Synergistic Action of Leukemogenic Agents

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Ionizing radiations (4), carcinogenic hydrocarbons (18), and estrogenic hormones (8) are leukemogenic for mice of specific genetic constitutions (8). Synergistic and additive effects of x-rays and methylcholanthrene have been demonstrated (1, 18). A preliminary paper has been published on the synergistic action of x-rays and an estrogenic hormone (9).

In the present report genetic requirements for synergism are considered, and the synergistic action of estrogenic hormone and a carcinogenic hydrocarbon is demonstrated.

MATERIALS AND METHODS

BALB/c (Bagg albino), CBA, and DBA/2 mice, equally divided between the sexes (unless otherwise indicated), were used in these experiments. Test animals were progeny of untreated controls maintained as breeding stock by brother-sister matings.

Animals were 42 days of age, unless otherwise indicated, when the experimental procedure was instituted. General body irradiation was accomplished by exposing the mice in cardboard boxes at 50 cm. t.d., 140 kv., and 2 mm. aluminum filter, HVL = 4.2 mm. Al. Methylcholanthrene was painted on the skin as a 0.25 per cent solution in benzene, a different site being utilized for each successive (3 times weekly) skin painting. Estrogenic hormone (estradiol dipropionate) was administered subcutaneously, either dissolved in peanut oil, or as a cholesterol pellet.¹

Mice were observed until they developed lymphoid neoplastic disease, i.e., either mediastinal (thymic) lymphosarcoma or leukemia, at which time they were killed and autopsied. Animals dying from other causes were autopsied within 24 hours after death or when death seemed imminent.

Specific experimental procedures will be described in connection with the report on results of each experiment.

OBSERVATIONS

BALB/c.—Mediastinal (thymic) lymphosarcoma did not appear spontaneously in 240 BALB mice. Lymphatic leukemia appeared infrequently before mice were 400 days of age. Leukemia, which was usually characterized by a very large spleen with macroscopic areas of lymphoid infiltration, occurred after 400 days more often in females (22 per cent) than males (4 per cent), although in either sex the incidence was no more than 1 per cent prior to the age of 400 days.

Induction of thymic lymphosarcoma and leukemia by estrogenic hormone in BALB mice.— Estradiol dipropionate, in the form of a 1–2-mg. pellet, was administered by trocar at 18, 36, or 72 days of age. Twenty-seven of 84 mice developed lymphomas, almost all thymic lymphosarcomas, before 400 days of age. The ultimate incidence of lymphoid neoplastic disease was at least 56 per cent (47 of 84 mice). Only males received pellets; females developed pyometra and do not survive long enough to develop leukemia.

Induction of thymic lymphosarcoma and leukemia by x-rays in BALB mice.—Three whole-body doses of 200 r given at weekly intervals caused seven cases of thymic lymphosarcoma to appear before 400 days of age in 32 irradiated mice.

Relative ineffectiveness of methylcholanthrene as a leukemogen for BALB mice.—Of 108 mice receiving 18 skin paintings (Chart 2, b), no cases of leukemia or lymphosarcoma appeared before 400 days of age. Of 55 animals receiving 36 skin paintings, three cases of lymphomatous disease (one thymic lymphosarcoma) developed prior to 400 days of age. This strain of mice, as contrasted with DBA/2 (Chart 4, b) is relatively, but not absolutely, resistant to the leukemia-inciting action of this carcinogenic hydrocarbon. The over-all incidence of leukemia was actually reduced because of the development of skin cancers.

Synergistic effects in BALB mice. X-rays and estrogenic hormone.—With a single dose of 200 r

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whole-body, only two cases of leukemia appeared before 400 days of age in 97 mice (Chart 1, a), the ultimate incidence being 29 per cent. Only one case showed heavy thymic infiltration. This dose of x-rays is considered (at least before 400 days of age) only mildly leukemogenic.

When 5 µg. of estradiol dipropionate were given weekly in one subcutaneous injection for 14 successive weeks, beginning at 42 days of age, with no further treatment, three of 47 (Chart 1, b) developed lymphomas before 400 days of age. Although the ultimate incidence in this group was increased to 30 per cent (fourteen of 47), only three of the cases were thymic lymphosarcomas. Using the first 400 days of life as the period for calculating incidence, estradiol dipropionate was, in this dose, only very moderately leukemogenic.

When whole-body radiation with 200 r was followed immediately by the hormonal treatment, sixteen cases appeared in 71 mice before they were 400 days of age (Chart 1, c), and in fourteen the thymus was involved either exclusively or was very heavily infiltrated. When 400 r of x-rays was combined with the hormonal treatment (Chart 2, e), the synergistic effect was revealed more conclusively. By 300 days of age only one of 34 mice receiving radiation alone (Chart 2, d), and only one of the 47 receiving hormonal treatment (Chart 1, b) had developed leukemia, whereas in the group in which radiation was supplemented by hormone, twelve of 30 had developed lymphomas by 300 days of age (Chart 2, e), ten of these being thymic. The over-all incidence was 65 per cent, higher than that achieved by the administration of large doses of any single leukemogen.

