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Synergistic effects of methyl methanesulfonate and X rays in inducing somatic mutations in the stamen hairs of *Tradescantia* clones, KU 27 and BNL 4430

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ABSTRACT

Young inflorescences of *Tradescantia* clones KU 27 and BNL 4430, the both of which are blue/pink heterozygotes and have been demonstrated to be highly sensitive to alkylating agents, were exposed either to aqueous solutions of methyl methanesulfonate (MMS) for 16 hr alone (at 0.005 to 0.02% for KU 27 and at 0.005% for BNL 4430) or to acute 150 kVp X rays alone (161 to 531 mGy for KU 27 and 501 to 976 mGy for BNL 4430), or in combinations (134 to 448 mGy for KU 27 and 458 to 865 mGy for BNL 4430 after the 0.005% MMS treatment). The induced somatic pink mutation frequencies per hair-cell division were studied and compared, and clone BNL 4430 was found to be nearly two times more sensitive to MMS than clone KU 27, while the X-ray-induced mutation frequencies in the latter was about 1.5 times higher than those in the former. The lower sensitivity to MMS of clone KU 27 (as compared with BNL 4430) was nevertheless about 5.6 times higher as compared with the responses of clone BNL 02 to MMS reported earlier, proving the high sensitivities of the two clones used in the present study. Clear synergistic effects of MMS and X rays were observed in the both clones, indicating that the mechanisms of inducing mutations are common at least in part between MMS and X rays.

1. INTRODUCTION

A synergistic effect of ethyl methanesulfonate (EMS), an alkylating agent, and X rays in inducing somatic mutations in *Tradescantia* stamen hairs has been reported by Cebulska-Wasilewska et al. (1981), after treating the cuttings of clone KU 9 either with 0.04 M EMS for 4 hr alone or with 200 to 600 mGy (20 to 60 rad) of X rays alone, or in combinations.

Such a synergism indicates that there must be some mechanisms of mutation induction which are common at least in part between alkylating agent and ionizing radiation. It seems therefore worthy to reconfirm the occurrence of synergism between other alkylating agents and ionizing radiation, for understanding further the mutation induction mechanisms. Reconfirming the occurrence of synergism is also important from another point of view; namely, the existence of synergistic effects of a chemical mutagen and ionizing radiation has implications for current

risk evaluation of individual mutagens, because simply summing the risks evaluated for individual mutagens will result in an obvious underestimation of the real risk (Ichikawa, 1992).

In the present study, the induction of somatic pink mutations in the stamen hairs of *Tradescantia* clones KU 27 and BNL 4430, the both of which are blue/pink heterozygotes, was investigated after treating them either with methyl methanesulfonate (MMS) alone or X rays alone, or in combinations. The stamen-hair system of *Tradescantia* heterozygous for flower color has proven to be one of the most suitable materials for detecting the genetic effects of ionizing radiations and various chemicals at low levels, as reviewd earlier (Underbrink et al., 1973; Ichikawa, 1981b; Schairer et al., 1983) and recently (Ichikawa, 1992). The two clones used in the present study have been demonstrated to be highly sensitive to alkylating agents, clone KU 27 being sensitive to EMS (Sanda-Kamigawara et al., 1991) and clone BNL 4430 to 1,2-dibromoethane (DBE) and EMS (Sparrow et al., 1974; Nauman et al., 1976).

2. MATERIALS AND METHODS

Materials and growing conditions

Clone KU 27 is a diploid segregant ($2n=12$) from clone BNL 02 ($2n=12$), a putative hybrid between *T. occidentalis* (Britt.) Smyth. and *T. ohiensis* Raf., and is a blue/pink heterozygote (Sanda-Kamigawara et al., 1991). The normal dominant blue color of this clone is somewhat darker than that of the parental clone, and the recessive mutant pink color is also generally deeper than that of the latter, facilitating the detection of pink mutant cells in the stamen hairs (Sanda-Kamigawara et al., 1991). This clone shows a low and stable spontaneous somatic mutation frequency and a radiosensitivity both comparable to those of clone BNL 02 (Sanda-Kamigawara et al., 1991; Ichikawa, 1992), but has been found to be two to four times more sensitive to EMS than the parental clone (Sanda-Kamigawara et al., 1991).

Clone BNL 4430 is a diploid hybrid ($2n=12$) between *T. hirsutiflora* Bush and *T. subacaulis* Bush (Sparrow and Sparrow, 1976; Emmerling-Thompson and Nawrocky, 1980), and its spontaneous somatic mutation frequency is about two to three times higher than those of clones BNL 02 (Sparrow and Sparrow, 1976; Ichikawa, 1984, 1992) and KU 27 (Ichikawa, 1992). This clone has been reported to be seven to nine times more sensitive to DBE than clone BNL 02 and also sensitive to EMS but to a lesser extent, although its sensitivity to ionizing radiation has been described to be almost comparable to that of the latter (Sparrow et al., 1974; Nauman et al., 1976).

The potted plants of clones KU 27 and BNL 4430 bearing young inflorescences just before initiating blooming were selected as materials from those which had been grown in a growth chamber (Sherer CEL 38–15), and these plants were

continuously grown in the growth chamber throughout the experimental periods. The environmental conditions in the growth chamber were $23\pm0.5^{\circ}C$ (for KU 27 in Experiments 1 to 3) or $22\pm0.5^{\circ}C$ (for BNL 4430 in Experiments 4 and 5) during the day and $20\pm0.5^{\circ}C$ at night, 60% humidity, and 16-hr day length with the maximum light intensity of 23 klx from Sylvania VHO cool white fluorescent tubes (22 klx) and incandescent bulbs (1 klx) for 12 hr.

