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Synergism of mutant frequencies in the mouse lymphoma cell mutagenicity assay by binary mixtures of methyl methanesulfonate and ethyl methanesulfonate

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Summary

The effect of mixed mutagen exposures on the rate and type of induced mutants was studied in the L5178Y/TK^{+/-} → TK^{-/-} mouse lymphoma cell mutagenicity assay. In this assay, exposure to ethyl methanesulfonate (EMS) results in more mutants that form large colonies than small colonies. Exposure to methyl methanesulfonate (MMS) results in more mutants that form small colonies than large colonies. Other reports in the literature suggest that large colony TK^{-/-} mutants appear to result from small-scale, perhaps single-gene mutations, and that small-colony TK^{-/-} mutants appear to be associated with chromosomal mutations. Treating cells for 4 h with simple, 2-component mixtures containing 6.45 µg/ml MMS and either 261, 392, 560 or 712 µg/ml EMS resulted in synergism of mutants at each mixture level. The frequencies of total mutants were synergized 12, 20, 35 and 72%, respectively, in mixed exposures with graded doses of EMS, above the sums of the mixture components. Small colony mutants were synergized to a greater extent than large colony mutants. The frequencies of small colony mutants in mixed exposures were increased 31, 54, 73 and 123%, respectively, while the frequencies of large colony mutants were increased -7, -6, 11 and 39%. Statistical analyses provide strong evidence of synergism (within the limits of the assay) for total and small-colony mutants at all doses of EMS tested, and for large-colony mutants above 400 µg/ml EMS. Similar magnitudes of synergism resulted when other constant levels of MMS (4.30 or 8.60 µg/ml) were mixed with the same graded doses of EMS. The degree of synergism was dependent on EMS concentration but not on MMS concentration.

Most mutagenicity studies evaluate the mutagenicity of single agents rather than mixtures of agents, a situation more representative of exposures in nature. Valuable information has come

from studies with single agents, but increasing interest in identifying and assessing environmental hazards points to the need for more information on mixed exposures. A review of mixed exposures and chemical interactions relative to mutagenicity has been published by Tennant et al. (1987). Synergism of mutants has been demonstrated in vitro and in vivo. Using the Ames assay, synergism results with mixtures of benzo[*a*]pyrene and

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benzo[*e*]pyrene (Hass et al., 1981), with mixtures of propyl gallate and *N*-hydroxy-2-acetylaminofluorene (Rosin, 1981), and from our laboratory, with mixtures of aflatoxin B₁ and butylated hydroxytoluene (Shelef and Chin, 1980). Synergistic effects have also been observed in mammalian cells (V79) with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and streptozocin (Harbach et al., 1982). Using the micronucleus assay, Watanabe et al. (1982) showed that Cd²⁺ enhanced the mutagenicity of dimethylnitrosamine in mice.

The L5178Y/TK^{+/-} → TK^{-/-} mouse lymphoma cell mutagenicity assay, as standardized by Turner et al. (1984), was used in the present study because it permits the recovery of two classes of thymidine kinase (TK) deficient mutants. By using EMS and MMS, each of which induces predominantly one class of mutant, L5178Y/TK^{+/-} cells afford a unique way of studying the genotoxic effects resulting from the interaction of these two mutagens. Clive et al. (1979) reported that TK^{-/-} mutant colonies in soft agar have a bimodal distribution for size. Mutants form large and small colonies, depending on the type of genetic damage (Clive and Moore-Brown, 1979; Moore-Brown and Clive, 1979; Clive et al., 1980; Clive, 1983; Moore et al., 1985a). Large colonies indicate small-scale, perhaps intragenic (single-gene), damage and small colonies represent larger incursions on the genome, perhaps in the form of intergenic (chromosomal) damage. Some doubt concerning the origin of small-colony mutants has been raised by Amacher and Paillet (1981), who suggested that small colonies can result from trifluorothymidine (TFT) selection or delayed toxicity. Their skepticism was recently proven to be unwarranted because: (1) Oberly et al. (1986) showed that induced and spontaneously formed small-colony mutants truly lack TK enzyme activity, and (2) additional cytogenetic data established that most small-colony mutants originate from cells that possess at least one chromosomal aberration in chromosome 11 (Hozier et al., 1981, 1983, 1985; Moore et al., 1985b; Blazak et al., 1986), which contains the mouse *tk* gene (Kozak and Ruddle, 1977).

