Proceedings of the National Academy of Sciences
Vol. 66, No. 4, pp. 1089–1095, August 1970

Immunological Alteration of Leukemic Cells *In Vivo* after Treatment with an Antitumor Drug*

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Communicated by Marshall Nirenberg, April 23, 1970

Abstract. L1210 leukemia was transplanted serially in CDF_1 mice treated with 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DIC, NSC 45388). After four different lines (C lines) had been treated for several generations, a marked increase in survival time of untreated mice was observed. In contrast, mice treated with DIC or immunosuppressed with cyclophosphamide succumbed earlier with generalized leukemia. Furthermore, a C line showed unusually high sensitivity to chemotherapeutic treatment with 1,3 bis(2-chloroethyl)-1-nitrosourea. The data suggest that C lines acquired strong antigenicity for CDF_1 and DBA/2 hosts. DIC treatment may have selected highly antigenic variants or induced somatic mutations resulting in the appearance of strong new transplantation antigen(s).

Introduction. The influence exerted by cytotoxic drugs on cell antigens has been studied *in vitro*[1] and *in vivo*.[2] Urethan[1] as well as thalidomide[3] seemed to produce antigenic simplification of normal tissue transplantation antigens *in vitro*. On the other hand, specific changes of tumor transplantation antigens were observed after treatment with 5-fluorouracil *in vivo*.[2]

These observations suggested the possibility that chemoresistant tumor lines[4] obtained after long-term treatment with antineoplastic agents might show altered antigenic properties as compared with the original sensitive line. Preliminary experiments with long-transplanted L1210 leukemic lines resistant to 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DIC, NSC 45388) and also to methotrexate indicated that both were antigenically different from the original sensitive leukemia. Thus, a number of L1210 lines resistant to these drugs has been developed *de novo* in order to characterize them with respect to tumor transplantation immunity.

The present paper describes the growth properties of four distinct DIC-treated lines under various experimental conditions *in vivo*. The data suggest that leukemic cells acquired rather strong transplantation antigens, foreign to the original host, after a few transplant generations in DIC-treated mice.

Materials and Methods. Animals: Mice of both sexes 2–4 months old of DBA/2 Cr and C57BL/10.A(5R) (abbrev. B10.A(5R)) strain, and hybrid mice CDF_1 (DBA/2 Cr male × BALB/c female) and BDF_1 (DBA/2 Cr male × C57BL/6 female) were used.

Tumors: *L1210-S*, L1210 leukemia ascites; sensitive line maintained in DBA/2 Cr male or in CDF_1 male mice.

C lines, DIC-treated L1210 ascites lines developed in CDF_1 mice; treated intraperitoneally with DIC daily in each generation from day 1 through day 10 after tumor transplantation. C_1, 10^5 leukemic cells implanted intraperitoneally (i.p.) in CDF_1 male mice; DIC 50 mg/kg. C_2, 10^6 leukemic cells i.p. in CDF_1 male mice; DIC 100 mg/kg. C_3, 10^6 leukemic cells i.p. in CDF_1 female mice (first transplant generation) and successively in CDF_1 male mice, DIC 100 mg/kg. C_4, 10^6 leukemic cells i.p., first generation from a methotrexate-resistant line derived from L1210 leukemia; DIC 100 mg/kg, no methotrexate treatment.

In each transplant generation, ascites tumor cells collected from DIC-treated mice were implanted intraperitoneally into two groups of mice: untreated or treated with DIC. When indicated, a third group of mice was immunosuppressed with cyclophosphamide (180–220 mg/kg)[5] given 24 hr before inoculation of the leukemic cells. This group was not treated with DIC. Mortality was recorded for at least 60 days after implantation. An autopsy was performed for all mice that died.

Drugs: 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DIC, NSC 45388) was suspended in a chilled solution of carboxy-methyl cellulose and protected from light. 1,3 Bis(2-chloroethyl)-1-nitrosourea (BCNU, NSC 409962) and cyclophosphamide (CY, NSC 26271) were dissolved in saline. Drug solutions or suspension were prepared immediately before use.

Plaque-forming cells test: The method described by Jerne and Nordin[6] was followed, with some minor modifications adopted by Shearer *et al.*[7]

Results. The influence of treatment with DIC over a series of generations for mice carrying the C lines is shown in Figure 1. In the first treatment generation (generation 0) a moderate antileukemic effect of DIC treatment as reflected in increased median survival times over controls was observed in mice carrying the L1210 sensitive cells.[8] After several generations had been treated with DIC, a marked increase in survival time of untreated control mice was observed and a number of animals survived for more than 60 days. In contrast, DIC-treated mice and mice immunosuppressed with cyclophosphamide succumbed earlier with generalized leukemia. From the 8th generation onward, for the C_1 line, an inoculum of 10^5 leukemic cells failed to grow in any of the untreated CDF_1 hosts. Additional experiments with C_1 and C_2 lines have shown that the differential growth properties in untreated as contrasted with immunosuppressed mice were not lost after five passages in immunosuppressed animals not subjected to DIC treatment.

