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Adoptive immunity in mice challenged with L1210/DTIC clones*

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Summary. New antigenic specificities, not detectable on parental cells, have been induced by many investigators in mouse lymphomas by treatment with the antitumor agent 5(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC). The antigens are transmissible, after withdrawal of the drug treatment, as an inheritable character. The mechanism of induction, the molecular nature, and the number of the new antigenic specificities have not been completely elucidated. Four clones from murine leukemia L1210 isolated and expanded in vitro were treated in vivo with DTIC and the new sublines were studied in detail. The four drug-treated sublines studied exhibited strong immunogenicity since they were rejected by syngeneic animals. Immunosuppressed animals challenged with 10^7 A/DTIC or P/DTIC cells were reciprocally protected by the adoptive transfer of spleen cells from donors that had rejected a lethal challenge of A/DTIC or P/DTIC clones. In a similar fashion, the adoptive transfer of spleen cells obtained from animals that had rejected the Q/DTIC or the R/DTIC clones protected immunosuppressed mice challenged with Q/DTIC or R/DTIC cells. No antitumor activity was observed in cross-protective schedules other than those indicated. It was been concluded that (a) the L1210 leukemia line does not have antigenic cells, (b) four DTIC-treated clone sublines were rejected by compatible hosts, and (c) two mutually exclusive sets of antigens were expressed in four antigenic clone sublines.

Introduction

Previous studies have demonstrated that immunologic alterations of experimental tumors may be induced by in vivo treatment with anticancer drugs [5, 11, 18]. One of the most active compounds effective in increasing the immunogenicity in a number of experimental lymphomas was 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC) [1, 2]. Syngeneic animals challenged with the drug-altered tumor sublines showed indefinite survival compared to death of animals challenged with parental, untreated tumor [14]. Animal survival was determined by antitumor immune response, since the inoculum of DTIC-treated tumor cells in immunosuppressed recipients was followed by progressive tumor growth and animal death [3]. More direct evidence was provided by in vivo transfer of immune lymphocytes [13, 16] and by in vitro assays, which indicated the presence of active immunity in the recipients of the modified tumors [9, 21]. The new immunologic properties not detectable on parental cells continue to be transmissible after withdrawal of the drug treatment [15].

The finding that highly immunogenic tumor sublines can be generated after treatment in vivo with DTIC raised obvious questions as to the possible mechanism(s) at the root of the phenomenon. One hypothesis postulates that highly immunogenic clones may arise spontaneously during tumor growth in vivo, possibly as a consequence of mutational events [8]. Therefore, DTIC as an immunosuppressive drug could give these cells a selective advantage. Another possibility was the DTIC activation of silent viruses as the origin of new antigenic properties. Finally, the mutagenic properties of DTIC might induce somatic mutations that allow drug-treated tumors to express altered or new antigenic structures responsible for increased immunogenicity [4, 7]. The number of antigenic specificities as well as the pattern of immunologic cross-reactivities might contribute to define the relations among drug-induced antigens and to understand the mechanism by which new immunologic properties can be induced.

As part of a program to elucidate some features of the drug-induced antigens, four clones of L1210 leukemia were treated in vivo with DTIC and, by an adoptive transfer assay, antigenic cross-reactivity was studied.

Material and methods

Mice and tumors. Hybrid Balb/c × DBA/2F_1 (hereafter called CD2F_1) mice, 8–10 weeks old of both sexes, from Chalres River Breeding Laboratories (Calco, Italy) were used in these studies. L1210 obtained from the National Cancer Institute (Bethesda, Md.), was maintained by weekly i. p. injection into CD2F_1 mice. L1210 cells were suspended in RPMI 1640 supplemented with 20% heat-inactivated fetal calf serum (Flow Labs, MacLean, USA), penicillin (100 units/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/ml and injected i. p. (0.2 ml/mouse) in 30 syngeneic CD2F_1 mice. The tumors

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that developed in 14 mice and four subclones (A, P, Q, R) were maintained by serial passages.

