Comparison of Lung Tumorigenesis Induced by Urethan in Athymic Nude Mice and Euthymic Littermates

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Athymic (*nu/nu*) and euthymic (*nu/+*) mice were intraperitoneally given doses of 0.25 to 1.5 mg/g body weight of urethan at the age of 14 to 16 days. Dose-response relationship and sequential changes in lung tumorigenesis induced by urethan in athymic mice were compared with those in euthymic littermates. The urethan dose-response relationship in lung tumorigenesis of the *nu/nu* mice was almost the same as that in the *nu/+* mice. Incidence and multiplicity of the lung tumors were investigated sequentially 28 days to 12 months after urethan injection. They showed similar indexes in the two phenotypically different mice at varying periods after 0.5 mg/g body weight urethan treatment (incidences of 4.8 and 4.4 tumors/mouse and 96 and 97% for *nu/nu* and *nu/+* mice, respectively, at 12 months after treatment). This means that the length of the latent period is similar in these phenotypically different mice. It may be concluded that the immunosurveillance mechanism mediated by T-cells does not function in the present model.—KEY WORDS : lung tumors, nude mice, urethan

Thomas [1] and Burnet [2] proposed that the immunosurveillance mechanism has an important role in suppressing tumor induction, with T-cells playing essential roles. According to this theory, it had been predicted that T-cell deficient animals would be susceptible to neoplastic diseases. However, earlier studies using athymic nude mice showed discrepant results depending on the experimental conditions. This immunosurveillance theory was generally supported in tumors induced by oncogenic viruses [3-6] and spontaneous lymphoreticular neoplasms [7,8]. However, this was not the case in chemical carcinogenesis; many investigators reported that the latent period and tumor incidence in nude mice treated with urethan or methylcholanthrene were not significantly different from those in euthymic control mice [8-12]. Recently, Anderson, Last-Barney and Budinger [13] and Anderson and Rice [14] described the carcinogenic response of nude mice as being more obvious than in euthymic counterparts when treated with ethylnitrosourea. In view of these conflicting results, we have attempted to reassess the

carcinogenic responsiveness of nude mice in comparison with their euthymic littermates. In this study, investigations were made of the dose-response relationship and sequential changes in the development of lung tumors after urethan treatment in nude mice (*nu/nu*) and their heterozygous littermates (*nu/+*).

Materials and Methods

Animals: Male athymic nude mice (*nu/nu*) and their female heterozygous littermates (*nu/+*) with a BALB/c background were supplied by the animal breeding facility of this institute, and then mated in our laboratory. Their offspring, *nu/nu* and *nu/+* mice, were housed in filter-topped metal cages, 6 or fewer per cage, and were maintained under specific pathogen-free conditions at 24°C, 45~60% humidity, and a 12-hour light-dark cycle. They were given a commercial pellet diet, MB-1 (Funabashi Farm Co., Funabashi, Japan) and chlorinated (12 ppm) and acidified water (pH 3) *ad libitum*. Both male and female mice were used throughout the experiments, as sex

difference does not influence lung tumorigenesis in these mice [3,15-18].

Urethan : Urethan (ethyl carbamate) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The 2.5% urethan solution dissolved in 0.9% NaCl solution (saline) and sterilized through a 0.22 μ m pore filter (Millipore Co., Mass., U. S. A.). Approximately 5-ml aliquots of the 2.5% urethan solution were distributed into glass tubes and stored at -20°C until use. The frozen solution was thawed prior to administration and used within 1 hour. All frozen stocks of the solution were used within 3 months.

Administration Route : The urethan solution was intraperitoneally (i.p.) administered. To prevent the possibility of leakage of the solution, the injector needle was inserted via the leg muscles into the abdominal cavity [17].

Experimental Design : In experiment 1, the dose-tumor incidence relationship was examined. The *nu/nu* and *nu/+* mice were divided into 5 groups, ranging in number from 31 to 65 mice for each group. Mice of each group were treated with urethan or saline at 14 to 16 days of age : Group 1 (control), 0.15 ml of saline (14 days of age) ; Group 2, 0.25 mg/g body weight (b.w.) of urethan (14 days) ; Group 3, 0.5 mg/g b.w. (14 days) ; Group 4, 2 doses of 0.5 mg/g b.w. (14 and 15 days) ; and Group 5, 3 doses of 0.5mg/g b.w. (14, 15 and 16 days). All mice were euthanized by ether anesthesia and autopsied 6 months after the treatment.

In experiment 2, the development of lung tumor was sequentially examined. Both *nu/nu* and *nu/+* mice were divided into two groups. Mice of the treated group were i.p. injected with a single dose of 0.5 mg/g b.w. of urethan at the age of 14 or 15 days. Age-matched mice given an i.p. injection of 0.15 ml of saline served as controls. Treated animals were sacrificed and autopsied 30 and 35 days, 2, 3, 4, 6, 8, 10 and 12 months after urethan-treatment. Control animals were sacrificed at 6 and 12 months after the start of the experiment.

