Prevention of Infectious Diseases in Experimental Mice.* (32581)

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(Introduced by L. H. Smith)
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Consequences of epizootic diseases in experimental mice are well known, such as those of salmonella or ectromelia(1), and considerable effort has been directed toward their control. Less attention has been shown enzootic diseases. Particularly important in this regard are the consequences of *Pseudomonas* sp. infection in irradiated animals. These animals sometimes exhibit the so-called "early death syndrome," a post X-irradiation septicemia(2). The consequences of enzootic virus infections of mice used in virus-isolation and oncology studies are well documented(3,4), and recently it has been found that 97% of 71 transplantable mouse tumors harbor at least one murine virus(5).

Infestations by internal and external parasites, a condition common to laboratory mice (6,7), are neglected by many users of experimental animals because their consequences are not always apparent without additional investigative effort. Congdon *et al* (unpublished data), during transplantation studies, have found *Hymenolepsis nana* lodged in mesenteric lymph nodes of mice, causing abscesses and chronic inflammation, and destroying the value of this organ for evaluation of experimental results.

The development of germfree and barrier-sustained breeding colonies has provided some new sources of mice free from most infectious diseases(8). However, these techniques often present serious impediments to investigators because of their effects on the animals themselves(9) and because of the difficulties involved in performing experiments under isolation conditions.

This paper reports studies on an approach to the prevention of diseases in experimental mice by using a filter-cap cage system relatively undemanding in expense or time, which could be applied to experiments under a variety of laboratory conditions.

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Materials and methods. A. Animals and maintenance conditions. The nucleus of breeding mice used in this study was from the C57BL/6 Caesarean-derived, specific-pathogen-free colony of Dr. H. E. Walburg, Jr.† 10 mated pairs were used to start the colony, and colony size was limited to 100 breeding pairs plus a requisite number of replacement breeding stock. During the experiment, the average litter size was 5.1 mice, of which 89.2% reached weaning age. The experiment covered a period of 18 months.

All mice were housed in polycarbonate shoebox cages covered with either Isocap‡ paper filters, or framed fiberglass filters§ (Fig. 1). The filter caps were changed at weaning time, paper caps being discarded, and the fiberglass caps sterilized in dry hot air for reuse if still serviceable. Wood shavings were found to be the most satisfactory bedding. Cages and bedding were changed twice weekly and used unsterilized. Both cages and water bottles were washed in automatic washing machines.

All mice were fed nonsterilized Purina Lab Chow‖ *ad lib*, and received fresh water bottles 3 times weekly. Water bottles were not sterilized, but water was hyperchlorinated to a mean level of 16 ppm. All mice were handled with clean forceps during servicing operations, and the average total-room environment exposure time per cage per week was about 3.5 minutes. Sexing and weighing weanling mice required about 2 minutes exposure time. Animal caretakers wore clean coveralls daily, but did have limited access to other containment-type animal quarters. The experimental colony quarters consisted of a single animal room in the Laboratory's Animal Receiving Facility. Room temperature was controlled at approximately 72-74°F, but humidity was not con-

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‡ Bioquest, Hackensack, N. J.

§ Thunder Corp., Greene, N. Y.

‖ Ralston Purina Corp., St. Louis, Mo.