MMS treatments

Young inflorescences of clone KU 27 were treated with 0.005, 0.01 or 0.02% (by volume) aqueous solutions of MMS (Nakarai Chemicals, Ltd., Kyoto) in Experiments 1 and 3, and those of clone BNL 4430 only with 0.005% MMS in Experiment 5, all for 16 hr at room temperature, using the same methods as in earlier studies (Ichikawa and Takahashi, 1978; Ichikawa et al., 1990; Sanda-Kamigawara et al., 1991). Describing the methods briefly, bracts of each inflorescence were cut off, and older flower buds (one or two days before flowering) were removed; small pieces of absorbent cotton were dipped into MMS solutions freshly prepared (the pH value adjusted to 7.0); and each inflorescence was carefully covered with the MMS-soaked cotton, covered by a small polyethylene bag (27×40 mm), and sealed tightly with an acetate tape. After the MMS treatments, inflorescences were washed with tap water for 3 min.

X-ray treatments

X-ray treatments of young inflorescences of clones KU 27 (in Experiments 2 and 3) and BNL 4430 (in Experiments 4 and 5) were performed using a Hitachi MBR-1505R X-ray generator. The treatments were conducted for 1 or 2 min at 150 kVp and 4 mA with a 0.5 mm Al+0.1 mm Cu filter at $23\pm0.5^{\circ}C$. The exposure data were obtained simultaneously with thermo-luminescence dosimeter (TLD) elements (National UD-170L) attached to individual inflorescences, and with a thermo-luminescence reader (National UD-502B). The exposure data obtained in R were then converted into absorbed doses in Gy with a converting factor of 9.57×10^{-3} (i.e., 1 R=9.57 mGy or 0.957 rad). The doses applied were 161 to 531 mGy for clone KU 27, and 501 to 976 mGy for clone BNL 4430.

Treatments in combinations

Treatments with MMS and X rays in combinations were performed by exposing the inflorescences of clones KU 27 and BNL 4430 in Experiments 3 and 5, respectively, first to 0.005% MMS solution and then to two or three different doses of X rays. Namely, the inflorescences for which the 16-hr treatment with 0.005% MMS had just been completed were immediately treated with X rays acutely. The X-ray doses applied were 134 to 448 mGy for clone KU 27, and 458 and 865 mGy for clone BNL 4430.

Scoring of mutations

The methods used for scoring pink mutations in stamen hairs in the present study are identical to those described elsewhere recently (Ichikawa et al., 1991; Sanda-Kamigawara et al., 1991; Ichikawa, 1992). That is, all the flowers that opened during the scoring period of three weeks after treatment were collected daily from each inflorescence; the numbers of stamen hairs and of pink mutant events were scored on each of six stamens; and the number of hair cells was also counted on ten representative hairs each of two oppositely located stamens per flower as described earlier (Ichikawa and Takahashi, 1977, 1978; Ichikawa and Ishii, 1991b), to estimate the average number of cells per hair for calculating mutation frequency per hair-cell division (Ichikawa and Takahashi, 1977, 1978; Ichikawa, 1984, 1992; Ichikawa et al., 1990, 1991; Ichikawa and Ishii, l99la, 1991b; Sanda-Kamigawara et al., 1991). A pink mutant event has been defined as a single pink cell, or two or more contiguous pink cells which are considered to have been derived from a single mutation (Ichikawa and Sparrow, 1968; Ichikawa et al., 1969). Two or more entirely pink hairs in a stamen, however, were scored as a single mutant event (Ichikawa, 1981a, 1992). A row of pink cells in a stamen hair, but separated by a single blue cell, was also regarded as one mutant event, since the occurrence of somatic recombination is considered to be more likely than simultaneous occurrences of two independent mutations (Ichikawa, 1981a, 1992; Sanda-Kamigawara et al., 1991).

The mutation frequency was expressed as the number of pink mutant events per 10^3 hairs as well as that per 10^4 hair-cell divisions. The mutation frequency per hair-cell division was obtained by dividing the mutation frequency per hair by one less than the average number of cells per hair, as described earlier (Sparrow and Sparrow, 1976; Ichikawa and Takahashi, 1977). The data were then pooled for the 4-day peak period for each treatment. The induced mutation frequencies per 10^4 hair-cell divisions (rather than those per 10^3 hairs) after subtracting each control frequency were exclusively used for the analyses of dose-response relationships, since the average number of cells per hair differs between clones and may differ after different mutagen treatments (Ichikawa and Takahashi, 1977; Ichikawa et al., 1991).

3. RESULTS

Induced mutation frequencies in KU 27

The somatic pink mutation frequencies induced by 16-hr treatments with 0.005, 0.01 and 0.02% MMS solutions are listed in Table 1 (Experiment 1), together with the data obtained after treating with water as the control. It is clear from these data that MMS caused severe loss of reproductive integrity (for this terminology, see Ichikawa et al., 1969) of the terminal cells of stamen hairs, which are

meristematic until hairs are fully developed (Ichikawa and Sparrow, 1968; Ichikawa et al., 1969, 1978; Ichikawa, 1981b, 1992), producing many short stunted hairs composed of much smaller numbers of cells than normal hairs. The decrease in the average number of cells per hair was larger with higher concentrations of MMS. Because of such great decreases in the cell number per hair, the mutation frequencies per 10^3 hairs are no longer reliable, in contrast to those per 10^4 hair-cell divisions, as seen in Table 1 (Experiment 1). When the MMS-induced mutation frequencies per 10^4 hair-cell divisions are plotted against MMS dose (%×hr) on a log-log graph, a regression line with a slope of 1.130 is obtained as shown in Fig. 1.