If the 200 r of x-rays and the estrogen given in 5-µg. doses weekly had operated in only additive fashion, then in 71 animals no more than seven lymphomas should have appeared before 400 days of age. The actual number was sixteen.

Thymic radiation followed by estrogenic hormone (Chart 1, f).—When the entire body except the thymus was shielded during exposure to 200 r,* followed by 14 weeks of treatment with estrogen, six of 34 mice developed thymic lymphomas.

* Shielding effected by lead .025 mm. thick.
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sarcomas by 400 days of age. Ultimately, thymic tumors appeared in eleven, whereas in 47 receiving the hormone alone only three thymic tumors were found (Chart 1, b). One thymic tumor was seen in 97 mice which received whole-body exposure to 200 r.

Radiation of the whole body except the thymus, followed by estrogenic hormone (Chart 1, c).—When the thymus was shielded during radiation with 200 r, and the 14-week treatment with estrogen was given, thymic tumors appeared in six of 31 mice by 400 days, although the thymus itself was not radiated. Although the total incidence of lymphomatous disease was only 39 per cent, as compared to 80 per cent in the group receiving hormone only, the onset of the disease was accelerated, and more cases (nine of 31 compared with three of 47) showed very large thymus glands.

Effect of thymectomy on leukemogenesis by synergistic action of x-rays and estrogenic hormone (Chart 1, d).—In thymectomized mice (urethan-anesthetized for thymectomy), the simultaneous leukemogenic action of x-rays and estrogen was abolished (none out of 31). Intact urethan-anesthetized controls developed thymic lymphosarcomas and leukemia to the same degree (seven out of 29 before 400 days), as did unanesthetized animals similarly treated (sixteen out of 71).

Lack of synergistic effect of x-rays and methylcholanthrene in BALB mice.—Eighteen skin paintings of 0.25 per cent methylcholanthrene caused no leukemias or lymphosarcomas to appear before 400 days in 102 mice so treated (Chart 2, b). In contrast, six of 131 DBA mice which received eighteen skin paintings developed lymphomas by 800 days of age (Chart 4, c), while only three cases appeared in 268 controls of similar age (Chart 4, a). As indicated above, 200 r of x-rays is mildly leukemogenic in BALB mice (Chart 2, a). When radiation with 200 r was followed immediately by the eighteen skin paintings (Chart 2, c), the incidence of lymphomas by 400 days was no greater (three of 98) than in mice receiving 200 r only (two of 97), nor was the total incidence of lymphomas increased. In DBA mice, however, x-rays and methylcholanthrene operated synergistically (Chart 4, b, c, d).

Combined action of methylcholanthrene and estradiol dipropionate in BALB mice.—Eighteen skin paintings of methylcholanthrene induced no
lymphomas in 102 BALB mice, but with 36 skin paintings, by 400 days of age, three of 35 animals developed lymphomas, one of which was a thymic lymphosarcoma. When combined with estradiol dipropionate, 36 skin paintings induced five thymic lymphosarcomas in 26 mice by 400 days (Chart 2, f). This is about twice the expected incidence if the effect were only additive, but it is questionable whether true synergism resulted. By contrast, in the DBA strain when eighteen skin paintings (Chart 4, c, six of 131 by 300 days) were combined with 5 μg. weekly of EDP (Chart 4, e, one of 47), seventeen of 55 had developed lymphomas (Chart 4, f). In this case, if the effect had been merely additive the expected incidence would have been 3.5, whereas the actual incidence was seventeen of 55.

CBA mice.—As in the BALB strain, spontaneous leukemia appears more frequently in females (fifteen of 105) than males (six of 112). Only one thymic lymphosarcoma appeared spontaneously in the 217 controls. Only three of the lymphomas (leukemia or lymphosarcoma) appeared before 400 days of age.

Induction of leukemia with large doses of leukemogenic agents in CBA mice.—In general, the response of CBA mice to the leukemogenic agents paralleled that observed in BALB mice. When pellets of estrogenic hormone were implanted at 70 days, five thymic lymphosarcomas had appeared by 400 days of age in fifteen mice, whereas in only three of 217 controls lymphomas had developed by this age. X-rays in heavy doses were likewise leukemogenic (13). Methylcholanthrene failed as a leukemogen. None of 38 with 36 skin paintings, and only one of 113 receiving seventeen skin paintings developed leukemia in the first 400 days.

