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TERMINAL PROGRESS REPORT: GRANT NO. 3R01 EC-00226-04S1

TITLE: MESOTHELIOMA IN RELATION TO ASBESTOS

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PERIOD: 03/01/66 - 08/31/70

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STATEMENT OF PROGRESS:

The aim of this project was to test 23 preparations of asbestos or other minerals for carcinogenicity by means of intrapleural injections into hamsters. Each sample was tested in a group of 50 hamsters. In addition, 2 groups of untreated control hamsters were followed for incidence of spontaneous tumors and other pathology. Ancillary experiments and observations are described at the end of this report.

All hamsters used for this work were LVG:LAK males obtained as weanlings from Lakeview Hamster Colony, Newfield, N.J.. When the hamsters reached a body weight of 100 ± 10 gms (about 2 months of age) each hamster to be treated was given a single injection into the right pleural cavity. Each injection consisted of 0.5 ml of a suspension of the selected material in 0.9% Na Cl solution.

The animals were followed to maximum periods of 650 or, where shown, 700 days after injection unless earlier spontaneous death occurred or unless they appeared moribund

and were sacrificed. At death, necropsies were performed which were complete except for the head. Gross observations were recorded. Thoracic contents were fixed in formalin as were pertinent lesions found elsewhere. Microscopic sections were cut and stained with hematoxylin and eosin. For recording and retrieval of data, we designed and used a punch card for individual hamsters as illustrated in Fig. 1.

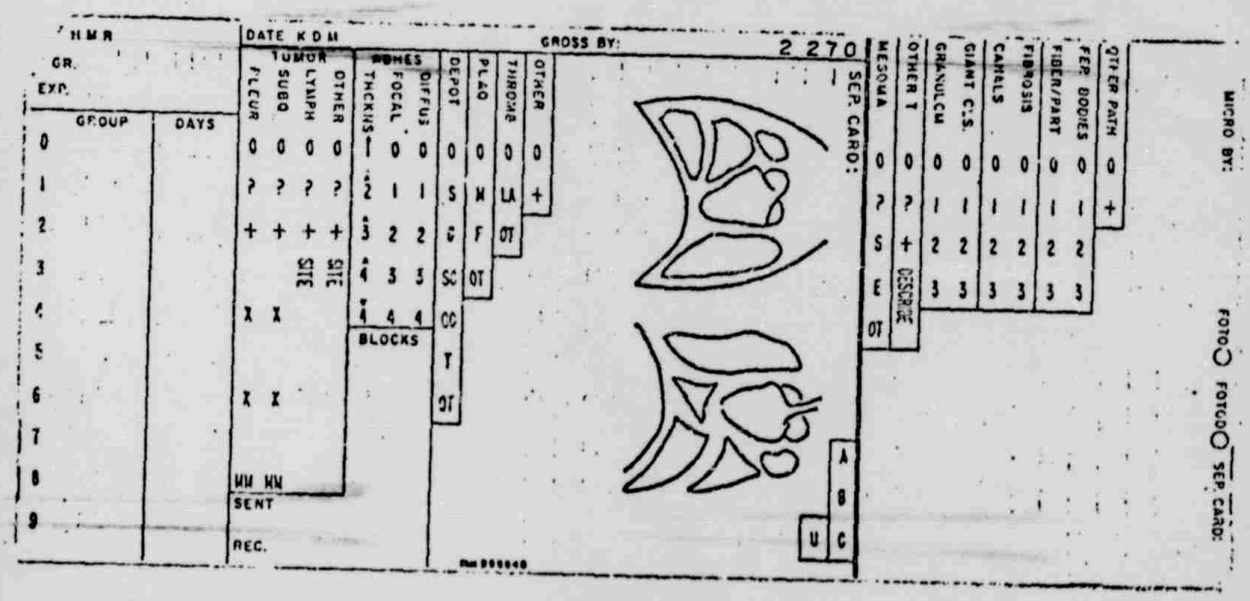


Fig. 1. Punch card used to record findings on individual hamsters.

