Therapeutic and Immunological Response of Mice with Meningeal Leukemia (L1210) to Challenge with an Antifolic-resistant Variant

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SUMMARY

Augmentation of the therapeutic effectiveness of 3'-bromo-5'-chloroamethopterin (BCM) against the BCM-resistant variant of leukemia L1210 was demonstrated in mice given preinoculations intracerebrally of the sensitive parental line of leukemia L1210. Substantial increases in survival time were obtained, and in some cases these increases were almost as extensive as those observed when mice with sensitive leukemia L1210 alone were treated. The results indicated that the defense mechanism of the host in response to the preinoculation of L1210 contributed to the therapeutic efficacy of the drug against the resistant variant.

Mice immunized with x-irradiated leukemia L1210 were capable of suppressing both subcutaneous and intracerebral inoculations of L1210. Substantial increases in survival time occurred, and several tumor-free survivors were obtained. Pathological study of meningeal leukemia showed that the leukemic deposits tended to be more localized in the immunized animals than in the nonimmunized animals. Leukemic cell infiltration into the retro-orbital tissues produced proptosis of the eyes in a few of the immunized mice.

When immunity was induced by the preinoculation of x-irradiated leukemic cells there was a considerable enhancement of the effectiveness of chemotherapy. The combination of immunization and chemotherapy produced more extensive increases in survival time and a higher percentage of apparently tumor-free survivors than did either chemotherapy alone or immunization alone.

Previous studies (5, 8) had shown that treatment with halogenated derivatives of amethopterin produced a high percentage of apparently tumor-free survivors among CDBA hybrid mice with subcutaneously inoculated advanced L1210 leukemia and that, when these survivors were given reinoculations of L1210, they were generally immune to the disease. Moreover, such survivors of systemic leukemia displayed cross-immunity to antifolic-resistant variants of L1210 (6). The degree of immunity exhibited by the successfully treated mice was directly related to the extent of leukemic infiltration when treatment was initiated (5, 7).

In contrast to the gratifying results of antifolic therapy in mice with subcutaneously inoculated advanced L1210, similar treatment did not produce tumor-free survivors among mice which had been given the tumor intracerebrally, although moderate to extensive increases in lifespan were obtained (2). When L1210 was inoculated intracerebrally, the leukemic cells infiltrated rapidly throughout the subarachnoid space and dura of the brain and into the spinal cord. The disease became systemic within 2–3 days after intracerebral (I.C.) inoculation. Cyclophosphamide (Cytoxan), an agent which is approximately as effective as amethopterin against subcutaneously inoculated L1210, was also considerably less effective in mice with intracerebrally inoculated tumor (1).

The failure of effective anti-leukemic agents to cross the “blood-brain” barrier in quantities that are sufficient to destroy leukemic cells was considered an important factor contributing to the relative difficulty encountered in the treatment of mice with intracerebrally inoculated disease. When intracerebrally inoculated mice were given cyclophosphamide subcutaneously, the moderate increase in survival was accompanied by the destruction of leukemic cells in the liver, spleen, bone marrow, and dura, whereas the leukemic cells in the subarachnoid space continued to proliferate (13). Thus, the therapeutic effect, as manifested by an increase in lifespan, appeared to be attributable to drug activity against the systemic disease and in the dura.

The current experiments were conducted to determine

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Received for publication August 28, 1963.
the extent to which mice given intracerebral inoculations of leukemia L1210 and treated with 3'-bromo-5'-chloroamethopterin (BCM) may display resistance to a subsequent I.C. or S.C. challenge with a BCM-resistant variant of L1210. Further, in view of the recent demonstration (4) that F1 hybrid mice can be immunized against subcutaneously inoculated L1210 by pretreatment with x-irradiated leukemic cells, an attempt was made to ascertain the role that such induced immunity might play in augmenting chemotherapy in the treatment of intracerebrally inoculated leukemia L1210.

MATERIALS AND METHODS

The general methods have been described previously (2, 6, 8). The experiments were conducted in CDBA1 male mice, 3–4 months of age, weighing 23–30 gm. The tumors employed were the sensitive line of the DBA/2 leukemia L1210 (10) carried as a stock tumor in DBA/2 male mice and the antifolic-resistant subline L1210-FR3 (3) carried as a stock tumor in CDBA male mice. Leukemic whole blood was employed for tumor inoculation. Methods employed for inoculation of the leukemia have been described previously (1,2). BCM2 was dissolved in 2 per cent sodium bicarbonate and administered at 0.01 ml/gm body weight. Treatment was administered subcutaneously in the scapular region on a daily schedule. The duration of treatment is indicated with the individual experiments.

Leukemic splenic tissue employed for immunization studies was obtained from DBA/2 male mice with systemic leukemia L1210. The mice were sacrificed 7 days post-inoculation, and a 20 per cent suspension of leukemic splenic tissue was prepared in physiological saline by passing the tissue several times through a 20-gauge needle. The average packed cell volume was 4 per cent when measured in a Wintrobe hematocrit. The leukemic tissue preparation was immediately exposed to 5000 r of x-ray2 and injected into mice within 1–2 hours. Four-month-old mice received twelve intraperitoneal (I.P.) inoculations (0.2 ml/mouse) of irradiated leukemic splenic tissue spaced 3–4 days apart. These mice were challenged subcutaneously or intracerebrally 3 days after the last injection of irradiated tissue.

