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Induction of Lung Tumours in Rats by i.v. Injection of *N*-Methyl-*N'*-Nitro-*N*-Nitrosoguanidine*

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Abstract—N-*Methyl*-N'-*nitro*-N-*nitrosoguanidine, when injected intravenously (i.v.) to BD-rats in one single dose of* 60 mg/kg, *induced lung malignomas in a high yield. With regard to the characteristic reactivity of this nitrosamide with thiols, this finding is consistent with a previously formulated hypothesis postulating that such nitrosamides which are decomposed* in vitro *by thiols under concomitant* N_2*-evolution should, on* i.v. *injection, induce lung cancer.*

INTRODUCTION

N-METHYL-*N*-NITROSOURETHANE and *N*-methyl-*N*-nitrosopropionamide, respectively, which, on i.v. application, selectively induce lung cancer in rats [1, 2], avidly react *in vitro* with cysteine, resulting in the evolution of N_2 [2, 3]. However, *N*-methyl-*N*-nitrosourea (MNU), a powerful neurotropic carcinogen [1], does neither react with this thiol [4] nor induce lung cancer [1]. It has therefore been concluded in a previous paper [2], that all those nitrosamides which react *in vitro* with thiols under concomitant liberation of elementary nitrogen, should be capable of inducing lung cancer upon i.v. application. Accordingly, by examining the reactivity *in vitro* of a given nitrosamide with thiols, it should be possible to predict whether or not an induction of lung cancer is to be expected upon i.v. application of this nitrosamide in a subsequent animal experiment.

N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was shown to react rapidly with cysteine with simultaneous liberation of N_2 [5, 6]. Thus MNNG seemed to be suitable for testing the validity of the hypothesis outlined above. Furthermore, if carcinogenesis is in fact an 'accelerated process' [1], the induction of lung cancer should be possible by a single high dose of MNNG. The main obstacle for the i.v. application of high doses of MNNG was its low solubility (0.1%) in water. This difficulty was overcome, however, by using dimethylformamide (DMFA) as solvent for MNNG.

MATERIALS AND METHODS

MNNG in crystallized form was obtained from Schuchard, München. For injections, a 5% solution of MNNG in a solvent consisting of equal volumes of DMFA (Merck, Darmstadt) and distilled water was prepared and injected immediately thereafter. DMFA has been shown to be noncarcinogenic [1, 7] and of very low toxicity; the acute LD_{50} of the i.v. administered compound amounted to 2000 mg/kg body weight [1].

For animal experiments female and male inbred BD X-rats [8] aged about 3 months were used; average body wt 220 and 350 g for females and males, respectively. Standard food was alternating Altromin maintenance diet 1320 and Latz biscuits; water *ad libitum*. BD-strain rats are characterised by a low spontaneous tumour rate. In our breeding colony at Freiburg the incidence of malignant and

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§Dissolved in 1973; the reported experiments were performed in 1971–1973 under the directorship of Professor Dr. med. H. Druckrey, to whom this paper is dedicated on the occassion of his 75th birthday.

benign tumours at the age of 2 yr was about 2 and 2%, respectively [8], due to a far-reaching exclusion of environmental carcinogens. Spontaneous lung tumours (incidence in BD X-rats: below 0.5%) or tumours of the heart were hardly ever seen. A comparable low spontaneous tumour rate of BD-rats is reported in a recently published paper [9].

Determination of the acute LD_{50} was performed on several groups of 6 rats, treated with single i.v. doses. Because of the homogeneity of the animal material used and consecutive very high slope of the lethality regression line, LD_{50} could be determined graphically [1].

For carcinogenicity test, 15 animals were given a single dose of 60 mg/kg MNNG, injected into the tail vein. The experimental animals were dissected immediately after the natural death; in some cases they were sacrificed in the final stage with chloroform. Organs showing abnormal macroscopic findings were fixed in 10% formalin and the slices stained with hematoxylin and eosin for microscopic examination.

For quantitative evaluation of the carcinogenesis experiment, the method of Miescher *et al.* [10], for the calculation of the cumulative percentage mortality, was used.

RESULTS

Acute toxicity

The value of LD_{50}, determined for MNNG by the i.v. route, was 80 mg/kg. Immediately after the application of lethal doses, cramps were observed and some animals died in convulsions. (By dividing the dose in 2 equal portions—the second given 1 hr after the first—it was, however, possible to alleviate the toxic effect upon the central nervous system thus reducing the mortality). Even with animals given sublethal doses cramps sometimes occurred. At the autopsy of the rats, which died 2–3 days after the application of single i.v. doses, haemorrhagic pulmonary oedema was found.

