Effect of 7,12-Dimethylbenz[*a*]anthracene and Splenectomy on Virus Titer and Blood Picture in Friend Virus Leukemia[1] (34512)

STEPHEN C. ELLIOTT,[2,3] WINIFRED K. KIEHN, CHRISTOPHER A. REILLY, JR.,[2,4] AND GERD T. SCHLOSS
(Introduced by Wayburn S. Jeter)

Department of Microbiology and Medical Technology, University of Arizona, Tucson, Arizona 85721

An inhibitory effect on Friend virus (FV) and Rauscher virus leukemia by the carcinogenic polycyclic aromatic hydrocarbons 7, 12-dimethylbenz[*a*]anthracene (DMBA) and 3-methylcholanthrene was recently reported (1). The parameters used to show this were latent period, survival time, and spleen weight. Interference with "indirect neoplastic effects" (development of hemorrhagic necrosis and proliferation of erythroblasts in the spleen) was considered to play a greater role than interference with a "direct neoplastic effect" (proliferation of the neoplastic reticulum cells). To investigate the mechanism of this inhibitory effect, the weekly FV titers in spleen and liver of untreated and DMBA-treated mice were compared. Also compared were the weekly liver weight and blood picture (erythrocyte volume, nucleated blood cell count, and differential blood cell count) of splenectomized, untreated and DMBA-treated, FV-infected mice with those of non-splenectomized mice.

Materials and Methods. The FV was the same as that used in the previous study (1). The titer of the virus pool was $10^{4.0}$ ID_{50}/ml. For the induction of leukemia, adult BALB/c mice, weighing approximately 25 g, received a single intraperitoneal injection of 0.2 ml of a 10^{-3} dilution of the virus in sucrose stabilizer. For the treatment with DMBA, 200 μg of the chemical dissolved in 0.05 ml of acetone were applied at weekly intervals to the depilated skin of the back, beginning on the day of infection.

For the determination of the FV titers, two groups of 120 BALB/c mice each were used, half males and half females. Group 1 received only the FV, group 2 the FV and DMBA. Ten animals from each group were sacrificed every week, males and females in alternating weeks. The spleens and livers were removed aseptically, weighed, pooled, frozen in a dry ice–alcohol bath, and stored at —70° until used for titration. Titrations were carried out for the first 6 weeks only, but spleen and liver weights were determined until the termination of the experiment at the end of week 12. The virus titer was determined by an extinction dilution bioassay. Spleen and liver homogenates (20%) were diluted in sucrose stabilizer in 10-fold steps from 10^{-1} to 10^{-6}, and 0.2 ml of each dilution injected intraperitoneally into a group of 10 normal adult BALB/c mice. After 35 days, these animals were sacrificed and considered as leukemic when the spleen weighed more than 0.5 g (2). Titers were then calculated by the method of Reed and Muench (3).

For the examination of the blood picture, four groups of 240 BALB/c mice each were used, half of which were splenectomized. Only female mice were used, since they showed a higher postoperative survival rate than males. The experiments were started 1–2 weeks after splenectomy. Group 1 served as untreated control, group 2 received only DMBA, group 3 only the FV, and group 4 the FV and DMBA. The experiment lasted

[1] This investigation was supported by Institutional Grants from the American Cancer Society to the University of Arizona.

[2] Trainees under USPHS Training Grant 5 T01 AI00122-09.

[3] Present address: Department of Biology, Drake University, Des Moines, Iowa.

[4] Present address: Experimental Radiation Pathology, Argonne National Laboratory, Argonne, Illinois.

