of positive LE cell tests. In mice, the agent provokes the formation of ANA. In addition, the canine LE virus may activate latent murine leukemia viruses. This could explain why some mice given the canine filtrate develop malignant lymphomas as well as ANA. Other mice do not develop tumors, perhaps because the number of activated RNA tumor viruses is small. However, when treated with the immunosuppressant ATS, these animals rapidly develop lymphomas of the same type found in the other recipients of the canine filtrate. We believe that the tumor contains two viruses: the canine LE virus and the murine RNA leukemia virus. When inoculated into newborn puppies, the tumor causes ANA formation and positive LE cell tests because of the presence of the canine LE virus. Neoplasms do not occur because the murine luekemia virus is incapable of transforming canine cells. When inoculated into mice, filtrates of the tumor induce both neoplasmas and ANA because of the presence of the two viruses. The inability of murine leukemia viruses alone to induce ANA formation in mice and dogs is shown by the control experiments listed in Table VII.

We stress that the preceding is an *interpretation* of results, and not a documentation of the presence of a virus in canine SLE. If this interpretation is borne out by further experiments, it will be necessary to consider the implications for man of a common house pet harboring a virus with the properties we have described. Its ability to cross the species barrier is striking in the case of the mouse, and its potential for horizontal spread is suggested by two experiments: in the first, filtrates from the spleens of two normal dogs living with the SLE colony induced ANA formation in mice; in the second, "control" littermates raised with canine recipients of a filtrate prepared from a colony dog developed ANA and positive LE cell tests. Furthermore, the absence of ANA and LE cells in four cesarean-derived, orphan-raised, germ-free, third generation offspring of the A breeding line argues strongly for the necessity of a transmissible agent to mediate the development of these antibodies. Although the gnotobiotic animals are currently only 14-mo old, it would appear that the high probability of a dog becoming serologically positive for SLE can be substantially reduced by avoiding some as yet unidentified pathway of infection by the proposed canine LE virus. Since these germ-free animals were reared by formula feeding, it is conceivable that a milkborne virus in involved. This possibility and its ramifications for the pathogenesis of SLE in human females is being investigated.

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