Eur J Cancer Clin Oncol, Vol. 22, No. 11, pp. 1401–1405, 1986.
Printed in Great Britain

0277–5379/86$3.00+0.00
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L1210/DTIC Antigenic Subline: Studies at the Clone Level

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Abstract— *Treatment of murine tumors with the anti-tumor agent* 5-(3,3 *dimethyl*-1-*triazeno)imidazole*-4-*carboxamide* (*DTIC*) *has resulted in the induction of antigenic specificities not found in parental cells. After the withdrawal of DTIC treatment, the antigenic sublines maintained the new properties indefinitely, as a heritable character although the mechanism of induction and the molecular nature of the new antigens are essentially unknown. Seven clones from the murine immunogenic leukemia L*1210/*DTIC were selected and studied in some detail. Three of the seven clones showed an increased immunogenicity* in vivo *since two clones* (*D*5 *and D*7) *were rejected by syngeneic mice and one* (*D*6) *prolonged the life span for* 3 weeks (*untreated tumours killed the mice in* 7 days). *The seven clones and the L*1210/*DTIC were recognized and lysed by* in vivo *primed,* in vitro *stimulated (with L*1210/*DTIC) lymphocytes. Therefore, the seven clones shared antigens with the L*1210-*DTIC immunogenic subline. Secondary stimulated lymphocytes to clone D*5, *and clone D*7 *were able to lyse D*5, *D*6 *and D*7 *cells, respectively, but were unable to recognize the remaining clones. From* in vivo *and* in vitro *studies, all the L*1210/*DTIC sublines are composed of cells carrying two or more distinct antigens. Each cell clone expressed one set of antigens. It was concluded that only a few antigens expressed in different cells were induced by a DTIC treatment of L*1210 *leukemia.*

INTRODUCTION

INCREASED tumor cell antigenicity has been demonstrated in several laboratories both in drug-resistant tumor sublines [1,2] and following *ad hoc* treatment with anticancer compounds [3]. New antigenic specificities, not detectable in parental cells, continue to be maintained after the withdrawal of drug treatment and elicit immune response in syngeneic hosts, as evaluated by classical rejection experiments [4, 5] and by transfer of immune lymphocytes [6, 7]. *In vitro* assays show that the drug-treated sublines elicit T cell-mediated primary and secondary immune response to drug-induced antigens [8–10], and also activated macrophages, which inhibit the *in vitro* growth of neoplastic cells [11].

The alkilating agent, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC), employed in these experiments was found to be particularly effective in increasing the immunogenicity in a number of experimental lymphomas [12,13].

There are several hypotheses about the DTIC mechanism for antigenic induction. For instance, antigenic clones can be present in a non-immunogenic tumor population [14]: DTIC could give these cells a selective advantage. A further theory has assumed that DTIC might activate silent viral genes, which in turn may induce the expression of viral-induced antigens on the cell membrane. Finally, as DTIC has mutagenic properties, the synthesis of altered or new antigenic structures responsible for increased immunogenicity has received widespread attention [15–17].

The number of antigenic specificities as well as the pattern of immunologic cross-reactivities might contribute to define the relationship among drug-induced antigens and to understand the mechanism by which new immunologic properties can be induced by drugs. The lack of cross reactivity among DTIC-treated tumor lines, either allogeneic or syngeneic, or among tumor lines obtained from the same tumor favors the mutagenic hypothesis [18].

In our laboratories, the characteristics of drug-induced antigens are under study by a number of approaches including the cloning of DTIC-treated L1210 leukemia. In the present study, seven clones from the murine leukemia subline L1210/DTIC

Accepted 1 July 1985.
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Research supported in part by Grant No.84.00492.44 from CNR, Rome, Italy.

were selected and some immunologic features studied.

MATERIALS AND METHODS

Mice and tumors

Hybrid Balb/c × DBA/2F_1 (hereafter called CD2F_1) mice, 8–10 weeks old of both sexes from Charles River Breeding Laboratories (Calco, Italy) were used in these studies. L1210 Cr obtained from the National Cancer Institute Bethesda, U.S.A., was maintained by weekly intraperitoneal (ip) injection into CD2F_1 mice.