In this publication, we apply binary mixtures of MMS and EMS to the mouse lymphoma cell mutagenicity assay and show that these mixtures

synergize the formation of mutant colonies. We introduce the term % change, as a measure of the magnitude of synergism relative to the mutant frequencies in separate exposures. Mixtures of MMS and EMS are synergistic in accordance with the definition proposed by the United Nations Scientific Committee (1982): the number of mutants obtained with mixtures of MMS and EMS exceeds the sum of the mutants obtained with separate exposures to MMS and EMS. At the doses tested, synergism was dependent on EMS concentration but not MMS concentration. Surprisingly, synergism was higher among small colony mutants – the predominant type produced by MMS – than among large-colony mutants, which are the predominant type induced by EMS.

Materials and methods

Cell culture

Mouse lymphoma L5178Y/TK^{+/-} –3.7.2C cells were obtained through the generosity of Dr. Donald Clive, Burroughs Wellcome Co., Research Triangle Park, NC. They were maintained in suspension culture in Fischer's medium (Gibco, Grand Island, NY), supplemented with 10% horse serum, 0.22 μg/ml sodium pyruvate, and 1.0 mg/ml Pluronic F68 (BASF Corp., Wyandotte, MI). Cultures were incubated at 37°C on a combination incubator-shaker (125 rpm) and cleansed weekly to kill spontaneously formed TK^{-/-} mutants.

Mutagens

MMS (Eastman Kodak Co.) and EMS (Eastman Kodak Co.) were purchased from Sigma Chemical Co., St. Louis, MO. MMS was dissolved in phosphate-buffered saline (PBS). EMS was dissolved in dimethyl sulfoxide (DMSO) and diluted with PBS. Each culture flask (treated and solvent controls) contained the same amount of DMSO (0.1%) and PBS. Both mutagens were prepared fresh and sterilized by filtration.

Mutation assay

The L5178Y/TK^{+/-} → TK^{-/-} mouse lymphoma cell mutagenicity assay was conducted in conformance with the protocol of Turner et al. (1984), with a 4-h exposure to mutagen followed by 48 h for expression of mutations. The selection medium for TK^{-/-} mutants contained 1.0 μg/ml trifluoro-

thymidine (Sigma Chemical Co.), 0.37% Noble agar (Difco, Detroit, MI), and 20% horse serum in Fischer's medium. TK^{-/-} mutants were selected after incubation for 14 d in the presence of TFT under 5% CO₂ in air at 37°C. Mutant colonies were counted by eye with the aid of a magnifying colony counter. Small and large colonies in soft agar were distinguished by size. The criteria for scoring a small colony were that the colony be round, intact, and in the range of 0.15–0.6 mm in diameter. Typical colony sizes were determined by a magnifying lens that contained a sizing grid. Most small colonies were approximately 0.25 mm in diameter. Large colonies were approximately 3–4 times the diameter of small colonies. A large colony, likewise, had to be round, intact, and in the range of 0.60–1.0 mm in diameter. Most large colonies were approximately 0.75 mm in diameter.

Statistical analysis of mixtures

Induced mutant frequencies (IMFs) were calculated as the number of induced mutants/10⁶ survivors for total mutants, small-colony mutants, and large colony mutants. IMFs were used to calculate % change:

$$\frac{\text{IMF}_{A+B} - (\text{IMF}_A + \text{IMF}_B)}{\text{IMF}_A + \text{IMF}_B} \times 100 = \% \text{ change}$$

where A and B are components of mixtures. Positive % change indicates synergism, zero % change indicates addition, and negative % change indicates antagonism.

