EFFECTS OF CHEMICAL
CARCINOGENS ON HEMOPOIESIS,
IMMUNOPOIESIS AND VIRAL ONCOGENESIS

Technical Progress Report
for Period December 1, 1977 - September 30, 1978

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"Effects of Chemical Carcinogens on Hemopoiesis,
Immunopoiesis and Viral Oncogenesis"

Abstract

During the past year we have concentrated on defining the circumstances under which methyl methanesulfonate (MMS), benzo[a]pyrene (BP) and 7,12-dimethylbenz[a]anthracene (DMBA) interact with Friend virus (FLV) to produce leukemia. Several experiments utilizing these chemicals have been carried out past 250 days, and therefore the pattern of erythroleukemia and lymphoma development in mice receiving virus alone or both chemical and viral carcinogens have been clarified. Evidence of virus potentiation can be observed within 100 days after virus injection. Experiments in which chemical carcinogen treatments were given at various times in relation to the virus were initiated and the optimum scheduling for each and also the effective dose levels of the chemicals have been partially determined.

It was found that there are at least three critical factors which govern whether or not a leukemogenic interaction can be shown between the chemical agents and the virus. These are chemical dose, virus dose and their relative time of administration. The most critical of these appears to be virus dose, as it has both an upper and lower threshold for demonstration of a combined effect. The optimum virus dose appears to be that which results in between 25 and 40% incidence of leukemia within 40 days after virus infection when virus is given alone. At higher doses all of the animals

quickly succumb to the leukemia and the potentiating effect of the chemical carcinogens is masked. At excessively low doses, although some potentiating effect of the chemicals can be observed, the incidence of a carcinogenic response during the observation period (260 to 365 days) is of a level so low as to render it difficult to attach a statistical significance to the effect with the number of animals being utilized. The chemical carcinogens appear to have a lower dose threshold, below which no significant potentiating effect can be observed. The only upper limit would appear to be that of acute drug toxicity. The third element, timing, appears to be equally critical and varies according to the chemical. This variation may reflect different mechanisms of action by the chemical agents and/or different pharmacology.

Interaction between these three chemicals and FLV have been seen when low doses of virus were used in virus-sensitive SJL/J mice and also when high doses of virus were used in virus-resistant B10SJF1 mice. Data on the effects of MMS, BP and DMBA on the immune system, as measured by the ability of mice to mount a humoral immune response to sheep red blood cells, have indicated that the viral enhancement is probably not dependent on this function. Techniques for the measurement of cellular immune response, such as cell mediated cytotoxicity via ^{51}Cr release and lymphocyte stimulation are being tested.

Further enhancement of the potentiation of viral leukemogenesis was observed using benzo[a]pyrene and caffeine when the caffeine was added four hours before virus, indicating that the inhibition by caffeine of the repair of lesions in DNA may be an important factor in

virus potentiation. To date an equivalent effect on viral leukemogenesis seen utilizing caffeine and benzo[a]pyrene has not been observed when either is used alone with the virus.

The standardization of our in vivo system in terms of virus doses employed and the leukemia symptoms monitored should make it possible to utilize it to assay new chemicals including mixtures of by-products from the manufacture or refining of hydrocarbon fuels.

Technical Progress Report
"Effects of Chemical Carcinogens on Hemopoiesis
Immunopoiesis and Viral Oncogenesis"

I. Introduction

The research goals which were set forth for this project last year include:

- 1) Determination of whether or not exposure to selected chemical carcinogens or mutagens could potentiate leukemogenesis in vivo in animals given a threshold or subthreshold dose of leukemia inducing virus.
- 2) Determination of whether or not such potentiation might involve an action of the chemical agents on the immune response.
- 3) Delineation of the conditions under which potentiation might occur, including time spread between chemical and virus and possible mechanisms of action.

Three agents were selected for emphasis in the period covered by this report. These were methyl methanesulfonate (MMS), benzo[a]pyrene (BP), and 7,12-dimethylbenz[a]anthracene (DMBA). Although it was proposed last year that 3-methyl cholanthrene (MCA) also be studied, animal space limitations precluded any extensive work with a fourth agent. During the current year the MMS studies have been nearly completed, and these will be phased out and replaced with MCA and possibly an additional hydrocarbon, propane sultone, which is thought to act on DNA in a manner similar to MMS. The sections below describe our progress in meeting the above listed goals during the past year.

II. Definition of the Assay System

Our experience during the past several months has emphasized that the dose of virus employed in our system is critical and must be standardized to permit the demonstration of an interaction between viral and chemical carcinogens. This is particularly

true when the virus-sensitive SJL/J mice are used, but it is also a factor when the virus-resistant B10SJF1 mice are used. Too large a dose of virus, when given to the virus sensitive mice, results in 100% incidence of leukemia, with all of the test mice dying rapidly within 40 to 60 days after virus exposure. Thus any possible potentiating effect of the chemical carcinogen is obscured by the action of the virus alone. Conversely, too low a dose of virus results in a subthreshold response for leukemogenesis in which not only does no disease develop, but the animals may show an enhanced immune response to the leukemia antigen and possibly build an immunity against the leukemia (1,2,3). Therefore, due to the inherent difficulties in obtaining an exact titer for number of leukemogenically active virus particles, each experiment must be carefully controlled, and the response to chemical carcinogen plus virus compared each time to that seen for the same preparation of virus injected alone into animals from the same randomized group. In doing so, those experiments in which the "virus only" controls show that the actual virus dose received by the animals was either too low or too high are rejected. The nominal dose of Friend virus which we have found to yield the most consistent results is 0.1 SED units. One SED unit is defined as that dose which will result in spleen size doubling in 50% of the animals at 14 days after virus injection.

After injection of 0.1 SED Friend leukemia virus (FLV) into SJL/J mice, the survival curve is biphasic, with two types of deaths occurring at different times after virus exposure.

The first of these is due to erythroleukemia and always occurs around day forty after virus injection. Mice with erythroleukemia have their hematocrits and white blood cell counts elevated and have enlarged spleens and livers. Their peripheral blood contains many immature red blood cells (normoblasts) and increased lymphocytes, many of which are fragile and appear ruptured on prepared slides (Fig. 1). Lymph nodes, on the other hand, are not enlarged at this time. Mice injected with 0.1 SED FLV that do not die around day 40 may die later, around day 200 after virus injection. Hepatosplenomegaly in these mice is generally less marked than those dying earlier of erythroleukemia, and, in contrast to erythroleukemia syndrome, there is enlargement of all lymphoid organs, including the thymus gland. This later death, therefore, appears to involve the development of a lymphoma. White blood cell counts may or may not be elevated in mice developing this lymphoma, and when they are elevated there is also a large population of myeloid cells and monocytes (Fig. 2). However, the above described erythroleukemia and lymphoma symptoms are not always mutually exclusive and some mice do show characteristics of both diseases. Treatment of the animals with the chemical carcinogen methyl methanesulfonate appears to shift the early deaths in favor of erythroleukemia and the late deaths in favor of the lymphoma, as will be illustrated later.

Because SJL/J mice have been reported to develop a spontaneous "Hodgkin's like" reticulum cell sarcoma after 8 months of age (4,5), which is also characterized by enlarged

Fig. 1. Wright-stained smear of peripheral blood obtained from an SJL/J mouse inoculated with FLV (0.1 SED) 99 days earlier, showing typical erythroleukemia signs. Immature red blood cells (normablasts) are indicated by arrows. 0 indicates a normal lymphocyte, and 1,2,3, and 4 indicate successive stages in lymphocyte conversion to a smudge cell.

Fig. 2. Wright-stained smear of peripheral blood from SJL/J mouse injected with 0.1 SED FLV 230 days earlier. The mouse had very enlarged lymph nodes. There are few, if any, immature red blood cells, and the number of smudge cells is less than that found in mice with erythroleukemia. Instead there are numerous other lymphoid elements such as polymorphonuclear monocytes (1) and lymphocytes (2), as well as myeloid cells (3).

lymphoid elements, it is important to distinguish this spontaneous disease from the lymphoma described above. The enlarged lymph nodes that we see in our experimental SJL/J mice appear homogenously packed with lymphoid cells in histological section (Fig. 3), whereas those from mice bearing reticuloendothelial disease are "mixed cell tumors" (4,5). Only a few of our control, vehicle-treated mice develop enlarged lymph nodes, and they do so at a substantially later time (about 100 days later) than the mice receiving virus. Although it is possible that the addition of FLV facilitates an earlier appearance of the reticuloendothelial disease, the histological difference mentioned above argues against this. When doses of Friend virus that were lower than 0.1 SED were used in SJL/J mice, few, if any, mice died of erythroleukemia, but a certain fraction of the mice, the size of which varied with the virus dose, died of lymphoma.

With our virus-resistant B10SJF1 hybrids, we have observed that 18% (4/22) of the mice succumbed to erythroleukemia after the injection of 100 SED FLV. As will be shown below, chemical pretreatment can produce up to 90% erythroleukemic deaths after the injection of 100 SED FLV into these hybrid mice. However, this potentiation decreases with decreasing virus dose. It should be noted that we have not observed any lymphoid symptoms in the B10SJF1 mice to date.

Since the virus dose is a prime critical factor in being able to demonstrate chemical potentiation of the leukemia, we have recently prepared a large amount of standardized virus that

Fig. 3. Section of an enlarged cervical lymph node from an SJL/J mouse injected with 0.1 SED FLV 177 days before its death. The uniform cell-type appearance shown was typical of the entire section. Stained with Hematoxylin-Eosin.

will be tested and stored at -60°C in small, individual aliquots at the optimum dilutions required for SJL/J and B10SJF1 mice to be used in future experiments.

