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# Potentiating Effect of Methyl Methane Sulfonate on Friend Virus Leukemogenesis *in Vivo* (40522)

R. B. RAIKOW, R. F. MEREDITH, B. J. BROZOVICH, P. R. SEEMAN, A. E. LIVINGSTON, AND J. P. OKUNEWICK

*Cancer Research Unit, Clinical Radiation Therapy Research Center, Allegheny General Hospital, Pittsburgh, Pennsylvania 15212*

Both chemical carcinogens and radiation have been shown to be potentiators of oncogenic viruses in various systems *in vitro* and in some studies *in vivo*. Toth (1) and Irino *et al.* (2) demonstrated as early as 1963 that cell free extracts obtained from chemically induced lymphoma would cause leukemia when injected into normal mice. Ball and McCarter (3) and Igel *et al.* (4) subsequently demonstrated reproducible activation and production of leukemia virus after treatment with chemical carcinogens. These findings have been confirmed in a number of *in vivo* and *in vitro* studies suggesting that interaction with exogenous (5–12) or endogenous (13–16) viruses may play an important role in the production of some tumors by chemical carcinogens (also see 17 for review). More recently, Pottathil *et al.* (18) have demonstrated the suppression of chemical carcinogenesis by administration of anti-viral antibodies to animals not known to already contain oncogenic virus, thus further substantiating the hypothesis of a viral intermediary in certain cases of chemical carcinogenesis.

To explain these observations, effects of chemical carcinogens on both the immune response (19) and the transforming target cell itself (5, 8) have been postulated. Even *in vitro* the mechanism by which chemicals potentiate the carcinogenic action of viruses remains uncertain, although all of the chemical agents thus far found to be active in this way have been shown to interact with DNA (20).

The present study is concerned with the action of one of these chemical agents, methyl methane sulfonate (MMS), on potentiation of Friend viral leukemia and on immune response. MMS has been shown to be carcinogenic in some animals (21) and to interact with adenovirus in cultured hamster cells (5). It is also known to cause short, easily-repaired, strand breaks in DNA similar to those caused by ionizing radiation (22). The objectives of the present study were: (a) To determine if MMS would act as a potentiating agent for an RNA cancer virus *in vivo*; (b) to determine if such a potentiation might be dependent on the relative time of administration of the chemical and the virus; and (c) to determine whether the humoral immune response of the host as measured by antibody-producing plaque-forming-cell (PFC) assay might be involved in such viral potentiation.

*Materials and methods. Animals.* Two strains of mice were used, the leukemia virus sensitive SJL/J (23) and a normally virus resistant B10SJF$_1$ hybrid (24). The SJL/J female mice employed in these studies were purchased from Jackson Laboratories (Bar Harbor, Me.) at 6–8 weeks of age and used when 10–14 weeks old. The B10SJF$_1$ female hybrids were bred by us from Jackson Laboratories stock by crossing C57Bl/10J females with SJL/J males. They were also used when 10–14 weeks old. The mice were housed in autoclaved plastic cages and autoclaved bedding with filter tops in an environmentally controlled room with a 12-hr light cycle. They were given autoclaved Purina Lab Chow and acidified water *ad libitum*.

*Virus.* Our Friend leukemia virus (FLV) stock was originally obtained from NCI in 1969 and has been stored at −60°. It was passaged once in SJL/J mice and a plasma preparation obtained for use in these studies. This FLV rich plasma was titered by the spleen enlargement assay (25). One spleen enlargement dose (SED) is defined as the amount of virus that causes splenomegaly in 50% of SJL/J mice by day 14. The virus containing plasma was diluted with phosphate buffered saline to give the appropriate SED of virus and injected intraperitoneally. Virus dose levels were chosen on the basis of



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their ability to induce leukemia in less than 100% but more than 20% of the animals, acting alone without chemical carcinogen.