At necropsy, the prosector entered identification and date of death of the individual animal on this card, punched the number of days on experiment, presence or absence of pleural adhesions, character of adhesions

(focal or diffuse), presence or absence of depots, pleural plaques, cardiac thrombi or other pathology. Gross findings in the thorax were then sketched on diagrams shown in the center of the card. Histopathologic findings were subsequently entered in the spaces provided to the right of the double line with special reference to diagnosis of any tumors, to characteristics of pleural adhesions, presence or absence of mineral particles, ferruginous bodies or other pathology. Where additional descriptions of gross or microscopic pathology were warranted, these were entered on a separate blank card.

All animals in these experiments have been examined at necropsy except for rare cannabalized individuals. Histopathologic studies have now been made on tissues saved from all animals except for rare autolyzed individuals. All tumors diagnosed as mesotheliomas have been confirmed microscopically. No tumors suggesting mesothelioma in the gross were in animals where autolysis precluded histopathologic study. Tumors listed in this report as mesothelioma resembled those illustrated and described in our previous work with intrapleural injection of chrysotile and amosite into hamsters. (Smith, W.E., L. Miller, R.E. Elsasser and D.D. Hubert. Tests for Carcinogenicity of Asbestos, Annals N Y Acad Sci 132 (Art 1):456-488, 1965), (Smith, W.E., L. Miller, J. Churg, and I.J. Selikoff, Mesotheliomas in Hamsters Following

Intrapleural Injection of Asbestos. Jour Mt Sinai Hosp (N Y) 32:1-8, 1965) Findings from the present experiments are summarized in Table 1.

Tumors listed in Table 1 under the heading "Mesothelioma" were with few exceptions large masses in the pleural space. They compressed but rarely invaded the lungs. Microscopically, they were composed of elongated or rounded cells that appeared malignant. Under a separate heading of "Lymphoid Tumors", Table 1 lists lymphomas, lymphosarcomas or reticulum cell sarcomas that were found in occasional animals of the various groups. These "Lymphoid Tumors" occured in mediastinal or mesenteric nodes, sometimes with leukemic features. Injection site sarcomas, also listed separately in Table 1, were tumors found subcutaneously on the chest wall in the area where intrapleural injection had been made. Mineral particles of the test samples were usually seen in microscopic sections of injection site sarcomas.

From yields of tumors designated as mesotheliomas in Table 1, information emerged on the following points:

1. The yield of mesotheliomas in response to 3 dose levels of chrysotile (Groups 2,7,8), 2 dose levels of amosite (Groups 9,10) and 2 dose levels of crocidolite (Groups 18,19) is dose related, hence

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hazards should be controllable by appropriate reductions in exposure (1). Chrysotile, amosite and anthophyllite (Groups 20,21) appear less carcinogenic than crocidolite.

2. Closely comparable yields of mesotheliomas resulted from soft (Group 1) and harsh (Group 2) chrysotile fibers prepared from Arizona ores, and from the same soft fibers heated to reduce their content of molecular water to the level of harsh fibers (Group 3). The tumors arose more rapidly in response to native harsh fibers (Table 2). Studies of pleural adhesions revealed that harsh chrysotile induced thicker and denser scars than soft chrysotile. Although heat treatment of soft chrysotile gave no appreciable enhancement of carcinogenicity, it did enhance fibrogenicity (2).

3. At the highest dose level tested (25mg), 3 samples of chrysotile with mean fiber lengths between 32 and 39 microns yielded 8, 9 and 10 mesothelioma-bearing animals (Groups 1,2,3). When fiber lengths of these chrysotiles were reduced to an extent where the large majority of fibers were less than 5 microns in length, fibrogenicity was reduced and carcinogenicity was eliminated (Groups 4,5,6).

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The above long fiber samples were prepared from ores by a wet grinding method, then sieved and washed by a technique described from this laboratory (Badollet, M.S., and W.A. Gantt. Preparation of Asbestos Fibers for Experimental Use. Annals of N Y Acad Sci 132 (Art 1):451-455, 1965). Fibers thus prepared were ground in a Wig-L-Bug to produce the short fiber samples. The Wig-L-Bug process was monitored by phase microscopy and stopped before destruction of the fibrous nature of the material.