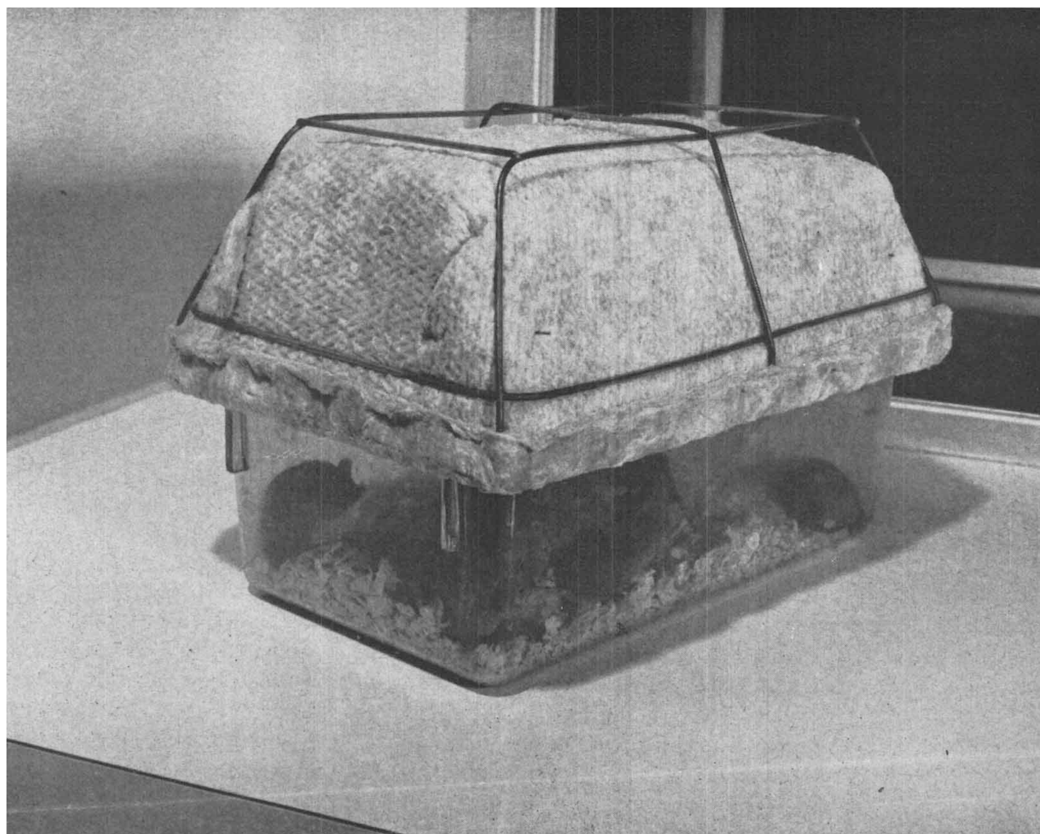

FIG. 1. Filter-top cage (D. G. Doherty and M. L. Simmons, unpublished data).

trolled. There was about 10% fresh air exchange per hour in the animal room.

B. Monitoring program. Periodically, mice which had attained the status of retired breeder (mean age ~200 days) were removed from the experimental colony and subjected to thorough examination. Animals were anesthetized with ether, exsanguinated through the jugular vein, and the blood collected in HA buffer¶ to a 1:5 final dilution. Serum was separated from cells with Sep-ar-aid.** centrifuged at 800 × *g* for 20 minutes, and stored at —20°C prior to serological examination.

a. Bacteriology. Segments of the small intestine, liver, and spleen were collected aseptically, cultured separately in tetrathionate broth for 24 hours at 37°C, then streaked to brilliant green agar for salmonella. 48 hours after initial culturing, the tetrathionate broth cultures were restreaked on brilliant green for Salmonella sp., and glycerol agar for *Pseudomonas*. The *tympanic bullae* were cultured for PPLO by inserting a sterile dissecting needle through the tympanic membrane, then aspirating any contents with a capillary pipette drawn to a fine point. This material was then placed in 0.01 M K_2HPO_4 as a dispersal medium(10) and poured directly on PPLO agar formulated according to J. B. Nelson (personal communication) with thallium acetate added to a final concentration of 1:4000. The right lung was removed aseptically ground in a Ten Broek grinder, and streaked directly on PPLO agar. Direct smears from the caecum and small intestine were examined microscopically for the presence of intestinal spirochetes. This procedure was usually performed on one mouse from each sample.

b. Parasitology. The caecum was removed,

¶ Difco, Detroit, Michigan

** Uni-Tech Chemical Mfg. Co., Panorama City, California.

TABLE I. Comparison of Test Results from Experimental Colony and Conventional Colonies.