*Methyl methane sulfonate.* MMS (Sigma) was diluted with sterile isotonic saline and injected intraperitoneally. Toxicity tests to determine maximum tolerable dose were carried out for single doses or two doses given 19 hr apart (Table I). On the basis of these tests the dose of 2 mg/mouse was selected for use in combination with the virus. Animals dying within 24 hr of MMS injection were considered to have died from chemical toxicity and are not included in the data presented in the other figures and tables of this report.

*Response parameters monitored.* Mice were kept for 250–300 days after virus treatment. All mice were checked daily and autopsied upon death. Autopsy examinations were directed at determining the existence of other tumors as well as evidence of leukemia as a probable cause of death. Leukemia development before death was monitored by periodic white blood cell counts, hematocrit determinations, and microscopic examination of blood smears.

*Plaque forming cell (PFC) assay.* To evaluate the level of humoral immune response the PFC assay was used as an index of the number of viable antibody producing cells. Effect of MMS on antibody producing PFC number was determined using the Kennedy-Axelrad modification (26) as previously employed by us (23, 24).

*Results. Effect of MMS and FLV on virus-sensitive mice.* Figure 1 shows the effect of 2 mg MMS given in conjunction with 0.1 SED FLV on the survival of SJL/J mice. With this dose of virus a significant enhancement of erythroleukemia occurred when MMS was given 5 hr before the virus. An even greater enhancement of viral leukemogenesis was seen when MMS was given as two injections at 24 and 5 hours preceding virus (Fig. 1 and Table II). However, only a slight indication of enhancement was seen when 24 hr elapsed between a single dose of MMS and the virus injection without a second injection of MMS. There was no enhancement at all when MMS was given 5 hr after the virus. To the contrary, there was some indication of a possible protective effect of the MMS against early deaths but not against later deaths.

TABLE I[a]

| MMS dose (mg/mouse) | % deaths |
|---|---|
| 0.1–1.0 (1×) | 0 |
| 0.1–1.5 (2×, 19 hr apart) | 0 |
| 2.0 (1×) | 10.6 |
| 2.0 (2×, 19 hr apart) | 26.3 |
| 3.0 (1×) | 44.8 |

[a] Drug-related deaths after injection of various doses of MMS to SJL/J mice. Animals died within 24 hr of the injection with no obvious pathological signs.

Two modes of death could be clearly distinguished by their symptomology and their time of maximum incidence (Table II). One was the erythroleukemia that is normally characteristic of Friend disease and results in early deaths (27). The second was a lymphoma that appeared later among the mice surviving beyond the peak of erythroleukemia incidence. Both these modes were evident in all groups that received virus, but absent in the controls and in the mice receiving MMS only. Although some deaths also occurred among the latter two groups, none could be associated with either the erythroleukemia or the above-mentioned lymphoma. Hepatosplenomegaly in the mice with the lymphoma was generally less marked than in those dying earlier of erythroleukemia, and in contrast to the erythroleukemia syndrome, there was enlargement of all lymphoid organs including the thymus gland. White blood counts were elevated in some of the mice developing this lymphoma. When elevated they were frequently associated with a large population of myeloid cells and monocytes. The above described erythroleukemia and lymphoma symptoms were not always mutually exclusive, and some mice showed characteristics of both diseases. Table II shows that MMS affects the expression of these two types of cancer by shifting the effect in favor of erythroleukemia in the period of less than 150 days after virus and toward lymphoma after that time, with no animals receiving MMS 5 hr before virus showing symptoms of both conditions upon death.

*Effect of MMS on virus-resistant B10SJF$_1$ hybrid mice.* Figure 2 shows the effect of MMS on our leukemia virus resistant mouse strain. Even when given a dose of virus 1000 times as large as that given to the SJL/J mice, only a very few of the B10SJF$_1$ mice developed erythroleukemia, and did so much later