Synergism of total, small, and large colony mutants was analyzed by weighted least squares regression analysis (Neter et al., 1985). A weighted approach was selected because the observed variability in % change increased with EMS dose. Inverse sample variances were used as the weights for regression analysis.

Results

Mutant colonies were synergized when mouse lymphoma cells were exposed to binary mixtures of MMS and EMS. Within an experiment, cells were exposed to a constant concentration of MMS (either 4.30, 6.45 or 8.60 µg/ml) and graded concentrations of EMS (261, 392, 560 and 712 µg/ml).

Tables 1 and 2 show the results of a typical, constant MMS:graded EMS mixed exposure experiment, in this case to 6.45 µg/ml MMS + 261, 392, 560 or 712 µg/ml EMS. Table 1 lists the data (colony counts, survival and mutant frequencies) used for the analysis of synergism, which is presented in Table 2. The dose of MMS was mildly toxic (83% relative total growth and mutagenic (IMF = 386; spontaneous frequency (f_s) = 55). In our laboratory, dose response for MMS appear to be linear to 18.4 µg/ml. Single doses of EMS were also moderately toxic (45–86% relative total growth) and mutagenic. Dose responses for EMS also appear to be linear, to 712 µg/ml (Fig. 1). A comparison of the actual mixture IMFs with the expected IMFs (determined by adding together the corresponding single agent IMFs; Table 2) resulted in positive % change values for total mutants: 9, 24, 59 and 66%, respective to increasing doses of EMS. Of the two colony types, the % change values were greater for small-colony mutants: 34, 67, 117, 158%, than large-colony mutants: -8, -3, 26, 16%. Total and small-colony mutants were apparently synergized at these levels of EMS. EMS was the variable component in this experiment, and more small-colony mutants were synergized than large-colony mutants. Cloning efficiencies show that cytotoxicity was not synergized.

Figs. 1a–c show the mean values, when the experiment described in Tables 1 and 2 was repeated 5 times. Fig. 1a is a plot of IMFs for total mutants against exposures to EMS, and shows that all mixtures appear to be synergistic. The mean values of % change were 12, 20, 35 and 72%, respectively, with increasing doses of EMS. Fig. 1b is a plot of IMFs for small-colony mutants against exposures, and shows higher levels of apparent synergism for all the mixtures. The mean values of % change were 31, 54, 73 and 123% respectively. Fig. 1c is a plot of IMFs for large-colony mutants against exposures, and shows apparent synergism for the two highest concentrations of EMS only. The mean values of % change were -7, -6, 11 and 39%, respectively.

Regression analysis of % change against EMS concentration was used to test the hypothesis of additivity, and to test the dependency of synergism on EMS concentration. Plots of % change

TABLE 1

COLONY COUNTS IN A TYPICAL MIXED EXPOSURE EXPERIMENT WITH A CONSTANT LEVEL OF MMS AND GRADED LEVELS OF EMS

($\mu\text{g/ml}$)		VC colonies ^a	% CE ^b	%Relative total growth ^c	Total		Small-colony		Large-colony	
EMS	MMS				mutants ^d	MF ^e	mutants	MF	mutants	MF
-	-	492	82	100	68	55	5	4	62	50
-	6.45	363	60	83	434	479	200	220	234	259
261	-	360	60	86	454	505	132	146	322	358
392	-	342	57	76	677	792	195	228	482	564
560	-	311	52	61	889	1144	255	327	634	815
712	-	252	42	45	989	1570	296	469	693	1100
261	6.45	293	49	67	740	1011	355	484	385	525
392	6.45	238	40	43	888	1493	440	739	448	753
560	6.45	150	25	23	921	2456	441	1176	480	1280
712	6.45	102	17	10	833	3267	449	1760	384	1506

^a Viable count (VC), number of colonies formed from 600 cells plated in non-selective medium.
^b Cloning efficiency (CE), VC/600.
^c % Relative total growth, (relative 2-day suspension growth) × (relative cloning efficiency).
^d Total mutants, number of mutant colonies formed from 1.5×10^6 cells plated in selective medium.
^e Mutant frequency (MF), number of mutant colonies/ 10^6 survivors.

vs. EMS concentrations are presented in Figs. 2a-c for total mutants, small-colony mutants, and large-colony mutants, respectively. Since levels of MMS (4.30, 6.45 and 8.60 $\mu\text{g/ml}$) had no apparent effect on synergism (see analysis below), results from experiments with these MMS levels were pooled in the regression analyses.