The somatic mutation frequencies induced by 161 to 478 mGy of X rays are also presented in Table 1 (Experiment 2). The mutation frequencies obtained in Experiment 3 in which the inflorescences were exposed either to 0.005% MMS for 16 hr alone, 423 or 531 mGy of X rays alone, or in combinations of MMS and X

Table 1. Somatic pink mutation frequencies in the stamen hairs of clone KU 27 treated with MMS alone (Experiments 1 and 3), X rays alone (Experiments 2 and 3), or in combinations (Experiment 3)

MMS (%)*	X-ray dose (mGy)	No. of hairs observed	No. of pink mutant events	No. of pink mutant events /10^3 hairs (±SE)	Minus control (±SE)	Average no. of cells /hair	No. of pink mutant events /10^4 cell divisions (±SE)	Minus control (±SE)
Experiment 1								
0	0	13,054	12	0.919±0.265	—	17.28	0.565±0.163	—
0.005	0	1,840	20	10.87 ±2.42	9.95±2.43	15.39	7.55 ±1.69	6.99±1.70
0.01	0	1,038	11	10.60 ±3.18	9.68±3.19	9.96	11.83 ±3.56	11.3 ±3.6
0.02	0	1,944	27	13.89 ±2.65	13.0 ±2.7	5.08	34.04 ±6.53	33.5 ±6.5
Experiment 2								
0	0	4,798	8	1.67 ±0.59	—	17.74	0.996±0.352	—
0	161	1,131	15	13.3 ±3.4	11.6 ±3.5	17.01	8.28 ±2.14	7.29±2.17
0	188	715	10	14.0 ±4.4	12.3 ±4.4	14.17	10.62 ±3.36	9.62±3.37
0	236	1,022	23	22.5 ±4.6	20.8 ±4.7	16.02	14.98 ±3.12	14.0 ±3.1
0	390	1,857	60	32.3 ±4.1	30.6 ±4.1	15.71	21.96 ±2.83	21.0 ±2.9
0	450	1,681	81	48.2 ±5.2	46.5 ±5.2	15.98	32.17 ±3.57	31.2 ±3.6
0	478	1,720	75	43.6 ±4.9	41.9 ±5.0	16.44	28.24 ±3.26	27.2 ±3.3
Experiment 3								
0	0	11,766	15	1.27 ±0.33	—	16.94	0.800±0.206	—
0.005	0	2,021	25	12.4 ±2.5	11.1 ±2.5	15.89	8.31 ±1.66	7.51±1.67
0	423	842	34	40.4 ±6.8	39.1 ±6.8	15.98	26.96 ±4.62	26.2 ±4.6
0	531	1,106	59	53.3 ±6.8	52.1 ±6.8	14.31	40.08 ±5.21	39.3 ±5.2
0.005	134	563	13	23.1 ±6.3	21.8 ±6.3	11.73	21.52 ±5.96	20.7 ±6.0
0.005	314	546	19	34.8 ±7.8	33.5 ±7.9	9.55	40.70 ±9.32	39.9 ±9.3
0.005	448	834	51	61.2 ±8.3	59.9 ±8.3	10.05	67.57 ±9.43	66.8 ±9.4

* Treated for 16 hr.