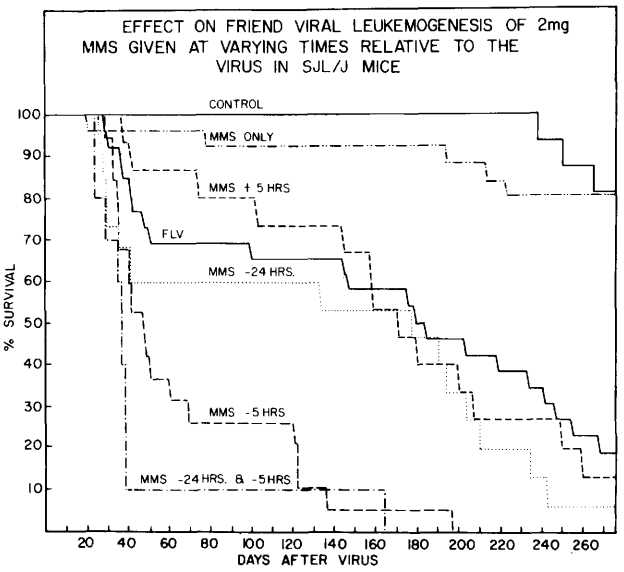


FIG. 1. Survival of mice treated at 10–12 weeks of age (day 0) with 2 mg MMS and/or 0.1 SED FLV as indicated. Sham injections of saline were also given at appropriate times so that all animals were injected 3 times. The results represent three experiments in which the survival curves were superimposable and hence were pooled. MMS given before virus is represented as −5 and/or −24 hr. MMS given after virus is represented as +5 hr.

TABLE II[a]

| | Erythroleukemia | Symptoms of both erythroleukemia and lymphoma | Lymphoma |
|---|---|---|---|
| Virus only | | | |
| Deaths before 150 days | 66.7 (5/9) | 22.2 (2/9) | 11.1 (1/9) |
| Deaths after 150 days | 33.3 (3/9) | 33.3 (3/9) | 33.3 (3/9) |
| MMS 5 hr after virus | | | |
| Deaths before 150 days | 42.9 (3/7) | 28.6 (2/7) | 28.6 (2/7) |
| Deaths after 150 days | 0 | 57.1 (4/7) | 42.9 (3/7) |
| MMS 24 hr before virus | | | |
| Deaths before 150 days | 88.9 (8/9) | 0 | 11.1 (1/9) |
| Deaths after 150 days | 0 | 57.1 (4/7) | 42.9 (3/7) |
| MMS 5 hr before virus | | | |
| Deaths before 150 days | 94.4 (17/18) | 0 | 5.5 (1/18) |
| Deaths after 150 days | 0 | 0 | 100 (2/2) |
| MMS 24 and 5 hr before virus | | | |
| Deaths before 150 days | 100 (7/7) | 0 | 0 |
| Deaths after 150 days | 0 | 0 | 100 (1/1) |

[a] Type of death seen in SJL/J mice after exposure to MMS (2 mg/mouse) and FLV (0.1 SED/mouse) taken from the survival curves for these mice plotted in Fig. 1. The percent dead showing the stated signs are given. The fraction in parentheses are the number of individuals with the stated signs over the total number in that group.

than their sensitive parent. However, following 2 mg of MMS 5 hr before 100 SED units of virus, 90% of these animals developed leukemia. A dose of 2 mg of MMS in combination with 50 SED units of FLV also resulted in increased incidence of erythroleukemia, but to a lesser extent, suggesting that experimental demonstration of the potentiating effect of chemical agents on viral cancer is probably dependent on the dose of virus as well as on the dose of the chemical carcinogen. All of the hybrid mice dying in these experiments had elevated white blood cell counts and gross hepatosplenomegaly at au-

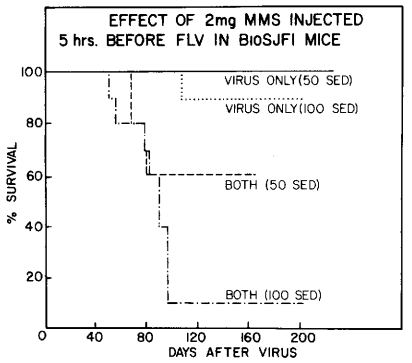


FIG. 2. Survival of B10SJF$_1$ mice injected at 10–14 weeks of age (day 0) with 2 mg MMS in saline or with saline only 5 hr before either 50 SED or 100 SED FLV. All of the deaths seen were due to erythroleukemia as indicated by elevated WBC counts and hematocrits before death and by a marked hepato-splenomegaly.