L1210/DTIC leukemia sublines. The four sublines were developed as previously described [1]. Briefly, 10^6 tumor cells were injected i. p. into $CD2F_1$ mice and treated for 7 consecutive days with DTIC (100 mg/kg per day i. p.) starting 1 day after tumor challenge. When the ascitic tumors developed in the DTIC-treated mice, the tumor cells were collected and 10^6 were transplanted into normal mice. Treatment was repeated as above for five transplant generations. Immunogenic sublines A/DTIC, P/DTIC, Q/DTIC, and R/DTIC were maintained in compatible total body X-irradiated (350 R Securix Compact CGD, 30 cm distance) mice.

Immune lymphocytes. The four DTIC-treated sublines were given to $CD2F_1$ mice as a single i. p. injection of 10^7 viable cells. Spleen cells from tumor-treated $CD2F_1$ mice were collected 15 days after the immunizing inoculum and washed cells (20×10^6/mouse per 0.2 ml i. v.) were inoculated in recipient mice as previously reported [13].

Drug. The citric salt of DTIC (Deticene, RBS Pharma, Milan, Italy) was dissolved in distilled water immediately before use.

Immunosuppression. The mice were immunosuppressed by 400 R total body X-irradiation (Securix Compact CGD, 30 cm distance), 24 h before the i. p. injection of the tumor cells.

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

Results

The occurrence of drug-mediated immunogenicity in the four sublclones treated in vivo with DTIC for five transplant generations was assayed by injection of tumor cells from DTIC-treated tumors into normal or immunosuppressed syngeneic hosts. The survival was then followed for 90 days.

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

R[a]	Tumor cells	MST[b]	D/T[c]
+	L1210	6	10/10
−	L1210	6.5	10/10
+	L1210/DTIC	6.5	10/10
−	L1210/DTIC	–	0/10
+	A/DTIC	7	10/10
−	A/DTIC	–	2/10
+	P/DTIC	6.5	10/10
−	P/DTIC	–	0/10
+	R/DTIC	7.5	10/10
−	R/DTIC	–	0/10
+	Q/DTIC	6	10/10
−	Q/DTIC	–	2/10

CDF_1 mice were challenged i.p. with 10^7 viavle cells on day 0.
[a] R = 400 R/mouse
[b] MST = mean survival time
[c] D/T = dead mice/treated mice
Parental untreated A, P, R, and Q, L1210 clones were not rejected by syngeneic mice

Table 2. Immunotherapy with lymphocytes immune to subline A/DTIC

R day −1	Tumor line day 0 10^6 i.p.	Treatment day +1 2×10^7 i.v.	MST	D/T
400 R	A/DTIC	–	12	8/8
+	+	N	12	8/8
+	+	I/A	25[a]	8/8
+	P/DTIC	–	13	8/8
+	+	N	14	8/8
+	+	I/A	22[a]	8/8
+	Q/DTIC	–	13	8/8
+	+	N	12	8/8
+	+	I/A	11.5	8/8
+	R/DTIC	–	10	8/8
+	+	N	10.5	8/8
+	+	I/A	14.5	8/8
+	A	–	10	8/8
+	+	N	9	8/8
+	+	I/A	9	8/8

N = spleen lymphocytes from $CD2F_1$ virgin mice
I/A = spleen lymphocytes from $CD2F_1$ mice immune to subline A/DTIC
[a] $P < 0.001$ by Mann-Whitney *U* test

As shown in Table 1, the treatment with DTIC induced marked immunogenic properties in the four L1210 subclones (A, P, Q, R). The sublines A/DTIC, P/DTIC, Q/DTIC, and R/DTIC were rejected by syngeneic hosts, and most of the mice survived indefinitely compared to X-ray immunosuppressed mice, which succumbed to challenge with the drug-modified sublines. The original L1210 leukemia was not rejected by syngeneic hosts, nor were parental A, P, Q, and R L1210 clones that did not undergo DTIC treatment (unpublished results).

Studies were conducted to establish the therapeutic effectiveness of adoptive transfer of immune spleen cells in tumor-bearing hosts. Spleen lymphocytes obtained from $CD2F_1$ mice previously (2–3 weeks) inoculated i. p. with 10^7 viable A/DTIC cells or spleen lymphocytes obtained from $CD2F_1$ mice challenged with lethally irradiated L1210 were injected i. v. into five groups of immunosuppressed mice bearing the four DTIC-modified sublines or the parental L1210 leukemia. As shown in Table 2, the adoptive transfer of immune lymphocytes to A/DTIC increased the survival time of mice bearing the subline A/DTIC and P/DTIC. In contrast, there were no therapeutic effects in mice bearing the subline Q/DTIC or the parental L1210 line [16].