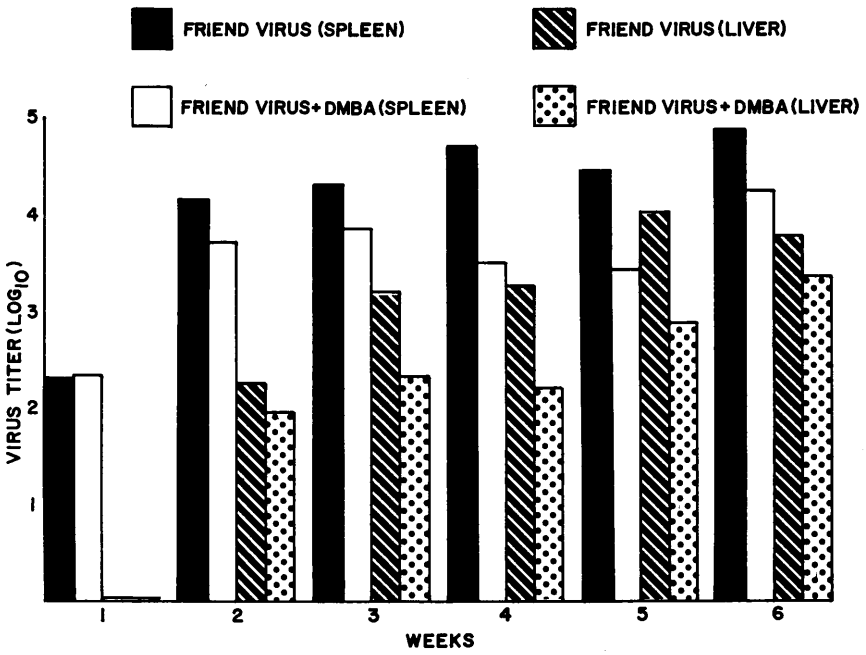

FIG. 1. Effect of DMBA on virus titer in spleen and liver of mice with Friend virus leukemia. Each bar represents the average for a group of 10 mice.

12 weeks. Ten splenectomized and 10 nonsplenectomized mice in each group were sacrificed every week after blood had been collected in heparinized capillary tubes from the retroorbital plexus. The erythrocyte volume was determined using Drummond microhematocrit tubes. The nucleated blood cells were counted in a Coulter counter. Differential smears were stained with Wright's stain. Spleens and livers were weighed and examined histologically after fixation in formalin and staining with hematoxylin and eosin.

Results. DMBA produced a decrease in virus titer by approximately 1 log, both in the spleen and in the liver (Fig. 1). This decrease is small, but statistically significant as determined by analysis of variance with a factorial design.[5] Virus was not detectable in the liver until week 2.

Splenomegaly was markedly inhibited throughout the course of the experiment (Fig. 2). Inhibition of hepatomegaly was not apparent until Week 10, but was quite marked during the next 2 weeks. At the end of week 12, the average weight for a group of 10 nonsplenectomized, infected, DMBA-treated mice was only 2.1 g as compared with 5.1 g for both nonsplenectomized and splenectomized, infected, untreated mice. The average weights for groups of 10 noninfected control mice were: normal 0.7-1.7 g ($\bar{x}$ = 1.2), DMBA-treated 1.3-1.9 g ($\bar{x}$ = 1.6), splenectomized 1.1–1.5 g ($\bar{x}$ = 1.3), splenectomized and DMBA-treated 1.0-1.3 g ($\bar{x}$ = 1.2).

As reported previously (1), DMBA greatly extends the life span of FV-infected, nonsplenectomized mice. Splenectomy had a similar effect. Whereas 80–100% of nonsplenectomized, infected animals were dead in 5–7 weeks (1), the mortality of splenectomized, infected mice at the end of week 7 in the present experiment was only 10%, and at the end of week 12 only 30%. The combination of DMBA and splenectomy, however, resulted in a rapidly fatal disease with weight loss and severe anemia, resembling the effect of drug toxicity.

[5] We thank Dr. Lee M. Kelley for help with the statistical analysis and Dr. Alvin G. Fiscus for technical advice.

