Koprowski, H., PROC. SOC. EXP. BIOL. AND MED., 1961, v107, 829.

2. Dubes, G. R., Wenner, H. A., *Virology,* 1957, v4, 275.

3. Koprowski, H., Norton, T. W., Jervis, G. A., *Bact. Proc.,* 1951, v92 (abst.).

4. Koprowski, H., Jervis, G. A., Norton, T. W., *Am. J. Hyg.,* 1952, v55, 108.

5. Koprowski, H., Jervis, G. A., Norton, T. W., Nelson, D. J., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 227.

6. Dane, D. S., Dick, G. W. A., Connolly, J. H., Fisher, O. D., McKeowan, F., *Brit. M. J.,* 1957, v1, 59.

7. Koprowski, H., *Proc. of 2nd Internat. Conf. Scientific Publication 50, Pan American Health Organization,* Washington, Sept. 1960, p. 5.

8. Eagle, H., *J. Exp. Med.,* 1955, v102, 595.

9. Reed, L. J., Muench, H., *Am. J. Hyg.,* 1938, v27, 493.

10. Carp, R. I., Koprowski, H., *Virology,* 1962, v16, 371.

11. Hayflick, L. H., Moorhead, P., *Exp. Cell Research,* 1961, v25, 585.

12. Hayflick, L. H., Plotkin, S. A., Norton, T. W., Koprowski, H., *Am. J. Hyg.,* 1962, v75, 2, 240.

13. Plotkin, S. A., Koprowski, H., Richardson, S. N., Stokes, J., Jr., *Acta Ped.,* 1960, v49, 551.

14. Carp, R. I., Plotkin, S. A., Cohen, B. J., LaLiberte, J., Koprowski, H., PROC. SOC. EXP. BIOL. AND MED., 1963, v112 —

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Oncogenic Effect of Methylcholanthrene in New-Born Germfree Mice.* (28009)

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Adenomas have appeared in the lungs of mice following inoculations with urethane and other carcinogenic chemical compounds(1-4). The mechanisms whereby such pulmonary neoplasms developed from remotely inoculated carcinogenic stimuli are not clear. Kelly and O'Gara(5) induced pulmonary adenomas rapidly by inoculating methylcholanthrene (MCA) subcutaneously into new-born mice. The response to MCA was quantitated by counting the tumors on and in each sectioned lung as observed by low-power microscopy.

Investigators have demonstrated clearly the involvement of viruses in tumor formation: some, such as leukemia, polyoma, papilloma, avian lymphoma, myeloblastosis, and sarcoma viruses exert a direct oncogenic effect on the host cell(6). Other viruses (adenovirus 12 and simian virus 40) are associated primarily with non-neoplastic lesions, but under special conditions they do elicit tumorous lesions in other species of animals(7,8). Virus-like inclusions have been observed in transplanted tumor cells of animals, which were initiated by carcinogenic chemical compounds (9,10); however, the role of such inclusions in tumor formation is uncertain. Perhaps the chemically-induced undifferentiated tumor cells provide a milieu more suitable for virus propagation than cells elsewhere in the body, and thus they manifest a selective proclivity for such cells.

Viruses can induce tumors in animals; however, there is uncertainty whether, as a group, they are the exclusive cause of such lesions. Tumors have been induced in rodents by numerous well-defined chemical agents, by physical agents, and by induced endocrine imbalances(11). All of the experiments described to date were performed in conventional animals. Since such animals are now known to harbor a wide range of viruses, some being carcinogenic, the mechanism(s) of oncogenesis by the above noted non-viral agents need clarification. The most direct approach to this problem would require the use of virus-free animals. Mice have been propagated and maintained in Lobund Laboratory under germfree conditions through 19 serial genera-

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tions. Bacteria, fungi, and parasites have not been detected in them. Tests for viral flora in such animals are still in progress. Since viruses may show some selective localization in tumor cells, 2 determinations may be attained through induction of tumors in germfree animals with MCA: (a) the ability of such animals to develop tumors, and (b) the viral flora of such tissues. MCA has been inoculated into germfree new-born mice and the lesions which developed subsequently are described here.