Pathological Examination : The lung was floated on saline to allow exsanguination for a few minutes and tumor nodules in each lung lobe were then counted macroscopically or under a dissecting microscope. To confirm the reliability of the gross count, histological semi-serial sections, 1 section every 100 μ m in

Fig. 1. Dose response of lung tumorigenesis by urethan administration in nude (*nu/nu*, shaded columns and solid line) and heterozygous (*nu/+*, open columns and broken line) mice. For details, see Materials and Methods. Each column represents average $\pm$ S. E. of the tumor number in 31 to 65 mice, on average 40 mice/column. Neither control *nu/nu* mice nor control *nu/+* mice, that had been injected with saline, bore lung tumors at the time of assessment.

Fig. 2. Lung-tumor incidence as a function of time after urethan administration in nude (*nu/nu*) and heterozygous (*nu/+*) mice. $\bullet$, Urethan administered *nu/nu* ; $\blacktriangle$, saline-injected control *nu/nu* ; $\circ$, urethan administered *nu/+* ; $\triangle$, saline-injected control *nu/+* mice. Each point represents 33 to 88 mice, an average of 49 mice/point. Only a single line is shown because the curve for *nu/nu* mice was not distinguishable from that for *nu/+* mice. *There was no significant difference between *nu/nu* and *nu/+* mice ($P > 1.2$ in each case, χ^2 -test).

thickness, of the lungs of a few mice were prepared, and the number of tumor nodules was counted under light microscopy. The number of nodules counted grossly coincided well with the microscopic count.

The lungs of all mice, and other gross lesions if present at autopsy, were fixed in 10% phosphate-buffered formalin. These sections were processed in a routine manner and stained with hematoxylin and eosin. A small number of mice died during the experiment and their carcasses were also examined as described above, but they were excluded from the present results.

Analysis of Data: Statistical analysis of the experimental data was performed using Student's *t*-test or the χ^2 -test.

Results

Dose-Response in Incidence of Lung Tumors: Lung tumorigenesis in both *nu/nu* and *nu/+* mice was closely linked to increases in the urethan dose (Fig. 1). Lung tumor multiplicity was proportional to the square of the urethan dose, and fit well with linear-quadratic curves in both *nu/nu* and *nu/+* mice; $Y = 2.56X^2 + 1.38X + 0.15$ and $Y = 3.13X^2 + 1.01X + 0.20$, respectively, where *Y* means the number of tumor nodules/mouse and *X* means the dose of urethan (mg/g b.w.). These two curves coincide except for the response at a dose of 1.5 mg/g b.w., but no significant difference was seen at this dose by *t*-test. This result may suggest that the frequency level of tumor initiation and of the ability to suppress tumor development did not differ between athymic and euthymic mice.

Sequential Observations of Lung Tumor Development: Time-dependent changes in the number of lung-tumor bearing mice and average multiplicity (number of tumor nodules/mouse) of the lung tumors after urethan treatment are shown in Figs. 2 and 3, respectively. No tumor nodule was observed at 28 days after urethan treatment. Tumors were first detected on the 35th day in both *nu/nu* and *nu/+* mice. The latent period in the present study was slightly longer than that reported by Shimkin and Polissar [19]. There was no significant difference in incidence between these two phenotypically different mouse groups for a period of up to 12 months (Fig. 2). The ratio of

Fig. 3. Mean lung tumor numbers as a function of time after urethan administration in nude (*nu/nu*) and heterozygous (*nu/+*) mice (same animals as for Fig. 2). Symbols are the same as in Fig. 2. Vertical bars mean average $\pm$ S. E. of the tumor number, solid line for *nu/nu* and broken line for *nu/+* mice. *There was no significant difference between *nu/nu* and *nu/+* mice ($P > 0.3$ by Student's *t*-test) at any time point. Only a single line is shown for the same reason as described in Fig. 2.

tumor bearing mice was 73% for *nu/nu* mice and 80% for *nu/+* mice at the 6th month, and 96% for *nu/nu* and 97% for *nu/+* mice at the 12th month of the experiment. Tumor multiplicity in *nu/nu* mice was also comparable to that in *nu/+* mice at all times of examination (Fig. 3). It seemed to increase almost linearly with time in both *nu/nu* and *nu/+* mice. Neither the incidence nor the multiplicity of lung tumors in athymic nude mice differed from those of euthymic mice at any time up to the 12th month. These results show no shortening of the latent period in lung tumor development after urethan administration. No lung tumors were observed in control mice injected with saline at 6 months, while there were lung tumors in more than 70% of urethan-treated mice in the same period.

All of the lung tumors examined at 6 months after the urethan treatment were adenomas. At twelve months after the treatment, about 10% of the lung tumors in both athymic and euthymic mice were carcinomas. Other observed lesions were hydrometras, thymic or non-thymic lymphomas, hepatomas and skin papillomas. Their incidences, however, were too low to allow statistical analysis.

