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The Intrapleural Route as a Means for Estimating Carcinogenicity*

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ABSTRACT

Intrathoracic tumors have been reported from several laboratories after intrapleural injection of various preparations of mineral substances into experimental animals. Application of information from such studies to problems of pulmonary carcinogenesis involves consideration of whether tumors so induced arise from mesothelial cells of the pleura or from other cells of the lungs or thoracic cage. Another question stems from the fact that direct introduction of test substances into the pleural space does not reproduce natural routes of exposure to inhaled carcinogens, however, the intrapleural route may serve a useful role as a convenient screening procedure in comparison to laborious, repeated intratracheal injections or the large space requirements of inhalation exposures.

In experiments with hamsters exposed to a variety of preparations of asbestiform and other minerals, we have given each animal only a single intrapleural injection. Multiple injections present undesirable variables, since early granulomatous responses to a single injection entail variations in deposition of subsequent injections.

After single intrapleural injection of hamsters, we have seen upward of 80 animals with intrathoracic tumors spreading along pleural surfaces in a manner characteristic of mesotheliomas. For purposes of this paper, these tumors will be designated as mesotheliomas, although we were able to trace their derivation from the mesothelium only in rare cases, most of the tumors being too large to identify their precise origin.

Data from these experiments show that tumor response to chrysotile, amosite, and crocidolite is related to dose. Tumors regarded as mesotheliomas arose in response to preparations of chrysotile containing numerous fibers visible by optical microscopy but not in response to preparations in which the great majority of fibers were visible only by electron microscopy.

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Introduction

Beginning in 1952, HUEPER and associates reported a series of experiments in which they attempted to utilize the pleural cavity for study of biologic responses to various minerals that had been associated with pulmonary carcinogenesis in epidemiologic studies of some occupational groups. Test materials were suspended in lanolin or gelatin and injected into animals through the supraclavicular fossa or implanted through thoracotomy. Epidermoid carcinomas, round cell carcinomas, adenocarcinomas, and sarcomas were reported in lungs of rats and mice after what was said to be intrapleural deposition of powdered chromite roast and a number of chromates, whereas no lung tumors were found in rats exposed by inhalation to chromite ore with particle size averaging less than 4 μm (HUEPER and PAYNE, 1959, 1960; HUEPER and CONWAY, 1964). Intrapleural deposition of powdered metallic nickel and uranium was said to induce sarcomatous responses from the lung and pleural tissues of rats (HUEPER and CONWAY, 1964). These sarcomas, however, appear to have been in the thoracic wall or subcutaneous tissues at sites of injection (HUEPER, 1952, 1955; HUEPER et al., 1952). For intrapleural experiments, multiple injections were often given over a period of months. Pleural adhesions resulting from early injections may have caused later injections to be deposited into the lungs, which might account for some tumors described as being found in the lungs.

HUEPER (1954) found no tumors related to treatment in 25 Osborne-Mendel rats, each of which received 6 monthly injections of about 15 mg of a preparation of asbestos described as a commercial-grade medium fiber. This material was suspended in lanolin.

HUEPER and PAYNE (1962) mention a pleural mesothelioma in 1 of 218 "Bethesda black rats" (from the National Institutes of Health) and state that it is a tumor that appears without any intervention in rats of that strain. In the same paper, they report an experiment in which chromic acetate in a gelatin capsule was implanted into the right pleural cavity of 42 rats and into thigh muscles of 35 rats of that strain. Aside from 1 tumor at the intramuscular site, they did not consider tumors occurring elsewhere in these animals to have any connection with the treatment. These other tumors included 3 mesotheliomas of the pleura.

WAGNER (1962, 1966) has developed a method for intrapleural injections of materials suspended in saline solution using a needle attached to a two-way tap introduced under ether anesthesia into the right axilla of rats at the level of the second teat. One end of the tap is attached to a capillary manometer, which gives a negative reading when the needle reaches the pleural cavity. A syringe containing the inoculum is then attached to the other arm of the tap and a volume of 0.4 ml is injected.