III. Effects of Chemical Carcinogens on Survival and Leukemia or Lymphoma Induction

Our studies during this year have concentrated on the effects in vivo of three chemical carcinogens chosen, as indicated last year, on the basis of reports in the literature of their in vitro mutagenic or carcinogenic action. The first was methyl methanesulfonate (MMS), which is a potent mutagen, demonstrated to be carcinogenic in animals (6), that is felt to act directly on the DNA, producing easily-repaired, single-strand breaks involving only a few bases (7-10). The second and third agents, benzo[a]pyrene (BP) and 7,12-dimethylbenz[a]anthracene (DMBA), are well-established polycyclic hydrocarbon carcinogens (11-14). BP is thought to require a metabolic activation before it can act on DNA. The reactive metabolites, one of which is a diolepoxide, are then felt to bind covalently with the nuclear DNA of the cell, distorting it (13,15-18). The remaining agent studied, DMBA, apparently acts in a similar manner to that of BP, being first metabolized to an activated form (19-23).

A) Effects of MMS in virus-sensitive SJL/J mice:

To determine the maximum amount of MMS which could be tolerated by the assay mice, toxicity tests for single doses or two doses given 19 hours apart were performed.

The results are shown in Table I. All doses tested of less

than 0.1 mg/mouse were easily tolerated. A single dose of 2 mg caused approximately 10% deaths. This was increased when 2 mg doses were given 19 hours apart. Since we wished to achieve the maximum probability of demonstrating the potentiating effects of the chemical, the dose of 2 mg/mouse was chosen. (Any animals dying within 24 hours of MMS injection were considered to have died from chemical toxicity and are not included in the figures presented herein).

Figure 4 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 can be demonstrated when MMS is given 5 hours before the virus. Notably, nearly all of the mice dying in the groups that received a 5 hour pre-treatment with MMS had hepatosplenomegaly and a lack of lymph node enlargement. Therefore their probable cause of death was erythroleukemia, as is illustrated in Table II. Furthermore, an even greater enhancement of viral leukemogenesis was seen when MMS was given two times at 24 and 5 hours preceding virus (Fig. 4 and Table II). However the MMS enhancement was apparently quickly reversed, possibly by rapid repair of DNA damage, as suggested by Regan and Setlow (7), for when 24 hours were allowed to elapse between MMS and virus injection, only a slight indication of enhancement remained. When MMS was given 5 hours after the virus, no enhancement whatsoever was seen (Fig. 4). However, there was some indication of a possible protective effect of the MMS against erythroleukemia development, but not

Table I

Drug-related deaths after injection of various doses of MMS to SJL/J mice. Animals died within 24 hours of the injection.

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

Table II

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 Figure 4. 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.

Treatment and Time Grouping	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 hrs 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 hrs 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 hrs 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 hrs before virus			
Deaths before 150 days	100 (7/7)	0	0
Deaths after 150 days	0	0	100 (1/1)

Fig. 4. SJL/J mice were injected intraperitoneally with 2 mg MMS in saline either before or after virus injection as indicated. The FLV only and MMS only groups also received second saline injections. The control group received saline only twice. The results of 3 experiments in which the FLV-only survival curves were superimposable were pooled. All FLV injections within a particular experiment were done with the same virus preparation within a 15 minute time span. The virus suspension was kept on ice and the FLV only group was injected first.

against the lymphoma (Table II). Lowering the dose of virus reduced the number of erythroleukemia deaths to virtually none, as mentioned above. However, in one experiment in which a very much lower virus dose of 0.015 SED was given in combination with 3 mg MMS 5 hours before, an increased incidence of lymphoma as well as erythroleukemia was seen (Figure 5).

For analysis of lymphoma versus erythroleukemia development, representative animals from the groups illustrated in Figure 4 were autopsied at time of death. Autopsy results were then correlated with the white counts from these same animals which had been periodically sampled before death to arrive at the diagnosis of the cause of death. Examination of Figure 4 shows the two separate death patterns referred to earlier: a rapid sequence of deaths through about the first 40 days; then a relative plateau with few deaths up to about 150 days, followed by further, relatively widely spaced, deaths. On the basis of this, the relative percentage of erythroleukemia to lymphoma deaths were determined for animals dying before and after 150 days. This is the data given in Table II. Administration of virus alone resulted in a rather even pattern of erythroleukemia versus lymphoma deaths for mice dying after 150 days. This was shifted in favor of the lymphoma by administration of MMS. Conversely, among the mice dying before 150 days, MMS treatment at 5 or 24 hours before virus shifted the pattern to erythroleukemia as the principal cause of death.

Fig. 5. SJL/J mice were injected i.p. with 3 mg MMS 5 hours before a low dose (0.015 SED) of FLV. Most of the deaths that occurred before 150 days, but none of the deaths that occurred after 150 days, involved erythroleukemia only.

The deaths in the control group of SJL/J mice that received no MMS or virus (Fig. 4) were due to a different cause. These displayed the spontaneous lymphoma that develops in SJL/J mice when they reach more than nine months of age, which we have referred to earlier (4,5). Whether MMS treatment (see MMS only group in Fig. 4) delays the onset of this different cancer, as seems to be indicated by the survival data, is questionable, since 7 of 15 mice assayed in this group had enlarged lymph nodes at the time of sacrifice and thus the numbers are not significantly different from the 8 out of 16 mice in the vehicles-only group that either died of this cancer or had enlarged lymph nodes at the time of sacrifice.

B) Effect of MMS on virus-resistant B10SJF1 hybrid mice:

By crossing virus-sensitive SJL/J mice with the virus resistant C57B1/10 we have been able to produce the B10SJF1, which is resistant to Friend leukemia virus under normal circumstances (1). However, after exposure to a very large dose of 100 SED units of Friend virus a small number (less than 20%) of the B10SJF1 do develop the leukemia at late times after virus infection (Figure 6). In these mice the potentiating effect of MMS on Friend virus induced disease is most significantly seen. Following 2 mg of MMS five hours before 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, further illustrating that demonstration

Fig. 6. Ten to fourteen week old female B10SJF1 mice were injected with 2 mg MMS in saline or with saline only 5 hours 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 hepatosplenomegaly.

of the potentiating effect of chemical agents on viral cancer is highly dependent on the dose of virus as well as on the dose of the carcinogen or mutagen. All of the hybrid mice dying in these experiments had elevated white blood cell counts and gross hepatosplenomegaly at autopsy. Since B10SJF1 mice are relatively resistant to FLV alone, the enhancement by MMS pretreatment produces a clearer contrast in comparison to the response of control groups treated with virus only than that seen in SJL/J mice. Therefore, this hybrid may prove to be the most useful in assessing the effect of chemical carcinogens on viral leukemogenesis.

C) Effects of BP in virus-sensitive SJL/J mice:

The initial dose of BP tested by us was 50 µg/mouse (equivalent to 2.5 mg/kg of body weight). This was chosen on the basis of the doses of BP employed in various other previous carcinogenesis studies (24-26). Intraperitoneal injections of SJL/J mice with this dose of BP either 5 hours before or 5 hours after FLV injection showed no potentiating effect on leukemogenesis. It should be observed that, although the nominal dose of virus used in the BP studies was the same as that used in the MMS studies (i.e. 0.1 SED_{50/14} units), the effective dose was actually much lower, as indicated by the survival patterns for the virus only groups in Figures 7 a,b, and c. This we feel is due to dilution effects incurred in preparing such small concentrations of virus in saline for injection. In preparing concentrations of this size there always occurs some loss of viral activity which cannot be predicted or compensated for the basis of physical dilution

factors alone. As we have already stated, in our future studies we shall try to avoid this problem through the production of relatively large batches of standardized virus individually packaged at the dose levels needed, as mentioned previously. Regardless of this problem the data for BP are relatively clear. As Figure 7a shows, all three lines: virus only, 50 μ g BP 5 hours before virus, and 50 μ g BP 5 hours after virus, show the same survival pattern, indicating no potentiation of leukemogenesis. When the same dose of BP was given a week before virus, somewhat different survival patterns were seen, with earlier deaths and a greater number of deaths than that found in the virus only control groups (Fig. 7b). These results are consistent with the proposed mechanism of action of BP as reported elsewhere in the literature (27). However, they represent only a relatively small number of animals and should be confirmed by additional work. Similarly, when the dose of BP was increased 10-fold (Fig. 7c) a slight enhancement in leukemogenesis during the first 150 days of the experiment was seen, even with BP injected at 5 hours before the virus. When both of these factors, i.e. increased dose of BP and increased length of time between BP and virus treatment were combined, the data suggests a positive potentiating effect of BP, but at greater time intervals before virus than those for MMS (Fig. 8, experiments still in progress). Here, in each of the time periods we have tested, 1,2,3 and 5 days, all of the mice given 500 μ g BP followed later by virus show an earlier or greater effect on survival than mice given virus alone. Also,

Fig. 7. The effect of 50 μ g BP injected within 5 hours of virus (Panel A) or 1 week before virus (Panel B), and the effect of 500 μ g BP injected within 5 hours of virus (Panel C) on the induction of leukemia and lymphoma in SJL/J mice. All deaths before 150 days were due to erythroleukemia. Most of the later deaths were due to lymphoma. There were no deaths in groups receiving BP only or vehicle-only. See text for details.

FIG.8) THE EFFECT OF 500 μ g OF
BP INJECTED INTO SJL/J MICE AT
VARIOUS TIMES

Fig. 8. BP (500 μ g/mouse) was injected at various number of days before FLV (0.1 SED) as indicated.

as of the present, none of the mice given BP only in this experiment have died. Although this study is still incomplete, the data to date suggest that a two day interval between BP and virus may result in the greatest potentiating effect, as opposed to the 5 hour interval that was optimal for MMS.

Although the carcinogenic action of BP may not involve the direct and immediate strand breaks of the type found with MMS, it may involve a type of DNA damage occurring over a longer term and which is also subject to repair (18,21,27, 28). It was therefore of interest to us to investigate what situation might occur if a third agent which inhibits DNA repair is introduced. In selecting an agent for this purpose it was our desire to also choose one to which many fuel production workers may be regularly exposed. The agent we chose for this purpose was caffeine, which has been frequently reported as an inhibitor of post-replication DNA repair (29, 30), and which is well known to be present in coffee, tea and soft drinks consumed by the general public and petroleum workers as well. The question posed was "Are the marginal potentiating effects of BP on viral leukemogenesis enhanced when caffeine is added to the system?".