The preparation of tissues for histopathological examination has been described previously (13).

RESULTS

Chart 1, A and B, shows that BCM therapy, initiated in mice with intracerebrally inoculated L1210 when the disease had become systemic augmented the therapeutic efficacy of BCM against a subsequent I.C. or S.C. inoculation of the leukemic variant, FR-3. In the experiment summarized in Chart 1, A, untreated mice given inoculations intracerebrally with equivalent inocula (1/100 dilution of leukemic blood) of L1210, or the variant FR-3, displayed a median survival time (M.S.T.) of 49.5 days (Group 2) but produced a 1-day increase in M.S.T. in mice with the variant FR-3 (compare Groups 6 and 9).

The mice in Groups 3 and 4 (Chart 1, A) were given I.C. inoculations of the parental tumor, L1210, and BCM therapy was begun 7 days later. The resistant variant, FR-3, was implanted intracerebrally into these mice 7 days after the initiation of treatment (14 days following L1210 inoculation). Treatment for Groups 5

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1 (Balb/cAn female × DBA/2 male)F1 mice.
2 3'-Bromo-5'-chloroamethopterin (BCM) was kindly provided by the Research Division, American Cyanamid Company, Pearl River, New York.
3 Irradiation was administered with two x-ray tubes placed opposite to each other, operated at 200 kvP and 15 ma. Filtration was 0.25 mm. Cu and 0.55 mm. Al. The distance from each focal spot to the center of the cell suspension was 25 cm., and the dose rate was 520 r/min.
and 6, which were given inoculations of FR-3 only, was begun 7 days prior to inoculation. Treated mice given inoculations of L1210 with the more concentrated FR-3 inoculum (Group 3) exhibited an M.S.T. of 17 days compared with 12 days for treated mice with FR-3 only (Group 5). Treated mice with L1210 and the less concentrated FR-3 inoculum (Group 4) displayed an M.S.T. of 21.5 days compared with 14 days for treated mice with FR-3 only (Group 6). Thus, the response to therapy of mice with both the sensitive and resistant tumors was greater than the response of mice with the resistant tumor only, but less than that of mice with the sensitive tumor only.

Similar results were observed when the parental tumor, L1210, was inoculated intracerebrally and the resistant tumor was given subcutaneously (Chart 1, B). Again, mice with only the FR-3 variant were relatively refractory to BCM therapy. However, treated mice which had received both tumors displayed increases in M.S.T. which approached those observed in treated mice with sensitive L1210 only. Moreover, a number of treated mice which had received both tumors succumbed with no evidence of local tumor at the site of S.C. FR-3 implantation.

In the experiments summarized in Chart 1, A and B, respectively, none of the mice which had received FR-3 at inoculum levels of 1/10-1/1000 survived. Approximately 84 per cent of untreated mice given inoculations of a 1/5000 dilution of leukemic FR-3 blood succumbed to tumor.

Further evidence in support of the hypothesis that a host defense mechanism is capable of suppressing the growth of a subcutaneous or intracerebral tumor challenge is shown by the two experiments summarized in Charts 2 and 3, respectively. In both experiments mice were given injections of x-radiated leukemic (L1210) splenic tissue. In the experiment summarized in Chart 2 the mice were subsequently challenged subcutaneously with L1210. The mice which had received the x-radiated leukemic cells were relatively refractory to the subsequent S.C. L1210 tumor challenge (Chart 2, Groups 5-8). Three of the eight mice which received the x-radiated tumor cells and the 1/10 dilution of leukemic blood (Group 6) succumbed with no evidence of local tumor at the site of inoculation, which observation suggested a host mechanism capable of suppressing local tumor growth without preventing the eventual development of systemic disease. The ability of such induced immunity to suppress both the local tumor growth and the systemic disease may be seen by comparing Groups 3 and 7. Of the eight mice in Group 7, three succumbed (two with local tumor and one without local tumor), and five survivors were apparently tumor-free on day 60. The survival of the mice in Group 9 shows that the x-radiated tissue per se was not capable of producing tumors.

Although pretreatment with x-radiated leukemic tissue conferred less resistance to an I.C. tumor challenge (Chart 3) than to the S.C. tumor challenge (Chart 2), substantial increases in the survival time of the pretreated intracerebrally inoculated mice were observed. The most pronounced effect of the pretreatment was observed in mice that were inoculated intracerebrally with a 1/1000 dilution of leukemic (L1210) blood (Chart 3, compare Groups 4 and 8). Six of the ten pretreated mice given this level of I.C. L1210 (Group 8) survived for more than 60 days and were apparently free of the disease. In contrast, all mice which were not pretreated succumbed to the 1/1000 I.C. tumor challenge (Group 4).