With this technique, WAGNER and BERRY (1969) injected 20 mg of preparations of amosite, crocidolite, chrysotile, and silica into specific pathogen-free (SPF) Wistar rats. The suspensions in saline were sterilized in an autoclave. There were 96 rats per sample, equally divided as to sex. With the 3 types of asbestos, they reported tumors that they diagnosed as mesotheliomas in 38, 61, and 55 rats, respectively. These tumors ranged in size from large masses enveloping the right lung to small nodules on the parietal pleura. They were equally distributed among males and females. Histologically, these tumors were said to be composed most frequently of spindle cells with occasional clefts lined by epithelial cells and were therefore considered as mixed-

pattern mesotheliomas. Purely spindle-cell tumors or tumors described as tubulopapillary were less common. In 9 of the rats injected with asbestos, subcutaneous sarcomas were found at injection sites. They were attributed to deposition of the inoculum in the chest wall rather than in the pleural cavity.

In the 96 rats injected with silica, with a particle size of less than 5 µm, about half developed intrathoracic tumors diagnosed as histocytic reticulum cell sarcomas. These tumors are discussed and contrasted with tumors diagnosed as mesotheliomas (WAGNER, 1966). No mesotheliomas were found in 96 control rats injected only with saline solution. These experiments with SPF rats were repeated with standard rats and generally comparable results were obtained.

From subsequent experiments with the same technique, WAGNER et al. (1973) reported that development of mesotheliomas in SPF Wistar rats was approximately proportional to dose of asbestos. In groups of 30 to 35 rats, they found no mesotheliomas in response to a sample of glass fibers, but single mesotheliomas in response to glass powder, nonfibrous aluminum oxide, and barium sulphate, 3 mesotheliomas in response to aluminium silicate fibers, and 18 in response to brucite. They state that their sample of brucite also contained chrysotile.

After intrapleural injection of 1 ml of 1:15 suspensions of asbestos fibers into 102 Sprague-Dawley rats, DONNA (1970) saw tumors in 12 animals after 13 to 20 months. These tumors were described as mesothelial in 3 rats treated with chrysotile, whereas in 4 rats treated with crocidolite and 5 rats treated with amosite, there were said to be undifferentiated pulmonary carcinomas. The possibility that some of the inoculum may have been deposited into the lungs must be considered.

A technique designed to achieve widespread deposition and retention of particulates in the pleural cavity has been developed by STANTON and WRENCH (1972). They suspended preparations of asbestos in liquid gelatin, which was then allowed to harden on a pledget of coarse glass fibers measuring about 30 x 20 x 2 mm. The pledgets were introduced into the left pleural cavity of female Osborne-Mendel (om) rats through a thoracotomy under ether anesthesia. In rats thus treated, they found numerous tumors that they described as pleural mesotheliomas of spindle cell or pleomorphic types, sometimes with tubulopapillary features. In groups of 30 rats treated with pledgets containing 40 mg of UICC (Union Internationale Contre le Cancer) standard reference samples (TIMBRELL, 1969) of chrysotile A, amosite, and crocidolite, tumors designated as mesotheliomas were seen in 15, 15, and 14 animals respectively. Milling of this sample of crocidolite to reduce particle size gave a product that induced mesotheliomas in only 8 animals.

With pledgets containing 10 mg or 1 mg UICC crocidolite, the number of mesothelioma-bearing rats was reported as 11 and 2, respectively. When 10 mg of the same crocidolite was suspended in saline and injected without pledgets of glass fibers, mesotheliomas were found in 9 rats. The closely similar yield of these tumors with and without pledgets indicates no advantage of this special method to influence distribution of particulates within the pleural cavity.

No tumors resulted from control implants of the coarse glass fiber pledgets. Single mesotheliomas were found in groups with pledgets impregnated with 40 mg of glass fibers 1 µm to 25 µm in diameter. In 2 groups treated with 40 mg of glass fibers, 0.06 µm to 3 µm in diameter, 3 and 5 tumors diagnosed as mesotheliomas were found.

Materials and Methods

Our group has carried out experiments in which various preparations of minerals have been injected into the pleural cavity of Syrian golden hamsters. Throughout, we have used male hamsters of the LVG:LAK strain obtained from Lakeview Hamster Colony, Newfield, New Jersey. Test materials have been suspended in 0.9% NaCl solution, sterilized in an autoclave, and injected in a volume of 0.5 ml with a syringe fitted with a 20-gauge needle, except for thick slurries for which an 18-gauge needle was used. Each animal received only 1 injection, which was made into the right chest in the mid-axillary line about 1/4 inch above the lower end of the sternum. Depending on the nature and dose of test materials, granulomatous responses can largely obliterate the pleural cavity within a few weeks. Accordingly, we have not used and do not recommend repeated injections.