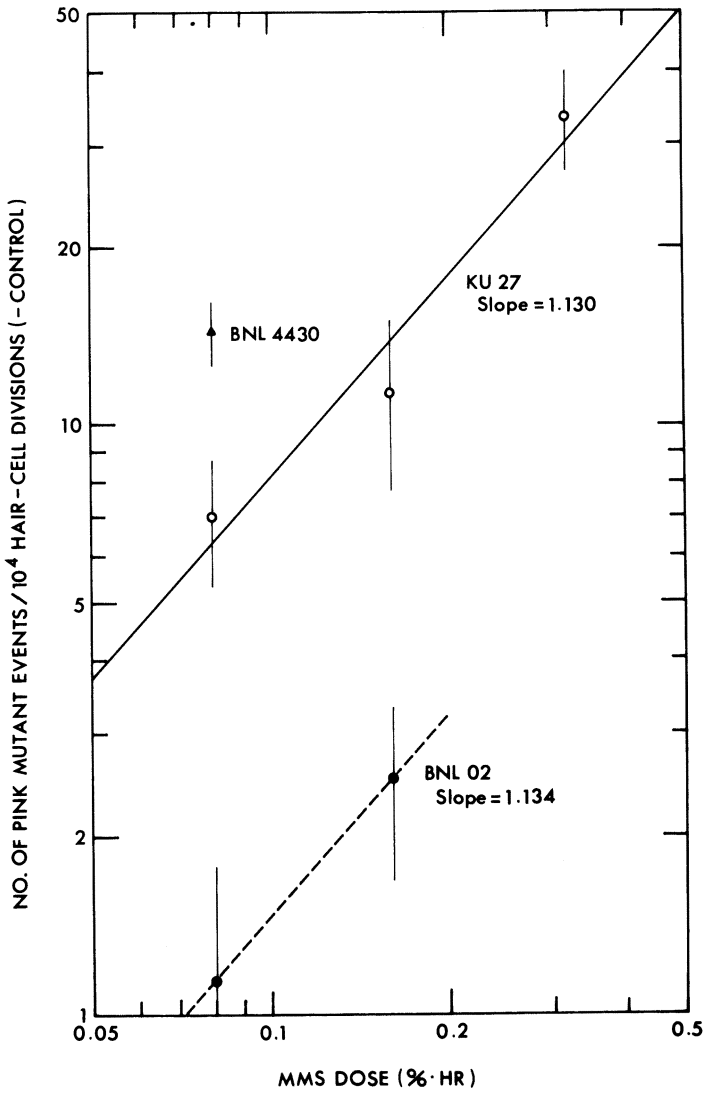

Fig. 1. The frequencies of pink mutant events per 10^4 hair-cell divisions in clones KU 27 and BNL 4430 treated with MMS for 16 hr. The data from clone BNL 02 for comparison were taken from those published earlier (Ichikawa et al., 1990) but were recalculated for the 4-day peak period. Vertical lines attached to the points plotted indicate standard errors.

rays (134, 314 or 448 mGy X rays immediately after 0.005% MMS for 16 hr) are also shown in the same table. The decreases in the average cell number per hair were not so great after treatments with X rays alone as seen in this table, while considerably decreased average cell numbers were observed after combined treatments with MMS and X rays. The X-ray-induced mutation frequencies per 10^4 hair-cell divisions obtained in Experiments 2 and 3 are plotted together

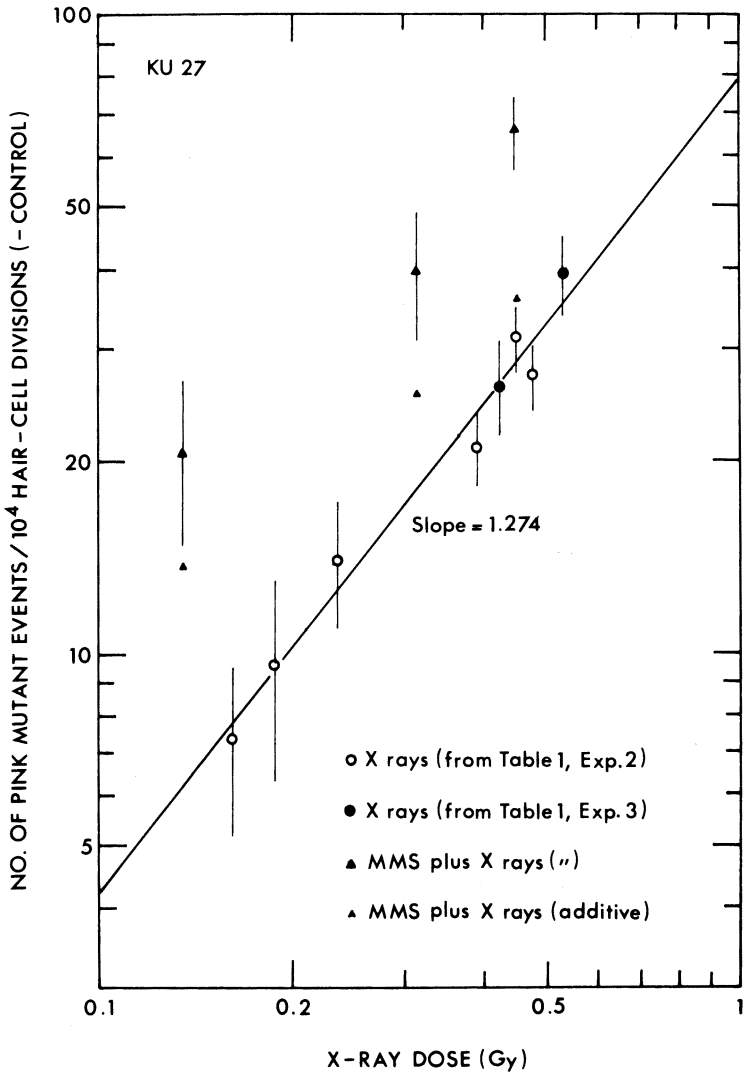

Fig. 2. The frequencies of pink mutant events per 10^4 hair-cell divisions in clone KU 27 treated with acute X rays alone or with 0.005% MMS for 16 hr plus acute X rays. Vertical lines attached to the points plotted indicate standard errors. The values expected from the additive effects of MMS and X rays are shown for comparison.

against X-ray dose on a log-log graph, and are shown in Fig. 2. The mutation frequency increased with X-ray dose with a slope of 1.274 on the log-log graph.

The mutation frequencies per 10^4 hair-cell divisions induced by MMS and X rays in combinations (in Experiment 3) are also plotted in Fig. 2 against X-ray dose for comparison. As seen in this figure, these frequencies were all higher than those expected from simply adding the frequency induced by 0.005% MMS (in Experi-

ment 3) and those by respective X-ray doses (calculated from the regression line in Fig. 2).