Tumor titration of the C_1 line at the 12th transplant generation (Table 1)

TABLE 1. *Survival of CDF_1 and B10.A(5R) male mice transplanted with graded doses of leukemic cells.*

Inoculum size	CDF_1 Mice				B10.A(5R) mice*	
	L1210-S Line		C_1 Line		L1210-S line	
	MST	D/T	MST	D/T	MST	D/T
10^8	6 (6–7)	8/8	5 (3–5)	8/9	7 (7–9)	7/8
10^7	7 (6–7)	8/8	... (8)	1/6	... (7–8)	3/7
10^6	8 (7–8)	8/8	... (24)	1/8	...	0/8

* = Host-tumor differing at multiple histocompatibility loci, including *H-2*.
MST = Median survival time. Range in parentheses.
D/T = Dead mice over total.
L1210-S = L1210 original line at 12th transplant generation in CDF_1 mice.
C_1 = C_1 line at 12th transplant generation.

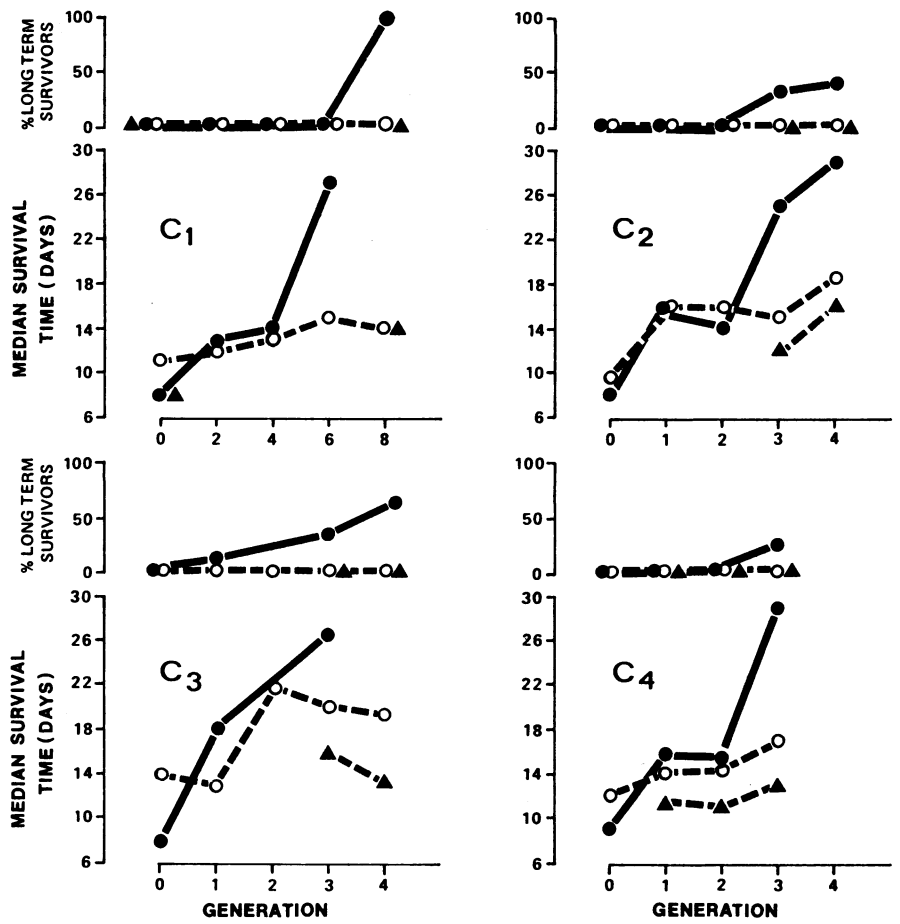

FIG. 1.—Median survival time and percentage of long-term survivors (>60 days) of CDF_1 male mice serially transplanted with C lines, untreated or treated with DIC, or immunosuppressed with cyclohosphamide before tumor transplantation. Six–eight animals per point. ●, untreated; ○, treated with DIC; ▲, pretreated with cyclophosphamide (180–220 mg/kg).

showed that an inoculum of 10^8 tumor cells still resulted in mortality similar to that observed in mice challenged with the same concentration of the L1210-S cells. However, the majority of the animals were resistant to growth of inocula of 10^6 or 10^7 C_1 cells. In this experiment essentially the same degree of resistance to tumor growth was also observed when L1210-S leukemia was transplanted into allogeneic B10.A (5R) mice, differing at multiple histocompatibility loci, including *H-2*.