	Experimental colony	CC No. 1*	CC No. 2†	IC‡	Rat colony No. 1	Rat colony No. 2
Salmonella	0/236§	0/40	0/12	2/5	0/10	0/19
Pseudomonas	0/236	0/40	3/12	NE‖	0/10	0/19
PPLO (middle ear)	0/236	0/40	0/12	NE	4/10	7/19
PPLO (lungs)	0/236	0/40	0/12	NE	0/10	1/19
External parasites	0/236	20/40	0/12	NE	0/10	0/19
Internal parasites	0/236	23/40	7/12	NE	5/10	0/19
Intestinal spirochetes	0/236	3/11	NE	NE	10/10	0/9
Protozoa	0/236	1/11	NE	NE	10/10	0/8

* Conventional mouse colony No. 1.
† Conventional mouse colony No. 2.
‡ Artificially infected mice (Salmonella). Held 20 days before exam. Fecal samples from these mice became negative on post-infection day 17.
§ No. infected/No. tested.
‖ NE = Not examined.

placed in a Petri dish with a small quantity of tap water, and opened longitudinally. The contents were gently mixed and examined for parasites under the microscope. Occasional spot checks for protozoa were made, in conjunction with spirochete exams, by employing direct smears from different levels of small intestine and caecum, with warm saline as the suspending medium. The remainder of the eviscerated carcass was placed in a plastic cup and covered with a soapy water solution. About 30 minutes later the carcass was removed and the major portion of the solution decanted, the sediment diluted in tap water and filtered through a black Whatman†† filter No. 29 mounted in a Millipore‡‡ filter apparatus. The filter was then examined under low power for external parasites.

c. Virology. Sera were examined for antibody to common murine viruses by techniques previously described(8). Hemagglutination-inhibition tests were performed with Reovirus type 3, pneumonia virus of mice (PVM), Theiler's GDVII, Sendai, K(newborn pneumonitis), and polyoma viruses. Complement-fixation tests were performed with mouse adenovirus and mouse hepatitis viruses. Serological reagents, viruses, and specific antisera were obtained through the Virology Research Resources Branch, National Cancer Institute, or purchased from Microbiological Associates, Inc., and all tests were performed in the *Microtiter* system. Positive and negative control sera, antigen, and complement titrations were performed with each test series. Tests were rejected when these varied more than 2-fold in dilution or greater than $2CH_{50}$ complement units(11).

d. Pathology. Gross observation was made of all mice during the course of the examination. Tissues collected from 27 experimental colony mice for histological examination included: lungs, thymus, heart, liver, spleen, kidney, adrenal, gonads, uterus, femur, intestine, thyroid, and salivary glands. All tissues were fixed first in neutral isotonic formalin, then transferred to Zenker's solution about 4 hours prior to trimming. Paraffin sections were prepared and stained with hematoxylin and eosin.

Results. a. Bacteriology and parasitology. A total of 263 mice (142 females and 121 males) was examined. During the last 6 months of the experiment the sample size tested at any given time represented about 5% of the total population. The results of the bacteriology and parasitology examinations were consistently negative for the specified organisms. The methods employed have been reliable in our hands in the diagnosis of these various agents in mice. Table I presents a comparison of results between the experimental colony and conventional colonies of mice and rats examined by the above procedures.

b. Virology. Each sample of mice examined for virus antibody represented about 5% of the breeder population. 50 retired breeders that were tested for antibody to the 8 viruses listed above had negative results.

†† W. and R. Balston, Ltd., (London) England.
‡‡ Millipore Filter Corp., Bedford, Mass.

TABLE II. Summary of Occurrence of Lymphoreticulocytic Lesions and Focal Hepatic Necrosis in Experimental, Barrier, and Germfree Colonies.

	Experimental colony (C57BL/6)	Barrier colony (C57BL/6)	Germfree (C57BL/6*)
Lungs	11/26†	21/32	0/6
Liver—Focal necrosis	15/28	23/28	5/6
Perivascular	11/28	13/28	2/6
Kidney (pelvis)	26/38	15/26	3/6

* Colony of Dr. H. E. Walburg, Jr., Biology Division, Oak Ridge National Laboratory.
† No. with lesions/No. examined.

TABLE III. Results of Bacterial Culturing of Experimental Colony (20 Mice).