The adoptive transfer of spleen lymphocytes immune to the P/DTIC subline (Table 3) exhibited a curative activity in immunosuppressed mice bearing the P/DTIC subline and significantly increased the survival time of immunosuppressed mice challenged with the A/DTIC subline. In contrast, lymphocytes immune to P/DTIC were not protective in immunosuppressed mice bearing R/DTIC or Q/DTIC sublines or the parental line L1210 leukemia.

As shown in Table 4, lymphocytes immune to Q/DTIC were effective, in adoptive transfer assays, against Q/

Table 3. Immunotherapy with lymphocytes immune to subline P/DTIC

R day −1	Tumor line day 0 10^6 i.p.	Treatment day +1 2×10^7 i.v.	MST	D/T
400 R	A/DTIC	–	13	8/8
+	+	N	12.5	8/8
+	+	I/P	26[a]	5/8
+	P/DTIC	–	12.5	8/8
+	+	N	13	8/8
+	+	I/P	–	1/8
+	Q/DTIC	–	14	8/8
+	+	N	14	8/8
+	+	I/P	16.5	8/8
+	R/DTIC	–	12	8/8
+	+	N	12.5	8/8
+	+	I/P	13	8/8
+	P	–	10	8/8
+	+	N	10.5	8/8
+	+	I/P	9	8/8

N = spleen lymphocytes from $CD2F_1$ virgin mice
I/P = spleen lymphocytes P/DTIC from $CD2F_1$ mice immunized with 10^7 cells i.p. of clone P/DTIC
[a] $P<0.001$ by Mann-Whitney U test

Table 4. Immunotherapy with lymphocytes immune to subline Q/DTIC

R day −1	Tumor line day 0 10^6 i.p.	Treatment day +1 2×10^7 i.v.	MST	D/T
400 R	A/DTIC	–	8	8/8
+	+	N	8.5	8/8
+	+	I/Q	8	8/8
+	P/DTIC	–	10.5	8/8
+	+	N	11	8/8
+	+	I/Q	11	8/8
+	Q/DTIC	–	9	8/8
+	+	N	11	8/8
+	+	I/Q	25[a]	6/8
+	R/DTIC	–	8.5	8/8
+	+	N	10	8/8
+	+	I/Q	20.5[a]	8/8
+	Q	–	8	8/8
+	+	N	7	8/8
+	+	I/Q	8.5	8/8

N = spleen lymphocytes from $CD2F_1$ virgin mice
I/Q = spleen lymphocytes from $CD2F_1$ mice immune to subline Q/DTIC
[a] $P<0.001$ by Mann-Whitney U test

DTIC or R/DTIC tumors and ineffective in A/DTIC or P/DTIC sublines.

The adoptive transfer of immune lymphocytes to R/DTIC cells was curative in immunosuppressed mice challenged with the relevant R/DTIC and the Q/DTIC sublines, no protection was observed in mice bearing the A/DTIC and the P/DTIC sublines (Table 5).

The immunologic cross-reactivity among clone DTIC sublines is outlined in Table 6. On the basis of increased survival time of tumor-bearing animals two sets of cross-reactive antigens could be distinguished.

Table 5. Immunotherapy with lymphocytes immune to subline R/DTIC

R day −1	Tumor line day 0 10^6 i.p.	Treatment day +1 2×10^7 i.v.	MST	D/T
400 R	A/DTIC	–	12	8/8
+	+	N	13	8/8
+	+	I/R	15.5	8/8
+	P/DTIC	–	9	8/8
+	+	N	8	8/8
+	+	I/R	8.5	8/8
+	Q/DTIC	–	12.5	8/8
+	+	N	12	8/8
+	+	I/R	37[a]	5/8
+	R/DTIC	–	10.5	8/8
+	+	N	12	8/8
+	+	I/R	–	0/8
+	R	–	12	8/8
+	+	N	12	8/8
+	+	I/R	11	8/8