Host resistance to a challenge of 10^5 or 10^3 C_1 cells (at the 8th transplant generation) was also found in BDF_1 or DBA/2 male and female mice. Immunosuppression with cyclophosphamide again abrogated the host resistance and the mice died with generalized leukemia.

These observations suggested that leukemic growth and accelerated death in

mice inoculated with the C lines and treated with DIC were not related to any direct DIC-dependence of the C lines but were rather a consequence of the immunosuppressant activity of the drug on the host.[9]

Additional studies were conducted to characterize the immunosuppressant properties of DIC. As reported in Table 2, the compound reduced the primary

TABLE 2. *Reduction of plaque-forming cells per spleen in CDF_1 male mice immunized with 4×10^8 sheep red blood cells intraperitoneally on day 0 and treated with DIC from day +1 through day +5 (four animals per group).*

	Days after Sheep Red Blood Cell Injection		
DIC mg/kg intraperitoneally	Day 2 Plaque forming cells/spleen $\times 10^3$	Day 4 Plaque forming cells/spleen $\times 10^3$	Day 6 Plaque forming cells/spleen $\times 10^3$
0 (controls)	3.6 (3.5–3.8)*	250 (223–280)	39.6 (33–47.5)
25	3.3 (2.7–4.0)	156 (121–201)	22.9 (20–26)
50	3.1 (2.3–4.2)	106 (82–137)	19 (16.1–22.4)
100	1.8 (1.4–2.5)	50 (35–70)	8.7 (6.5–11.7)

* Geometric mean. In parentheses the values of the mean minus or plus one standard error calculated after logarithmic transformation of the data.

humoral antibody response to sheep erythrocytes. The drug also reduced transplantation immunity, as evidenced by experiments conducted with L1210 leukemia transplanted into allogeneic C57BL/10.A, C57BL/10.A(2R), and C57BL/10.A(5R) mice. While no tumor growth occurred in untreated allogeneic recipients, high incidence of mortality with generalized leukemia was observed in the mice treated with DIC (100 mg/kg i.p. daily from day 1 through day 10 after tumor transplantation).

The possibility of bacterial or viral contamination of the C lines, which could account for reduced growth rate, has been considered. All tests for bacterial contamination were negative. The survey for the presence of various mouse viruses[10] in the C_1 line also gave negative results. Moreover, 10^5 intact or 10^7 frozen and thawed cells of the C_1 line (10th generation) added *in vitro* to 10^5 or 10^3 L1210-S cells, failed to retard the growth or reduce the incidence of takes in CDF_1 mice. A second transplant generation of tumor cells collected from mice inoculated with the mixture of sensitive and C_1 cells again gave regular growth patterns, as the sensitive line, in CDF_1 mice. Thus, it would appear that no ordinarily transmissible biological agent capable of changing the growth properties of L1210-S cells is present in the C_1 line.

A chemotherapy experiment was performed in BDF_1 mice inoculated with L1210-S or with the C_1 line and treated with a single dose of BCNU. The mice were treated with cyclophosphamide 1 day before tumor inoculation in order to allow growth of the C_1 line. It was considered that cyclophosphamide administration would not complicate any observation of therapeutic effect with BCNU since its half life is so short.[5] The results of this experiment are reported in Table 3 and show that therapy with BCNU was considerably more effective in increasing the life span of mice inoculated with the C_1 line than of animals bearing the original L1210-S leukemia.

TABLE 3. *Antileukemic activity of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) against L1210-S and C_1 lines in BDF_1 male mice partially immunosuppressed with cyclophosphamide.*

Inoculum size Day 0	Treatment: Day −1 cyclophosphamide mg/kg intraperitoneally	Treatment: Day +6 BCNU mg/kg subcutaneously	L1210-S Line* MST	L1210-S Line* D/T	C_1 Line† MST	C_1 Line† D/T
10^5	180	0 (controls)	10 (9–10)	8/8	12 (10–13)	8/8
"	"	10.8	15 (9–19)	8/8	... (18–51)	2/8
"	"	18	19 (10–20)	8/8	...	0/8
"	"	30	21.5 (9–27)	8/8	... (29)	1/8

* 14th transplant generation in CDF_1 male mice.
† 10th transplant generation.
MST = median survival time, range in parentheses.
D/T = Dead animals over total mice after 120 days of observation period.

Discussion. The results of this study indicate that profound immunological alterations can be induced in leukemic L1210 cells on treatment with DIC. With increasing generations of treatment the leukemic cells (C lines) became progressively less capable of proliferation in the untreated host, while they retained the capability to grow and kill mice either immunosuppressed with cyclophosphamide or treated with DIC. These findings indicate that DIC treatment supports leukemic growth of C lines through an immunosuppression mechanism rather than through any direct biochemical dependence of C cells for the drug.