These samples were furnished to Dr. J. Leineweber at Johns-Manville Research & Engineering Center for electron micrography. From his photographs it was learned that the long fiber samples contained relatively few fibers less than 5 microns in length, whereas the short fiber samples contained relatively few fibers more than 5 microns in length. Dr. Leineweber noted that the short fiber samples had not been reduced to amorphous material but preserved the fibrous structure of chrysotile. Further discussion of length measurements is offered later in this report.

4. Tests of long and short fiber anthophyllite gave results consistent with an hypothesis that reduction in fiber length reduced carcinogenicity,

though findings were not statistically significant. These samples were not tested at the 25mg level. At the 10mg level, long fiber anthophyllite induced 3 mesotheliomas (Group 20), short fiber anthophyllite induced 1 mesothelioma (Group 21).

The short fiber sample was prepared by grinding the long fibers in a Wig-L-Bug, the process being stopped before the fibrous nature of the material was destroyed.

5. At the 10 mg dose level, mesotheliomas resulted from a sample of microquartz with mean fiber length of 56.7 microns (Group 12), but not from glass fibers with or without phenol-formal binder (Groups 16, 22). Mean fiber lengths of these two samples were 24.9 and 16.9 microns, respectively.

6. At our highest (25mg) dose level, mesotheliomas were induced in hamsters by diatomaceous earth and by calcined diatomaceous earth, but not by talc, nemalite or, except for a single case, by serpentine rock dust (Groups 15, 15A, 14, 13, 11). The sample of talc contained a large amount of tremolite.

7. Mesotheliomas were induced by powdered nickel tested at a 10mg dose level. The yield of mesotheliomas in response to nickel was closely comparable to the yield

obtained with the same dose level of chrysotile and of amosite, and less than the yield obtained with that level of crocidolite.

8. No mesotheliomas have been found in the untreated control groups.

9. Lymphoid tumors occurred in 14 of the 23 treated groups, and in 1 of the 2 untreated control groups. These tumors were regarded as spontaneous tumors unrelated to treatment.

10. Tumors of the adrenals were common in both untreated control groups and in treated groups. Hepatomas were found in 2 control and in 4 treated animals. Adenocarcinoma of the intestine was found in single animals in groups 2,6, 13, 15, 16 and 18.

11. Thrombi in the left cardiac atrium were a common cause of death in hamsters in our experiments. Such lesions occurred in untreated control groups, but in some of the treated groups they were found earlier and more frequently, as shown in Table 2 of addendum A (3).

Computer programming and analyses of data from necropsy cards for animals in groups shown in Table 1 has been done by Dr. Thomas Hayton, our Consultant in Statistics. For each

group, he has made life table analyses broken down into 100 and 50 day intervals as follows:

Li = number of hamsters at start of period

Di = dead in period

Vi = lost in period

Wi = withdrawn in period

Ei = effective number at risk in the period = $Li - \frac{1}{2}(Di + Vi + Wi)$

Qi = probability of dying in the period

Pi = probability of surviving in the period

Ri = survival rate

Si = standard deviation

From these analyses, the group size used for tests in Table 1 (50 hamsters per group) seems satisfactory for comparison of relative carcinogenicity of the tested samples in the 100 day periods.

Dr. Hayton's report on his statistical studies is attached as Addendum A.

In the preceding text, fiber lengths cited for our samples 1 and 2 (Table 1) were determined by light microscopy using a 40X objective and 10x ocular. These measurements were made in the laboratory of the Occupational Health Program, Dept. of Health, State of New Jersey, at the start of our tests of these samples in animals. Also at the start of our animal tests, very similar measurements were reported to us for

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these samples by the U.S.P.H.S. Occupational Health Research & Training Facility in Cincinnati where 40X objective, 8X ocular and phase contrast illumination were used. Recently, these samples were furnished to Johns Manville Research and Engineering Center, Manville, N. J., where they were studied with a 12X objective and a 5X ocular by dark field microscopy, images being projected to a final magnification of 100X, photographed, then enlarged 2½X for measurement of lengths and diameters. Mean lengths reported under these latter conditions were considerably longer. Measurements of samples studied under the 3 optical conditions are shown in Table 3.