These plots of % change vs. EMS concentration suggest that % change of total, small, and large colony mutants increased approximately linearly with EMS concentration. We therefore fit the simple linear regression model $E(Y) = a + bx$ (where

$E(Y)$ represents the expected % change and x the EMS level) using weights as described in the Materials and Methods section. The resulting regression equations were:

$$\hat{Y} = -12.9 + 0.0977x \quad (\text{total mutants})$$

$$\hat{Y} = 1.4 + 0.1315x \quad (\text{small-colony mutants})$$

$$\hat{Y} = -24.1 + 0.0766x \quad (\text{large-colony mutants})$$

where \hat{Y} represents the predicted % change.

The corresponding F statistics for significant regressions of % change on EMS concentration were: 25.1 (df = 1 and 35, $p < 0.00002$, total

TABLE 2

SYNERGISM OF MUTANT COLONIES IN A TYPICAL MIXED EXPOSURE EXPERIMENT WITH A CONSTANT LEVEL OF MMS AND GRADED LEVELS OF EMS

($\mu\text{g/ml}$)		Total mutants			Small-colony mutants			Large-colony mutants		
EMS	MMS	Actual IMF ^a	Expected IMF ^b	% change ^c	Actual IMF	Expected IMF	% change	Actual IMF	Expected IMF	% change
261	6.45	956	874	9	480	358	34	475	517	-8
392	6.45	1438	1161	24	735	440	67	703	723	-3
560	6.45	2401	1513	59	1172	539	117	1230	974	26
712	6.45	3212	1939	66	1756	681	158	1456	1259	16

^a Induced mutant frequency (IMF), number of induced mutants per 10^6 survivors.
^b Expected IMF based on adding together the single agent IMFs.
^c % change = $\frac{\text{IMF}_{A+B} - (\text{IMF}_A + \text{IMF}_B)}{\text{IMF}_A + \text{IMF}_B} \times 100$