Methods and materials. As background to the use of germfree rodents in this study, 100 randomly-selected adult mice were tested serologically for polyoma virus, with negative results. Cells cultured from embryos and from kidneys appeared normal microscopically. The germfree animals were maintained in flexible plastic isolators and fed *ad libitum* on sterile diet during the experiment(12). They were negative for bacteria by frequent tests using standard bacteriological technics(13) performed at regular intervals during the experiments. All of the germfree animals had an enlarged cecum.

3-Methylcholanthrene‡ was dissolved in olive oil, and sterilized in glass-sealed ampoules by dry heat at 165°C for 3 hours. The inoculum was introduced into the sterile isolator through a lock which had been sterilized by a spray containing 2% peracetic acid.

Sixty-one non-inbred mice (Swiss-Webster strain) of the nineteenth generation in germfree environment were inoculated subcutaneously, in the interscapular region, with 0.1 mg of MCA in olive oil. The animals were inoculated as soon after birth as possible, and at the latest under 18 hours of age. Fifty-four conventional mice of the same strain and age received the same inoculum, but were maintained at all times in an open animal room. Control animals, germfree and conventional, were inoculated with the oil vehicle, or they were not inoculated.

Of 12 litters of germfree mice inoculated, 7 were examined 8 weeks later; 3 at 10 weeks and 2 at 15 weeks after inoculation. Each animal was killed by cervical section, the lungs were quickly exposed and inflated with Zenker's fixative introduced through the trachea by syringe and needle. The lungs were fixed in Zenker's fluid for 24 hours, washed in tap water for 24 hours, and stored in 70% ethanol. The lungs were examined by low power magnification (3×) and all surface tumors were counted. Selected areas of each lung were dehydrated and imbedded in paraffin; sections were stained with hematoxylin and eosin and examined microscopically. Before fixing the lung in Zenker's solution, selected areas of tumorous tissue were excised, fixed in osmic acid, and embedded in epon. Ultrathin sections were stained with uranyl acetate for 1 hour and lead according to Karnovsky Method A(14), and examined with an electron microscope (RCA EML). The cells were screened carefully for evidence of viral particles. Each animal was examined for evidence of lesions in extrapulmonary areas.

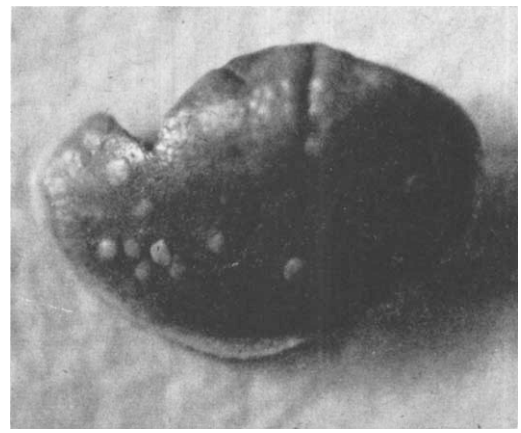

FIG. 1. Multiple adenomas in lung of germfree mouse at 10 wk after subcutaneous inoculation of 0.1 mg methylcholanthrene.

Results. Forty-seven (78%) of the germfree and 42 (83%) of the conventional mice survived the effects of MCA inoculation. One germfree mouse showed dyspnea at 15 weeks, due to an enlarged thymus. In all the germfree mice, circumscribed, white, vesicular tumorous foci were observed in the lung parenchyma, many of which were projected against the pleurum, above the surface of the lung (Fig. 1). The lesions ranged from 0.1 to 3 mm in diameter. The tumors visible on the surface of the lung were counted and recorded (Table I). More tumors were observed in the lungs of germfree than in conventional mice. All inoculated germfree animals had lung adenomas; 11% of the conven-

‡ 3-Methylcholanthrene, Eastman Kodak Co.