Also shown in Table 3 are measurements made on these and other samples by electron micrography at JMR&E. For EM work, the original photograph was made at 2000X and was then enlarged 2½X for measurements. Fibers extending beyond the EM field were excluded from measurements, hence mean lengths reported in Table 3 for samples 1 and 2 are much smaller than mean lengths reported for those samples by optical microscopy. In EM photographs, fibers less than 5 microns in length were relatively scarce in samples 1 and 2, which were samples that proved highly carcinogenic in hamsters.

In samples 4,5 and 6 which proved non-carcinogenic in hamsters, most fibers were too short for meaningful measurement by optical methods. EM measurements of sample 4 gave mean fiber length of 0.76 micron. EM photographs of samples 5 and 6 look about the same as sample 4, but have not yet been measured.

Table 1. FINDINGS IN HAMSTERS AFTER INTRAPLEURAL INJECTION
50 hamsters per group
(Cumulative data to 650 days except where noted)

SAMPLE Group No.		MEAN FIBER LENGTH microns	DOSE mg.	MESOTHELIOMA	SARCOMA INJECTION SITE	LYMPHOID TUMORS	REMARKS
1	SOFT CHRYSOTILE	32.54	25	8	2	1	
2	HARSH CHRYSOTILE	38.85	25	9	3	1	
3	SOFT CHRYS. (Heated)	32.54	25	10	0	0	
4	SOFT CHRYSOTILE	.76	25	0	0	2	700 days
5	HARSH CHRYSOTILE	1 (approx.)	25	0	1	3	700 days
6	SOFT CHRYS. (Heated)	1 (approx.)	25	0	1	0	700 days
7	HARSH CHRYSOTILE	38.85	10	4	1	1	
8	HARSH CHRYSOTILE	38.85	1	0	1	0	700 days
9	AMOSITE	17	10	3	2	0	
10	AMOSITE	17	1	0	0	0	
11	SERPENTINE ROCK DUST (ANTIGORITE)		25	1	0	0	700 days
12	MICROQUARTZ FIBERS	56.7	10	4	1	3	
13	NEMALITE	53.1	25	0	0	2	

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Table 1 (Cont.) FINDINGS IN HAMSTERS AFTER INTRAPLEURAL INJECTION
50 hamsters per group
(Cumulative data to 650 days except where noted)

Group No.	SAMPLE	MEAN FIBER LENGTH microns	DOSE mg.	MESOTHELIOMA	SARCOMA INJECTION SITE	LYMPHOID TUMORS	REMARKS
14	TREMOLITE TALC		25	0	0	0	700 days
15	DIATOMACEOUS EARTH		25	3	2	2	
15A	DIATOMACEOUS EARTH (Calcined)		25	4	2	1	
16	FIBERGLASS WITH PHENOL FORMAL BINDER	24.9	10	0	0	5	
17	NICKEL POWDER	8	10	4	4	0	
18	CROCIDOLITE U.I.C.C.		10	10	5	1	
19	CROCIDOLITE U.I.C.C.		1	2	0	4	700 days
20	ANTHOPHYLLITE	Long (UICC)	10	3	0	2	
21	ANTHOPHYLLITE	Short (3.7)	10	1	0	0	
22	GLASS FIBERS	16.9	10	0	0	5	
23	UNTREATED		none	0	0	0	700 days
24	UNTREATED		none	0	0	4	660 days (survivors are being followed to 700 days)

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TABLE 2. YIELD OF MESOTHELIOMAS IN HAMSTERS AT SITES OF INJECTION OF CHRYSOTILES

Group	Sample	151 days	340 days	372 days	450 days	500 days	550 days	600 days	650 days
2	B2 (Harsh)	1/41	4/26	4/21	7/16	7/13	7/9	8/5	9/2
1	C2 (Soft)	0/44	0/33	1/30	1/20	4/11	4/9	7/3	8/1
3	C2H1 (Heated Soft)	0/37	1/35	1/32	1/27	4/17	5/15	8/4	10/1

Numerator: Cumulative number of hamsters having mesotheliomas.

Denominator: Number of surviving hamsters.

N. B. All survivors at 650 days were killed on that day. Pleural adhesions, but no tumors, were found in them. For statistical treatment, see Addendum A.