Synergistic effects in CBA mice.—X-rays and estradiol dipropionate acted synergistically in increasing the incidence of lymphomatous disease in the first 400 days of life (Chart 3, a, b, c). If the 200 r of x-rays and the 5-μg. weekly dose of estrogen had acted in additive fashion, the expected incidence would have been about six in 40 mice, whereas actually it was seventeen (Chart 3, c). From the standpoint of total incidence, the effect was not so striking, since the total incidences of lymphomas were 23 per cent for 800 r, 45 per cent for estradiol dipropionate, and 55 per cent for the combination. In the last group the majority of the cases were thymic lymphosarcoma, in contrast to the first two. When the disease appears early in life, the thymus is heavily infiltrated. When 400 r
was combined with the estrogen, fifteen of 27 mice had developed lymphomas by 400 days (Chart 3, d). Combining x-rays and methylcholanthrene (Chart 3, e) or methylcholanthrene and estrogen (Chart 3, f) did not accelerate the onset of lymphomas.

The thymus and partial-body radiation with reference to induction of lymphomas in CBA mice.—Of 28 CBA mice which were thymectomized prior to receiving 200 r plus estrogen, no cases of lymphocytic neoplastic disease appeared. Two cases of chlorotic granulocytic leukemia appeared between 400 and 600 days of age. If only the thymus was radiated with 200 r, followed by 5 μg. of EDP for 14 weeks, lymphomas were not induced in high frequency (one in 32 before 400 days). If the entire body except the thymus was radiated and similar estrogenic treatment given, two of 32 developed thymic lymphosarcomas by 400 days of age, which is approximately the same incidence as that obtained with the estrogen alone (Chart 3, b). Thus, it appears that in CBA mice partial-body radiation was not effective even when used together with estrogen in inducing lymphocytic neoplasia.

DBA mice.—In this strain the spontaneous disease occurs more frequently in females (31 out of 146) than males (seventeen out of 128), although the age distribution is similar for the two sexes. Whether lymphomas occur spontaneously or are induced, the age of appearance is earlier in DBA's than in the other two strains. Consequently, 300 days of age was used as the base-line age for determining synergistic effects in accelerating the onset of lymphomas. Other respects in which the DBA's differ from the other two strains are the following:

1. In DBA mice, methylcholanthrene is leukemogenic (Chart 4, a, c, h).

2. Estradiol dipropionate decidedly potentiated the leukemogenic action of methylcholanthrene (Chart 4, c, e, f). With the dose of estradiol dipropionate used, no independent leukemogenic effect was obtained (Chart 4, e), whereas the addition of this dose to eighteen skin paintings of methylcholanthrene increased the incidence of lymphomas in the first 300 days from 4 1/2 per cent (six out of 181) to 31 per cent (seventeen out of 55). The over-all incidence was increased from 20 to 46 per cent.

3. The accelerating effect of x-rays and methylcholanthrene was more than additive (Chart 4,
b, c, d), with seventeen cases appearing in 98 mice by 300 days, whereas the calculated incidence by addition of independent accelerating action is twelve. The over-all incidence exceeded that calculated by adding the incidences expected in 98 mice by the independent action of methylcholanthrene and x-rays (47–41).

As in the CBA's and BALB mice, estrogenic hormone potentiated the leukemogenic action of x-rays (Chart 4, b, e, g).

DISCUSSION

By their combined action agents may be more strongly leukemogenic than when used independently. This is especially true for the early induction of lymphomatous disease. If the incidence for the first 300–400 days of life is considered, then the combined action in accelerating onset is in many cases synergistic, i.e., the incidence exceeds the calculated additive expectancy.

The primary site of action for this accelerated development of lymphomas in young mice is the thymus. When this organ was removed prior to leukemogenic treatment, leukemia was not induced. In young mice only the thymus may be involved in the lymphomatous process, or the disease may be systemic with the thymus heavily infiltrated, suggesting that the initial involvement was in this organ. When leukemia appears in CBA or BALB mice beyond 500 days of age, even when the incidence is increased due to the leukemogenic activity of administered agents, the thymus is only very infrequently involved. Whether the thymus has served as the primary source of leukemic cells even in these older mice, without its being appreciably enlarged, is not known. If BALB or CBA mice receive leukemogenic treatment after thymic involution has taken place, then susceptibility is negligible.4 The effect upon susceptibility in older mice of delaying thymic involution by gonadectomy is now being tested.