When caffeine alone was given in conjunction with virus two to four hours before the virus it had no effect. This was as expected, in as much as the mechanism of caffeine action is directed against DNA repair rather than DNA damage. However, when the mice were first exposed to 500 μ g BP, between 2 and 5 days before caffeine and virus, there was

a significantly greater fraction which developed leukemia as compared to those given BP alone plus virus (Figure 9). Although these experiments are also still in progress, and the data must be taken as preliminary, the combined effects of BP and caffeine on leukemogenesis are substantiated by white blood counts which have been taken on the mice. Table III shows these counts, the elevated WBC data being shown as shaded blocks. Generally, the maximum point for increased WBC was at one month after exposure to BP, FLV and caffeine. The apparent regression to lower values after this time reflects the death of the individual animals with elevated WBC rather than a recovery from the disease. It should also be noted that in the experiments represented by this data the actual dose of virus received was less than the nominal 0.1 SED dose as determined by dilution of the higher titer stock virus. This is indicated by lack of deaths and increased WBC among the virus only group. Nonetheless, the combination of BP and caffeine given to these animals in conjunction with the virus did result in a significant increase in erythroleukemia, which is all the more striking because of the lack of the disease among the recipients of virus alone. Although we emphasize that these results are preliminary and must be confirmed by us, the implications of them should be obvious. The suggestion is readily apparent that, while fuel production workers exposed to agents such as BP alone may show no ill effects, if they are also simultaneously exposed to another agent which may of itself be normally non-carcinogenic, a significant effect may occur.

Fig. 9. SJL/J mice were injected with 500 μ g BP i.p. at various number of days before they were injected with caffeine and virus, as indicated (---). Other groups were given BP only (—), virus only (—) or BP and virus only (.....). The caffeine (100 mg/kg body weight) was injected 4 hours before FLV (0.1 SED).

Table III

Effect of 500 µg BP, 0.1 SED FLV and 100 mg/kg Caffeine (2-4 hours before virus) in SJL/J mice. Mean white blood cell counts ($\times 10^3$); (range); no. elevated over 25,000.
Total

Days After Virus	BP Only	Virus Only	Caff. Only	BP 1d Before Virus	BP 1d Before Virus + Caff.	BP 2d Before Virus	BP 2d Before Virus + Caff.	BP 3d Before Virus	BP 3d Before Virus + Caff.	BP 5d Before Virus	BP 5d Before Virus + Caff.
13	12.8 (7.5-13.3) 0/4	11.4 (10.5-13.4) 0/6	13.0 (12.2-14.6) 0/7	13.6 (9.0-17.2) 0/8	34.1 (19.6-120.0) 4/9			14.4 (10.9-18.4) 0/8	17.8 (14.7-25.3) 1/9	18.6 (4.1-24.0) 0/5	12.6 (9.4-16.0) 0/0
17	N.D.	22.0 (18.5-24.5) 0/4				9.1 (3.2-12.0) 0/5	13.3 (8.7-17.3) 0/10	12.1 (6.1-20.3) 0/10	12.1 (6.1-20.3) 0/10		
30	10.7 (6.6-18.7) 0/4	11.671 (8.797-22.189) 0/11	11.3 (8.6-15.4) 0/7	9.2 (8.4-16.8) 0/8	17.6 (10.3-23.7) 0/9	15.0 (15.3-26.9) 1/5	93.4 (10.9-184.2) 6/9	20.2 (7.0-60.1) 2/14	45.0 (12.6-155.5) 6/19	16.8 (15.9-26.0) 1/5	47.0 (13.8-160.9) 4/10
51	N.D.	10.8 (7.0-13.4) 0/5				16.4 (8.2-35.6) 1/5	14.8 (8.2-21.5) 0/2	11.5 (7.5-13.3) 0/6	11.5 (7.5-13.3) 0/6	26.8 (12.2-73.8) 1/5	11.6 (9.7-13.8) 0/5
60	11.6 (8.2-14.0) 0/4	12.4 (10.3-14.2) 0/6	8.7 (8.5-10.7) 0/7	11.7 (7.7-19.7) 0/8	10.2 (6.7-10.8) 0/9			16.1 (13.6-19.4) 0/5			
100	13.6 (11.6-15.5) 0/4	7.3 (6.4-9.4) 0/6	9.4 (5.2-11.7) 0/7	11.8 (8.5-16.7) 0/8	11.2 (5.4-16.5) 0/8			13.3 (9.0-14.1) 0/5			

D) Effect of DMBA:

The effects of DMBA (50 µg/mouse) injected within a few hours of virus also seem to depend on the dose of FLV employed. When the nominal dose of 0.1 SED FLV was used the effect of a 5 hour DMBA pretreatment was not significant and a 5 hour DMBA post-treatment was somewhat protective (see our technical report COO-4800-2, May 1978). With doses of virus higher than 0.1 SED, the protective effect of DMBA was indicated even when DMBA was given 5 hours before the virus. These findings were consistent with earlier reports of a protective effect of DMBA against Friend and Rauscher leukemia viruses (31,32). Conversely, if the actual effective dose of virus was lower, the 5 hour pretreatment seemed to be somewhat potentiating. However, all of the above mentioned effects using a single dose of DMBA were small and therefore of marginal significance.

A more significant potentiating effect was seen when two 50 µg injections of DMBA were given at 8 days and 5 hours before the virus (Figure 10). The addition of a third DMBA injection at 7 days after the virus had no significant additional effect. This suggests that DMBA, like BP, requires several days in vivo before its effect on the enhancement of FLV leukemogenesis is evident.

DMBA, like BP, is a polycyclic, aromatic hydrocarbon derivative of fossil fuels (33,34) and, like BP, its metabolites form adducts to DNA (20-23,28). The mechanism of action by which these carcinogens cause DNA damage is controversial (27, 35-37). However, it has been suggested that their metabolic

Fig. 10. SJL/J mice were injected with 0.1 SED FLV at day 0. DMBA (50 μ g/mouse) was injected once, twice or three times at various times in relation to the virus, as indicated. The DMBA only control received DMBA 3X.

end products may act by causing DNA breaks (38) analogous to those caused by UV light. The fact that DMBA and BP require periods of time that are longer than those required by MMS before some type of an effect on viral leukemogenesis is demonstrable probably reflects the necessity for these compounds to become metabolically activated by aryl hydrocarbon hydroxylase enzyme complexes (16,17,19,20,24-27). It may also reflect the necessity for an accumulation of carcinogenic damage (14), which if unrepaired may facilitate viral oncogenesis.

IV. Immune Response Following Exposure to MMS, BP and DMBA

A possible mechanism of action which has been proposed for chemical carcinogens in vivo is suppression of immune response, and immunosuppression has been claimed as a property for many of these agents (37,39,40). The leukemia viruses themselves likewise have been shown to be immunosuppressive (41-43). The question we have asked in the present studies is: "Is there a relation between the immunosuppressive action of the chemical agent and the enhancement of viral leukemogenesis?".

Our efforts in this during the current period have been centered on the humoral immune response levels as measured by the numbers of antibody producing plaque-forming cells (PFC) in the spleens of our assay mice after exposure to MMS, BP and DMBA. PFC response has been measured by the Kennedy-Axelrad (44) modification of the Jerne technique (45). At specified times after exposure to the chemical the mice are sensitized by injection of washed sheep red blood cells (SRBC). Five days later

the mice are sacrificed, the spleen removed and the splenocytes tested for their ability to lyse sheep red blood cells plated on a petri dish. Each lytic cell is indicated by a clear plaque occurring in the lawn of SRBC's as the result of diffusible lysing antibody emanating from one spleen cell. The mounting of the humoral response involves both B-cells and T-cell helpers, so that this reaction measures the activities of at least two cells of the immune system (46).

A) Effects of MMS on PFC response:

The effect of MMS on antibody producing PFC have been measured in both SJL/J and B10SJF1 mice, without concurrent exposure to virus. The results are summarized in Figure 11. MMS reduced immune response in both strains, with the lowest level of response being found 2 to 4 days after virus exposure. Comparing this response curve to the leukemogenesis data of Figure 4, it is evident that the timing for the depression of PFC response does not parallel the timing for the carcinogenic potentiating effect of MMS. The PFC response is depressed by day 2 after MMS injection and remains at a low level through day 14, while the leukemogenesis enhancement effect is no longer evident 24 hours after MMS treatment.

B) Effects of BP on PFC response:

In our previous progress report we indicated that 50 µg of BP appeared to have a stimulatory effect on humoral immune response. Using a higher dose of 500 µg BP we have confirmed this earlier finding (Fig. 12). Following an i.p. injection of 500 µg of BP, plaque forming cell response to SRBC increases as a function of time and by 10 days is between 1.5X and 2X normal.

Fig. 11. SJL/J or B10SJF1 mice were injected with 2 mg MMS at different times before sensitization by sheep red blood cell (SRBC) antigen. The ability of splenocytes from these mice to lyse SRBC in vitro was tested 5 days after sensitization.

Fig. 12. SJL/J mice were injected with 500 μ g BP and then with sheep red blood cells (SRBC) as sensitizing antigens at the time indicated on the abscissa. Five days later the proportion of SRBC-lysing units was estimated in isolated splenocytes. See text for details.

C) Effects of DMBA on PFC response:

In contrast to MMS and BP, in these studies DMBA appeared to have no effect on the level of PFC response, even when given in multiple injections prior to exposure to SRBC antigen. The mean values for PFC response after DMBA exposure in this instance did not differ from that for the controls (Table IV).

V. Discussion

It is apparent from the data on leukemia/lymphoma incidence and survival, and from the data on immune response, that the three principal agents which we have reported in this period act in markedly different ways relative to their effects on viral carcinogenesis and on immune response. MMS appears to have a pronounced potentiating effect on viral leukemogenesis, and those mice which survive the early effects also develop a later lymphoma, which also occurs in greater proportion to that found in animals not exposed to MMS. It is clear that the time of exposure to MMS and to virus is a critical factor, with a greater effect being seen when MMS is given shortly before virus. Regan and Setlow (7) have proposed that MMS produces a type of DNA damage which is quickly repaired, and they compare this damage to that produced by x-ray. If this is the case, then the mechanism by which MMS is acting may involve the production of short single-strand breaks in the DNA which facilitate the entry of the viral genome. Such a mechanism would be consistent with our observations of the criticality of time, carcinogen dose, and virus dose in the demonstration of a potentiating effect on viral leukemogenesis. Other chemicals that can be derived from

Table IV

The effect of 50 μ g DMBA given to SJL/J mice 3 times, at 2, 9 and 16 days before sensitizing SRBC, on the humoral immune response. PFC/ 10^6 spleen cells of individual mice.