The immunological changes found in C lines after DIC treatment can be interpreted in two ways: (*a*) increased immunosensitivity of the cells to the antibodies elicited by the weak transplantation antigens of L1210 leukemia,[11] and (*b*) appearance of strong transplantation antigen(s) in the cells.

The results of the experiments suggest that a strong immune reaction is elicited in the host by C lines and these support the second hypothesis. The parental L1210-S leukemia does not possess transplantation antigens strong enough to elicit a demonstrable primary allograft reaction in DBA/2 or related hybrid hosts so that an inoculum of one leukemic cell is capable of killing the animals within 16 days.[12] Weak transplantation antigens can be demonstrated only after preimmunization with leukemic cells in CDF_1 mice.[11] In the case of derived C_1 line, the immunological barriers of CDF_1 mice were capable of preventing the growth of 10^7 cells (Table 1). A similar degree of protection has been obtained when the L1210-S line was transplanted into mice differing in multiple histocompatibility loci including the strong *H-2* locus (Table 1). Therefore, these data are in agreement with the hypothesis of the appearance of strong transplantation antigen(s) in the C lines.

Several possible mechanisms to account for an increase in antigenicity may be considered. The presence of DIC-protein complexes acting as transplantation antigen(s) can be ruled out since the antigenicity of C_1 cells was maintained after five passages in immunosuppressed hosts not treated with DIC.

The data could be consistent with a mutation-selection hypothesis. It is possible that highly antigenic spontaneous mutants occur in the population of L1210 leukemic cells and that they are ordinarily immuno-selectively destroyed by the host. If such antigenic variants are less susceptible to DIC treatment, they could have been selected by drug administration. The immunosuppressant effect of the drug would then permit survival of these highly antigenic clones.

Drug-induced somatic mutation responsible for the appearance of new transplantation antigen(s) must also be considered. Nontransplantable mutant clones of a mammary carcinoma were induced *in vitro* by treatment with 4-nitroquinoline 1-oxide by Koyama and Ishii.[13] The clones elicited an immune response resembling an allograft reaction in the original host. In addition it is well known that chromosomal alterations[14,15] and mutations at specific loci[16] can be induced by alkylating compounds and antimetabolites in various biological systems. Furthermore, malignant transformation *in vitro* as a result of an inductive phenomenon rather than selection has been found by Mondal and Heidelberger[17] in mouse cell lines treated with methylcholanthrene. DIC has recently been demonstrated to have carcinogenic properties inducing a high incidence of mammary carcinoma and sarcomas of the thymus, lymph node, and spleen in rats.[18] After administration of ^{14}C-methyl-DIC to rats, radioactivity was localized in the 7-methylguanine fraction of DNA and RNA and the suggestion was made that DIC may be carcinogenic because of alkylation action on nucleic acids.[18] These data support the possibility that strong transplantation antigens of the C lines are the consequence of mutagenic action of DIC.

It is well known that any immune response of the host against transplantable tumor cells may enhance the antitumor effect of chemotherapeutic agents.[11,19] Thus, the C lines might be expected to be more sensitive to treatment with chemotherapeutic agents than the original essentially nonantigenic L1210-S line. This did occur since the C_1 line responded dramatically to BCNU treatment (Table 3). Although increased biochemical sensitivity of this line to treatment with the alkylating agent may have occurred, the results suggest that a residual immune response of the host after cyclophosphamide treatment may have served to potentiate the therapy with BCNU.

Analogous observations have been made with a radiation-induced leukemia transplanted in congenic[20] mice differing at the C-K subregions of the *H-2* locus. The animals were partially immunosuppressed by administration of cyclophosphamide before tumor transplantation and treated with graded doses of BCNU 5 days after leukemic challenge.[21] In this system BCNU therapy was capable of inducing long-term survival of congenic mice in a very high percentage of the animals. The enhanced therapeutic response was attributable to the contribution to therapy of a residual immune response of the host against the antigens specified by the C-K subregions of the *H-2* locus of lymphoma cells.

In conclusion, the results reported in the present communication demonstrate that treatment with DIC altered the immunological properties of L1210 leukemia. The observations suggest that treatment with DIC increased the tumor antigenicity of leukemia L1210 to such extent that it became highly antigenic for DBA/2 and related hybrid mice. The reproducibility of the change and its

heritability suggest that the effect is the result of a specific directed action of the drugs on the leukemic cells. Whether a selection or mutation mechanism was operative has not been clarified. If antigenic changes are indeed induced by DIC treatment and are tumor specific, drug induction of antigenic targets in leukemic cells *in vivo* could provide new perspective in tumor chemotherapy.

* Supported in part by contract PH43-68-1283 from Chemotherapy, National Cancer Institute, National Institutes of Health.

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