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Table 3. Size Measurements of Selected Samples.
(For measurements of other samples see Table 1)

Sample*	Lab+	OPTICAL MICROSCOPY Mean fiber length microns	ELECTRON MICROSCOPY Mean fiber length microns
1	A	30	
	B	32.54	
	C	52.5	6.9
2	A	30	
	B	38.85	
	C	63.3	5.3
4	C	#	0.76
12	C	56.7	7.38

* Sample nos. same as those in Table 1

+ Lab A: 40X objective, 8X ocular (phase contrast)
Lab B: 40X " 10X " (dark field)
Lab C: 12X " 5X " " "

Fiber lengths of sample 4 were too small for measurement by optical methods.

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Table 3. Size Measurements of Selected Samples.
(For measurements of other samples see Table 1)

Sample*	Lab+	OPTICAL MICROSCOPY Mean fiber length microns	ELECTRON MICROSCOPY Mean fiber length microns
1	A	30	
	B	32.54	
	C	52.5	6.9
2	A	30	
	B	38.85	
	C	63.5	5.3
4	C	#	0.76
12	C	56.7	7.38

* Sample nos. same as those in Table 1

+ Lab A: 40X objective, 8X ocular (phase contrast)
Lab B: 40X " 10X " (dark field)
Lab C: 12X " 5X " " "

Fiber lengths of sample 4 were too small for measurement by optical methods.

For optical and EM studies of these samples at JMRcE we are indebted to Drs. James Leineweber, Alan Burns, Don Bailey and Tom Brown.

Samples of soft and harsh chrysotile prepared in our laboratory from Arizona ores were sent to Dr. Cralley in 1967 for analysis of trace elements by atomic absorption spectroscopy. The following report was received from him on these samples, which were the "long fiber" samples that subsequently proved highly carcinogenic in our intrapleural tests in hamsters (Groups 1 and 2).

Sample No.	Material	Microgram element/ gram sample			
		Ni	Cr	Co	Mn
B2b	Chrysotile (harsh)	N.D.	N.D.	N.D.	420
C2c	Chrysotile (soft)	N.D.	N.D.	N.D.	190

From these results, it can be seen that no detectable amounts of nickel, chromium or cobalt were found in the samples as injected into hamsters.

Subsequently, Dr. Cralley's group developed a microsieve method for separating "longer than 10 micron" (+10μ) and "Less than 10 micron (-10μ) fractions of asbestos and found certain trace elements in higher concentration in the short fractions. In 1969, we resubmitted our samples to Dr. Cralley. They were fractionated by the microsieve method in his laboratory and he has reported the following analyses:

Sample	Microgram element/ gram sample						
	Co	Cr	Mn	Ni	Ca	Mg	Fe/g sample
Harsh Ariz. +10μ	<10	<10	595	<10	710	8.8	
chrysotile -10μ	<60	414	480	230	1800	8.7	
Soft Ariz. +10μ	<80	<9	310	<9	185	4.6	
chrysotile -10μ	<50	157	230	46	118	2.9	

In these analyses, the symbol < means not detectable at the stated limit of sensitivity of tests. From these data, it is seen that detectable amounts of chromium and nickel were not found in the +10μ fractions but were found in the -10μ fractions. The -10μ fractions constituted 6 to 7% by weight of the samples. These fractionations were made on our "long" (32-39 mean fiber length) samples that proved highly carcinogenic in hamsters. If trace elements in the "less than 10 micron" fractions were important in carcinogenesis, then high carcinogenicity would have been expected in our "short" (1-5 micron) preparations of these ores, but such was not the case, our short preparations proving essentially non-carcinogenic.

The following studies were not part of the aims stated for this project in our original proposal but were carried out as ancillary work:

a. In a previous project, we had carried out life-span studies on hamsters given repeated intratracheal injections of chrysotile with and without benzo(a)pyrene. Histo-

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pathologic studies of lungs from those animals were completed in the course of the present project with results presented in an attached publication (4).

b. Hamsters given a single intratracheal injection of chrysotile were sacrificed at intervals for examination of lungs by electron micrography with results described (5), (6), (7).

c. For chemotherapeutic trials, a fast-growing mesothelioma induced by chrysotile and a slow-growing mesothelioma induced by crocidolite have been established and are being carried in serial transfer in new hosts (hamsters).

d. Squamous cell carcinomas and adenocarcinomas were induced from mouse pulmonary tissue exposed to chrysotile (8). For chemotherapeutic trials, 2 lines of these pulmonary squamous cell carcinomas and one line of pulmonary adenocarcinoma have been established and are being carried in serial transfer in new hosts (RALB/c mice).