	No. of specimens showing growth	Isolates: Aerobic	Isolates: Anaerobic
Lungs	11/17*	Coagulase-negative staphylococcus (4) Bacillus sp. (3) Enterococcus (7)	Coagulase-negative staphylococcus (1) Bacillus sp. (10) Enterococcus (9) Corynebacterium sp. (2)
Liver	3/2	Coagulase-negative staphylococcus (2) Bacillus sp. (2) Corynebacterium sp. (1)	Bacillus sp. (2)

* Aerobic/Anaerobic.

No virus isolations have been attempted but the absence of antibody in a large sample of the population over a period of time has been a reliable measure of the absence of these agents(4).

c. Pathology. Histologically 11 of 27 of the mice examined from the experimental colony had pulmonary vascular lesions (Table II). This consisted of a cuff of infiltrating leukocytes, primarily lymphocytes and a few mononuclear cells, around the respiratory venules (Fig. 2). Occasionally the infiltrate involved the entire thickness of the venule wall, and sometimes caused bulging of the endothelium. Similar lesions were seen in 21 of 32 barrier-maintained C57BL/6 mice. Bacteriological examination of 20 lungs from the experimental colony gave varied results, and it is doubtful if the organisms recovered were the cause of this particular lesion. Most isolates are common inhabitants of the gastrointestinal tract, and are also commonly found in the respiratory tract (Table III) (R. C. Allen *et al,* unpublished data). Initial attempts to transmit this lesion to neonatal mice of the C57BL/6 and C3H strains have met with no success when employing the intraperitoneal, intracerebral, and intranasal routes. Inoculated neonates were held 21 days before being killed. The absence of similar lesions in 6 germfree C57BL/6 mice (Table II) could be due to chance. Many mice considered to be affected had only 1 or 2 cuffed venules, and only a limited number of lung sections was examined from any of the mice studied, although attempts were made to examine the same general areas of the lungs from each mouse.

Crystals were found to be the apparent cause of aspiration pneumonia in the left lung of 1 female breeder that died during the course of the experiment. Large extracellular and small intracellular crystals similar to those described by others(12-15) were seen in this mouse, and occasionally in the lungs of other mice from the experimental colony. Such crystals are in fact common in epithelium-lined organs in the mouse, and are occasionally seen in gall bladders, bile ducts, the uterus, and very commonly in the mucous glands lining the trachea of old mice where at least some are probably formed (personal observations). The cause of such crystals has been speculated upon by some(13,15) as being the products of abnormal epithelium, *i.e.,* inflammation, etc. Crystal aspiration pneumonia was found in the caudal lobe of 2 of the 6 C57BL/6 germfree mice that were