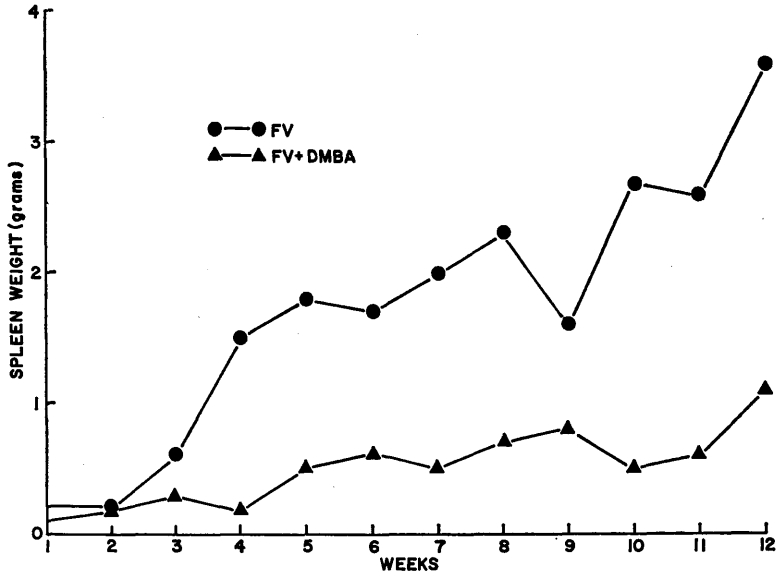

FIG. 2. Effect of DMBA on spleen weight of mice with Friend virus leukemia. Each point represents the average for a group of 10 mice. Spleen weight of normal and DMBA-treated mice: 0.1 g.

DMBA alone did not affect the erythrocyte volume, nucleated blood cell count, differential blood cell count, and the histologic appearance of spleen and liver in nonsplenectomized and splenectomized, noninfected mice.

Erythrocyte volume. In nonsplenectomized, infected animals, there was a progressive anemia during the first 8 weeks, but then a recovery until the normal level was reached at the end of Week 12 (Fig. 3). Treatment with DMBA did not alter the anemia during the first 6 weeks, but produced a somewhat greater recovery from then on than occurred spontaneously. In splenectomized, infected animals, the anemia was profound during the first 6 weeks, but became less so during the last 6 weeks. When splenectomized, infected mice were treated with DMBA, there was no recovery from the anemia, and all animals were dead by the end of week 8.

Nucleated blood cell count. The results were the same for nonsplenectomized and splenectomized, infected mice (Fig. 4). Treatment with DMBA kept the number of nucleated cells in the peripheral blood at a normal level, while a marked rise of these cells occurred throughout the course of the experiment without treatment. Analysis of variance with a factorial design showed the statistical significance of the values for erythrocyte volume and nucleated blood cell count.

Differential blood cell count. Since no general agreement about the nature of the various immature cells in the peripheral blood in Friend and Rauscher virus leukemias has been reached, the reported results of differential blood cell counts are by necessity subjective. Nevertheless, treatment with DMBA had a markedly antagonistic effect on the abnormal blood picture produced by the FV both in nonsplenectomized and splenectomized animals. Erythroblasts were absent, anisocytosis, poikilocytosis, and polychromasia were much less marked, and the number of white blood cells was markedly decreased.

Histologic examination. In the spleen of infected animals, DMBA delayed the first signs of neoplastic infiltration by approximately 1 week, from the end of week 1 to the end of week 2. Thereafter, the progress of infiltration was much slower than in untreated animals, and the architecture of the spleen remained intact much longer. De-