L1210/DTIC leukemia

The subline was developed as previously described [3]. Briefly, 10^6 tumor cells were injected ip into CD2F_1 and treated for 7 consecutive days with DTIC (100 mg/kg/die, ip) starting 1 day after tumor challenged. When the ascites tumors developed in the DTIC-treated mice, the tumor cells were collected and 10^6 cells were transplanted into untreated mice and the same treatment as above was given for five transplant generations. The immunogenic L1210/DTIC subline (D) was maintained in compatible total body X-irradiated mice (350, Securix Compatct CGD, 30 cm distance). D cells were suspended in RPMI 1640 supplemented with 20% heat-inactivated fetal calf serum (Flow Laboratories), penicillin (100 U/ml) streptomycin (100 μg/ml) and L-glutamine (2×10^{-3}M) the cell suspension was diluted to a final concentration of 1–4 cells per ml and seeded in microplates (LIMBRO). Seven cloned lines were obtained and upon passage (10^6cells/CD2F_1 mouse in immunosuppressed as above) were used to rejection assay and for ^{51}Cr release assays.

Cytotoxic T lymphocytes (CTL) and ^{51}Cr release assay

Spleen cells were obtained from CD2F_1 mice that had previously rejected 10^7D viable cells injected 3–5 weeks before. After gently squeezing the spleen tissue, the spleen cells were dispersed and washed in balanced salt solution and resuspended in RPMI 1640 containing 10% heat-inactivated fetal calf serum penicillin (100/U/ml), streptomycin (100 μg/ml), L-glutamine (2×10^{-3}M), and 2-mercaptoethanol (2×10^5/M). Responder cells (7×10^6) were seeded in 24 well plates (Costar, Cambridge, MA) with a varying number of treated D cloned cells (stimulating cells) in a final volume of 2.5 ml/well. Stimulating cells had been previously incubated for 45 min at 37° C with mitomycin C (33 μg/ml) (Kiowa Hakko Kogyo C.O, Tokyo, Japan). After 5 days incubation in a moist atmosphere of 95% air and 5% CO_2 the effector CTL were harvested with Pasteur Pipettes and their lytic activity against several D clones was evaluated by the ^{51}Cr release microassay [19]. Briefly, 10^4 ^{51}Cr-labelled L1210 cells in 100 μl were mixed with 100 μl of effector cells at different concentrations in microtiter plates and incubated for 4 hr at 37° C in a moist atmosphere of 95% air and 5% CO_2. Control samples were incubated with detergent (5% aqueous Brij 35, BDH, Milan). After 4 hr, the plates were centrifuged, and 100 μl of the supernatant was counted in an automatic gamma-counter. The percentage of specific lysis was calculated as follows:

$$\% \text{ lysis} = \frac{\text{cpm experimental sample-cmp controls}}{\text{cpm in detergent sample/cpm in controls}} \times 100^{\text{cpm}}$$

Statistics

The significance of differences in survival time were compared with the Mann–Whitney *U* test.

RESULTS

Treatment of L1210 leukemia with DTIC resulted in the induction of antigenic specificities not found in parental cells. The new properties were maintained indefinitely as heritable characteristics, although the mechanism of antigen induction is essentially unknown. An immunogenic subline of the L1210 leukemia L1210/DTIC(D) has been cloned *in vitro* and seven clones were isolated and studied (D_1 to D_7).

Table 1 reports the mean survival times of CD2F_1 mice challenged with 10^7 cells of the different clones derived from the subline D. The tumors were injected intraperitoneally into X-ray immunosuppressed mice and in normal mice. Clones D_5 and D_7, were rejected by unprimed syngeneic mice. Clone D_6 was rejected by only some of the mice, and they show a significantly increased survival time.

Although the other clones grew and killed the mice, the apparent significant lack of immunogenicity might be dependent on reduced antigenicity as a consequence of *in vitro* passage (unpublished observations). In mice, immune responses become effective 7–8 days after antigen challenge therefore, a minor loss of immunogenicity might and ultimately is responsible for the death of the mice. All clones proliferated and killed immunosuppressed mice in 8–9 days.

Lymphocytes from spleen cells of CD2F_1 mice immunized ip 3–5 weeks previously with viable 10^7 D cells were restimulated *in vitro* with mitomycin C-blocked D cells. The activity of activated lymphocytes was tested in the cell-mediated cytotoxic assay against cells derived from the seven D clones. Immune D lymphocytes were capable of recognizing and lysing the relevant target cells as well as the seven clones with similar patterns of cytotoxicity.