Control 1 (Untreated)	Control 2 (Trioctanoin Injected 3X)	Experimental (DMBA in Trioactanoin Injected 3X)
1117	776	1033
623	712	926
686	751	1072
	1126	562
$\bar{X}(\pm SE)$ 809(653-963)	814(745-937)	900(785-1015)

fossil fuels which have been shown to produce the same type of quickly repaired damage include ethyl methanesulfonate and propane sultone. It would be of interest to see if these yield the same type of results.

From the data on the BP studies, it is clear that such potentiation that occurs requires a greater time interval between exposure to the chemical and the virus for its demonstration. This in itself would suggest a different mechanism than that for MMS. The requirement of metabolic conversion of BP to another carcinogenically active compound has been well documented (13,15-17,19,20,26,27). The binding of the metabolites of BP to DNA is also thought to be different from that for MMS, although at least one author (38) has suggested that the ultimate effect on DNA may be identical to MMS. The data from the experiments in which caffeine was also introduced into the system seem to imply that repair of DNA damage may be another critical element governing whether or not co-carcinogenesis with a viral agent may occur. It also brings out the importance of considering other factors which workers might be commonly exposed to that are otherwise overlooked, but which if they occur in the right combination may have a lethal potential.

With regard to DMBA, still other elements are evident. Both our earlier results and those of other researchers (31,32) have suggested that low doses of DMBA may inhibit the process of viral oncogenesis when given close to the time of viral injection. Two possible mechanisms by which this may occur would be the blocking of the cellular DNA or inactivation of the viral produced DNA by the chemical, thus preventing entry of the viral genome.

DMBA is also a carcinogen which is thought to act in a manner similar to BP, requiring the same types of metabolic action to produce the active form (19-23, 27). The results reported by us of a potentiation using the higher dose of DMBA administered several days before virus would be consistent with this.

With respect to the immune response, results on the effects of these compounds on the humoral immune system are not consistent with the hypothesis that potentiation of viral leukemogenesis by these agents is mediated by immunosuppression. Only one of the three, MMS, was clearly immunosuppressive; however, at the time of maximum potentiation of leukemogenesis for this compound did not coincide with that of immunosuppression. BP appeared to have an immunostimulating effect, also inconsistent with leukemogenesis potentiation, and DMBA evidenced no effect on PFC response whatsoever. Although these data appear to rule out the effect on humoral immune response as a mechanism by which exposure to these agents may lead to cancer, they do not totally eliminate immunological factors from consideration. Whether or not effects on other, cellular, immune response mechanisms may play a role remains to be determined.

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TECHNICAL REPORT

**LEUKEMIA INDUCTION BY FRIEND VIRUS IN NORMALLY LEUKEMIA VIRUS
RESISTANT MICE AFTER TREATMENT WITH METHYL METHANE SULFONATE**

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Prepared for
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ABSTRACT

F1 offspring of virus-resistant C57B1/10 females and virus-sensitive SJL/J males are normally resistant to Friend leukemia virus. However, after treatment of these hybrids with methyl methane sulfonate (MMS), Friend leukemia virus given within 5 hrs of the treatment caused many individuals to succumb to erythro-leukemia with characteristic elevated white counts, hepatomegaly, splenomegaly, and high hematocrit. Although MMS was found immunosuppressive in these mice when given alone, it was found to enhance humoral immune function measured by a plaque forming assay against sheep red blood cells, when given in combination with the virus. These results suggest that MMS enhances viral leukemogenesis by some mechanism other than immunosuppression.

INTRODUCTION

Sensitivity to leukemia induction by Friend leukemia virus varies in different mouse strains (1,2). While the molecular mechanisms involved in the resistance to this virus are only partially understood, the resistance is known to be inheritable (3).

C57B1/10 mice are one of the types that have been designated as absolutely resistant by Stutman and Dupuy (1), meaning that they could not be made leukemic even by relatively high virus doses. In the present study we have used hybrid mice, which we have designated as BD10SJF1, that result from crossing of C57B1/10 females and virus-sensitive SJL/J males. This hybrid is intermediate to the two parents in its response to leukemia virus and seems to develop a transient leukemia after injection of Rauscher or Friend leukemia virus (4, and unpublished data). The intent of the present study was to evaluate the response of this mouse which contains genes for both leukemia resistance and leukemia sensitivity to the combined exposure to the leukemia virus and a chemical carcinogen.

Based on previous reports concerning its mechanism of action (5,6), our choice for a chemical carcinogen was the alkylating agent, methyl methane sulfonate (MMS). In previous studies, Casto et al (6) have shown in vitro that MMS potentiates adenovirus-dependent transformation of hamster cells in culture. From this it could be inferred that MMS facilitates viral integration into the host genome. In the present study we have asked the question as to whether a 5 hour pretreatment with MMS also might facilitate in vivo the leukemogenic action of FLV in B10SJF1 mice. We have also measured the effect of MMS on the humoral immune response of these mice, both alone and in combination with FLV, to determine whether immunosuppression is important in MMS action.

MATERIALS AND METHODS

Animals - C57B1/10 female and SJL/J male mice were purchased from Jackson Laboratories. Their F1 hybrids were bred in our facilities. Ten to fourteen week old female F1 hybrids were used. All animals were maintained in temperature and humidity controlled rooms with a 12 hour light cycle. They were given Purina lab chow and acidified water ad libitum.

Virus - Friend leukemia virus (FLV) preparations were made by passaging Friend virus stock obtained from NCI through mice of the SJL/J parental strain, obtaining the virus rich plasma and diluting it with saline. The virus dose to be administered was measured in spleen enlargement units (SED), which are defined as that amount of virus which causes spleen enlargement in 50% of injected SJL/J assay mice within two weeks. The B10SJF1 mice were given the virus by intraperitoneal injection.

MMS - Methyl methane sulfonate (MMS) (Sigma) was diluted for injection with isotonic saline. Two mg were injected intraperitoneally per mouse. Preliminary experiments indicated that this dose is close to the highest tolerated by the mice without any obvious toxic affects. MMS was given 3.5-5 hrs before FLV, because our experiments with SJL/J mice (7) indicated that pretreatment within 24 hours of virus injection is most effective in augmenting FLV leukemogenesis.

Plaque forming assay - The ability of splenocytes to lyse sheep red blood cells (Sacks Farms) was measured by the Jerne plaque forming technique as described previously (4).

RESULTS

Table I illustrates the effect of exposure of B10SJF1 mice to MMS only, virus only, or virus plus MMS, as compared to untreated controls and as a function of time after virus injection. Mice that received 50 SED units of virus 5 hrs after a dose of 2 mg of MMS had increased white blood cell counts (WBC) when compared to mice that received FLV only. The mice that received both carcinogens had a greater elevation in WBC at 30 days than those receiving either agent alone. Mice with both virus and MMS also had significantly higher hematocrits: 77 and 80 at the forty-fourth day after virus injection, as compared to 54 in the one mouse with elevated WBC in the virus only group and 45 for normal mice. Coupled with these changes in the peripheral blood, survival in the group receiving combined exposure to MMS and FLV was poorer than any other group, and those mice which died had gross hepato- and splenomegaly. On day 169 the experiment was terminated and all animals were autopsied. Two of the three remaining mice in the MMS and FLV exposed group had enlarged spleens of 2.6170 and 0.6210 g, while all of the mice in the other groups, including those which had received virus only, had normal spleens of less than 0.3 g in size.

Figure 1 shows the results of a second experiment in which B10SJF1 mice received 2 mg MMS 3.5-4 hrs before 100 SED FLV, and illustrates that survival was greatly shortened when compared to mice receiving virus only. There were no deaths in the group receiving MMS only. All of the mice that died had leukemia, as judged by elevated WBC and hepato- and splenomegaly at death.

Table I

Effect of MMS Pre-treatment on the Leukemogenic Action of FLV in B10SJF1 Mice.

2 mg MMS was given IP to B10SJF1 mice on day 0 5 hrs before IP injection of 50 SED FLV. Average white blood cell counts, their range and the number of mice with elevated white blood cell counts per total number of mice per group are given.

Day	Control	Elevated Total	MMS Only	Elevated Total	Virus Only	Elevated Total	Both	Elevated Total
16	10,550 (6,763-14,210)	0/5	10,814 (6,454-13,719)	0/5	12,646 (10,184-17,305)	0/5	16,630 (9,154-38,335)	1/5
30	9,000 (8,361-9,646)	0/5	44,294 (9,846-183,278)	1/5	12,833 (8,897-17,234)	0/5	24,285 (7,419-48,537)	2/5
44	8,565 (6,397-11,362)	0/5	7,378 (6,234-9,002)	0/5	39,214 (6,806-156,258)	1/5	43,734 (8,241-138,827)	2/5
70	8,779 (6,419-12,913)	0/5	16,907 (4,004-62,062)	1/5	7,900 (7,554-8,433)	0/5	19,362 (8,820-57,177)	1/4
100	9,634 (5,922-13,086)	0/5	5,924 (4,411-9,344)	0/5	8,968 (7,922-11,705)	0/5	10,782 (8,933-12,630)	0/3
123	13,014 (11,012-14,948)	0/5	12,338 (6,259-14,891)	0/5	12,667 (10,616-15,107)	0/5	16,015 (14,270-17,090)	0/3
Survival at day 169	100%		100%		100%		60%

Fig. 1. 10-14 week old B10SJF1 female mice were given MMS intraperitoneally 3.5-4 hrs before 100 SED FLV. Other groups were given one or none of these carcinogens. There were 4 mice in each group, except in the group that received both carcinogens which consisted of 10 mice.