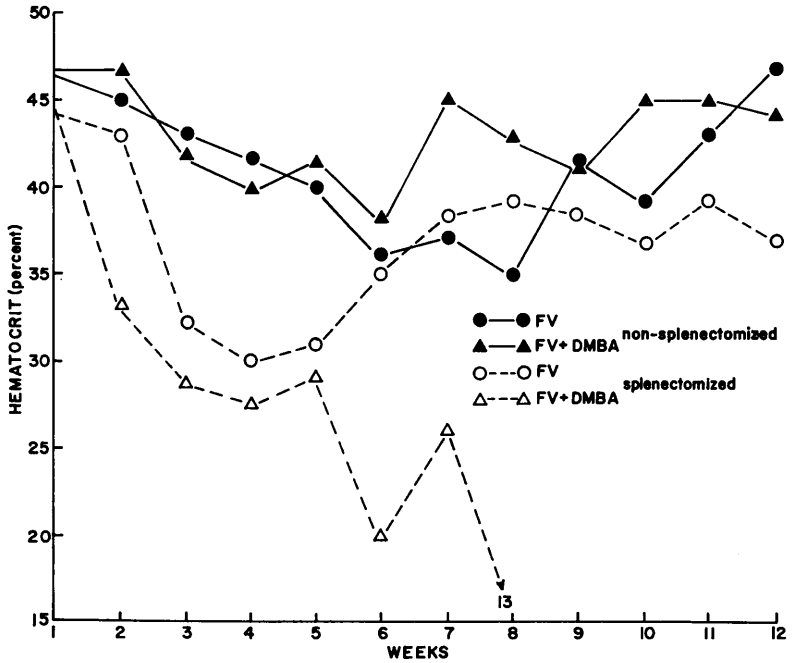

FIG. 3. Effect of DMBA on erythrocyte volume of nonsplenectomized and splenectomized mice with Friend virus leukemia. Each point represents the average for a group of 10 mice. Values for noninfected controls (in %): normal 44–52 ($\bar{x}$ = 50), DMBA-treated 39–50 ($\bar{x}$ = 47), splenectomized 48–52 ($\bar{x}$ = 49), splenectomized and DMBA-treated 46–49 ($\bar{x}$ = 47).

velopment of hemorrhagic necrosis was completely prevented. In the liver, DMBA delayed the first appearance of neoplastic infiltration by approximately 3 weeks, from the end of week 2 to the end of week 5. This infiltration remained mild and progressed only very slowly. In splenectomized animals, the effect was similar, but less pronounced.

Discussion. In the Friend and Rauscher virus-induced erythroleukemias, the neoplastic cell is an erythrocyte precursor, developing from a primitive reticulum cell to an erythroblastic cell (4–8). Whether the maturation goes so far that erythrocytes derived from these erythroblastic cells are actually released into the peripheral circulation has not yet been demonstrated conclusively, but some evidence for this exists (9). The Rauscher virus and most strains of the Friend virus, like that used in our experiments, induce anemia, whereas infection with the "Mirand strain" of the Friend virus results in polycythemia (10–12). Although the difference in the virus strain used makes a comparison of our results after splenectomy with those of previous reports (10–12) difficult, it seems that splenectomy has a depressive effect on the number of circulating erythrocytes in both strains, since it intensified the anemia in our experiments and prevented the onset of polycythemia in the experiments using the "Mirand strain."

The examination of the relationship between the neoplastic process in the spleen and the number of circulating erythrocytes is complicated by additional factors: (1) hemorrhagic necrosis in the spleen; (2) active erythropoiesis in the spleen; and (3) the role of the bone marrow in splenectomized mice. The results of the various combinations of splenectomy and treatment with DMBA in our experiments lead to the following conclusions.

1. Sequestration of erythrocytes in the spleen resulting from hemorrhagic necrosis does not seem to contribute to the anemia which was much more severe in splenecto-