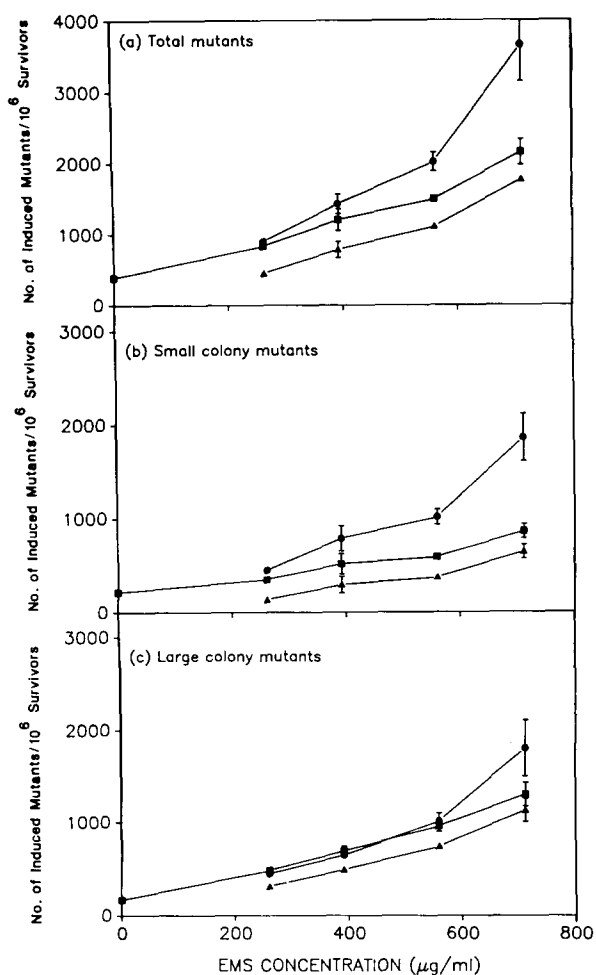


Fig. 1. (a) Synergism in total mutants. (b) Synergism in small-colony mutants. (c) Synergism in large-colony mutants. (●) Mixed exposures to MMS and EMS; (■) arithmetically summed separate EMS and MMS exposures; (▲) single agent exposures to EMS. The point at which the summed single agent exposure curve intersects 0 on the X-axis represents the IMF of 6.45 $\mu\text{g}/\text{ml}$ MMS. The error bars are ± 1 standard error from the mean in 5 independent Expts., except for the points at 392 $\mu\text{g}/\text{ml}$ EMS for which there are only 3 replicate trials. Error bars were omitted when the standard error was too small to permit an accurate display.

mutants), 21.4 ($df = 1$ and 35, $p < 0.0001$, small-colony mutants), and 19.4 ($df = 1$ and 35, $p < 0.0001$, large-colony mutants). Based on this linear model, the estimate curves in each of the plots (the solid lines) represent estimated mean % change values. The increase in % change was greatest for small-colony mutants.

We calculated 95% confidence bands (the

dashed lines) for the regression curves. These bands were chosen to have a 95% prior probability of covering the entire regression curve. They are also plotted in Figs. 2a-c.

The estimate curves suggest that synergism occurs for total and small-colony mutants at all EMS doses tested, and for large-colony mutants at EMS doses above 315 $\mu\text{g}/\text{ml}$. The lower 95% confidence band provides strong evidence of synergism for total and small-colony mutants at all

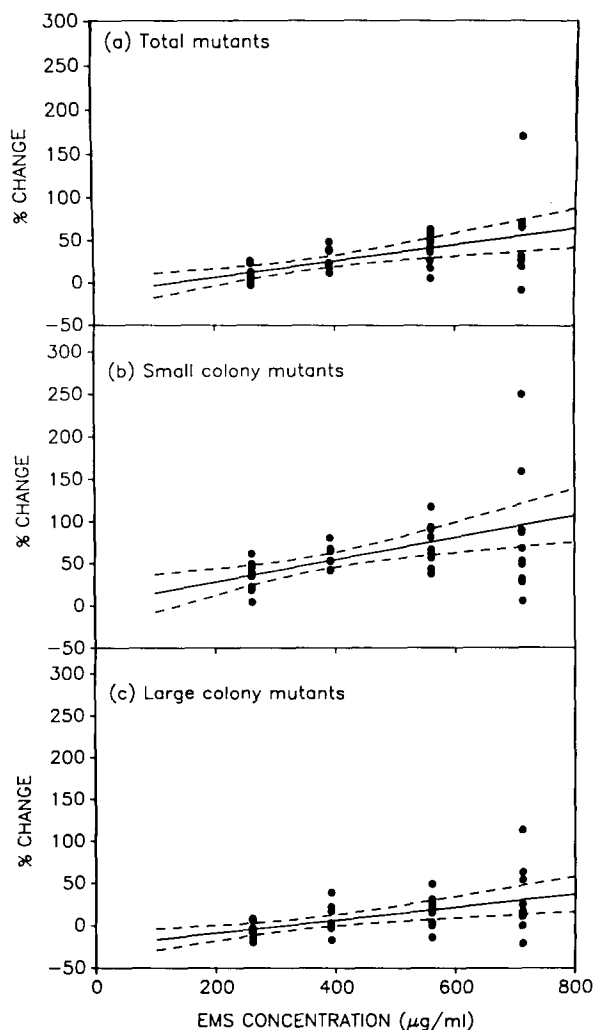


Fig. 2. (a) Estimate curve (solid line) for total colony mutants. (b) Estimate curve (solid line) for small-colony mutants. (c) Estimate curve (solid line) for large-colony mutants. Solid circles represent the % change value obtained in each experiment. Each Figure shows the 95% confidence bands (broken lines) for the estimate curve. These estimates were obtained from 10 independent Expts.