Fig. 2. B10SJF1 mice were injected with 2 mg MMS IP. Then on the days indicated they were injected with SRBC. Five days later their splenocytes were isolated and tested for ability to lyse SRBC in vitro. The insert shows the same function measured in B10SJF1 mice that were injected with 100 SED FLV alone or with 2 mg MMS plus 100 SED FLV 3 days before SRBC sensitization. The values represent the average of 3 mice.

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Figure 2 shows the effect of MMS on the ability of B10SJF1 mice to mount a humoral immune response to sheep red blood cells (SRBC) as determined by the plaque forming technique (4). Plaque forming cell (PFC) response decreased as a function of time after MMS given alone, while when virus was given in conjunction with MMS there was an apparent increase (box, fig. 2).

DISCUSSION

Our results show that MMS pretreatment increases the sensitivity of normally FLV-resistant B10SJF1 mice to the leukemogenic action of this virus. This implies that the juxtaposition of these two different carcinogens can have a synergistic effect. Normal B10SJF1 hybrid mice seem to be able to control leukemia virus action, as is evident by a lower mortality and the transient nature of leukemia symptoms such as WBC elevation. Since exposure of the Fl to leukemia virus actually results in an increased humoral immune response, as measured by the plaque forming response to SRBC (4), it was of interest to measure the effect of MMS on this immune response and in relation to the observed effect of MMS on leukemogenesis. Although MMS did decrease the level of plaque forming response when given singly, when FLV was given following MMS the PFC response was elevated above control levels by the third day, and above the level of FLV exposure alone. In the previous study with this hybrid (4), elevated PFC response was not seen until the eighth day after virus infection. Thus the data from this study suggest that not only is the PFC response enhanced when the two agents are given together, but that this enhancement occurs earlier. Additional studies will have to be performed to confirm this.

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However, the preliminary results do suggest that this observed increase in leukemogenesis following combined administration of MMS and FLV may not be related to humoral immunosuppression.

Results of in vitro studies of adenovirus in cultured hamster cells suggest that MMS may act directly on the virus target cells. The fact that our data show an effect as early as 3.5 to 5 hours would be in agreement with that. It has also been postulated (8,9) that virus enhancement by radiation and chemicals that mimic radiation damage to DNA might be accomplished during post replication repair of DNA damage. Whether the same is true in our system can be tested by the use of inhibitors of such repair, for example caffeine (9). Such experiments are now in progress.

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TECHNICAL REPORT

SOME EFFECTS OF 7,12-DIMETHYLBENZ(A)ANTHRACENE AND
BENZO(A)PYRENE ON FRIEND VIRUS LEUKEMOGENESIS

R.B. Raikow
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ABSTRACT

The effects of the chemical carcinogens, 7,12-dimethylbenz(a)-anthracene (DMBA) and benzo(a)pyrene (BP), on leukemia induction by Friend leukemia virus (FLV) have been evaluated in SJL/J mice. In the viral carcinogen controls, FLV doses of 0.1 SED injected IP caused early leukemia in up to 50% of the recipient animals, which died with characteristic leukemia induced splenomegaly around day 40 after virus injection. The remainder died between 120 and 260 days after FLV, with some splenomegaly but also notably with other symptoms, such as enlarged lymph node and thymus glands. In the chemical carcinogen controls, a single dose of DMBA or BP (up to 50 µg DMBA and 500 µg BP were tested) was not found to have any significant tumorigenic effect by itself in this study, except in one experiment where DMBA was dissolved in olive oil instead of the usual synthetic oil, trioctanoin. However, similar doses of DMBA, given in conjunction with FLV, were found to be unexpectedly protective with regard to the leukemogenic effect of FLV, particularly when given 5 hrs after the virus. On the other hand, pretreatment with BP had an opposite effect, resulting in increased leukemogenesis in SJL/J mice and also in normally virus resistant B10SJF1 mice.

INTRODUCTION

A number of synergistic interactions between viral and physical (1,2) and also between viral and chemical carcinogens (3-6) have been reported. Most of these studies, however, utilize radiation or chemicals which mimic radiation effects on DNA and/or in vitro systems. In the present studies we have been investigating in vivo effects of the polycyclic hydrocarbons, 7,12-dimethylbenz(a)anthracene (DMBA) and benzo(a)pyrene (BP), on leukemogenesis induced by injection of low doses of Friend leukemia virus (FLV). DMBA (8,9) and BP (10,11) are known to produce sarcomas and carcinomas in adult mice, mostly at the site of application or injection. DMBA also causes an increased incidence of breast tumors in certain rat strains (12). Although a single injection of these hydrocarbons is effective when given to neonatal animals (13), in adults repeated administration is normally required to produce an effect (7,10,11), or they must be given in conjunction with promoters of carcinogenic action (9). The purpose of the present study was to determine if these agents also showed a synergistic effect when given in single dose to normal adult mice in conjunction with a known viral carcinogen.

MATERIALS AND METHODS

Animals - Four to six week old female SJL/J and C57B1/10 mice were purchased from Jackson Laboratories and held in our facilities until use at 10-12 weeks. The B10SJF1 hybrids were produced in our facilities by crossing C57B1/10 females and SJL/J males. Female

hybrid mice were used for experiments when they were 10 to 14 weeks old. All animals were maintained in plastic cages with filter tops and were given acidified water and sterilized food ad libitum. The animal room was temperature and humidity controlled and maintained at a 12 hr light cycle.

Virus - FLV obtained from NCI was maintained by passaging in SJL/J mice. FLV rich plasma of leukemic SJL/J mice was titered for virus by a spleen enlargement assay. One spleen enlargement dose (SED) is defined as that amount of virus that causes splenomegaly in 50% of SJL/J mice by day 14. The plasma was diluted with phosphate buffered saline to give the appropriate SED. The virus was injected intraperitoneally.

Carcinogens - 7,12-DMBA or BP (Sigma) was dissolved in trioctanoin, a pure synthetic oil (Eastman Kodak), and injected intraperitoneally as indicated. In the experiment of figure 1, olive oil was used as the vehicle instead of trioctanoin.

Parameters monitored - Mice were kept for 250 to 300 days after treatment. They were checked for deaths daily and their condition at autopsy was noted. Leukemia was monitored with periodic white blood cell counts, hematocrit determination, and microscopic examination of blood smears.

RESULTS

In these studies the effects of DMBA or BP given 5 hours before or 5 hours after FLV were contrasted with the effects of the virus alone or the chemical alone. For control purposes an additional set of mice not exposed to either carcinogen was also maintained

and regularly checked for evidence of spontaneous cancer development.

DMBA - Figure 1 shows the effect of 10 µg of DMBA given by intraperitoneal injection 5 hours before or 5 hours after 0.1 SED FLV. When given 5 hours after the virus, DMBA had a significant protective effect when the survival of these mice is compared to that of mice given 0.1 SED of virus alone from the same preparation. This protective effect is illustrated by a generally longer survival time than that seen for FLV given alone, and a smaller number of animals dying with the normal symptoms of Friend leukemia. When given 5 hours before FLV, this protective effect is still suggested but the difference is not significant. All of the mice that died after day 120, and none of the mice that died before this time, had enlarged lymph nodes and thymus glands, as well as enlarged spleens. The incidence of the later deaths with enlarged lymphoid organs appears to be unaffected by DMBA. Of the 4 mice in the DMBA only group that died, 3 had large tumors at the injection sites, while 1 appeared to die of infection. It should be noted that the experiment of figure 1 was the only instance where olive oil was used as the carcinogen vehicle, and it was also the only instance where solid tumor development at the site of carcinogen injection in the group receiving carcinogen only was observed.

Figure 2 shows the results of an experiment where a lower dose of FLV (0.015 SED) was used in conjunction with three doses of 50 µg DMBA in trioctanoin. The lower dose of FLV used in this experiment had only minimal early leukemogenic effect, and only those mice that died before day 100 were free of lymphoid organ enlargement. The fact that DMBA had no effect on survival in this

Fig. 1. 10 μ g DMBA dissolved in olive oil was injected 5 hrs before or 5 hrs after 0.1 SED FLV into SJL/J mice. The groups that received both viral and chemical carcinogens consisted of 15 mice each. All of the other groups consisted of 10 mice. A control group that received the carcinogen vehicles only had 100% survival by day 300 (not shown in graph).

Fig. 2. 50 μ g DMBA dissolved in trioctanoin was injected IP into SJL/J mice. 5 hrs later 0.015 FLV was injected. The DMBA injection was repeated again 7 days and 14 days after FLV injection. There were 5 mice in the control group that received vehicles only; 10 mice in each of the groups that received either carcinogen alone and 20 mice in the group that received both chemical and viral carcinogens.

experiment agrees with the results of figure 1, since nearly all of the deaths in this experiment were of the type which is unaffected by DMBA.

In the experiment of figure 3 the dose of FLV used was higher than the usual 0.1 SED, as is apparent from the increased number of leukemic deaths in the group that received virus only. The results of DMBA treatment in conjunction with this virus dose confirm the conclusion drawn from figure 1 above that DMBA is protective against the early leukemogenic action of FLV. In this case a single exposure to DMBA was as effective as 3 exposures given a week apart.

BP - In the experiment illustrated by figure 4, 500 µg BP was given to SJL/J mice 5 hours before or after 0.1 SED FLV. The results indicate that pre-treatment with BP potentiated FLV carcinogenesis. This conclusion is confirmed by the experiments reported in Table 1, where various doses and schedules of BP increased leukemogenic death in normally resistant B10SJF1 mice. With B10SJF1 mice all of the deaths were due to erythroleukemia, characterized by hepato- and splenomegaly with no instances of lymphoid organ involvement.

DISCUSSION

Evidence was obtained that hydrocarbon carcinogens affect Friend viral leukemogenesis. BP increased this effect of FLV, while DMBA apparently decreased it. These effects were observed when FLV produced symptoms of early erythroleukemia as originally described for this virus (15), i.e. gross hepato- and splenomegaly, increased white blood cell counts and hematocrit. However, in the group of

Fig. 3. 50 µg DMBA in triolein was injected IP to SJL/J mice seven days before, 5 hrs before or 7 days and 5 hrs before. All the deaths observed involved erythroleukemia as judged by spleno- and hepatomegaly. There were ten mice per group.