In our early work (SMITH et al., 1965b), we "stabbed" the right chest 2 days before injection in an attempt to induce pneumothorax and thus favor intrapleural deposition and spread of inoculum. With experience, we found that comparable deposition and spread can be achieved by direct injection. Hence, preliminary "stabbing" has been omitted in all of our intrapleural work since 1965.

Following attempts at intrapleural injections, part of the inoculum was sometimes found subcutaneously in the chest wall. Sarcomas occasionally arose at such sites. We have not included such subcutaneous tumors in comparing yields of intrathoracic tumors in response to different test materials. To avoid this problem, the occasional animal that shows a subcutaneous swelling at the time of injection is discarded and replaced. With this precaution, examination of trial animals immediately after injection has shown the inoculum in the pleural space. The technique of intrapleural injection is thus not more difficult than the commonly used technique of intraperitoneal injection. No anesthesia is required. The operator holds the animal in 1 hand and makes the injection with the other, holding a finger on the xiphoid to assure proper positioning of the needle.

Results

After intrapleural injection of a carcinogenic dose, we have occasionally seen small tumors whose origin from mesothelium could be traced. The majority of intrathoracic tumors found in our experiments, however, have been large masses whose origin could not be precisely ascertained. Such masses compress the lung, sometimes largely fill the right chest, spread along pleural surfaces, but invade the lungs only superficially. Some penetrate the diaphragm. Occasionally, metastases are found. Gross and microscopic appearance of such tumors in hamsters have been described and illustrated (SMITH et al., 1965a; SMITH et al., 1965b).

In man, mesotheliomas have been described as tumors that spread along mesothelial surfaces and they have been subdivided into epithelial-like, sarcomatous, or mixed types (CHURG and SELIKOFF, 1968). The majority of intrathoracic tumors in our experiments with hamsters appear sarcomatous while others resemble the so-called epithelial or mixed types. Use of the term "mesothelioma" for many experimentally induced tumors reported in animals has been questioned by GROSS (1973)

in an exchange of views including STANTON's contribution. For the present, we use the term "mesothelioma" for purposes of comparing experimental yields of malignant tumors that appear to be primary in the chest, spread along mesothelial surfaces, and histologically bear some resemblance to one or another type of tumor presently diagnosed as mesothelioma in man.

Thus defined, we found that the yield of mesotheliomas after intrapleural injection of hamsters was related to the dose of chrysotile or amosite. In groups of 50 hamsters, the number of tumors considered to be mesotheliomas were 9, 4, and 0 in response to 25 mg, 10 mg, and 1 mg chrysotile; 4 and 0 in response to 10 mg and 1 mg amosite (SMITH et al., 1968). No mesotheliomas were found in response to 25 mg of a preparation of talc that contained 50% fibrous tremolite (SMITH, 1973). We saw no mesotheliomas in 100 untreated hamsters maintained as control groups in these experiments.

3 preparations of chrysotile with mean fiber length of 5.3 µm to 6.9 µm, as measured by electron microscopy, were milled to reduce fiber size to mean lengths of 0.37 µm to 0.86 µm. These 6 preparations were tested at a dose level of 25 mg in groups of 50 hamsters each. The 3 original preparations induced extensive pleural adhesions and yielded 8, 9, and 10 mesotheliomas, whereas the 3 samples that had been subjected to further milling induced relatively thin pleural adhesions and no mesotheliomas (SMITH et al., 1972). Fiber diameter in the original preparations averaged 0.18 µm to 0.2 µm and in the milled preparations 0.03 µm to 0.07 µm.

This evidence that carcinogenicity of asbestos is related to physical properties is supported by an as-yet unpublished experiment of ours in which 50 hamsters were given an intrapleural injection of 25 mg serpentine rock dust (antigorite). This material has the chemical composition of chrysotile but is not crystallized in fibrous form. Only 1 mesothelioma resulted.