Some of the nonimmunized and immunized mice which received the 1/100 tumor inoculum intracerebrally (Chart 3, Groups 3 and 7) were sacrificed for pathological study at intervals following I.C. inoculation. The nonimmunized mice were killed on days 3, 7, 9, and 11, and

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immunized mice were killed on days 3, 7, 9, 11, 14, 18, 21, and 25 following I.C. inoculation. Histological examination of the brain of immunized mice revealed that I.C. inoculation of leukemic blood failed to produce leukemia in a number of the animals. In others leukemia developed after the I.C. inoculation, but proliferation of the leukemic cells was markedly retarded, and the cells tended to be localized. In contrast, there was a diffuse spread of the leukemia throughout the meninges in non-immunized animals. Infiltration of leukemic cells around the large sinuses adjacent to the olfactory bulbs was seen in several of the immunized animals, and when this infiltration became massive and spread to the retro-orbital tissues (Fig. 1), it produced enlargement and anterior displacement of the eye. This proptosis has been referred to as an “eye tumor,” and mice that displayed it are indicated in Chart 3, groups 5 and 7. In the nonimmunized animals diffuse involvement of the dura and arachnoid occurred, and leukemic cells spread by the blood stream to systemic organs and tissues. Spread of leukemic cells to systemic organs and tissues occurred only rarely in immunized mice.

Chart 4 illustrates the results obtained when immunized mice were challenged intracerebrally with leukemia L1210 and treated with BCM. Drug therapy of nonimmunized mice increased the M.S.T. from 10 days (controls, Group 1) to 38 days (Group 2). Immunization produced some degree of refractoriness in the mice to an I.C. tumor challenge, as demonstrated by the extension in survival time exhibited by Group 3. When immunization and therapy were combined (Group 4) a superior therapeutic response was obtained. Two out of nine mice succumbed after an extended period, and seven mice survived for greater than 60 days. These leukemia-free survivors represent a rare example in which I.C. leukemia was apparently eradicated. In this experiment immunization alone was also very effective against a S.C. tumor challenge (Chart 4, Groups 5 and 6).

**DISCUSSION**

The current study shows that the response to therapy of an antifolic-resistant variant of L1210 was increased when the variant tumor was inoculated into BCM-treated animals bearing the antifolic-sensitive parental subline of L1210. This augmentation of therapeutic efficacy against the resistant tumor was observed when the sensitive L1210 was inoculated intracerebrally and the resistant leukemia was inoculated intracerebrally or subcutaneously. Similar findings have been reported (5–7) in a system in which both the sensitive leukemia and the drug-resistant subline were inoculated subcutaneously. The increased therapeutic effect obtained in the above systems appears to be attributable to the combined effects of therapy and the host's immune response to the sensitive L1210 cells. When both the sensitive and the resistant leukemia were inoculated concomitantly and treatment was initiated on the same day or 6 days prior to tumor inoculation, there was no augmentation of effect against the resistant variant (7). Studies on the nature of the antigenicity responsible for this immune response have been presented in detail elsewhere (4, 11).

The present study demonstrates that immunity induced by the injection of x-radiated tumor cells extends to...
intracerebrally inoculated L1210. This leukemia failed to develop in a number of the immunized mice that were given inoculations intracerebrally of a 1/1000 dilution of leukemic blood, whereas the same inoculum level resulted in a progressive growth of the leukemia and death in 100 per cent of the nonimmunized mice. The I.C. implantation of a more concentrated leukemic cell inoculum (1/100) into immunized mice did result in meningeal leukemia, and pathological examination showed that the leukemic deposits tended to be more localized than in nonimmunized animals. Occasionally, immunized mice exhibited proptosis of the eyes as a result of leukemic cell infiltration in the retro-orbital tissues. The ultimate cause of death of immunized mice challenged with the 1/100 inoculum level (Chart 3, Group 7) is, at present, not clear. Histological examination of the animals which were sacrificed showed no evidence of leukemia in the brain, meninges, or systemic organs in 50 per cent of the cases, even though all the animals died.

Of particular interest in the current study was the degree to which intracerebrally inoculated leukemia was controlled by the combined effects of chemotherapy and host immunity (Chart 4). In the experience of this and other laboratories (1, 12) intracerebrally inoculated L1210 has been more refractory to therapy than subcutaneously inoculated L1210. For example, indefinite survivors have been obtained employing dihalogenated derivatives of amethopterin in the treatment of advanced subcutaneously implanted L1210. In contrast, such treatment has not produced indefinite survivors among mice with intracerebrally inoculated disease.

The possibility still remains open that factors other than immunity may have contributed to the augmented response with BCM. However, the immunity obtained with x-radiated leukemia L1210 would indicate that immunity was the prime contributor to therapy. Also such nonspecific factors as food restriction did not result in appreciable increases in survival time with leukemia L1210 (9).

Although not in a completely isogenic system, the observation that immunity can contribute to the chemotherapeutic management of intracerebrally inoculated leukemia lends impetus to the study of the role of host defense mechanisms in the treatment of meningeal leukemia. With the advent of more effective chemotherapeutic agents for the treatment of meningeal leukemia, the role of immunity in the autochthonous host could be an important factor.

REFERENCES
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