Carcinogenesis experiments

Before the appearance of the first tumour, one of the 15 treated animals died of pneumonia. The first lung malignoma was found at the autopsy of a rat which died 387 days after treatment. In total, 7 rats with lung tumours were registered; the last animal with lung cancer died 625 days after treatment. Dissection revealed solitary tumour nodes or tumours of multicentric origin. In some cases a whole lung was occupied by a large tumour mass (Fig. 1). Metastases in the mediastinal lymph nodes were observed in 4 cases.

On microscopic examination all tumours proved to be malignomas. Squamous cell carcinomas (with or without cornification) were found in 3 cases; alveolar cell carcinomas were registered in 3 cases. Finally, an osteosarcoma of the lung was observed; this tumour could be successfully transplanted into rats of the same strain and retained its macroscopic and microscopic characteristics over many passages.

In view of the fact that spontaneous lung tumours hardly ever occur in BD-rats, these findings clearly show carcinogenic action of i.v. applied MNNG to the lungs.

Beside lung tumours a malignoma of the right ventricle of the heart (polymorphocellular sarcoma) and a cornified squamous cell carcinoma of the eyelid respectively were registered in two other rats. No brain tumours were observed; 5 animals died intercurrently of lung infections.

After eliminating the influence of incidental mortality and plotting in a logarithmic probability paper the cumulative percentage mortality against individual survival times of rats with tumours, a linear regression was obtained (Fig. 2). The standardized total tumour yield [10] was 85.8% (67% lung tumours). The mean survival time of tumour bearing rats was 530 days.

DISCUSSION

The carcinogenicity of MNNG has been known since 1966; on s.c. application, local sarcomas have been induced [11]. Upon oral application, carcinomas in the forestomach of rats [9, 12] and in the glandular stomach of rats [9, 13] and dogs [14] have been observed.

In accordance with the hypothesis outlined previously, i.v. applied MNNG, even at a single dose, induced lung cancer in a high yield. MNU, which on the other hand, does not react with thiols, showed no pulmotropic carcinogenic activity, given either in high single i.v. doses or in weekly repeated small i.v. doses [1]. In contrast to MNU, however, no brain tumours were induced by MNNG although it does penetrate the blood–brain barrier, as evidenced by convulsions which appeared immediately after i.v. injection

The reaction of MNNG with cysteine pro-

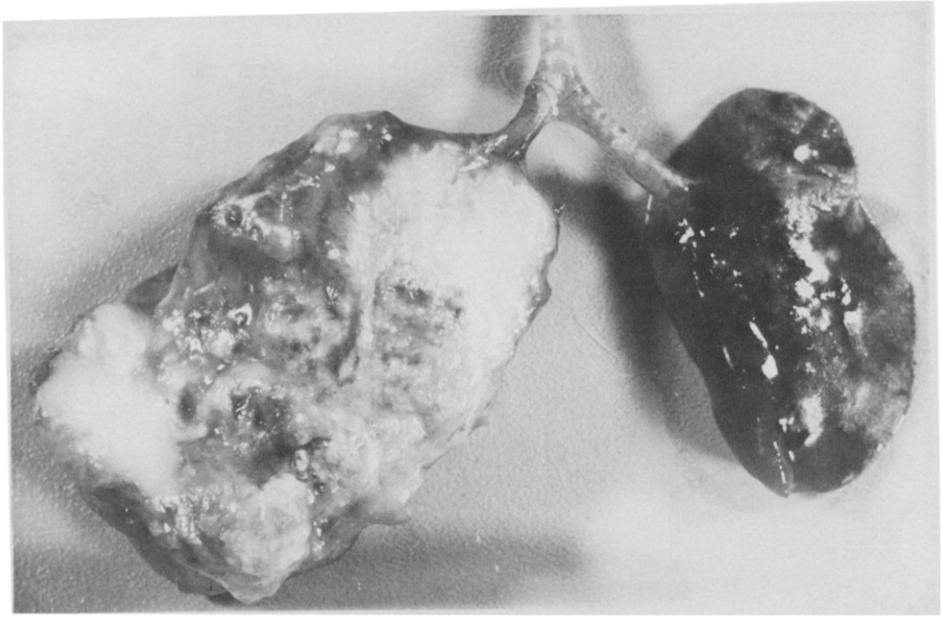

Fig. 1. Lung cancer, induced by a single i.v. dose of MNNG (60 mg/kg). Death 391 days after treatment. The whole right lung occupied by the tumour tissue. Histologically, the tumour was a squamous cell carcinoma, partly cornified.