Induced mutation frequencies in BNL 4430

The somatic pink mutation frequencies induced by 506 to 772 mGy of X rays are listed in Table 2 (Experiment 4). The mutation frequencies obtained in Experiment 5 in which the inflorescences were treated either with 0.005% MMS for 16 hr alone, 501, 716 or 976 mGy of X rays alone, or in combinations of MMS and X rays (458 or 865 mGy X rays immediately after 0.005% MMS for 16 hr) are also presented in Table 2. As seen in this table, the decreases in the average number of cells per hair after X-ray treatments were not so great, and those after combined treatments with MMS and X rays were also much less than the cases in clone KU 27 (see Table 1). The X-ray-induced mutation frequencies per 10^4 hair-cell divisions obtained in Experiments 4 and 5 are plotted together against dose on a log-log graph as shown in Fig. 3. The regression line calculated with the least square method had a slope of 1.112.

The mutation frequencies per 10^4 hair-cell divisions obtained after treatments with MMS and X rays in combinations (in Experiment 5) are also plotted in Fig. 3 for ready comparison. The both points plotted are located obviously above the

Table 2. Somatic pink mutation frequencies in the stamen hairs of clone BNL 4430 treated with X rays alone (Experiments 4 and 5), MMS alone, or in combinations (Experiment 5)

MMS (%)*	X-ray dose (mGy)	No. of hairs observed	No. of pink mutant events	No. of pink mutant events /10^3 hairs (±SE)	Minus control (±SE)	Average no. of cells /hair	No. of pink mutant events /10^4 cell divisions (±SE)	Minus control (±SE)
Experiment 4								
0	0	9,566	40	4.18± 0.66	—	25.53	1.70±0.27	—
0	506	1,482	79	53.3 ± 5.8	49.1± 5.9	25.32	21.9 ±2.5	20.2±2.5
0	555	754	44	58.4 ± 8.5	54.2± 8.6	22.77	26.8 ±4.0	25.1±4.0
0	669	1,458	79	54.2 ± 5.9	50.0± 6.0	23.73	23.8 ±2.7	22.1±2.7
0	738	1,528	106	69.4 ± 6.5	65.2± 6.5	23.07	31.4 ±3.0	29.7±3.1
0	772	1,135	84	74.0 ± 7.8	69.8± 7.8	23.06	33.5 ±3.7	31.8±3.7
Experiment 5								
0	0	14,112	56	3.97± 0.53	—	24.67	1.68±0.22	—
0.005	0	2,425	81	33.4 ± 3.6	29.4± 3.7	21.76	16.1 ±1.8	14.4±1.8
0	501	1,344	66	49.1 ± 5.9	45.1± 5.9	22.81	22.5 ±2.8	20.8±2.8
0	716	1,097	81	73.8 ± 7.9	69.9± 7.9	21.73	35.6 ±4.0	33.9±4.0
0	976	786	70	89.1 ±10.2	85.1±10.2	19.97	46.9 ±5.6	45.3±5.6
0.005	458	1,026	89	86.7 ± 8.8	82.8± 8.8	19.84	46.0 ±4.9	44.4±4.9
0.005	865	761	106	139.3 ±12.6	135 ±13	18.72	78.6 ±7.6	76.9±7.6

* Treated for 16 hr.