Table 1. Growth pattern of D clones in normal or immunodepressed mice

[a]RAD	Tumor cells	[b]MST	[c]D/T
+	–	–	0/10
+	L1210	5	20/20
–	"	6	20/20
+	D	6.5	20/20
–	"	–	0/20
+	D_1	5.5	20/20
–	"	9.0	18/20
+	D_2	6	20/20
–	"	8.2	19/20
+	D_3	7.0	20/20
–	"	9.0	20/20
+	D_4	6.0	20/20
–	"	7.5	18/20
+	D_5	9.3	20/20
–	"	–	1/20
+	D_6	8	20/20
–	"	38[d]	14/20
+	D_7	11.0	20/20
–	"	–	0/20

CDF_1 mice were challenged i p with 10^7 viable cells at day 0.
[a]RAD = 350 rad/mouse.
[b]MST = Mean survival time.
[c]D/T = Dead mice/treated mice.
[d] = $P < 0.001$ by Mann–Whitney U test.

The findings reported in Table 2 show that D clones expressed antigenic specificities leading to *in vitro* susceptibility of CTL immune to the parental D subline. This was found in spite of the lack of obvious immunogenicity as revealed by *in vivo* studies. It remained to be established whether clones were endowed with the same set of antigens as the parental D subline, or whether the latter was composed of a number of cells that carried different antigens.

Table 2. Lysis D clone cells by CD^2F_1 immune lymphocytes restimulated in vitro *by D cells*

Target cells	% ^{51}Cr Release 10 : 1	20 : 1	40 : 1
L1210	2.1	2.0	3.0
D	20.1	50.1	56.1
D_1	29.7	40.3	51.3
D_2	28.6	39.2	52.0
D_3	25.2	37.8	41.9
D_4	30.1	41.2	52.1
D_5	32.7	39.3	54.2
D_6	25.6	32.4	55.7
D_7	30.4	40.3	53.2

Responder cells were spleen cells from $CD2F_1$ mice injected i p 3–5 weeks before with viable 10^7 D cells and restimulated *in vitro* with D cells treated with mitomycin C (see Materials and Methods).

Table 3. Lysis of D clone cells by CD^2F_1 immune lymphocytes restimulated in vitro *with D_5 clone*

Target cells	% ^{51}CR Release 10 : 1	20 : 1	40 : 1
L1210	1.8	2.2	3.4
D	25.2	29.6	35.2
D_1	2.2	4.6	5.0
D_2	4.0	5.2	5.4
D_3	2.1	3.2	4.6
D_4	1.4	2.4	3.6
D_5	50.6	51.4	55.8
D_6	38.7	42.2	45.5
D_7	34.8	39.4	41.2

Responder cells were spleen cells from CD^2F_1 mice injected ip 3–5 weeks before with viable 10^7 D_5 cells and restimulated *in vitro* with D_5 cells treated with mitomycin C (see Materials and methods).

To study this hypothesis, cross lysis experiments were performed in which CTL to D_5 clone or to D_7 were matched with other cloned cells. The experimental results are reported in Tables 3 and 4. It was found that anti D_5 and anti D_7 CTL were able to lyse D_5, D_6, D_7 clones in addition to parental D cells. D_1 to D_4 cloned cells were not susceptible to anti D_5 or anti D_7 CTL. The untreated L1210 cells were not lysed by CTL to drug-induced antigens.

Although there was a trend toward a preferential killing of primed cells with respect to other target cells, conclusions with regard to the amount of surface antigens in different clones could not be drawn. The findings shown in Tables 3 and 4 favor the hypothesis that non-cross-reacting sets of antigens are present in cells from the D subline.

Table 4. Lysis of D clone cells by CD^2F_1 lymphocytes restimulated in vitro *with D_7 clone*

Target cells	% ^{51}Cr Release 10 : 1	20 : 1	40 : 1
L1210	2.3	2.4	3.1
D	28.5	36.3	39.5
D_1	2.1	3.7	3.9
D_2	3.0	4.2	5.6
D_3	1.9	3.2	4.5
D_4	1.6	2.4	3.2
D_5	32.5	36.4	32.2
D_6	41.6	48.1	49.2
D_7	49.7	52.4	55.2

Responder cells were spleen cells from CDF_1 mice injected ip 3–5 weeks before with viable 10^7 D_7 cells and restimulated *in vitro* with D7 cells treated with mitomycin C (see Materials and Methods).