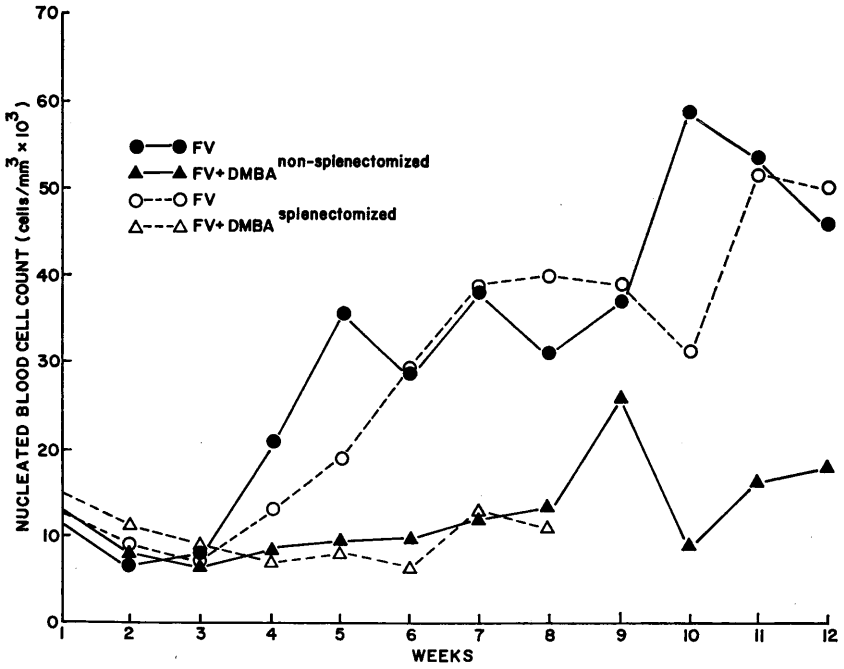

FIG. 4. Effect of DMBA on nucleated blood cell count of nonsplenectomized and splenectomized mice with Friend virus leukemia. Each point represents the average for a group of 10 mice. Values noninfected controls (in mm³): normal 5,000–10,500 ($\bar{x}$ = 7,600), DMBA-treated 6,300–11,200 ($\bar{x}$ = 8,300), splenectomized 7,500–22,000 ($\bar{x}$ = 13,800), splenectomized and DMBA-treated 11,000–19,800 ($\bar{x}$ = 14,600).

mized than in nonsplenectomized, infected, DMBA-treated and untreated animals. Although DMBA prevents hemorrhagic necrosis in the spleen of mice with Friend and Rauscher virus leukemia (1), recovery from the anemia during the second half of the experiment occurred in untreated as well as in DMBA-treated mice.

2. Since the spleen of the normal adult mouse has been described as an active erythropoietic organ (13), a portion of the circulating erythrocytes which is not produced in the bone marrow could be derived from normal splenic erythropoiesis, from neoplastic splenic erythroblasts, or from both sources. DMBA prevents splenic erythroblastosis in Friend and Rauscher virus leukemia (1), possibly by interfering with the maturation of the neoplastic primitive reticulum cells to erythroblastic cells. Since DMBA-treated, infected mice showed a more rapid increase in erythrocyte volume and an almost normal differential blood cell count as compared with untreated, infected mice, suppression of maturation of neoplastic reticulum cells and, implicitly, further maturation to erythrocytes cannot account for the anemia. Splenectomy aggravated the anemia in untreated infected mice. This has also been reported by others in Friend virus (14) and Rauscher virus (8, 15) leukemia and has been attributed to the absence of the compensatory increase in normal splenic erythropoiesis.

3. Our results can be explained best when spleen and bone marrow are correlated. The bone marrow must be able to compensate completely for the loss of normal splenic erythropoiesis, since splenectomy did not influence the blood picture of our control mice. The increase in total erythropoiesis in the spleen of mice infected with the "Mirand strain" of the Friend virus (10), with an anemia-producing strain of the Friend virus (14), and with the Rauscher virus (15) has been explained by increased normal splenic erythropoiesis. This assumption seems to be

supported by our results in DMBA-treated, infected mice. Although the erythrocyte volume was not influenced, the morphologic abnormalities of the erythrocytes were less than in untreated, infected mice, possibly due to an inhibitory effect of DMBA on the proliferation of the neoplastic reticulum cells which interfere with normal splenic erythropoiesis. Splenectomy aggravated the anemia, probably because of the loss of the compensatory splenic erythropoiesis. The recovery from the anemia during the second half of the experiment in splenectomized, infected, untreated and in nonsplenectomized, infected, untreated and DMBA-treated mice could be attributed to action of the bone marrow which compensates now for the spleen which is either absent or impaired in its normal erythropoiesis by the increasing neoplastic infiltration. Only when the bone marrow is severely damaged, does this recovery not take place, and the animals die from the profound anemia and not from the leukemia. This happened in our DMBA-treated, splenectomized, infected mice. Although DMBA in our experiments, in which it was applied in repeated small doses, had no effect on the blood picture in normal controls and an ameliorating effect on the anemia in nonsplenectomized, infected mice, it is a potential depressor of erythropoiesis when used in a single large dose (16, 17). It seems likely that in splenectomized animals the bone marrow collects that fraction of DMBA which is otherwise taken up by the spleen, and that this increased concentration combined with the effect of the Friend virus results in an irreversible depression of the erythropoiesis in this organ.