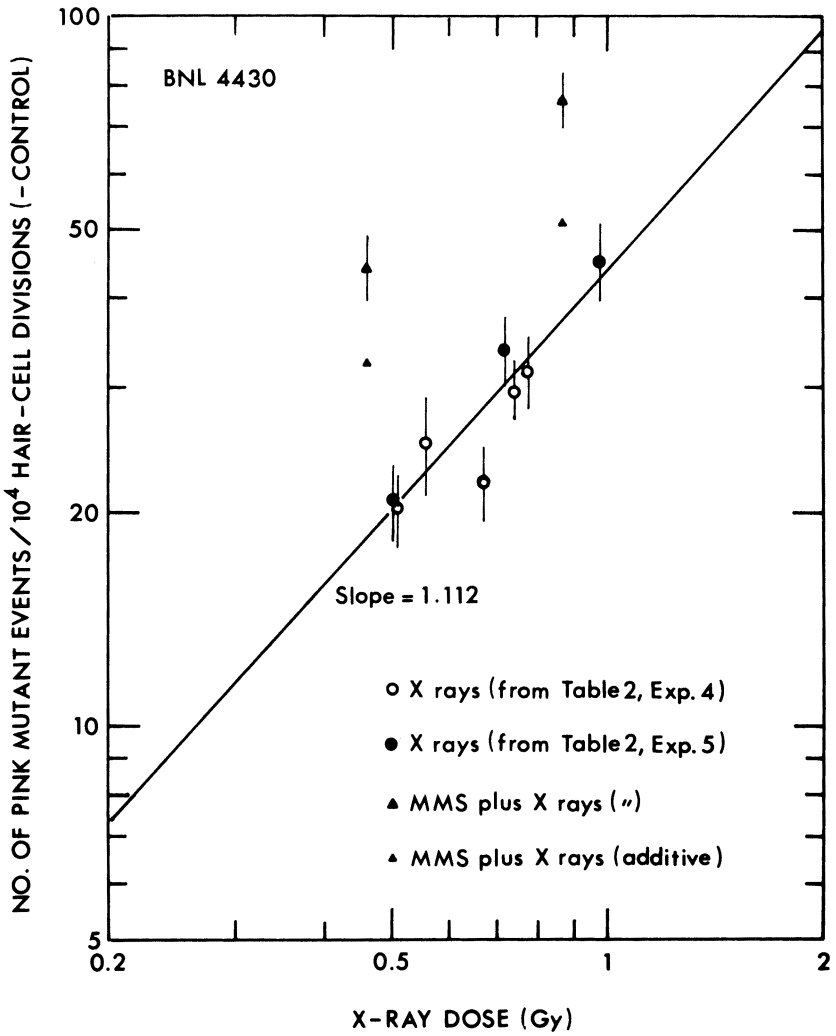

Fig. 3. The frequencies of pink mutant events per 10^4 hair-cell divisions in clone BNL 4430 treated with acute X rays alone or with 0.005% MMS for 16 hr plus acute X rays. Vertical lines attached to the points plotted indicate standard errors. The values expected from the additive effects of MMS and X rays are shown for comparison.

expected values obtained by simply summing the effect of MMS (in Experiment 5) and those of X rays (based on the regression line in Fig. 3).

4. DISCUSSION

MMS sensitivities of KU 27 and BNL 4430

The responses of clone KU 27 to 0.005, 0.01 and 0.02% aqueous solutions of

MMS (in Experiment 1, see Table 1) showed that the induced pink mutation frequency per 10^4 hair-cell divisions increased with the MMS dose (%×hr) with a slope of 1.130 on a log-log graph (Fig. 1). The values of slopes on log-log graphs reported earlier for the mutation frequency per 10^4 hair-cell divisions are 1.309 (Ichikawa and Takahashi, 1978) or 1.202 and 1.209 (Ichikawa et al., 1990) for EMS in clone BNL 02, 1.194 for EMS in clone KU 27 (Sanda-Kamigawara et al., 1991), and 1.441 for MMS in clone BNL 02 (Ichikawa et al., 1990). The value of 1.130 obtained in the present study is slightly or apparently smaller than those earlier values. It should be noted, however, that all the earlier values were those calculated for the data pooled for the 7-day peak periods, while the present value is that for the 4-day peak periods (for evaluating the mutation frequencies induced by the 16-hr MMS treatments together with those induced by the acute X-ray treatments which bring sharper peaks than semi-chronic treatments with mutagens; see Sanda-Kamigawara et al., 1991).

In fact, the value of slope of 1.441 for MMS in clone BNL 02 calculated for the data pooled for the 7-day peak period (Ichikawa et al., 1990) decreases to 1.134 when recalculated for those for the 4-day peak period, as shown in Fig. 1 for comparison. This is due to the fact that the values of mutation frequencies for the 4-day peak period are, as a matter of course, higher than those for the 7-day peak period, thus, when the data for the 4-day peak period were used instead of those for the 7-day peak period, the increase of the value after subtracting control frequency becomes relatively greater at smaller doses.

It is clear in Fig. 1 that clone KU 27 is about 5.6 times more sensitive to MMS than its parental clone BNL 02 in terms of induced mutation frequency. In Fig. 1, the induced mutation frequency in clone BNL 4430 treated with 0.005% MMS for 16 hr (in Experiment 5, see Table 2) is also plotted for comparison, and it is seen that clone BNL 4430 exhibited an induced mutation frequency which is about twice as much higher than that in clone KU 27.

Clone BNL 4430 has been demonstrated earlier to have higher sensitivities to two alkylating agents, DBE and EMS, as compared with clone BNL 02, i.e., to be seven to nine times more sensitive to DBE and also to EMS to a lesser extent (Sparrow et al., 1974; Nauman et al., 1976). It has been also reported that clone KU 27 is two to four times more sensitive to EMS than clone BNL 02 (Sanda-Kamigawara et al., 1991). The present study reconfirms the higher sensitivities of clones BNL 4430 and KU 27 to another alkylating agent, MMS, in comparison with clone BNL 02, but clone BNL 4430 being about two times more sensitive to MMS than clone KU 27 in terms of mutation frequency.

Responses of KU 27 and BNL 4430 to X rays

Based on the somatic pink mutation frequencies per 10^4 hair-cell divisions in clone KU 27 exposed to 161 to 531 mGy of X rays (in Experiment 2, see Table 1) and on those in clone BNL 4430 after 501 to 976 mGy X-ray treatments (in

Experiment 4, see Table 2), the dose-response regression lines on log-log graphs were calculated to have slopes of 1.274 and 1.112 for clones KU 27 and BNL 4430, respectively (Figs. 2 and 3). The slope for clone KU 27 agrees well with the value of 1.237 reported earlier for this and the parental BNL 02 clones treated acutely with X-ray doses ranging higher than in the present study (Sanda-Kamigawara et al., 1991). The value of 1.274 is also very close to the slope of 1.24 determined earlier for clone BNL 02 after acute gamma-ray treatments which also covered a higher dose range (Ichikawa et al., 1978), but this earlier value was obtained for the mutation frequency per hair. The slope of 1.112 determined for clone BNL 4430 in the present study is slightly smaller than 1.22 reported earlier for this clone treated with 100 to 1,000 mGy of acute X rays (Sparrow et al., 1974), but the earlier value was also that for the mutation frequency per hair. It should be noted here that the slope on a log-log graph for the mutation frequency per hair-cell division should be somewhat larger than that for mutation frequency per hair, because greater reductions in cell number per hair occur with higher doses (Sanda-Kamigawara et al., 1991).