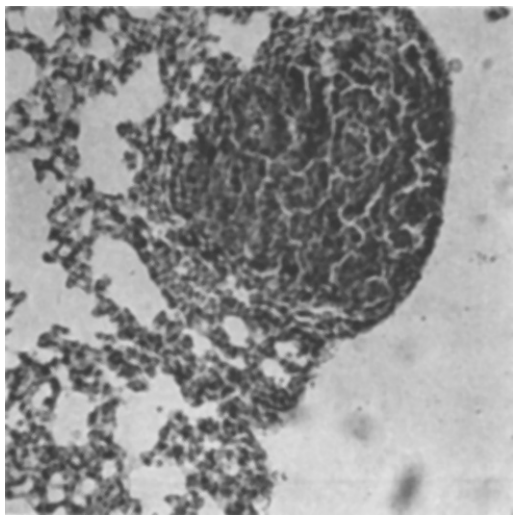

FIG. 2. Adenoma in lung shown in Fig. 1. Hematoxylin and eosin stain, ×100.

tional mice were tumor free. Microscopic examinations of affected lungs revealed focal areas of adenomatous structure in all areas of the lungs. The majority of lesions were located at the lung surfaces; however, they varied in size from those barely detected as thickened prominent alveolar septa to round dense masses of enlarged cells. The histopathology of lung lesions was the same as that described previously (4,5). Mitoses were rare, and metastases were not observed. The lung lesions in conventional and in germfree mice could not be differentiated morphologically. The lungs of mice inoculated 10 and 15 weeks previously contained increased numbers of adenomas, many of which were confluent; however, the histopathology was unchanged. At 15 weeks, individual germfree mice contained in excess of 300 tumors per lung.

TABLE I. Development of Adenomas in Lungs of Mice Inoculated at Birth with Methylcholanthrene.

	Weeks after inoculation	Surface tumors per lung	Avg
Germfree	8	11, 3, 12, 17, 21, 29, 19, 26, 20, 32, 70, 61, 15, 42, 13, 13, 22, 7, 1, 8, 2, 10, 144, 108, 85, 89, 79	35
	10	30, 12, 25, 20, 80, 75, 123, 25, 19	45
	15	200, 203, 300, 310, 263, 100, 60, 55, 133, 177, 67	169
Conventional	8	3, 1, 3, 9, 3, 3, 6, 4, 11, 8, 21, 151, 131, 10, 58, 26, 148, 0, 0	22
	10	1, 44, 3, 8, 60, 52, 148, 0, 0, 140, 60, 58, 3, 140, 5, 26, 80, 17, 102, 53, 0, 23, 35, 4, 2, 17	41

The lungs of uninoculated 8-week-old germfree and conventional mice were free of lesions, as were the lungs of mice which had been inoculated only with the oil vehicle.

Neither viral inclusions nor any other suggestive structures have as yet been detected in the adenoma cells by electron microscopy.

Discussion. Animals may respond to carcinogenic stimuli with lesions which may eventually appear as a consequence of senescence (4). The occurrence of lung lesions in aging Swiss-Webster germfree mice has not yet been determined. White Swiss mice manifest a predisposition for lung tumors; possibly the MCA stimulus accelerates evolution of such lesions. Conventional mice had a greater range of lung tumor counts (0-151) than the germfree animals. As high as all tumor counts were, they would have been higher if the lesions in the central areas of the lung were included.

Viral agents have not been detected in the germfree colonies of mice at Lobund Laboratory, but we cannot preclude their existence, either masked, latent, or otherwise indistinguishable by conventional technics. The search for viral contaminants continues by methods involving cytology, serology, tissue cultures, animal inoculations, and electron microscopy. The data reported here may be interpreted in two ways: (a) that tumors can develop without viral agents, or (b) if the latter are required they must be sought out. Thus far viral agents have not been detected.