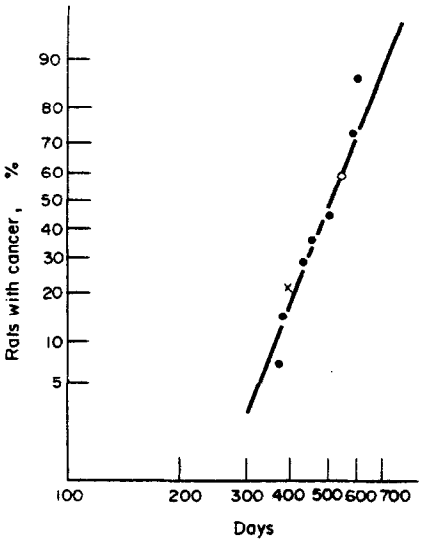

Fig. 2. Survival times of tumour bearing rats. Each point represents an animal, which died of a tumour. (●) lung tumours; (×) heart tumour; (○) tumour of the eyelid. Ordinate: cumulative percentage mortality. Abscissa: individual survival times (days).

ceeds simultaneously in two fundamentally different ways [5, 6]. The main route is represented by the nucleophilic attack of the ionised thiol on the electron-deficient imino carbon of MNNG; thereby methyldiazonium ions are generated which in turn decompose to methylcarbonium ions and N_2. The remaining part of the MNNG molecule and cysteine yield 2-nitramino-thiazoline-4-carboxylic acid [5, 6]. The second pathway (quantitatively of lesser importance) involves attack by the thiol on the nitroso group, resulting in denitrosation of MNNG to 1-methyl-3-nitroguanidine, evolution of N_2O and formation of cystine [5, 6]. No alkylating fragments are produced in the course of this decomposition route; due to the main way of reaction with the thiol, however, the extent of methylation of DNA by MNNG *in vitro* is substantially enhanced in the presence of cysteine [5, 6]. Furthermore, DNA of cultured mammalian cells treated with MNNG is rapidly methylated, the extent of methylation being the greater the higher the intracellular acid-soluble thiol content [6]. Cell-proteins are less methylated [6], indicating that the sulfhydryl groups of proteins, known otherwise to be very susceptible to alkylation, are blocked by reaction with the imino carbon of MNNG [6] thus splitting the nitrosamide to alkylating fragments.

In accordance with these experiments it was recently shown that rat tracheal epithelium could be transformed by *in vitro* exposure to MNNG and produced palpable tumours upon injection into immunosuppressed isogenic recipients [15]. Similar observations were made on a cell line derived from canine embryo cells, which underwent neoplastic transformation upon treatment with MNNG [16]. However, no analyses of the intracellular SH-groups were performed in these experiments.

Considered together with the carcinogenic activity of the i.v. applied MNNG to the lungs, these observations seem to suggest that an alkylation of DNA, triggered by SH-groups, is the first link in the chain of macromolecular events which finally result in the induction of lung cancer by i.v. applied MNNG. However, any information concerning concentration and/or spatial arrangement of SH-groups in the lungs different from that in other organs, which could explain the preferential induction of pulmonary tumours by i.v. injected MNNG, is lacking at present (such differences should perhaps be sought at the level of nuclear proteins, the interaction of which with MNNG has been recently demonstrated [17]). Thus further experimental work is necessary before the question as to whether or not SH-groups play a role in the induction of lung cancer by MNNG can be decided. Furthermore, it is not to be overlooked that because of the very low solubility in water, MNNG could be partially precipitated immediately after i.v. injection (the solution in 50% DMFA being diluted with the blood) and might then be trapped in the lungs. This would be an alternate explanation for the specific production of lung tumours by i.v. applied MNNG. However, one has to consider that the precipitated particles must have an appropriate size to be retained in the lung capillaries. The growth of the precipitated particles is a time-consuming process; it is, therefore, not clear whether this size could be attained in the short time interval between injecting the solution and its reaching the lungs. In the opposite case, the particles would pass the lungs and finally be trapped in various organs. The mechanism of specific induction of lung tumours by i.v. applied MNNG thus remains unclear.

To our knowledge the carcinogenic activity of MNNG to the lungs has not been hitherto described. It remained reserved to Druckrey's school to add a new quality to the list of carcinogenic activities of this interesting nitrosamide.

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