These slopes larger than 1 seem to be the results of involving two-break deletions in the mutational events, as discussed earlier (Ichikawa and Takahashi, 1978; Ichikawa et al., 1978, 1990).

It has been demonstrated that clone KU 27 exhibits X-ray-induced mutation frequencies per 10^4 hair-cell divisions comparable to those in clone BNL 02 (Sanda-Kamigawara et al., 1991). Clone BNL 4430 has been also described to show a radiosensitivity almost identical with that of clone BNL 02 in terms of X-ray-induced mutation frequency per hair (Sparrow et al., 1974; Nauman et al., 1976). In the present study, however, the mutation frequencies per 10^4 hair-cell divisions in clone KU 27 (Fig. 2) were obviously higher than those in clone BNL 4430 (Fig. 3). The apparent discrepancy between the earlier reports (Sparrow et al., 1974; Nauman et al., 1976) and the present results plus our recent finding (Sanda-Kamigawara et al., 1991) is due to the fact that the earlier comparison between clones BNL 4430 and BNL 02 were made on the basis of the mutation frequency per hair. Clone BNL 4430 has longer hairs (larger numbers of cells per hair; see control values in Table 2) than clones KU 27 (see controls in Table 1) and BNL 02 (see Ichikawa, 1984; Ichikawa et al., 1990; Ichikawa and Ishii, 1991b). Thus, even if the mutation frequency per hair in clone BNL 4430 was almost identical with that in clone BNL 02, the mutation frequency per hair-cell division in clone BNL 4430 should be lower than that in clone BNL 02. In fact, the X-ray-induced mutation frequencies per 10^3 hairs in clones KU 27 (Table 1) and BNL 4430 (Table 2) are almost identical as shown in Fig. 4, being quite different from Figs. 2 and 3. It is clearly seen in Fig. 4 that comparison of the mutation frequencies per hair between these two clones gives a false impression as if the both clones show almost identical responses.

Therefore, comparison of mutation frequencies in *Tradescantia* stamen hairs

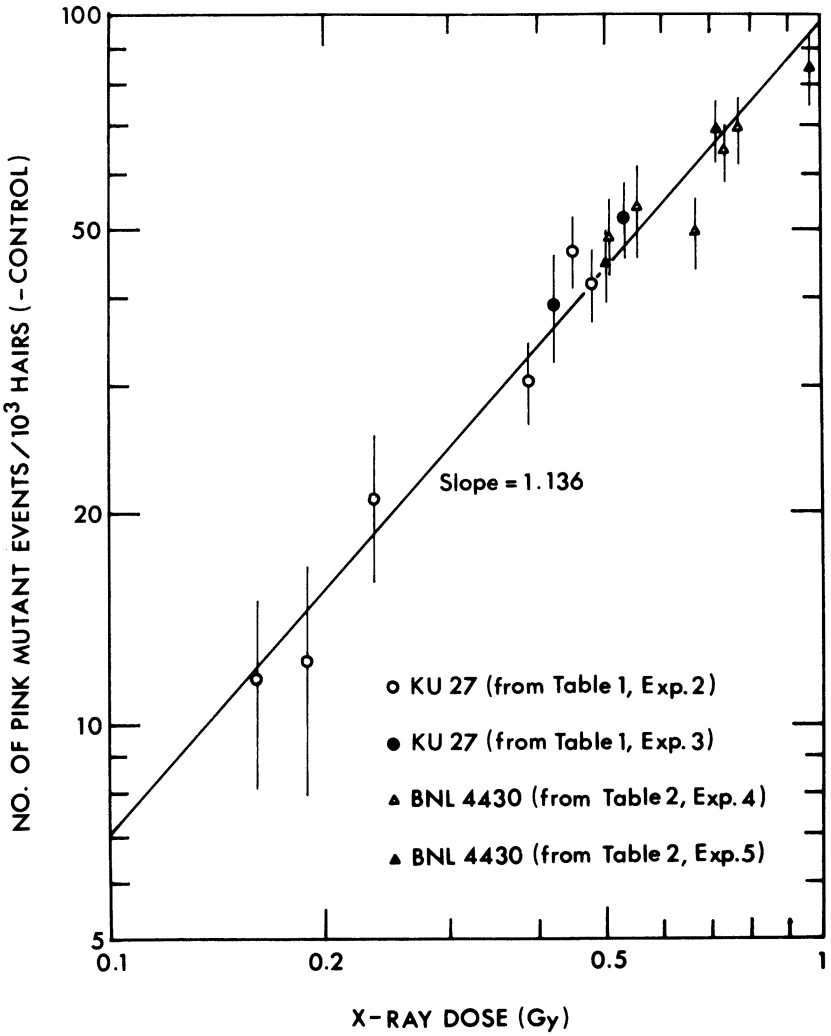

Fig. 4. The frequencies of pink mutant events per 10^3 hairs in clones KU 27 and BNL 4430 treated acutely with X rays. Vertical lines attached to the points plotted indicate standard errors. Comparison of the mutation frequencies per hair between these two clones gives a false impression as if the both clones show almost identical responses.

between different clones should be made based on the number of mutant events per hair-cell division, but not on that per hair. Comparing the mutation frequencies per 10^4 hair-cell divisions in Figs. 2 and 3, at 500 mGy, it can be concluded that clone KU 27 is about 1.5 times more radiosensitive than clone BNL 4430.