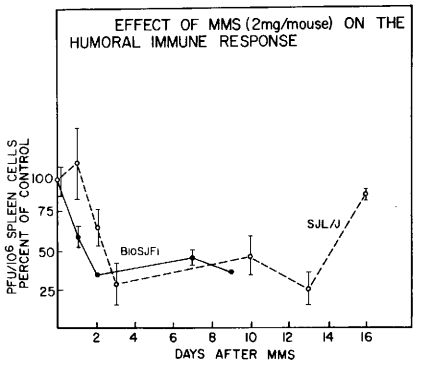


FIG. 3. Ten to fourteen-week old SJL/J or B10SJF$_1$ mice were injected with 2 mg MMS at different times before sensitization by sheep red blood cell (SRBC) antigen (as indicated on the abscissa). The ability of splenocytes from these mice to lyse SRBC *in vitro* was tested by the PFC technique described by us previously (23, 24).

topsy. None showed evidence of the lymphoma.

*Effect of MMS on PFC response.* It has been previously demonstrated that Friend virus alone will severely repress PFC response in retrovirus sensitive mice (28, 29). It is also known that mice can be induced to develop antibodies to leukemia viruses (30) and that the SJL/J mouse can be thus immunized against murine viral leukemia (31). The studies illustrated in Fig. 3 were performed to determine whether MMS might reduce the capability of the animals to produce antibody forming cells, rendering them more susceptible to the virus. Within 2–3 days after MMS injection both SJL/J and B10SJF$_1$ mice showed a fourfold reduction in their ability to mount a humoral immune response to sheep red blood cells. This reduction was maintained for at least 13 days (Fig. 3). On the other hand, as described above, the leukemogenesis enhancement by MMS was strongest at 5 hr but barely evident at 24 hr after MMS injection (Fig. 1). Thus the timing for the depression of the PFC response did not parallel the timing of the carcinogenic potentiating effect of MMS.

*Discussion.* The possibility that chemical carcinogens may interact with oncogenic viruses in a potentiating manner has important implications for cancer etiology. Even though both types of carcinogens may produce malignancies while acting alone and unaided, evidence that the two may also interact in animals to produce cancer should be considered when attempting to extrapolate chemical carcinogenesis studies from animals to man. The recent work of Pottathil *et al.* (18) serves to emphasize this point. In the system studied by these authors, suppression of chemically induced carcinogenesis by antibody directed against murine leukemia virus strongly suggested a viral intermediary, even though no virus could be demonstrated by direct tests.

Previous studies have shown that in certain systems potentially oncogenic viruses could be activated by exposure to chemical carcinogens *in vivo* (3, 4, 6, 13–16, 32, 33), or could interact with chemical carcinogens *in vitro* (5–12). The purpose of the present studies was to examine *in vivo* some of the conditions under which interactions may occur and to determine if given in common the two agents might produce cancer in animals otherwise resistant to induction of the disease. From the data obtained in the SJL/J mice it is apparent that relative timing of the virus and chemical exposure is an important condition for demonstration of a potentiating interaction. The data suggest that with MMS there is a relatively narrow window available for potentiating interaction to occur. Regan and Setlow (22) examined the effects of this chemical on chromosomal DNA and report that it produces relatively short single-strand breaks similar to those produced by X-irradiation, and which are relatively quickly repaired.

The high level of interaction seen in the present studies when MMS is given at 5 hr before the virus or in two doses 5 and 24 hr before virus suggests that the potentiation observed may also be dependent on the existence of such strand breaks at the time of exposure to the virus.