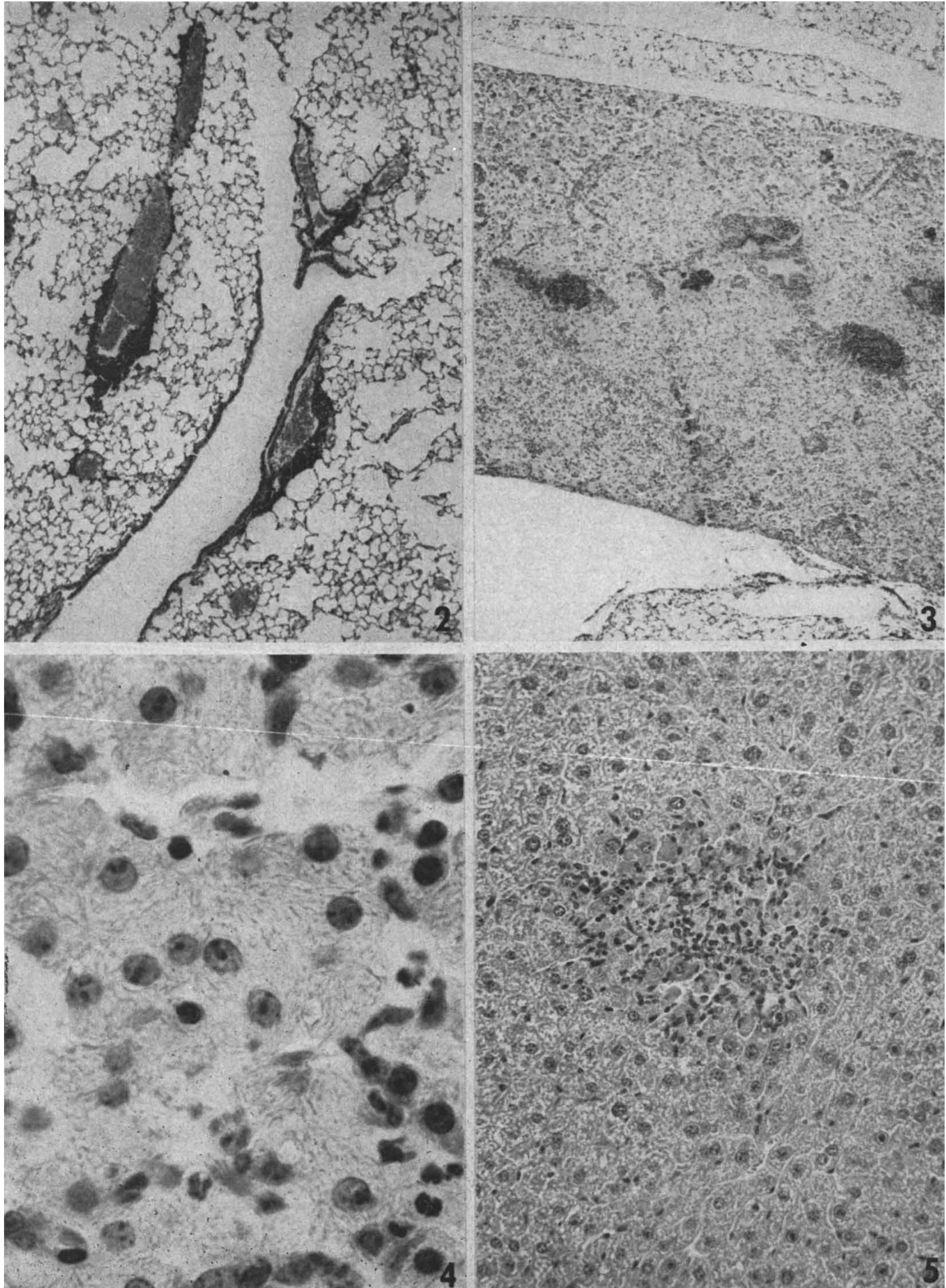

FIG. 2. Heavy cuffs of lymphocytes and reticulocytes around pulmonary venules. 200-day-old retired breeder. × 65 (hematoxylin and eosin).

FIG. 3. Consolidation of post caval lobe in a germfree C57BL/6 mouse with crystal phagocytes. × 65 (hematoxylin and eosin).

FIG. 4. High-power view of Fig. 3 showing crystalline spicules packed in the cytoplasm of phagocytes. × 1000 (hematoxyline and eosin).

FIG. 5. Focal necrosis, liver, 200-day-old retired breeder from experimental colony. × 250 (hematoxylin and eosin).

examined as experimental controls (Figs. 3, 4).

2 types of lesions were seen in the liver. The 1st, consisting of microfoci of necrosis scattered throughout the parenchyma, was seen in 15 of 28 mice examined from the experimental colony. These foci were accompanied by leucocytic infiltration, usually containing neutrophils (Fig. 5). The 2nd type of liver lesion consisted of perivascular focal infiltrations of lymphocytes and mononuclear cells (Fig. 6) and was seen in 11 of 28 mice examined (Table II). Occasionally other cell types were mixed in with the lymphocytes and monocytes, *i.e.*, neutrophils, eosinophils, plasma cells, much like the lesions described by Cosgrove and Upton(16). In mice of this age, the lesions were widely scattered and small. Others have detailed the morphologic and cellular characteristics of similar lesions in various inbred strains of mice(17-20). Additional effort is necessary to clarify and relate these lesions as well as the perivascular lesions in the lungs. Retired breeders held to 300 days of age and then killed show a marked increase in the size of the lung and perivascular liver lesions (Figs. 6, 7). Cytotypically the lesions in this age group were almost purely reticulocytic and appeared as low-grade sarcomas.