N = spleen lymphocytes from $CD2F_1$ virgin mice
I/R = spleen lymphocytes from $CD2F_1$ mice immune to subline R/DTIC
[a] $P<0.001$ by Mann-Whitney U test

Table 6. Cross-reactivity between the four sublines

Sublines	Immune lymphocytes			
	I/A	I/P	I/Q	I/R
A/DTIC	++	++	–	–
P/DTIC	++	+++	–	–
Q/DTIC	–	–	++	++
R/DTIC	–	–	++	+++

I = spleen lymphocytes from $CD2F_1$ mice immune to the four sublines A/DTIC, P/DTIC, Q/DTIC and R/DTIC
+++ = complete protection
++ = partial protection $P<0.001$
– = no protection

Discussion

Appropriate chemotherapeutic treatment of murine lymphomas can result in an immunologic alteration of tumor cells. The antigenically altered cells implanted in mice compatible with the parental tumor elicit a host-allograft reaction comparable to that observed with an allogeneic inoculum [1, 8, 12]. The mechanism(s) responsible for the drug-induced antigen(s) has not yet been elucidated. Over the past years this laboratory, while studying the properties of the DTIC-induced antigens [3, 15, 21], has proposed several mechanisms to explain the appearance of new antigenic properties on leukemic cells.Decreased oncogenicity of drug-modified tumor cells, DTIC positive selection of immunogenic clones arising spontaneously in the parental line, DTIC activation of latent viruses in turn responsible for virus-dependent membrane antigen(s), or DTIC induction of somatic mutations with the expression of new or altered antigens.

Loss of oncogenicity was ruled out [3, 14, 17, 19]., since parental and DTIC-treated tumors showed similar cell

kinetic characteristics and were equally tumorigenic in immunodepressed mice. Therefore, the present study was carried out to elucidate the other three hypotheses.

As DTIC is endowed with marked immunosuppressive activity [2, 14, 20], a selection favoring the gradual emergence of immunogenic cell clones may be hypothesized [6]. Previous studies [10] and the present one have not shown obvious antigenic properties among L1210 clones. In contrast, evident antigenicity was exhibited by the clones following DTIC treatment (Table 1). It is noteworthy that DTIC-treated clone cells were lethal to immunosuppressed mice. Moreover, similar findings were obtained in the other DTIC clones derived from the L1210 parental line and not considered for further studies (data not reported).

Our experimental data do not completely support the hypothesis of a viral infection, which frequently accompanies transplantable tumors and which may be activated by DTIC treatment. In fact, the four L1210 clone sublines, upon treatment by exactly the same modalities, did not share antigenic properties, as would be expected if there was modification of surface membranes by DTIC-activated virus [4].

In vivo DTIC treatment may produce "at random" somatic mutations in tumor cells, resulting in novel or altered antigenic specificities apparently without affecting the genetic loci that regulate the normal histocompatibility profile of the cell (O. Marelli, unpublished). Such an hypothesis is more compatible with our results.

The results reported in Tables 2–5 and summarized in Table 6 show that two sets of antigens were expressed in the DTIC clones. The adoptive transfer of immune lymphocytes cross-protected the immunosuppressed mice challenged with the relevant or other clone. Spleen cells immune to the A/DTIC sublines or to the P/DTIC subline were reciprocally effective against A/DTIC tumors and P/DTIC tumors. However, anti-Q/DTIC or anti-R/DTIC lymphocytes cross-protected the correspondent tumor sublines. Therefore, four nonimmunogenic L1210 clones following parallel in vivo treatment with the compound DTIC were induced to express two major sets of antigens in a mutually exclusive fashion.

The findings reported here exclude the presence of antigens in L1210 tumor cells before DTIC treatment. In that case, DTIC clones would share antigenic properties not found here. Moreover, the number of antigens inducible by the DTIC treatment in L1210 leukemia would be quite limited, in agreement, with recent studies [10]. If these findings are confirmed, DTIC-induced mutations in very limited regions of the cell genoma would restrict the random mutation hypothesis. Whether they are new antigens or antigens preferentially expressed on DTIC-treated cells is still unkonw, and studies are in progress to elucidate this problem with the production and aid of monoclonal antibodies specific to the different DTIC-induced antigens.

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