In related *in vitro* studies rat embryo cells injected with Rauscher leukemia virus (RLV) have been used by several authors (10–12) to show that the presence of virus enhances the cells' susceptibility to chemical carcinogenesis. Results reported by Waters et al. (34) using these cells indicate that postreplication repair of DNA damage caused by chemical carcinogens may be interfered with by the presence of oncogenic virus leading to oncogenic transformation. With rat embryo cells *in vitro*, Price and his co-workers (12) have shown that RLV must be present when the cells are exposed to the chemical carcinogen if cocarcinogenesis is to be demonstrable. Chemical carcinogens given prior to subsequent RLV infection of rat embryo cells were ineffective. Conversely, pretreatment by chemical carcinogens has been reported to enhance adenovirus transformation of hamster embryo cells in vitro (5, 9). In our *in vivo* system, a 5-hr pretreatment with MMS in relation to FLV injection enhanced leukemogenesis, but posttreatment did not. The reason for the apparent discrepancy between these findings and the RLV infected rat embryo cells is not known at this time. However, the fact that RLV given alone does not transform rat embryo cells, while FLV alone does produce murine leukemia and adenovirus alone does transform hamster cells, may be significant.

The strongest evidence in our system for an *in vivo* viral-chemical oncogenic interaction as opposed to simple activation of a latent virus comes from the studies with the B10SJF$_1$ mice. Crossing the virus-sensitive SJL/J mice with the virus-resistant C57Bl/10J mice produces this hybrid, which is comparatively resistant to FLV under normal circumstances. Yet a large fraction of these mice developed Friend leukemia upon exposure to MMS in conjunction with the virus. The present studies, in which the level of MMS was kept constant, also suggests that demonstration of the potentiating effect may be dependent on the size of the virus dose. Since B10SJF$_1$ mice are relatively resistant to FLV alone, the enhancement of viral leukemogenesis by MMS pretreatment is more clearly seen in this hybrid than in SJL/J mice. Therefore, this hybrid may prove to be the most useful in assessing the effect of chemical carcinogens on viral leukemogenesis.

The timing of the effect of MMS on PFC response does not fit however with the presently observed maximum potentiation of viral leukemia induction. Little or no effect on PFC response was observed until two days after MMS injection, while maximum potentiation of leukemogenesis occurred with a separation between chemical and virus of five hours. Although more definitive studies need to be done on the role of the immune system in relation to viral-chemical carcinogen interaction, the level of this immune response does not seem to be a critical factor governing the potentiation reported here. Nevertheless, immunosuppression by both oncogenic viruses (28, 29) and by chemical carcinogens (35) have been previously reported, and may be of critical importance in promoting tumor growth in otherwise marginal cases of *in vivo* cancer development. However, for the present studies it would appear that possible interaction of chemical and viral carcinogens at the level of the target cell nucleus was probably a more critical factor in determining initial transformation and potentiation.

*Summary.* Methyl methane sulfonate (MMS) given ip five hours before Friend Leukemia Virus (FLV) injection enhanced the leukemogenic action of FLV in virus-sensitive SJL/J mice and also in relatively virus-resistant B10SJF$_1$ mice. MMS also decreased the humoral immune response, as measured by plaque forming cell assay. However, the timing of the effect of MMS on the immune system did not coincide with the timing of the MMS related potentiation of leukemia. Hence, it is suggested that the potentiating effect of this chemical on viral leukemogenesis is more likely due to events occurring at the intracellular level than at the level of humoral immune response.

1. Toth, B., Proc. Soc. Exp. Biol. Med. **112**, 824 (1963).
2. Irino, S., Ota, Z., Sezaki, T., and Suzaki, K., Gann **54**, 225 (1963).