The importance of the thymus in mouse leukemogenesis (10, 11) is reaffirmed by these studies. It has been demonstrated that susceptibility to spontaneous (10) and carcinogen- (11) and x-ray-induced (5) leukemia is diminished by thymectomy. Estrogen-induced leukemia is characterized by a very large thymus (9), and thymectomy reduces the incidence of leukemia in mice subjected to the combined action of x-rays and estrogenic hormone, suggesting that the susceptible locus for the estrogenic-induced disease is the thymus. Preliminary experiments indicate that if the BALB thymus is grafted into thymectomized F1 hybrids between the BALB and Strong A strains, the grafted thymus may become lymphomatous if the F1 host is subjected to the combined action of x-rays and estrogenic hormone.

“Co-leukemogens” analogous to co-carcinogens have not been demonstrated. A co-leukemogen would be an agent which augments the action of a leukemogen, but is not in itself leukemia-inciting. For the DBA strain, estrogenic hormone may be a co-leukemogen, since in these mice there is no evidence that estrogenic hormone is leukemogenic. Large doses are not tolerated, but in doses that are at least mildly leukemogenic for the BALB and CBA strains, estrogenic hormone does not induce leukemia in DBA's. Its potentiating effect on methylcholanthrene leukemogenesis is very decisive (Chart 4, f), and there is a significant accelerating influence on x-radiation leukemogenesis. Since androgenic hormone opposes the leukemogenic action of x-rays (2, 7) and of estrogen (9), its effect on methylcholanthrene leukemogenesis is now being tested.

If any area of the body of C57BL mice was shielded during exposure to leukemogenic doses of x-rays, then leukemia was only infrequently induced, suggesting that nonirradiated tissue may in some way inactivate a humoral leukemogen emanating from the irradiated portion of the body (6). Shielding of a portion of the body did not in BALB mice inhibit the acceleration of leukemogenic action resulting from the combined effects of x-rays and estrogen. This suggests that the estrogen may have prevented the inactivation and indeed enhanced the potency of the theoretical humoral agent.

Since the thymus was the primary locus of origin of leukemia even in BALB mice in which the thymus had been shielded during irradiation, the evidence points again toward a humoral factor which can be potentiated by estrogen, and which may actually implement neoplastic alteration by secondary effects in a thymus not radiated by x-rays (Chart 1, e).4 This phenomenon did not occur in CBA mice where shielding of a part of the body apparently prevented the x-rays from operating in conjunction with exogenous estrogen to induce leukemia. Thymic radiation of estrogen-treated CBA mice did not induce thymic lymphosarcoma as in BALB mice.

Leukemogens may be defined only in terms of the biological material within which they may operate effectively to induce leukemia. Methyl-

4 A. Kirschbaum, unpublished data.
cholanthrene induces leukemia in DBA but not CBA mice. On the other hand, ionizing radiations are probably universally leukemogenic for mice, but with varying strain susceptibilities.

Genetic factors determine the reaction to the combined effects of leukemogenic agents. In DBA mice methylcholanthrene augments the leukemogenic potency of x-rays and acts synergistically with estrogenic hormone, whereas in CBA mice this carcinogenic hydrocarbon exerts neither effect.

SUMMARY
1. Although susceptible to the leukemogenic action of x-rays or estrogenic hormone, BALB and CBA mice were relatively refractory to methylcholanthrene. X-rays and estrogenic hormone acted synergistically in accelerating the onset of induced leukemia. Methylcholanthrene did not act synergistically with either x-rays or estrogenic hormone in these strains.
2. In DBA mice lymphosarcoma and leukemia were induced by either methylcholanthrene or x-rays. Estrogenic hormone enhanced the leukemia-inciting potency of either the carcinogenic hydrocarbon or the ionizing radiations, although independent leukemogenic activity of estrogenic hormone in the dose used was not demonstrable in DBA's.
3. Genetic factors determine susceptibility to specific leukemogens acting either independently or in conjunction with others.
4. "Synergism," as used here, denotes increasing, by the simultaneous administration of leukemogens in relatively low doses, the incidence of lymphomas in the first 300–400 days of life beyond the sum of the incidences obtained by the independent action of the leukemogens.
5. The thymus was the most important locus for the synergistic effects of leukemogens (x-rays, methylcholanthrene, estrogen) in accelerating the onset of induced leukemia.
6. In BALB mice radiation restricted to the thymus accelerated the onset of thymic lymphosarcoma when given in conjunction with estrogen, whereas in CBA's thymic radiation was ineffective under the same conditions.
7. Thymic lymphosarcomas developed pre-cociously in BALB mice after the whole body, except the thymus, was subjected to radiation and to relatively small doses (from the standpoint of leukemogenesis) of estrogenic hormone. Since thymectomy abolishes the leukemogenic activity of these agents in BALB mice, it is probable that the radiation effects on the thymus were secondary, or humoral, and were elicited by the action of estrogenic hormone.

REFERENCES
7. ———. Inhibition by Testosterone of Radiation-induced Lymphoid Tumor Development in Intact and Castrate Male Mice. Ibid., p. 292.