The following information is available from additional as-yet unpublished experiments with groups of 50 hamsters:

Since the external surface of chrysotile fibers is thought to consist of magnesium hydroxide (SPEIL and LEINEWEBER, 1969), an experiment was designed to ask whether such a surface might play a role in asbestos carcinogenesis. For this purpose, a fibrous magnesium hydroxide (nemalite) was tested. At the 25-mg dose level, no mesotheliomas resulted.

Another experiment was designed to test whether carcinogenic activity might be shown by fibers comparable to asbestos fibers in size but different in chemical composition. For this purpose, we used silicon dioxide fibers with an average diameter of 0.75 µm. At a dose of 10 mg, mesotheliomas developed in 4 hamsters. No mesotheliomas occurred in 50 hamsters injected with borosilicate glass fibers with an average diameter of 5 µm, or in 50 hamsters injected with such glass fibers coated with phenolformaldehyde (a binder used in some preparations of fiberglass).

A standard reference (UICC) sample of crocidolite (TIMBRELL, 1969) was tested in 2 groups of 50 hamsters each. At a dose of 10 mg, it induced mesotheliomas in 10 hamsters. At a dose of 1 mg, it induced mesotheliomas in 2 hamsters. 3 of 50 hamsters developed mesotheliomas after 10 mg of a standard reference (UICC) sample of anthophyllite.

These findings show that intrapleural injection of hamsters with various mineral substances suspended in saline provides a convenient means for

comparison of relative carcinogenicity. In response to the principal types of asbestos, hamsters have shown a lesser incidence of mesotheliomas than rats, but, as might be expected from the shorter life span of hamsters, mesotheliomas tend to appear earlier in them than in rats.

The earliest tumor that we considered a mesothelioma was found in a hamster examined 151 days after injection of 25 mg chrysotile. In contrast, the earliest mesothelioma reported in rats by WAGNER and BERRY (1969) was at 353 days after injection of 20 mg chrysotile, and the earliest mesothelioma seen in rats by STANTON and WRENCH (1972) appears to have been at about 350 days after implantation of a glass pledget containing 40 mg amosite. In tests of UICC crocidolite at 10 mg, STANTON and WRENCH's data from rats show the earliest mesothelioma at about 560 days when the material was injected in saline and about 525 days after implantation in a glass pledget, whereas our test of 10 mg UICC crocidolite in hamsters gave the first mesothelioma at 418 days. By the intrapleural route, the hamster thus appears to afford a more rapid test model than the rat.

Since tests for carcinogenicity are customarily run over the life span of test species, the rate at which results can be obtained is of practical importance. To learn whether mice might give useful information more rapidly than hamsters, we made intrapleural injections of 5 mg UICC crocidolite suspended in saline into 40 male BALB/c mice. Intrathoracic tumors were found in 3 of these mice at 301, 301, and 340 days. These 3 tumors resemble plasma cell tumors that have been reported in BALB/c mice after intraperitoneal injection of Plexiglass borings, Plexiglass discs, Millipore filters, or mineral oil (POTTER, 1968). Plasma cell tumors have been reported in White Leghorn fowls after introduction of tremolite into axillary air sacs (PEACOCK and PEACOCK, 1966).

Both pleural and peritoneal mesotheliomas have been reported in female CBA mice after subcutaneous injection of massive (60-mg) amounts of asbestos in divided doses (ROE et al., 1967). Among 5 groups of 20 mice treated with crocidolite, amosite, or chrysotile there were 6 mice with sarcomas at injection sites and 10 mice with mesotheliomas. These were distributed among the treatment groups. The lesions diagnosed as mesotheliomas appear to have been small papillomatous proliferations of mesothelial cells. Heavy deposits of fibers were seen in submesothelial tissues of mice with mesotheliomas. It was thought that fibers had been actively transported from the subcutaneous site to the submesothelial site, but subsequent studies by the same group did not provide evidence for this. Passive movement of fibers along tissue planes or as a consequence of inflammation and necrosis is now thought the more probable explanation (ROE, personal communication, 1974).

Discussion

The literature on tumors induced by intraperitoneal injection of various plastic, metallic and other materials has been reviewed by HUEPER (1964a) and HUEPER and CONWAY (1964). Discussions of mechanisms of carcinogenesis by this route and comparison of results of tests by other routes has been presented by BISCHOFF and BRYSON (1964) and BRYSON and BISCHOFF (1967, 1969). Most of the tumors reported as experimentally induced at intraperitoneal sites have been sarcomas in

rats. After intraperitoneal implantation of polyurethan foam into Bethesda black rats, HUEPER (1964b) reported tumors including an unspecified number of mesotheliomas, but he also states that peritoneal mesotheliomas occur spontaneously in rats of this strain.