Fig. 4. 500 μ g BP was injected IP to SJL/J mice 5 hrs before or 5 hrs after 0.1 SED FLV. There were 10 mice in each of the groups that received both viral and chemical carcinogen; and there were 5 mice in the group that received virus only as well as in the control group that received saline injections only. There was 100% survival in the control group (not shown on graph).

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Table I
B10.SJF1 Mice Were Treated With BP Dissolved in
Triolein and FLV as Indicated. Control Mice Received FLV Only.

	Number of Leukemic Deaths	
	Control	Experimental
Exp. 1 50 μ g BP 5 hrs before FLV	0/6	1/6
Exp. 2 50 μ g BP 5 hrs before FLV plus 500 μ g BP 7 days after FLV	1/6	6/6

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SJL/J mice which survived the initial wave of deaths, the survivors often died several months later with different symptoms, more resembling a lymphoma. Neither BP or DMBA given alone seemed to have any effect on this later type of mortality. The lower incidence of late deaths with lymphoid involvement in the group receiving FLV alone, as opposed to both viral and chemical carcinogen (Fig. 1), probably reflects the fact that most of the recipients of FLV alone died before such symptoms usually develop.

We have observed the relatively late development of lymphoid involvement only in these present experiments with hydrocarbon carcinogens and not with others involving the water soluble carcinogen, methyl methane sulfonate (15,16). However, since lymph node enlargement was seen in groups that were also treated with virus only, it is possible that the oil vehicle given these mice for control purposes was instrumental in its induction. The synthetic oil, trioctanoin, was used in all experiments except our first one reported in figure 1. In one set of studies the development of local solid tumors at the site of injection was also observed. However, this occurred only when olive oil was used as the solvent for DMBA. This suggests that the olive oil may have acted as a promotor (9). Histological examination of the solid tumors as well as of enlarged lymphoid organs are now in progress.

Development of a thymic lymphoma has been previously observed to occur in the late course of Friend disease (17), following an early splenic response. A similar situation was also described for the pathogenesis of the closely related Rauscher murine leukemia virus (18). Our data suggest that the early leukemic and late lymphoma effects are differentially sensitive to carcinogen

interaction. Interestingly, the enlarged lymph nodes and thymus glands that are characteristic of late deaths in SJL/J mice infected with FLV were never seen in B10SJF1 mice, despite the fact that many of the deaths occurred around day 150 after FLV injection. It was also the case in this hybrid strain that even the late deaths were influenced by BP injection. Since B10SJF1 mice are fairly resistant to FLV action alone, this observation indicates that the development of lymphoid involvement requires a high degree of virus sensitivity.

The basis for the protective effect of DMBA is not clear and we are presently monitoring plasma titers of virus in mice treated with FLV and DMBA in order to determine whether it may be the result of virus inactivation. Further experiments utilizing various doses of both DMBA and BP are necessary to determine whether the apparent fundamental difference between the effect of DMBA and BP is real.

Another interesting observation involving B10SJF1 mice is that they develop temporarily elevated white blood cell counts in about 30% of animals given 100 SED FLV alone (unpublished). Thus it is possible that some immunological control of the virus occurs in B10SJF1 mice. Experiments to determine whether BP effects the immune system are in progress. Also in progress are other experiments utilizing different timing schedules of hydrocarbon and virus exposure as well as the use of various inhibitors of DNA repair. The latter may determine whether effects on the hosts' DNA are involved.

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TECHNICAL REPORT

LEUKEMIA INDUCTION IN SJL/J MICE TREATED WITH
METHYL METHANE SULFONATE AND FRIEND VIRUS

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ABSTRACT

Methyl methane sulfonate (MMS) given to SJL/J mice in conjunction with Friend leukemia virus (FLV) enhanced leukemogenesis. The time of MMS administration in relation to FLV inoculation was varied. MMS was effective in leukemogenic enhancement only if given within 24 hrs preceding the virus. MMS given 5 hrs after the virus was slightly protective. MMS was also found to have an immunosuppressive effect as measured by a depressed plaque forming response to sheep red blood cells. However, the nadir of this response did not correspond to the peak of leukemia virus enhancement.

INTRODUCTION

Several studies have shown that chemical agents as well as irradiation enhance the effect of carcinogenic viruses in vitro (1-5). One such chemical is the alkylating agent, methyl methane sulfonate (MMS), which has been shown to enhance adenovirus transformation of hamster cells (6). The mechanism by which this occurs seems to depend on a direct effect of this agent on DNA, with the degree of virus co-carcinogenic enhancement being directly correlated with the extent of DNA damage. Possibly the virus becomes more easily integrated at such damaged sites.

In the present study we have asked the question whether MMS was effective in enhancing viral leukemogenesis in vivo; and having found this to be the case, we asked whether this activation involved immunosuppression.

MATERIALS AND METHODS

Animals - SJL/J female mice were purchased from Jackson Laboratories and held until 10-12 weeks of age. They were housed in plastic cages with filter tops in a temperature humidity and 12-hr light cycle controlled room, and were given acidified water and Purina lab chow ad libitum.

Methyl methane sulfonate (MMS) - MMS (Sigma) was diluted with sterile isotonic saline and injected intraperitoneally into 10 to 12 week old mice, at various doses and times in relation to virus, as indicated.

Virus - Friend leukemia virus (FLV) preparations were made by passaging Friend virus stock obtained from NCI through SJL/J mice,

obtaining the virus rich plasma and diluting it with saline. The virus dose to be administered was measured in spleen enlargement units (SED), which are defined as that amount of virus which causes spleen enlargement in 50% of the injected SJL/J assay mice within two weeks. The mice were given the virus by intraperitoneal injections.

Plaque forming assay - The ability of splenocytes to lyse sheep red blood cells (Sacks Farms) was measured as described previously (7).

RESULTS

In figure 1 the survival of mice that were injected with 2 mg MMS 5 hrs before or after 0.1 SED FLV is compared to that of mice that were given the same doses of FLV or MMS alone. All of the deaths, with one exception, in this and all experiments described below, were due to erythro-leukemia as indicated by hepato- and splenomegaly at death, elevated white blood cell counts, elevated hematocrits and increased percentages of abnormal red cells in the peripheral blood. The one exception was a single mouse in the MMS only group that died on day 148 which had no splenomegaly but did have enlarged lymph nodes. The cause of death in this instance is unknown.

The experiment illustrated by figure 2 is similar to that of figure 1, except that a lower dose of virus (0.015 SED) and a higher dose of MMS (3 mg) was used. This dose of MMS proved to be toxic, and 44% of the mice receiving it died within 24 hrs of its injection. As these deaths were clearly due to the chemical toxicity of MMS,

Fig. 1. SJL/J mice were injected IP with 0.1 SED FLV and with 2 mg MMS either 5 hrs before or 5 hrs after the virus. There were 15 mice in the two groups that received both viral and chemical carcinogens. All of the other groups, including a control group that received saline injection only, which is not shown, consisted of 10 mice each. There was 100% survival in the control group.

Fig. 2. SJL/J mice were injected intraperitoneally with 0.015 SED FLV and with 3 mg MMS either 5 hrs before or 5 hrs after the virus. There were 5 mice in the virus only group, 7 mice in the group that received MMS before virus and 15 mice in the group that received MMS after virus.

they are not included in the survival data of figure 2. However, in the remaining mice the leukemogenic action of FLV was enhanced by MMS pretreatment in agreement with the data of figure 1. In contrast, MMS given 5 hrs after FLV in this study also enhanced leukemogenesis. In the experiment of figure 3, two mg of MMS were injected at various times, up to 5 days, before 0.1 SED of the virus. The survival data indicate that MMS is equally effective when given 5 or 24 hrs before FLV. However, when 48 hrs or more is allowed to elapse between the two carcinogen treatments, MMS has no enhancing effect and, in fact, appears to be slightly protective. When two 2 mg doses of MMS were given prior to 0.10 SED of FLV (figure 4), a greater enhancement was obtained than when a single dose was given at 24 hrs before FLV.

As shown in figure 5, MMS suppressed the splenic plaque forming response when given to SJL/J mice without virus. The table insert of figure 6 shows plaque forming response was also decreased by FLV injection, as previously reported (8), and when MMS was given in combination with FLV, this immune function was further suppressed.

DISCUSSION

MMS enhancement of viral leukemogenesis - Two different doses of virus were used in this study, both of them substantially below the 100% threshold of 50 SED units previously established by this laboratory to be the lowest dose to cause 100% leukemic deaths in SJL/J mice by day 40. These were 0.1 SED units and 0.015 SED units. The FLV dose of 0.1 SED was sufficient to kill 90% of the mice by

Fig. 3. SJL/J mice were injected IP with 2 mg MMS at 5 hrs, 1 day, 2 days or 5 days before 1.0 FLV. There were 6 mice per group.

Fig. 4. 2 mg MMS was injected into SJL/J mice IP 24 hrs and/or 5 hrs before 0.1 SED FLV. There were 10 mice in each of the groups, except in the control group that received saline injection only which consisted of 5 mice.

Fig. 5. SJL/J mice were injected with 2 mg MMS IP and then with SRBC on the designated days. Five days after SRBC injection the mice were sacrificed and their splenocytes were tested for ability to lyse SRBC in vitro. To obtain the data in the insert, SJL/J mice were injected with 2 mg MMS and then 5 hrs later with 50 SED FLV. Three days after the FLV injection these mice were injected with SRBC, and 5 days after SRBC they were sacrificed and their splenocytes isolated for in vitro assay.

day 300, with some leukemic death occurring as early as 40 days (figure 1). The lower dose of 0.015 SED used in the experiment of figure 2 appeared to be totally without effect when used alone until day 180. With either FLV dose the injection of MMS 5 hrs before the virus enhanced leukemogenesis, i.e. it decreased the number of long-term survivors. When 2 mg was injected 5 hrs after 0.1 SED of FLV, it appeared to have a slightly inhibitory effect on FLV leukemogenesis. However, when 3 mg MMS was given 5 hrs after 0.015 SED FLV, there was a small enhancement of viral action. It is unclear at present whether this discrepancy is due to the doses of virus or chemical carcinogen employed. However, it is clear that pretreatment with MMS is the most effective way of enhancing FLV leukemogenesis by this chemical.