The microfocal areas of necrosis appear to be strictly inflammatory, and these lesions were equally common in germfree mice (Table II). It is possible that foreign material may gain entrance to the portal circulation directly from the gut, and cause a reaction when it lodges in the liver, or that some unknown infectious agent may be the cause. The latter possibility seems unlikely in view of the presence of the lesions in the germfree mice. Table III presents a summary of bacteriological findings from the livers of 20 mice from the experimental colony. The large number of negative results and small number of isolates suggest that the latter could be contaminants or that small numbers of these organisms may gain entrance to the mouse liver as in other species(21).

16 of 28 mice examined from the experimental colony had small collections of lymphocytes in the peripelvic renal tissues (Fig. 8). It is probable, as with the other lesions, that these collections would be seen in an even higher percentage of kidneys (Table II) if serial sectioning were to be performed.

Discussion. The filter-cage concept of Kraft has been applied successfully in quenching infections of epizootic diarrhea of infant mice (22) and mouse hepatitis(23). The studies reported here extend these observations to many other types of infectious agents and determine some minimal operational requirements for this system in a mouse breeding colony.

Mice which were free of common pathogens were reared under filter caps in a conventional animal cell and maintained in unsterilized cages free of specific-pathogenic bacteria, viruses, parasites, and PPLO for at least 18 months. Instestinal spirochetes and protozoa are common inhabitants of the gastrointestinal tract of wild rodents, as well as of those of many laboratory rodents(24,25, and C. B. Richter, personal observations), and although not often regarded as pathogenic they are an easily detectable measure of contamination.

Gross lesions were extremely rare in these mice, no significant number being found, and the mortality rate among adults was nil.

Histologically these mice did not demonstrate lesions qualitatively or quantitatively different from those seen in barrier-maintained mice or the limited number of germfree mice of the same strain which were examined. Coincidentally, they did not experience the physiologic and morphologic characteristics of germfree mice such as lymphatic atrophy and caecal distention. The lymphoreticular lesion observed in lungs, liver, and kidney appears to be a strain-related, leukemia-like disease. The mechanism of focal hepatic necrosis in these and germfree mice needs further study. In terms of full-life expectancy of this strain, the mice examined here were relatively young (~200 days).