Synergistic effects of MMS and X rays

The mutation frequencies per 10^4 hair-cell divisions induced by 0.005% MMS

plus two or three different doses of X rays in combinations were all higher than those expected from additive effects of MMS and X rays in the both clones, KU 27 (Fig. 2) and BNL 4430 (Fig. 3). By testing the significance of the differences between the observed and the expected (additive) frequencies, it was found that the mutation frequencies observed after treatments of clone KU 27 with 0.005% MMS plus 448 mGy X rays and of clone BNL 4430 with 0.005% MMS plus 865 mGy X rays were highly significantly higher than those expected from the additive effects, as shown in Table 3. The present results of detecting such clearly significant synergistic effects in inducing mutations were obtained by applying X rays immediately after the 16-hr MMS treatment at 0.005%. In our recent experiments with clone BNL 4430, highly significantly synergistic effects in inducing somatic mutations have been clearly detected after three different combinations of MMS and X-ray treatments, namely, X-rayed either 30 min before, at the just middle time during, or immediately after a 4-hr MMS treatment at 0.02%, the extents of the synergisms being almost identical in all the three cases (Shima and Ichikawa, in preparation).

Table 3. The differences between the mutation frequencies observed after treatments with MMS and X rays in combinations and the mutation frequencies expected from the additive effects

Clone	Treatment	No. of pink mutant events /10^4 cell divisions observed (O)	No. of pink mutant events /10^4 cell divisions expected from			O/E	Significance
			MMS	X rays	Total (E)		
KU 27	MMS+134 mGy	20.7±6.0	7.51[1)]	6.15[2)]	13.7	1.51	$0.3<p<0.5$
	MMS+314 mGy	39.9±9.3	7.51	18.1[2)]	25.6	1.56	$0.2<p<0.3$
	MMS+448 mGy	66.8±9.4	7.51	28.5[2)]	36.0	1.86	$p<0.01$
BNL 4430	MMS+458 mGy	44.4±4.9	14.4[3)]	18.3[4)]	32.7	1.36	$0.05<p<0.1$
	MMS+865 mGy	76.9±7.6	14.4	37.1[4)]	51.5	1.49	$p<0.01$

[1)] From Experiment 3 in Table 1.
[2)] Based on the regression line in Fig. 2.
[3)] From Experiment 5 in Table 2.
[4)] Based on the regression line in Fig. 3.

A synergistic effect of EMS and X rays in inducing mutations in the stamen hairs of *Tradescantia* clone KU 9 has been reported by Cebulska-Wasilewska et al. (1981), and a possible synergistic effect has been also suggested by Nauman et al. (1979) between DBE and ^{3}H beta rays in the stamen hairs of clones BNL 4430 and BNL 02 treated with ^{3}H-labeled DBE. The confirmation of the synergistic effect of MMS and X rays in inducing mutations in the stamen hairs of clones KU 27 and BNL 4430 in the present study supports these earlier studies, adding new evidences for the combinations of MMS and X rays, and indicates that such

synergistic effects must be common between ionizing radiations and alkylating agents.

Another synergistic effect observed between MMS and X rays in the present study was that in decreasing the number of cells per hair especially in clone KU 27 (see Table 1), suggesting that MMS and X rays act synergistically also in causing the loss of reproductive integrity of the hair cells. Similar synergistic effects in reducing the cell number per hair have been reconfirmed in our recent experiments mentioned above (Shima and Ichikawa, in preparation).

These synergisms indicate that some common mechanisms exist between alkylating agents and ionizing radiations in causing DNA damages which lead to induction of mutations (Ichikawa, 1992) and also to the loss of reproductive integrity of cells. It has been reviewed that the majority of mutations induced by ionizing radiations are due to chromosomal breaks (Nauman et al., 1975; Ichikawa et al., 1978; Ichikawa, 1981b) resulting from DNA double-stranded breaks. A double-stranded break may result from simultaneous breaks of two strands of DNA or from two independent single-stranded breaks occurring closely nearby. The loss of reproductive integrity of cells has been also demonstrated to be associated with chromosomal breaks (Ichikawa et al., 1978; Ichikawa, 1981b). Even as for the somatic pink mutations in *Tradescantia* stamen hairs induced at lower dose rates and/or with small doses of ionizing radiations, the linear dose-response curves demonstrated repeatedly (Sparrow et al., 1972, 1974; Nauman et al., 1975; Ichikawa et al., 1978, 1981, 1991; Ichikawa, 1981b, 1992) indicate that predominantly one-hit events occur at such low dose rates and with small doses (Ichikawa et al., 1978; Ichikawa, 1981b), the one-hit events being those such as single chromosomal breaks leading to base-deletion type mutations (Ichikawa, 1981b). On the other hand, alkylating agents have been shown to induce DNA single-stranded breaks in higher plants (Veleminsky and Gichner, 1978). Therefore, it seems reasonable to conclude that the single-stranded breaks induced by alkylating agents and those by ionizing radiations are interacting together to produce the synergistic effects.

Synergistic effects as observed clearly in the present study has implications for current risk evaluation of individual mutagens (Cebulska-Wasilewska et al., 1981; Ichikawa, 1992). That is, if a synergistic effect exists between any two (or more) different mutagens, then simply summing the risks evaluated for the individual mutagens will result in an obvious underestimation of the real risk. Therefore, more intensive studies are urgently needed for such synergistic effects between chemical and physical mutagens, and probably also between different chemicals.

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