Discussion

The results of the present study demonstrate that lung tumorigenesis by urethan in BALB/c background nude mice, assessed by dose-response relationship, latent period, tumor incidence and multiplicity dose not differ from that of euthymic littermates. These results strongly suggest that the T-cell mediated immunosurveillance function is not operational in this model. In addition, it was assumed that NK cells and macrophages also do not exert any effect on carcinogenesis, because the activities of these cells are usually fairly high in nude mice in comparison with normal mice [20,21], and there was no detectable decrease of tumor incidence or tumor multiplicities in our urethan-treated nude mice. It may be concluded that the immunosurveillance system mediated by not only T-cells but also NK cells and macrophages is not operating during urethan induction of lung tumors in mice. This coincides with the findings reported in the earlier literature on tumor development by urethan or methylcholanthrene in nude mice [8-12].

On the other hand, oncogenic virus-induced carcinogenesis [3-6] and oncogenesis by some carcinogenic chemicals, such as methyl-nitrosourea, ethylnitrosourea and dimethylbenzanthracene [13,14], support the concept of the immunosurveillance theory. In those studies, athymic nude mice developed more tumors than euthymic mice. From the results on experimental skin tumorigenesis by nitrosourea alone or combined with 12-*o*-tetradecanoyl-phorbol-13-acetate (TPA) and by UV light, Anderson and Rice assumed that the skin anomalies in nude mice were a reflection of the higher susceptibility to tumorigenesis compared with normal mice [14]. The development of spontaneous lymphoreticular neoplasms has also been reported to be predominant in athymic mice [7,8]. It is still unclear why the immunosurveillance mechanism acts in virus-induced and spontaneous oncogenesis but not in urethan-induced tumorigenesis. More information is needed to elucidate the mechanism of tumor-development suppression.

Although the immunosurveillance mechanism did not function in the present system, more dominant tumor regulatory mechanisms

may operate in mice of different phenotypes with the same genetic background. The most probable regulatory mechanism for lung tumorigenesis is the expression of gene(s) determining the susceptibility to certain oncogenic conditions. As major lung tumor regulatory genes, the "*ptr*" (pulmonary tumor resistance) gene [22], and the "*Pas-1*, -2 and -3" (pulmonary adenoma susceptibility-1, -2 and -3) genes [23], as well as other genes [15,18,24], have been proposed. The H-2 complex [25], and particularly the I-A and I-E subregions [26,27], have also been suggested to influence lung tumorigenicity, although their roles could not be demonstrated in our present model.

Linear-quadratic lung tumorigenic responses with increasing doses of urethan were observed in the present experiment. Similar results have been reported by other investigators [28]. Based on a comparative analysis between the urethan concentration in serum and the development of adenoma, Sichak and O'Flaherty proposed that the quadratic/linear-quadratic tumorigenic response can be explained by a single-stage mutation model rather than a two-stage model [28]. The amounts of certain proximal or ultimate metabolites and the resulting nucleic acid adduct formation are closely related to urethan tumorigenesis [17,29,30]. In other words, carcinogenic mutation is strongly influenced by the metabolism of the chemical. It is well known that younger or newborn mice are more susceptible to urethan, in terms of the induction of lung adenoma, than older mice. This was explained by the difference in the rate of urethan elimination, i.e., the rate of urethan elimination is lower in younger than in older mice [17,18]. Therefore, information about not only the activating mechanisms but also the entire metabolic profile, including inactivating mechanisms and repair mechanisms of induced DNA damage, is required to fully elucidate the dose-response relationship.

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ヌードマウスと正常マウスにおけるウレタン誘発肺腫瘍発生の比較

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化学物質による肺腫瘍の発生に対する免疫監視機構の役割を解析する目的で、14～16日齢のT細胞機能を欠くBALB/cヌード(*nu/nu*)マウスと、その機能を有する同腹のBALB/c(*nu/+*)マウスにウレタンを腹腔内投与し、肺腫瘍発生の用量－反応相関性および経時的变化を比較検討した。用量－反応性の検討には*nu/nu*マウスと*nu/+*マウスをそれぞれ5群に分け、体重1g当たり0, 0.25, 0.5, 1.0および1.5mgのウレタンを投与し、6ヵ月後に全動物を屠殺し、肺腫瘍発生数を調べた。その結果、各投与量に対する反応性(肺腫瘍発生)は*nu/nu*マウスと*nu/+*マウスではほぼ同じであった。次に0.5mg/g体重のウレタン投与後、28日から12ヵ月にかけて経時的にマウスを屠殺

し、肺腫瘍の発生率と腫瘍数を調べた。その結果、*nu/nu*マウス群および*nu/+*マウス群での肺腫瘍発生率は、6ヵ月目ではそれぞれ73%, 80%であり、12ヵ月目では96%, 97%で、双方のマウス群間に差は見られなかった。1匹当たりの平均肺腫瘍数は、両マウス群共に経時的に同様の値で加算的に増加し、12ヵ月後では*nu/nu*マウスでは4.8個、*nu/+*マウスでは4.4個であった。以上の結果から、T細胞機能を欠くヌードマウスは*nu/+*マウスに比べて、ウレタンによる肺腫瘍発生の頻度は高くなく、今回のモデル実験からは、いわゆる免疫監視機構説を支持する結果は得られなかった。