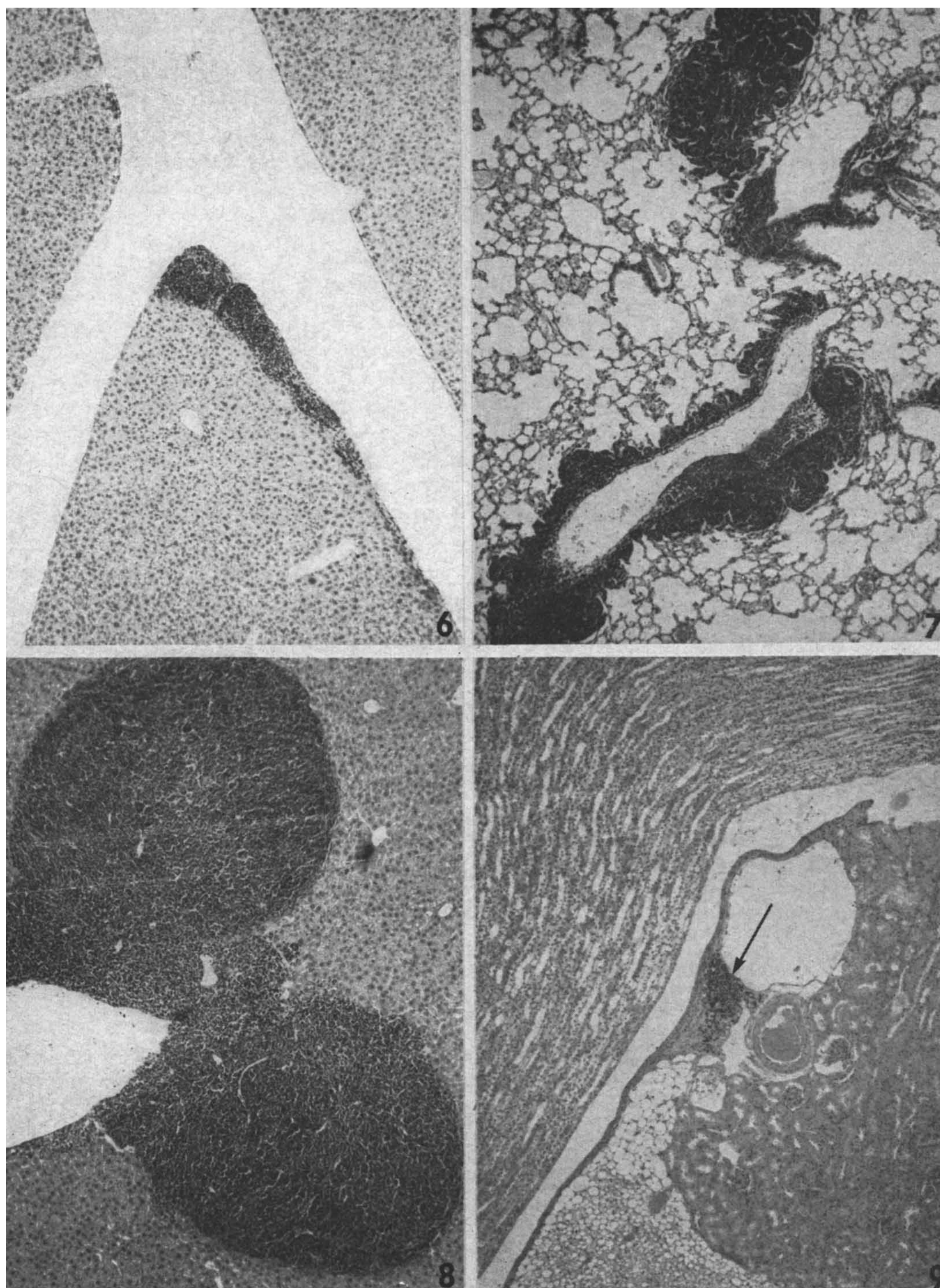

FIG. 6. Perivascular lymphoreticulocytic lesion in liver of 200-day-old retired breeder. × 65 (hematoxylin and eosin).

FIG. 7. Heavy sarcomatous cuffs around pulmonary venules in 300-day-old retired breeder. Cuff is primarily reticulocytic. × 65 (hematoxylin and eosin).

FIG. 8. Perivascular sarcomatous lesion in liver of 300-day-old retired breeder. × 65 (hematoxylin and eosin).

FIG. 9. Small peripelvic lymphoreticulocytic focus in 200-day-old retired breeder (arrow) × 65 (hematoxylin and eosin).

Storer(26) determined that the C57BL/6 Jax had a mean life expectancy of ~700 days.

As with other mouse-care techniques the system described does not exclude the presence of any agents transmitted vertically, as suggested by the leukemia-like lesions described, or of those agents for which tests are not available. These studies likewise suggest that commercial feed and bedding may be uncommon sources of contamination by pathogenic agents. Furthermore, it appears that conventional cage-and-bottle-washing equipment may also be adequate.

It is expected that more experience with this system will provide information on the minimum level of colony monitoring necessary to detect and eradicate infections should they occur. The filter-cage system is also used in our laboratory to isolate experimentally infected mice, and to restrict the dissemination of allerogenic animal dander.

Summary. By employing conventional husbandry techniques and filter-capped cages, a breeding colony of mice from Caesarian-derived stock was maintained free from many infectious agents common to laboratory mice through seven generations. The system described prevented infections with pathogenic bacteria, viruses, parasites, and PPLO. Mice housed under these conditions remained healthy and fecund while being free of the morphological and physiological consequences of germfree environment. In addition, the expense and restrictions of conventional barrier-type operations were minimized.

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