DISCUSSION

Drug-induced antigens in tumor cells are associated with a number of genetic, biochemical and immunological alterations with respect to the original untreated cells [3, 20, 21]. The way these modifications occur at the molecular level is still unknown, and only in recent years has it received sufficient attention. This laboratory has been involved for a number of years in attempts to elucidate the immunological modifications associated with a proper drug treatment. Specifically, we are studying the nature of the DTIC-induced antigens in cancer cells capable of eliciting specific immune reactivity and leading to the rejection of drug treated sublines by hosts compatible with the parental tumor lines. The true mechanism of action of DTIC remains poorly defined. Moreover, a variety of leukemias and lymphomas with different sensitivities to the antitumor activity of DTIC have been shown to undergo a drug-mediated increase in immunogenicity on exposure to DTIC [22, 23].

Several hypotheses have been suggested: (a) selection of antigenic clones; (b) activation of silent viral genes; (c) induction of new proteins as a result of a mutagenic action.

As previously discussed [24], one of the possible mechanisms to explain the nature of antigenicity of the DTIC-treated lines was the selection of antigenic clones pre-existing in a tumor line. According to this hypothesis, most of the antitumor compounds, including DTIC, exhibit strong immunosuppressive activity, which allows antigenic clones originated by spontaneous mutation to escape immune surveillance, proliferate and become antigenic lines [12, 13]. Our data seem in contrast with this hypothesis, since increased, antigenic properties have never been found in clone tumor lines, including L1210 cloned cells. Recent findings have shown that L1210 cells from a single clone-originated non-cross-reacting antigenic lines following the same DTIC treatment (unpublished results). Moreover, antigenic determinants in drug-treated tumors demonstrated by *in vitro* assays might not be easily recognized by *in vivo* studies in syngeneic animals. Therefore, the hypothesis of *in vivo* immunoselection has not found experimental support.

The second hypothesis, namely the possible activation of silent viral genes or virus-induced cellular genes following DTIC treatment, does not completely fit with experimental observations. For instance viral activation should lead to a limited number of antigenic specificities, occurrence in contrast with experimental findings. The failure of DTIC treated tumors to react with antisera to murine viral proteins further supports the above claim (unpublished observation).

The third hypothesis, that the DTIC treatment induces a 'random' mutation on the cell genome that expresses either altered or new proteins, is favored by our experimental results.

In fact the antigenic diversity among DTIC-treated tumor lines, the mutagenic properties of DTIC, and similar findings obtained by treatments with frankly mutagenic compounds such as MNNG, are in keeping with the mutagenic hypothesis. However, the number of specificites expressed within each antigenic line remains unknown. In theory, a DTIC line might possess either cells that carry a common antigen or cells endowed with mutually exclusive antigens.

The present study was carried out to check this latter hypothesis. It was found that (1) all seven clones were endowed with antigenic properties, although clones D_1 to D_4 were not rejected by syngeneic mice: (2) clones D_5 to D_7 and line D were lysed by CTL to D_5 or to D_7 (3) clones D_1 to D_4 were not lysed by CTL to D_5 or to D_7 although they were lysed by anti-D CTL. The first observation is supported by the finding that anti-D CTL were able to lyse D cells as well as D clones. Therefore, in spite of the lack of *in vivo* immunogenicity in the assay used here, D_1 to D_4 clones expressed DTIC antigens that were also demonstrated by specific CTL (data not reported).

Since clones D_5 to D_7 were cross reactive at CTL analysis, it follows that 3 out of 7 D clones share antigenic properties. In contrast, D_1 to D_4 clones were not lysed by either anti D_5 CTL or by anti-D_7 CTL. Therefore, in L1210/DTIC line at least two antigenic specificities were identified, and they were expressed at the clonal level in a mutually exclusive fashion.

Until specific antibodies are found, studies to define the nature of drug-induced antigens in experimental tumors will present some problems. In addition to the present approach, CTL clones will help to understand some characteristics of DTIC-induced antigens.

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