LIST OF RESULTS

1. After single intrapleural injection of hamsters, tumors with gross and microscopic characteristics of pleural mesotheliomas were found after injection of soft chrysotile, harsh chrysotile, amosite, crocidolite, anthophyllite, diatomaceous

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earth, nickel powder and microquartz fibers, but not after injection of tremolite talc, nemalite or glass fibers. Serpentine rock dust (antigorite) induced only a single case of mesothelioma.

2. Native harsh chrysotile induced tumors more rapidly than soft chrysotile. Heat treatment of soft chrysotile increased fibrogenicity but not carcinogenicity.

3. With reduction in dose, there was reduction in fibrogenic and carcinogenic response to chrysotile, amosite and crocidolite. Hence, hazards associated with exposures to asbestos should be controllable by measures to reduce amounts of exposure.

4. At the lowest dose level tested (1mg), no tumors arose in response to chrysotile or amosite, but tumors were found in response to crocidolite.

5. With each of 3 samples of chrysotile, treatment to reduce fiber length reduced fibrogenic responses and eliminated carcinogenic responses. Electron microscopy revealed that the treated samples were fibrous in nature and had not been reduced to amorphous material. These findings afford information pertinent to design of masks and to monitoring of dusts for industrial hygiene purposes.

6. Polyvinylpyridin-N-oxide did not inhibit fibrosis induced by asbestos injected intrapleurally into hamsters.

7. After intratracheal injection of chrysotile into hamsters, electron micrography revealed phagocytosis of asbestos fibers

not only by macrophages but also by epithelial cells. Changes in fibers and in epithelial cells were described.

8. After intratracheal injection of benzo(a)pyrene plus chrysotile into hamsters, the incidence of lung tumors was greater than after benzo(a)pyrene alone.

9. After exposure of fetal mouse lung tissue to chrysotile by tissue transplant technique, squamous cell carcinomas and adenocarcinomas resulted.

10. For chemotherapeutic trials, two lines of transplantable mesotheliomas were established in serial transfer in hamsters, two lines of pulmonary squamous cell carcinomas and one line of pulmonary adenocarcinoma were established in serial transfer in mice.

11. Statistical treatment of responses of hamsters to intrapleural injection of tested minerals is described.

12. Additional manuscripts are in preparation for publication.

PUBLICATIONS (10 copies of each are attached):

1. Smith, Wm.E., D.D. Hubert, L. Miller, M.S. Badollet and J. Churg. Tests for Threshold Levels of Carcinogenicity of Asbestos. II Internation. Conf. on Biol. Effects of Asbestos, Dresden, German Democratic Republic, 1968, in press. 2646

2. Badollet, M.S., Physical Properties and Technical Applications of Asbestos Minerals. Canad Mining & Metal Bull, 62:432-437, April 1969.

3. Hubert, D.D., R. Zaldivar and Wm. E. Smith, Increased Incidence of Cardiac Thrombosis in Hamsters Treated with Asbestos and Talc. Am J Path 55:31a, 1969.

4. Smith, Wm.E., L. Miller and J. Churg. An Experimental Model for Study of Co-carcinogenesis in the Respiratory Tract. Atomic Energy Commission Symposium Series, in press. 3336

5. Suzuki, Y., J. Churg and Wm. E. Smith. Development of Asbestos Bodies in Experimental Asbestosis. Amer J Path 52:20a, 1968

6. Suzuki, Y., J. Churg and Wm. E. Smith, Phagocytosis of Asbestos. Laboratory Investigation 18:335, 1968.

7. Suzuki, Y., J. Churg and Wm. E. Smith, Behavior of Alveolar Epithelial Cells in Experimental Asbestosis. II Internation Conf. on Biol. Effects of Asbestos, Dresden, German Democratic Republic, 1968, in press. 1680

8. Smith, Wm. E. and E. Yazdi, Induction of Carcinomas from Mouse Lung Transplanted with Asbestos. Amer Assoc Cancer Research 10:84, 1969.