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Expression of Normal Histocompatibility Antigens in Murine Lymphomas Treated with 5-(3, 3′-Dimethyl-1-Triazeno)-Imidazole-4-Carboxamide (DTIC) *In Vivo**

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Abstract—*Novel transplantation antigens have been detected in a Moloney virus-induced LSTRA lymphoma of BALB/c origin* (H-2^d), *or in a chemically-induced* L5178Y *lymphoma of* DBA/2 *origin, following treatment of tumor-bearing hosts with* 5-(3, 3′-*dimethyl*-1-*triazeno*-)-*imidazole*-4-*carboxamide* (*DTIC*), *for* 4–8 *transplant generations. Marked cell-mediated cytotoxic responses against DTIC-treated LSTRA or* L5178Y *lines were found by primary* in vivo *and secondary* in vitro *sensitization of histocompatible mice. Moreover, preliminary data show that no obvious antigenic cross-reactivity can be found among DTIC-treated sublines derived from distinct parental lymphomas. To test whether DTIC lines would express variable levels of normal histocompatibility antigens, cytotoxic lymphocytes were generated* in vitro *against alloantigens of* H-2 *complex or sub-regions of it and tested against parental or DTIC-treated lymphomas in a short-term* ^{51}Cr-*release assay. A cold-inhibition test was performed with LSTRA or* 4 *LSTRA/DTIC sublines. The results showed that little or no difference in the expression of* H-2 *antigens recognized by cytotoxic lymphocytes could be detected between parental and DTIC-treated sublines. Moreover, no foreign* H-2 *specificities of* H-2^b *or H-2*^{k} *haplotypes detectable by cytotoxic lymphocytes could have been found in* L5178Y *or* L5178Y/*DTIC lymphomas.*

INTRODUCTION

A LARGE amount of experimental evidence is now available indicating that a number of murine lymphomas acquire strong immunogenicity following treatment with DTIC *in vivo* [1, 2]. Drug-mediated tumor antigens (DMTA, [2]), not detectable in the tumor line of origin (parental line), have been demonstrated in DTIC-treated sublines.

Specific cell-mediated cytotoxic responses against DMTA were detected in histocompatible mice by sensitization with immunogenic sublines either *in vivo* [3, 4] or *in vitro* [5–7]. However, no detailed studies have been performed on the possible relationship between DMTA and the expression of normal histocompatibility antigens. Therefore, preliminary investigations have been designed to test whether the presence of DMTA on tumor cell membrane would affect the reactivity of the alloantigens specified by the *H-2* complex, or would promote the appearance of foreign *H-2* specificities [8] as detectable in a cell-mediated cytotoxicity assay.

In the present study, parental and DTIC-treated sublines were tested for the reactivity of *H-2* antigens, using cold-cell inhibition of *in vitro* immune cytolysis. The results showed that no substantial differences could be detected between the original and DTIC-treated lymphomas. Moreover, the induction of DMTA was not associated with the ap-

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Abbreviations: DTIC, 5-(3, 3′-Dimethyl-1-triazeno)-imidazole-4-carboxamide; DMTA, drug mediated tumor antigens; Cy, cyclophosphamide; CMI, cell-mediated immunity.

pearance of foreign *H-2* alloantigens specified by the $H\text{-}2^b$ or $H\text{-}2^k$ haplotype. The results of *in vivo* or *in vitro* sensitization experiments with DTIC lines showed also that no cross-reactivity could be found among DMTA associated with lymphoma sublines derived from different parental tumors.

MATERIALS AND METHODS

Animals

Inbred mice of both sexes of BALB/c Cr ($H\text{-}2^d$), DBA/2 Cr ($H\text{-}2^d$), C3H/Sn Cr ($H\text{-}2^k$), B10(pd/cz) Cr ($H\text{-}2^b$) strains, congenic-resistant B10.A ($H\text{-}2^a$), B10.A(2R) Cr ($H\text{-}2^{h2}$), B10.A(4R) Cr ($H\text{-}2^{h4}$), B10.A(5R) Cr ($H\text{-}2^{i5}$), mice and hybrid (BALB/c Cr × DBA/2 Cr) F_1, (CD2F_1, $H\text{-}2^d/H\text{-}2^d$) mice were obtained from the Mammalian Genetics and Animal Production Section, Division of Cancer Treatment, National Cancer Institute (NCI), National Institutes of Health (NIH), Bethesda, Maryland, U.S.A., and maintained under standard conditions.