EMS doses tested, and for large-colony mutants above 400 $\mu\text{g}/\text{ml}$.

We tested the dependency of synergism on MMS concentration by substituting other constant doses (4.30 and 8.60 $\mu\text{g}/\text{ml}$ MMS) into mixtures containing the same graded levels of EMS (261, 392, 560 and 712 $\mu\text{g}/\text{ml}$). When the % change values were compared for the 3 MMS levels (4.30, 6.45 and 8.60 $\mu\text{g}/\text{ml}$) by analysis of variance, no significant differences were obtained for total ($F = 0.91$, $df = 2$ and 34 , $p > 0.40$), small ($F = 0.52$, $df = 2$ and 34 , $p > 0.60$), or large-colony mutants ($F = 1.17$, $df = 2$ and 34 , $p > 0.30$). In this analysis of constant MMS:graded EMS experiments, 3 replicate experiments contained 4.30 $\mu\text{g}/\text{ml}$ MMS, 5 replicate experiments contained 6.45 $\mu\text{g}/\text{ml}$ MMS, and 2 replicate experiments contained 8.60 $\mu\text{g}/\text{ml}$ MMS. Synergism was dependent on EMS concentration but not on MMS concentration.

Discussion

This paper is part of a systematic study of mixed exposures to mutagens. The identification of a mixture that significantly synergizes mutants in a standard mammalian cell assay points to the need for more information on the incidence, mechanism, and properties of synergism and on the risks that mixed exposures pose for health.

We have established the phenomenon of synergism by showing that mutant frequencies of mixed exposures are greater than the mutant frequencies expected by adding together the corresponding single agent exposures, i.e. $\text{IMF}_{A+B} > \text{IMF}_A + \text{IMF}_B$. Testing this simple comparison is permissible because our single agent dose responses follow linear functions (United Nations Scientific Committee, 1982). Fig. 1 shows that the dose response of EMS is linear, within the range that was tested, and our MMS dose response, agreeing with the data of Moore et al. (1985a), also fits a linear function. Even though the absolute number of mutant colonies was not synergized, our data, when corrected for cytotoxicity, show that the induced mutant frequencies were synergized.

One advantage of using the mouse lymphoma cell assay for studying mutagenesis is that these cells produce two types of $\text{TK}^{-/-}$ mutants. One

type forms large colonies, reflecting single-gene mutations, and the other type forms small colonies, reflecting chromosomal mutations. A good example of the assay's usefulness is the demonstration that simple methyl alkylating agents favor the formation of small-colony mutants, whereas their ethyl analogues favor the formation of large-colony mutants (Clive, 1983; Turner et al., 1984).

These characteristics are exhibited to good advantage in our demonstration of synergism in mixtures that contain a constant dose of MMS and graded doses of EMS. Our analysis shows that the frequency of total mutants is synergized. This frequency is composed of small and large colony mutants. Of the two colony types, small-colony mutants are synergized to a greater extent and at lower EMS doses than large-colony mutants. Given that EMS favors the formation of large-colony mutants, we expected that if synergism were to occur, increasing the EMS dose would result in increasing synergism of large-colony mutants. Our data shows the opposite effect. Increasing exposures of EMS resulted in increased synergism of small-colony mutants.

We used % change as a mathematical expression for the magnitude of synergism. Our data show that the % change values in each experiment were greater than zero for total and small-colony mutants. The significance of the effect was confirmed using a statistical analysis that incorporated all the available data. This analysis suggests that the % change values, at the doses tested, were significantly greater than zero for total and small-colony mutants, and greater than zero for large-colony mutants at the highest EMS level tested. The statistical analysis confirms that the variability in the % change values was low enough to conclude that the mixtures of MMS and EMS were synergistic.