MMS dose - As mentioned above, the 3 mg/mouse dose of MMS used in the experiments of figure 2 proved to be toxic. However, when a total of 4 mg was given in two doses separated by 24 hrs (figure 4), there were no early deaths, and such a double treatment with MMS was more effective than a single 2 mg dose. Thus the MMS effect does appear to be dose dependent.

Time of MMS pretreatment - Having established that pretreatment by MMS was effective in enhancing viral leukemogenesis, we varied the interval between the MMS pretreatment and the injection of virus in order to determine whether the MMS effect was reversible. As shown above, when given 5 hrs or 24 hrs before virus, MMS was approximately equally effective. However, MMS given 2 to 4 days before FLV did not enhance viral leukemogenesis at all. Thus it appears that the MMS caused lesion is repaired by 48 hrs.

Humoral immune function - The plaque forming assay used in the present study measures the ability of mice to produce lymphocytes that synthesize antibody against sheep red blood cell antigens. This function is apparently dependent not only on B cells but also requires T helper cells (9). It has been shown that FLV causes pronounced suppression of this function in virus susceptible mice (8), and, because the degree of this immunosuppression has been correlated with the degree of susceptibility of viral leukemia in various mouse strains, it was postulated that the virally induced immunosuppression may be an etiological factor in this disease. It was therefore of interest that MMS was also found to be an effective immunosuppressant by the plaque forming assay, and that when MMS was given in combination with FLV a lower level of immunosuppression was seen than when either agent was given alone. However, this effect does not appear to be the prime cause of the presently observed potentiation of viral leukemogenesis. We draw this conclusion from the following two points: 1) MMS is effective in enhancing leukemogenesis only if given within the 24 hrs preceding the virus, although the peak of immunosuppressive activity is not reached until 5 to 7 days after MMS; 2) Cytosan, which is equally or more immunosuppressive when compared to MMS (10), has no effect on viral leukemogenesis (unpublished results). Moreover, the short-lived nature of the MMS effect suggests that the MMS induced damage is relatively quickly repaired. Thus it is reminiscent of DNA damage repair. There is evidence that MMS-augmented, adenovirus transformation of hamster cells is also dependent on short-lived DNA damage (6). Thus we suggest that such an MMS effect, as

previously demonstrated in vitro (3,6), is probably also the most important MMS action responsible for viral enhancement in vivo.

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DNA-dependent-DNA-polymerase: Possible limiting influence on cell reproduction during viral leukemogenesis*

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Summary. Evidence is presented that during viral leukemogenesis spleen cell nuclei show an increase in labelling index and mean grain count, that is not accompanied by any changes in the nuclear level of DNA-polymerase- α . It is suggested that polymerase production remains under the control of the normal cell mechanisms and the virus may affect cell proliferation by altering the primer-template levels.

Cancer development in both humans and animals is often characterized in the initial growth phase by a population of rapidly proliferating cells and in the later growth phase by a large compartment of slow growing or resting, G_0 cells¹. Previous studies in our laboratories have shown this also to be the case in mice given Rauscher leukemia virus, RLV²⁻⁴, for which the hematopoietic stem cell has been identified as a leukemia virus target cell⁵⁻⁷. In these mice, even though the first response to the virus is to increase the number of DNA synthesizing (S-phase) cells^{8,9}, measurement of the splenic stem cell G_0 compartment at the 14th day after virus administration has shown it to be 20 times the normal size for a non-leukemic control¹⁰. Since at the same time, and at still later disease stages, Rauscher leukemic mice are also known to have massive serum virus titers¹¹, it can be inferred that although RLV does initially increase the number of proliferating S-phase stem cells it cannot of itself maintain an increased rate of proliferation in the target stem cells, even at these high virus titers. Hence, the possibility is suggested that there may be a limiting necessary factor for DNA synthesis, even in the leukemic cell, which cannot be influenced by the virus. To further investigate this possibility in the Rauscher leukemia model we have utilized the primer-dependent-polymerase (PDP) assay technique recently developed by Schiffer et al.¹². This technique allows one to study not only the changes in the fraction of proliferating cells during leukemogenesis through DNA labelling, but further to discriminate within the labelled populations as to whether the changes in labelling are a consequence of

changes within the cell nucleus in DNA-dependent-DNA-polymerase concentration, or in primer-template, or both¹³. **Materials and methods.** The animals used were female SJL/J mice injected with 30 SED₅₀ units of RLV¹⁴ at 12 weeks of age. As controls normal, non-injected, mice selected from the same lots were used. To allow for correlation of the present studies with our previous G_0 studies¹ the mice were sacrificed 14 days after RLV injection. The spleens were removed, single cell suspensions prepared in Hank's solution supplemented with fetal calf serum, and dry film smears prepared on acid cleaned

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slides. The unfixed preparations were dipped into a 0.25% agar solution at 40°C to strip away the cytoplasm, leaving the bare nuclei adherent to the slide, and air dried. Glass incubation chambers were affixed to the slides with paraffin, and 0.5 ml of incubation mixture containing tritiated thymidine triphosphate (³HTTP) was added. The samples were then incubated and autoradiogrammed in the manner previously described, with positive ³HTTP labelling indicating the simultaneous presence of primer-template in conjunction with DNA-polymerase- α within the cell nucleus¹²⁻¹⁴. Further evaluation of the relative levels of DNA-polymerase- α in the nuclei of leukemic spleen cells as compared to normal was accomplished by enzyme inactivation using para-hydroxymercuribenzoate (p-HMB), a potent sulfhydryl binding agent and inhibitor of DNA-polymerase- α ¹⁵. For this varying concentrations of p-HMB, ranging from 1 mM to 10⁻⁴ mM, were added to the PDP incubation mixtures in otherwise duplicate slides at the beginning of the assay. DNA-polymerase- α inactivation was determined by the reduction in labelling achieved as a function of the p-HMB concentration.

Results. At sacrifice the leukemic mouse spleens were 4 times larger than normal, 395.3 $\pm$ 45.4 mg as compared to 93.2 $\pm$ 9.8 mg. The results for PDP labelling index and mean grain count for normal and leukemic nuclei incubated without p-HMB inhibitor are shown in the table. All nuclei were evaluated except those obviously belonging to differentiating myeloid cells. The results show that at 14 days after RLV injection, the inflection point for the growth phase Rauscher leukemia development¹⁴, the overall PDP labelling index is 4.8 times that

for normal control spleen cells, indicating that a greater proportion of leukemic cells than normal contain the 2 minimal requisites for PDP assay and DNA synthesis, primer and polymerase. Further, the mean grain count is increased by a factor of 2, suggesting that the concentration either of one or both of these is also increased on a per cell nucleus basis.

The figure shows the inactivation of DNA-polymerase- α in normal and leukemic spleen cell nuclei as a function of p-HMB concentration. Inspection of the curves indicates that, proportionally, the polymerase inhibition for cells of the leukemic mice is similar to that of normal mice, with a virtually identical 50% inactivation point in terms of p-HMB concentration. The similarity of these curves suggests that in both cases the DNA-polymerase- α enzymes being evaluated were analogous, and that the average amount present per labelled cell nucleus was equivalent for the leukemic and normal spleen cells.

Discussion. In previous studies, Schiffer et al. have shown that the PDP technique yields a value for total labelling quite analogous to the growth fraction¹², and has an additional advantage in that one can directly evaluate the activity within the nuclei of the individual cells of 2 of the essential components which determine the capability for DNA synthesis, primer-template and DNA-polymerase- α ¹⁶. Although by 14 days the fraction of Rauscher leukemic mouse stem cells in S-phase has returned to a value similar to that for non-leukemic mice⁴, the present results show that the overall growth fraction for the general undifferentiated and differentiating spleen cell population is still substantially increased at this time. However, within that population, the average amount of DNA-polymerase- α per leukemic cell is similar to that in non-leukemic control spleen cells.

Schiffer et al.¹² have also reported that normal cell nuclei commonly contain a more than adequate amount of DNA-polymerase- α at the beginning of S-phase and have postulated available primer-template as a limiting factor governing the entrance of normal cells into DNA synthesis. This was based on their observation that addition of exogenous DNA template primer to the PDP system in normal cells resulted in an increased incorporation of ³HTTP radiolabel. In light of this, the findings of the present study that DNA-polymerase- α levels in Rauscher leukemic nuclei are unchanged from the normal suggests by inference that the increase in the mean grain count, and hence radiolabel incorporation, in the PDP labelled leukemic cells may be the result of an opposite situation. Specifically, the action of the oncovirus may result in leukemia-related excess of available primer-template per labelled nucleus, with the level of DNA-polymerase- α now becoming the limiting factor. This interpretation would be consistent with Temin's pro-virus hypothesis¹⁷ and with other earlier reports on the production of both new linear and circular types of DNA by leukemia viruses^{18,19} and their subsequent migration to the nucleus and incorporation into the host cell genome²⁰.

Comparison of PDP index parameters in spleen cell nuclei of normal and Rauscher leukemic SJL/J mice 14 days after virus infection

	Normal	Rauscher
Labelling index ($\pm$ 1 SE)	13.09 $\pm$ 1.10	62.89 $\pm$ 3.33
Mean grain count ($\pm$ 1 SE)	7.30 $\pm$ 0.24	13.36 $\pm$ 2.54

Extinction of PDP response in normal and viral leukemic cells as a function of concentration of the DNA-polymerase- α inhibitor, p-hydroxymercuribenzoate, in the PDP incubation medium. Loss in percent of control level reflects the reduction in mean grain count in terms of the percent value for either normal cell nuclei or leukemic cell nuclei not exposed to the inhibitor. Data indicates identical concentration of the polymerase in the normal and leukemic nuclei. The extinction curves are also similar in shape, within the limits of 2 SE, suggesting that the enzymes in the 2 types of nuclei are not significantly different.