In our previous report (1), the prevention of splenic erythroblastosis in DMBA-treated, infected mice was attributed to the often reported depressive effect of this chemical on hematopoiesis and, therefore, thought to be interference with an "indirect neoplastic effect." This explanation seems now less likely, not only because DMBA did not have such an effect on normal control mice, but also because the splenic erythroblasts in the two viral erythroleukemias have to be considered now as descendants of virus-transformed neoplastic reticulum cells and, therefore, as quite different from the cells of normal splenic erythropoiesis. That DMBA interferes with the "direct neoplastic effect" of the virus is also supported by the histologically demonstrable delay in neoplastic infiltration and concomitant decrease in virus titer in spleen and liver. This might be due to interference with viral replication or with neoplastic transformation of reticulum cells. The effect of DMBA would then be 2-fold, delay in the neoplastic transformation of the reticulum cells and suppression of maturation of the transformed reticulum cells to erythroblastic cells.

The neoplastic infiltration of the liver in the two viral erythroleukemias has been considered as being metastatic from the spleen (4), or, in splenectomized animals, from the bone marrow (8). Results of carbon-clearance studies which show that the Kupffer cells are not transformed into neoplastic cells (18) support this view.

Summary. In Friend virus leukemia, 7,12-dimethylbenz[a]anthracene (DMBA) delays the neoplastic transformation of the reticulum cells in the spleen, as shown by histologic examination, and decrease in virus titer, and suppresses their maturation to erythroblastic cells as shown by histologic examination and absence of erythroblasts in the peripheral blood. DMBA also lowers the elevated white blood cell count. Splenectomy alone prolongs the survival time in Friend virus leukemia, but it produces a rapidly fatal profound anemia when combined with DMBA, probably because of damage to the bone marrow.

1. Fiscus, A. G., Schloss, G. T., and Wertman, K. F., Proc. Soc. Exp. Biol. Med. **125**, 1035 (1967).

2. Fieldsteel, A. H., Dawson, P. J., and Bostick, W. L., Proc. Soc. Exp. Biol. Med. **108**, 826 (1961).

3. Reed, L. J. and Muench, H., Amer. J. Hyg. **27**, 493 (1938).

4. Siegler, R. and Rich, M. A., Cancer Res. **24**, 1406 (1964).

5. Siegler, R. and Rich, M. A., Nat. Cancer Inst. Monogr. **22**, 525 (1966).

6. Friend, C., Patuleia, M. C., and de Harven, E., Nat. Cancer Inst. Monogr. **22**, 505 (1966).

7. Patuleia, M. C. and Friend, C., Cancer Res. **27**, 726 (1967).

8. Yokoro, K. and Thorell, B., Cancer Res. **26**, 536 (1966).

9. Reilly, C. A., Jr. and Schloss, G. T., to be published.

10. Mirand, E. A., Prentice, T. C., Hoffman, J. G., and Grace, J. T., Proc. Soc. Exp. Biol. Med. **106**, 423 (1961).

11. Mirand, E. A., Hoffman, J. G., Grace, J. T., and Trudel, P. J., Proc. Soc. Exp. Biol. Med. **107**, 824 (1961).

12. Mirand, E. A., Nat. Cancer Inst. Monogr. **22**, 483 (1966).

13. Brodsky, I., Dennis, L. H., Kahn, S. B., and Brady, L. W., Cancer Res. **26**, 198 (1966).

14. Brodsky, I., Dennis, L. H., and Kahn, S. B., Cancer Res. **26**, 1887 (1966).

15. Brodsky, I., Kahn, S. B., Ross, E. M., Petkov, B., and Braverman, S. D., J. Nat. Cancer Inst. **38**, 779 (1967).

16. King, M. A. R., Brit. J. Haematol. **11**, 411 (1965).

17. Cawein, M. J. and Sydnor, K. L., Cancer Res. **28**, 320 (1968).

18. Elliott, S. C. and Schloss, G. T., to be published.

Received Sept. 8, 1969. P.S.E.B.M., 1970, Vol. 133.