Induction of sarcomas in rats after intraperitoneal injection of asbestos was reported by SCHMÄHL (1958).

KLOSTERKÖTTER and ROBOCK (1970) reported experiments in which they made intraperitoneal injections of 50 mg asbestos into groups of 30 rats. With preparations containing fibers up to 50 µm in length, they found fibrosis in response to chrysotile, amosite, and crocidolite. Mesotheliomas were said to have been found in 2 rats of the group on amosite and in 4 rats of the group on crocidolite. No tumors were found in the chrysotile group. In other groups treated with these 3 samples of asbestos after further milling, there was little fibrogenicity and no tumors. Most particles in these milled products were said to be less than 1 µm in size. The animals were observed for periods of 6 to 12 months.

POTT et al. (1972) reported results from groups of 30 female Wistar rats given 4 intraperitoneal injections of 25 mg UICC chrysotile A or the same material after further milling. Samples were suspended in saline and injected at weekly intervals. The animals were followed for periods up to 2 years. Extensive adhesions were found in the peritoneal cavity of animals given UICC chrysotile but only slight fibrosis in those that had received the milled product. The incidence of tumors was about 40% in both groups, but the first tumor was found at 7 months in the group on UICC chrysotile in contrast to 13 months for the milled product. Most of the tumors were diagnosed as sarcomas but 7 were listed as mesotheliomas.

After intraperitoneal injections of groups of 40 female rats with 6.25 mg, 25 mg, and 100 mg chrysotile, POTT and FRIEDRICHS (1972) found 17, 19, and 16 animals, respectively, with abdominal tumors within 530 days. This apparent plateau of response suggests to us that dose-response studies using intraperitoneal injection may require lesser doses than the intrapleural route. Since the surface area of the peritoneum is much more extensive than the surface area of the pleural cavity, larger numbers of mesothelial cells are at risk.

In testing materials for carcinogenicity in animals, evaluations depend upon observation of tumors of types or at sites not found in controls, or at a frequency or speed of appearance greater than seen "spontaneously" in untreated control animals. For studies on mesotheliomas, there is only limited information on their spontaneous incidence or pathology in animals. A transplantable mesothelioma has been reported in a mouse (SHAPIRO and WARREN, 1949). A mesothelioma was listed among tumors seen in untreated control hamsters (FORTNER, 1961). As mentioned above, both pleural and peritoneal mesotheliomas have been said to occur spontaneously in Bethesda black rats (HUEPER and PAYNE, 1962; HUEPER, 1964b). MORRIS et al. (1961) state that papillary mesotheliomas of the testes or epididymis occurred in 3 Buffalo rats fed N-2-fluorenylacetamide and in 2 untreated control Buffalo rats. Pathologic material from these and other Buffalo rats was studied by H.L. STEWART (personal communication, 1974) who advises that he had not seen spontaneous pleural mesotheliomas but that he had seen mesotheliomas arise spontaneously in that strain from an extension of the peritoneal mesothelium (tunica vaginalis of the testis). He states that these tumors are papillary, sometimes tubular, and they occasionally spread to the peritoneal cavity. In a discussion of spontaneous lesions of rats, RIBELIN and MCCOY (1965)

state that they have seen small papillary tumors along the genital omentum and serosal surface of the testis or epididymis. The legend for their photograph of one such tumor describes it as a mesothelioma. They state that histologically these tumors appear benign and that they may be found in rats of a number of strains, but most often in ACI, Buffalo, and om strains.

The cited reports of spontaneous mesotheliomas emphasize need for untreated controls and open avenues for exploration of etiologic mechanisms in testing chemicals for carcinogenicity in cavities lined by mesothelial tissues. Multiple mesotheliomas have been described in chickens after injection of MC29 avian leukosis virus (CHABOT et al., 1970). Resemblance of these virus-induced mesotheliomas in chickens to cases of multiple mesotheliomas seen in hamsters after injection of asbestos suggests the possibility that carcinogenic effects of chemicals associated with mesotheliomas may depend upon stimulation of a virus (SMITH, in press).

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