Tumors

Chemically-induced ascitic leukemia L5178Y [9] of DBA/2 origin and Moloney virus-induced LSTRA leukemia of BALB/c origin [10], both carrying the $H\text{-}2^d$ haplotype, were maintained in histocompatible mice by weekly i.p. injections of 10^6 cells. The DTIC treated sublines (LSTRA/DTIC-1, LSTRA/DTIC-2, LSTRA/DTIC-3 and LSTRA/DTIC-4) were obtained in BALB/c recipients following daily treatment of mice bearing parental tumor (LSTRA, 10^6 i.p.) with DTIC (100 mg/kg, i.p.) from day 1 through day 5 after challenge, for 5–8 transplant generations [11]. Ascites L5178Y/DTIC and L1210Ha/DTIC were obtained by DTIC treatment of the original lines, L5178Y and L1210Ha, respectively, as previously described [12, 13]. Lymphoma LBD-1 was obtained after DTIC-treatment of a chemically-induced lymphoma (LSBM-1) of C57BL/10 ($H\text{-}2^b$) origin [14]. These tumor sub-lines were maintained in histocompatible mice immunodepressed with Cyclophosphamide (Cy, 180 mg/kg, 6 hr before challenge). Rauscher virus-induced leukemia RBL-5 of C57BL/6 origin [15] and ascites lymphosarcoma 6C3HED [16] induced by α-estradiol dipropionate in C3H mice were maintained in histocompatible mice by weekly i.p. injections of 10^5–10^6 cells.

Generation of cytotoxic lymphocytes against alloantigens

In vitro. Spleen cells to be sensitized were cocultured *in vitro* with allogeneic spleen cells in RPMI-1640 medium (Difco Lab., Detroit, Mi.) supplemented with 25% Fetal Bovine Serum (Microbiological Assoc., Bethesda, Md.), glutamine, sodium pyruvate, 2-mercaptoethanol, non-essential amino acids, penicillin and streptomycin as described by Mishell and Dutton [17]. Responder spleen cells (7×10^6) were placed in 2 ml of medium per well, in Limbro culture plated (Limbro Scientific, Inc., Hamden, Conn.) together with 3.5×10^6 sensitizing irradiated (2000 R in a ^{60}Co irradiator, Hot Spot MKIV, Harwell, U.K.) allogeneic spleen cells. The suspensions were cultured for 5 days at 37°C in a humidified atmosphere of 5% CO_2.

^{51}Cr-*Microcytotoxicity* test. After 5–6 days of incubation the effector lymphocytes were harvested, washed once and resuspended in medium RPMI-1640 containing 20% Fetal Calf Serum (FCS), 10 mM Hepes Buffer, penicillin (100 units/ml) and streptomycin (100 μg/ml), and used as 'attacker cells' in a ^{51}Cr-release microcytotoxicity test [10, 18]. Tumor cells (5×10^6) suspended in 1 ml of RPMI-1640 supplemented as above were incubated for 45 min at 37°C with 200 μCi of $Na_2\ ^{51}CrO_4$ (sp. act. 200 mCi/mg, Radiochemical Centre, Amersham, U.K.), washed twice, counted and resuspended in the same medium. The spontaneous ^{51}Cr release (target cells incubated in medium alone) ranged from 8 to 13%. Of the labeled cell suspension 2.5×10^4 cells (0.1 ml) were mixed with 0.1 ml of the attacker cells suspension at the proper dilution.

After 4 hr of incubation in a CO_2 incubator at 37°C the microplates were centrifuged at 800 ***g*** for 10 min and 0.1 ml of the supernatant was collected and counted in a γ-scintillation counter (Packard model 5142) for 5 min.