The pulmonary adenomas induced by MCA are morphologically benign. If an adenoma is interpreted as a "pre-cancerous" lesion, would a superimposed viral infection provide the stimulus for development of malignancy? If such is the prospect, why does it not occur in conventional mice, in view of the reported frequent detection of viruses in colonies of so-called "normal" mice? Perhaps the time sequence of exposure is inconsistent with such a cell transformation.

How can the apparent acceleration of adenoma-formation in "germfree" animals be explained? Can the poorly developed reticuloendothelial system of such mice be related to increased susceptibility to tumorigenesis? Data accumulated thus far indicate that subcutaneously-inoculated MCA induces localized fibrosarcomas more rapidly in germfree than in conventional adult rats and mice(15). Possibly the strains of germfree mouse and rat tested are more uniformly susceptible to MCA than other strains. Do the results here reported suggest that the microbial flora in the conventional animal may induce some relative state of resistance or interference to the carcinogenic mechanism? The germfree animal provides a mechanism for fruitful exploration of carcinogenic stimuli and the alterations which they induce in the host.

Summary. Methylcholanthrene was inoculated subcutaneously into new-born germfree and conventional mice (Swiss-Webster strain). Pulmonary adenomas were thereby induced more frequently and in greater numbers in the germfree animals. Viral agents have not been detected in the adenoma tissues by electron microscopy.

1. Low, L. W., *Science,* 1940, v91, 96.
2. Larsen, C. D., *J. Nat. Cancer Inst.,* 1947, v8, 63.
3. Shimkin, M. B., *Arch. Path.,* 1940, v21, 479.
4. Smith, W. E., Rous, P., *J. Exp. Med.,* 1948, v88, 529.
5. Kelly, M. G., O'Gara, R. W., *J. Nat. Cancer Inst.,* 1961, v26, 651.
6. Gross, L., *Oncogenic Viruses,* 1961, Pergamon Press, New York.
7. Trentin, J. J., Yabe, Y., Taylor, G., *Science,* 1962, v137, 835.
8. Eddy, B. E., Borman, G. S., Berkeley, W. H., Young, R. D., PROC. SOC. EXP. BIOL. AND MED., 1961, v107, 191.
9. Oberling, C., Guerin, M., *Bull. du Cancer,* 1950, v37, 5.
10. Granboulan, N., Riviere, M. R., Bernhard, W., *ibid.,* 1960, v7, 291.
11. Shubik, P., Hartwell, J. L., *U. S. Dept. H. E. W., P. H. Service Publ. 149,* Suppl. 1, 1957.
12. Trexler, P. C., Reynolds, L. I., *Appl. Microbiol.,* 1957, v5, 406.
13. Wagner, M., *Ann. N. Y. Acad. Sci.,* 1959, v78, 29.
14. Karnovsky, M. J., *J. Biophys. Biochem. Cytol.,* 1961, v11, 729.
15. Pollard, M., unpublished material.

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Cholesterol Content of Human Serum Lipoproteins Obtained by Dextran Sulfate Precipitation and by Preparative Ultracentrifugation.* (28010)

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The formation of insoluble complexes between lipoproteins and various sulfated polysaccharides was described by Bernfeld(1), Oncley(2), and Burstein and Samaille(3). The subject was thoroughly reviewed by Cornwell and Kruger(4).

Although it is generally assumed(4) that sulfated polysaccharides precipitate β and lower density lipoproteins ($d<1.063$ g/ml), no comparison of the cholesterol content of the fractions obtained by precipitation and by preparative ultracentrifugation of human serum has been reported. Recently Sakagami and Zilversmit(5) compared the dextran sulfate precipitable lipoprotein in sera from dogs with those obtained by ultracentrifugation and indicated that this method could be used as a means of quantitatively precipitating low density lipoproteins. Advantage was taken, therefore, of an opportunity to compare the cholesterol content of ultracentrifugally isolated α lipoproteins ($d>1.063$ g/ml)

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