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Stimulation of Immune Response in Hybrid Mice Following Rauscher Virus Infection¹ (40074)

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Suppression of plaque former cell (PFC) response to sheep red blood cells (SRBC) following Rauscher leukemia virus (RLV) or Friend leukemia virus (FLV) infection has been well documented in several mouse strains sensitive to these viruses (1-3). Likewise, a transient suppression of PFC response has been reported for the virus-resistant C57B1/6 mouse during the first week after FLV infection (4). Ceglowski and Friedman (5) compared PFC immunosuppression by FLV in several strains 8 days after infection and reported that the degree of immunosuppression roughly paralleled that of virus susceptibility, with the only hybrid tested (the B6D2F1 of the C57B1/6 and the DBA) showing even more immunosuppression than its sensitive DBA parent. However, in that study as in most of the previous studies the prime concern was with the immune response of strains known to be sensitive to the virus. Further, many of these studies have been limited in their observation periods to the rather short duration after infection in which confirmation of immunosuppression is usually obtained. On the other hand, recent data of at least one study (4) suggest that stimulation of PFC response by leukemia virus might also be demonstrable in resistant strains if the observation period is sufficiently long. In the present study we have compared the level of PFC response as affected by RLV in the sensitive SJL/J mouse, the resistant C57B1/10 mouse and their resistant hybrid (SJL/JxC57B1/10)F₁. Evidence for stimulation of PFC response in the resistant mice between the second and fourth week after

infection was obtained, with the immune response of the hybrid showing even greater enhancement than that of its resistant parent.

Materials and methods. *Mice.* The SJL/J and C57B1/10 mice were females obtained from Jackson Laboratories (Bar Harbor, ME) at the age of 6-8 weeks and quarantined before use. The F₁ hybrids were females bred in our own facilities from parents obtained from Jackson Laboratories. All mice were maintained five per cage in sterilized filter-top cages and allowed autoclaved food and water *ad libitum*.

Virus. The NB-tropic strain of Rauscher leukemia virus used was obtained from NIH in 1968 and has been maintained in our laboratories in the SJL/J mouse. Assay for the virus activity was done by a variation of the spleen enlargement dose (SED) technique of Chirigos *et al.* (6), as previously employed by us (7-9). Virus was prepared from a cell free plasma filtrate, buffered with sodium citrate and kept at -72° until use. 0.1 ml of saline diluted virus suspension containing 50 SED_{50/14} units of RLV was injected ip into the mice at approximately 12 weeks of age. Control mice were sham injected with a similarly prepared material not containing virus.

PFC Response. A modification of the Kennedy-Axelrad technique (10) was used to evaluate PFC response. This technique has been shown to yield a greater number of plaques per unit of cells than the Jerne and Nordin technique (11). At the times indicated in the results, either before or after RLV administration, washed SRBC were injected into the virus infected mice and into paired age-matched control mice of the same strain. Five days later the mice were sacrificed, their spleens removed, weighed, gently mashed, and single cell suspensions prepared in PBS. Total nucleated cell number per spleen was determined and then the concentration of the

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suspension was adjusted to 2.5×10^6 cells per ml. Two milliliters of this, mixed with guinea-pig complement, was then carefully layered over a monolayer of SRBC affixed to a grided plastic Petri dish with poly-L-lysine. After a 45-min incubation at 37°, the supernate was carefully decanted off and the plates gently rinsed in PBS. The plaques per plate were counted on the damp plates and the number of PFC per spleen calculated. The plaque forming ability of the virus injected mice was compared directly with that for the normal control mice of the same strain run in parallel on each of the experimental days, and the results are reported in terms of percent of the control animal plaque forming ability.

Statistics. For each data point given a minimum of four virus infected animals was run against the controls. Most points were repeated two or more times and the data averaged in the production of the figures. Mean values and standard errors were calculated using the Student's *t* test as described by Dixon and Massey (12). The error limits as given in the text and figures are ± 1 SE.

Results. The mean level of PFC per spleen in the normal SJL/J, C57B1/10 and F₁-hybrid mice varied according to the strain, being $204,600 \pm 9,000$, $293,000 \pm 15,700$ and $431,000 \pm 24,500$ respectively, which differ significantly from each other at an α level of better than 0.05. At the same time there were no significant differences in the spleen size of these normal mice, indicating that the observed variation in PFC numbers reflected a true difference in the relative proportion of plaque formers per spleen between these strains.

Figure 1 gives the data for PFC response of the virus injected animals, normalized to their respective control values for each strain and day, and presented for comparison purposes in percent of the respective controls. The RLV sensitive SJL/J mouse showed a brief elevation in response when the virus was injected 1 day before or immediately before SRBC administration. This was followed by a depression in response, reaching minimum levels at the 21st day. The C57B1/10 showed contrasting behavior, with only a slight depression in PFC response at day 2, which was much less than that previously reported for FLV infected C57B1/6 (4).

FIG. 1. Change in splenic PFC response as a function of time after RLV infection. Data is normalized to the control values for each strain taken as 100%. Error bars indicate ± 1 SE of the mean. Half error bars are shown where they would otherwise overlap.

Following this there was a recovery and overshoot which first became significant at the $\alpha = 0.05$ level at day 12. The most striking difference seen was that in the hybrid, which showed a PFC response as affected by RLV that was unlike either of its parental strains. First there was a significant immunosuppression, evident at day 1 after virus infection. This was followed by a rapid recovery in PFC, peaking at a level twice the normal by day 8, with a subsequent drop-off but a sustained immunostimulation throughout the remainder of the observation period.

Figure 2 illustrates that the observed levels of PFC response were not a direct function of spleen size following RLV infection. The C57B1/10 showed no significant change in spleen size throughout the duration of the study. The hybrid did show a transient three-fold increase, but returned to normal by the termination of the study. Further, the peaks of splenomegaly for the hybrid did not coincide with the peaks of PFC activity. However, it would be correct to say that some of the increases in PFC response were due to the overall elevated number of spleen cells in the infected hybrid. By contrast, although the SJL/J developed the greatest splenomegaly, this strain showed virtually complete suppression of total splenic PFC response.

Discussion. While several authors have previously shown that murine leukemia viruses are immunosuppressive in virus-sensitive

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FIG. 2. Variation in spleen weight as a function of time after RLV infection. Error bars indicate ± 1 SE of the mean. Where no error bars are given the SE was less than the size of the data symbols.

hosts (1-3, 5, 13, 14), evidence for a possible stimulatory effect had been limited to the C57B1/6 strain (4, 5), and only suggestive at best. The data of this paper gives positive evidence that enhancement of the humoral antibody plaque forming cell response can be demonstrated following leukemia virus infection. The significance of this lies in the fact that humoral antibody formation is known to play a role in the induction of immunity to leukemia virus in otherwise sensitive mice following the injection of killed virus and leukemia cells (15-17). For other strains, including the group to which the C57B1/10 belongs, it has also been established by *in vitro* studies that one control of resistance to infective oncornavirus rests directly at the level of the target cell, unmediated by immune response (18-20). In that regard the level of PFC response of the hybrid is of particular interest, in that the F_1 possesses alleles for both sensitivity and resistance to the virus. The much greater stimulation of plaque formers suggests that the influence of the virus is more pronounced in the hybrid than in its resistant parent, perhaps strongly activating an immune response to the leukemia antigens. The elevated spleen weights also indicate an effect of the virus in the hybrid resulting in some cellular proliferation, but not to the extent observed during leukemogenesis in the SJL/J mouse, and clearly reversible. This suggests that some initial oncogenic activity may have occurred

which the animal was able to successfully overcome. Full leukemogenesis did not result, however, as peripheral white counts done at the terminal point of the study showed only a slight elevation, and none of the animals died of Rauscher disease. In contrast to the B6D2F₁ hybrid of the DBA and C57B1/6, which shows immunosuppression and succumbs to leukemia virus (5), we have found this hybrid survives RLV infection (21).

Because Rauscher virus is NB-tropic (22), the Fv-2 locus would be expected to play the primary role in the genetic determination of resistance to the virus. This gene governs spleen focus formation and splenomegaly, with the sensitivity allele, Fv-2^s, being dominant over Fv-2^r. The C57B1/10 is Fv-2^r (18), while the SJL/J is felt to be Fv-2^s, similar to other Swiss-Webster derived mice (18, 23). Therefore, like the B6D2F₁, one would expect this F_1 hybrid to be almost as sensitive to Rauscher virus as its SJL/J parent. Since it is not, it would seem likely that other genes in this cross may be masking the effect of the Fv-2 locus. In the related Friend virus system, Chesebro and Wehrly (24) have reported that hybrids of C57B1/10 with either BALB.B or A.BY, which are also Fv-2^r, likewise show a high incidence of recovery from splenomegaly and leukemia. From their results they postulated that this may be due to other non-H-2 genes associated with the H-2^b configuration, but not found in H-2^d mice. Whether any of these other susceptibility genes are also present in the SJL/J mouse, which is H-2^d, is as yet unknown. However, if they were it might account for the observed resistance of the hybrid used in this present study to Rauscher leukemia and its recovery from splenomegaly. Additional work remains to be done to establish this point, as well as the specific response of this hybrid to the spleen focus forming virus component of the Rauscher complex (25). In so doing, this hybrid may prove to be a useful model, analogous to that of Chesebro and Wehrly (24), for the study of genetic factors governing RLV sensitivity and the development of immune response to viral leukemia antigens.

Summary. The effect of Rauscher leukemia virus injection on the splenic PFC response to SRBC was measured in a mouse strain sensitive to the virus (SJL/J), one resistant to

the virus (C57B1/10), and their F_1 hybrid (SJL/JxC57B1/10) F_1 . In contrast to previous findings of a suppressive effect of Friend virus on PFC response in another hybrid of different resistant and sensitive strains, a significant immunostimulation was seen. Immunostimulation was also seen in the resistant parent when the PFC response level was measured 12 days or later after RLV infection, while the PFC response of the sensitive parent was nearly completely suppressed. Evaluation of the effect of RLV on the splenic masses of the three strains indicated a mild transient splenomegaly in the hybrid; a pronounced splenomegaly in the sensitive parent; and no effect on the resistant strain. Comparison of the data suggests that the change in splenic PFC number in the hybrid following RLV infection occurs as a function partially independent of changes in the total splenic mass.

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