3. Ball, J. K., and McCarter, J. A., J. Nat. Cancer Inst. **46,** 751 (1971).
4. Igel, H. J., Huebner, R. J., Turner, H. C., Kotin, P., and Falk, H. L., Science **166,** 1624 (1969).
5. Casto, B. C., Pieczyaski, W. J., and DiPaolo, J. A., Cancer Res. **34,** 72 (1974).
6. Salerno, R. A., Ramm, G. M., and Whitmire, C. E., Cancer Res. **33,** 69 (1973).
7. Price, P. J., Suk, W. A., Peters, R. L., Gilden, R. V., and Huebner, R. J., Proc. Natl. Acad. Sci. U.S.A. **74,** 579 (1977).
8. Mishra, N. K., Pant, K. J., Thomas, F. O., and Price, P. J., Int. J. Cancer **18,** 852 (1976).
9. Hatch, G. G., Balwierz, P. J., Casto, B. C., and DiPaolo, J. A., Int. J. Cancer **21,** 121 (1978).
10. Freeman, A. E., and Igel, H. W., *In Vitro* **11,** 107 (1975).
11. Rhim, J. S., and Huebner, R. J., Cancer Res. **33,** 695 (1973).
12. Price, P. J., Bellew, T. M., King, M. P., Freeman, A. E., Gilden, R. V., and Huebner, R. J., Proc. Natl. Acad. Sci. U.S.A. **73,** 153 (1976).
13. Karande, K. A., Taskar, S. P., and Ranadive, K. J., Brit. J. Cancer **31,** 434 (1975).
14. Bibby, M. C., and Smith, G. N., Brit. J. Cancer **32,** 660 (1975).
15. Nexo, B. A., and Ulrich, K., Cancer Res. **38,** 729 (1978).
16. Huebner, R. J., Turner, H. C., Kotin, P., and Falk, H. L., Science **166,** 1624 (1969).
17. Roe, F. J. C., and Rowson, K. E. K., Int. Rev. Exp. Pathol. **6,** 181 (1968).
18. Pottathil, R., Huebner, R. J., and Meier, H., Proc. Soc. Exp. Biol. Med. **159,** 65 (1978).
19. Schaller, J. P., Mathes, L. E., Hoover, E. A., Koestner, A., and Olsen, R. G., Cancer Res. **38,** 996 (1978).
20. Brookes, P., Life Sci. **16,** 331 (1975).
21. Tomatis, L., Agathe, C., Bartsch, H., Huff, J., Montesano, R., Saracci, R., Walker, E., and Wilbourne, J., Cancer Res. **38,** 877 (1978).
22. Regan, J. D., and Setlow, R. B., Cancer Res. **34,** 3318 (1974).
23. Meredith, R. F., OKunewick, J. P., Brozovich, B. J., and Weaver, E. V., Dev. Comp. Immunol. **2,** 133 (1978).
24. OKunewick, J. P., Meredith, R. F., Brozovich, B., and Weaver, E. V., Proc. Soc. Exp. Biol. Med. **157,** 449 (1978).
25. Chirigos, M. A., Luber, E., March, R. W., and Pettigrew, H., Cancer Chemother. Rep. **45,** 29 (1965).
26. Kennedy, J. C., and Axelrad, A. A., Immunol. **20,** 253 (1971).
27. Siegler, R., and Rich, M. A., Cancer Res. **24,** 1406 (1964).
28. Ceglowski, W. S., and Friedman, H., J. Nat. Cancer Inst. **40,** 983 (1968).
29. Ceglowski, W. S., Campbell, B. P., and Friedman, H., J. Immunol. **114,** 231 (1975).
30. Thomson, S., and Axelrad, A., Cancer Res. **28,** 2105 (1968).
31. OKunewick, J. P., Phillips, E. L., and Brozovich, B. J., Amer. J. Hematol. **1,** 443 (1976).
32. Links, J., Calafat, J., Buijs, F., and Tol, O., Europ. J. Cancer **13,** 577 (1977).
33. Weiss, R. A., Friis, R. R., Katz, E., and Vogt, P. K., Virology **46,** 920 (1971).
34. Waters, R., Mishra, N., Bouck, N., DiMayorca, G., and Regan, J. D., Proc. Nat. Acad. Sci. **74,** 238 (1977).
35. Levy, R. L., Barrington, M. H., Lerner, R. A., Griffin, G. F., and Whitmire, C. E., Cancer Res. **37,** 3892 (1977).

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