When this assay is conducted properly, the identification of small- and large-colony mutants reflect lesions in the genome rather than extragenetic factors. Competition for nutrients appears not to be a factor, since the number of mutant colonies that we counted in the mixture and single exposure plates were similar. For example, the number of total mutants counted after exposure to 560 $\mu\text{g}/\text{ml}$ EMS (Table 1) was 889 colonies. The

number of total mutants after exposure to 6.45 $\mu\text{g}/\text{ml}$ MMS + 560 $\mu\text{g}/\text{ml}$ EMS was 921 colonies. The similarity in the total number of mutant colonies speaks against the possibility that the increase in small colony mutants in mixed exposures results from "starving" of large-colony mutants by nutrient depletion from other colonies.

Cloning efficiencies (Table 1) show that cytotoxicity was not synergized. The viable count (VC) colonies listed in Table 1, column 3, represent the number of colonies formed from plating 600 cells. For the mixture (6.45 $\mu\text{g}/\text{ml}$ MMS + 560 $\mu\text{g}/\text{ml}$ EMS), the viable count was 150 colonies, implying that the mixture was toxic for 450 cells (i.e., 600-150 = 450). The viable count for 6.45 $\mu\text{g}/\text{ml}$ was 363; this exposure was toxic for 237 cells. For 560 $\mu\text{g}/\text{ml}$ EMS, the viable count was 311; this exposure was toxic for 289 cells. The cytotoxicity of the mixture (450 cells) was less than the sum of toxicities for MMS (237 cells) and EMS (289 cells).

We monitored the ratio of small to large mutant colonies in the 5 replicate experiments to assess the variability in colony type from experiment to experiment and to address the concern of an inadvertent counting bias in favor of small-colony mutants in mixed exposure experiments. It is important to show that errors in colony counting are not responsible for the apparent synergism of

small colony mutants. Table 3 shows the counts of small and large colony mutants, as well as the ratios in each of the experiments, for 6.45 $\mu\text{g}/\text{ml}$ MMS, 560 $\mu\text{g}/\text{ml}$ EMS, and 6.45 $\mu\text{g}/\text{ml}$ MMS + 560 $\mu\text{g}/\text{ml}$ EMS. Some variability in the ratios of small to large colonies is evident; however, the ratios for mixtures and single agent exposures within an experiment were always displaced in the same direction (either above or below the mean ratios) in each of the 5 experiments. For example, in Expt. No. 2 (Table 3), the MMS ratio (0.99) was below its mean ratio (1.30), the EMS ratio (0.44) was below its mean (0.51), and the mixture ratio (0.70) was below its mean (1.02). This pattern was consistent for all 5 Expts., except for Expt. No. 4, where the experimental ratios closely approximated the mean ratios. This same pattern was observed in the other single and mixed exposures as well. Since the variabilities in the ratios for mixture and single agent exposures were consistently in the same direction within an experiment, bias in counting appears unlikely.

This paper demonstrates synergism of mutant colonies resulting from exposure of mouse lymphoma cells to binary mixtures of EMS and MMS. Synergism was more apparent in small rather than large colony mutants. These results imply that binary mixtures synergize intergenic (chromosomal) mutations. We have further studies

TABLE 3

SMALL AND LARGE MUTANT COLONY COUNTS AND THE RATIO OF SMALL TO LARGE MUTANT COLONIES IN 5 INDEPENDENT Expts.

($\mu\text{g}/\text{ml}$)			Expt. No.					Mean	SE ^b
EMS	MMS	CT/Ratio ^a	1	2	3	4	5		
-	6.45	S	244	131	264	180	200	204	23.6
-	6.45	L	171	158	214	175	235	191	14.5
-	6.45	S/L	1.42	0.83	1.23	1.03	0.85	1.07	0.113
560	-	S	280	205	321	311	255	274	20.9
560	-	L	551	479	532	599	634	559	26.8
560	-	S/L	0.51	0.43	0.60	0.52	0.40	0.492	0.035
560	6.45	S	484	266	528	444	441	433	44.6
560	6.45	L	410	391	455	437	480	435	15.8
560	6.45	S/L	1.18	0.68	1.16	1.02	0.92	0.992	0.091

^a Colony type (CT): small (S), large (L).

^b Standard error (SE).

in progress to more directly demonstrate that binary mixtures do synergize chromosomal mutations.

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