Experimental results were expressed as percentage specific lysis in the experimental group. The percentage specific lysis was calculated as follows:

$$\%\ ^{51}\text{Cr specific release} = \frac{\text{cpm test} - \text{cpm AC}}{\text{cpm TC} - \text{cpm AC}} \times 100$$

(cpm = counts per minute;

AC = autologous control: labeled target cells incubated with cold autologous tumor cells, in place of equal numbers of

attacker lymphocytes. The ^{51}Cr release of labeled target cells of AC did not exceed 15% of total release, in all experiments;

TC = total count, 80% of the total radioactivity incorporated by the target cells).

All tests were performed in quadruplicate and the mean and the standard error were calculated.

Cold inhibition test. In order to test the specificity of the cytotoxic reaction [19], graded numbers of unlabeled tumor cells were added to the mixture of attacker–target cells at different effector–inhibitor cell ratios. Experimental results were expressed as the percentage of lysis in the presence of inhibitor cells.

RESULTS

Generation of highly-immunogenic DTIC-treated sublines of LSTRA lymphoma

Male BALB/c mice were inoculated with 10^6 LSTRA lymphoma cells, i.p., and treated with DTIC (100 mg/kg, i.p.), daily from day 1 through day 5 after challenge. On day 15 after tumor transplantation, ascitic lymphoma cells were collected from DTIC-treated mice, which survived 8 days longer than untreated controls. The tumor was inoculated (transplant generation 1) into 4 groups of 10 BALB/c mice each, in order to generate 4 DTIC-treated sublines, namely: LSTRA/DTIC-1, LSTRA/DTIC-2, LSTRA/DTIC-3 and LSTRA/DTIC-4. Non-treated control mice or mice immunodepressed with Cy (180 mg/kp, i.p., 6 hr before challenge) were included.

Following 4–8 transplant generations (Fig. 1) the majority of non-treated recipients (untreated controls) survived more than 60 days, whereas all mice treated with DTIC or immunodepressed with Cy died with generalized lymphoma.

All subsequent studies were therefore conducted using fully immunogenic DTIC sublines obtained after 8 transplant generations.

Primary and secondary cytotoxic responses against DTIC-treated sublines

Male BALB/c or $CD2F_1$ mice were sensitized i.p. with a single injection of 10^7 cells of LSTRA/DTIC-4 or L5178Y/DTIC, respectively. Immune spleen cells were collected on

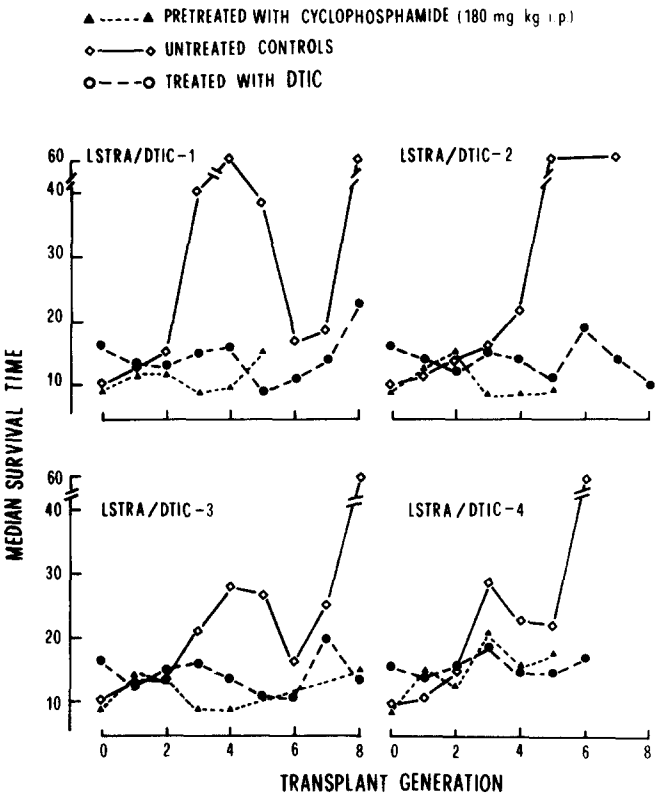

Fig. 1. Median survival time of BALB/c male mice serially transplanted with LSTRA/DTIC lines, untreated or treated with DTIC, or immunodepressed with CY before tumor transplantation. Six animals per point.

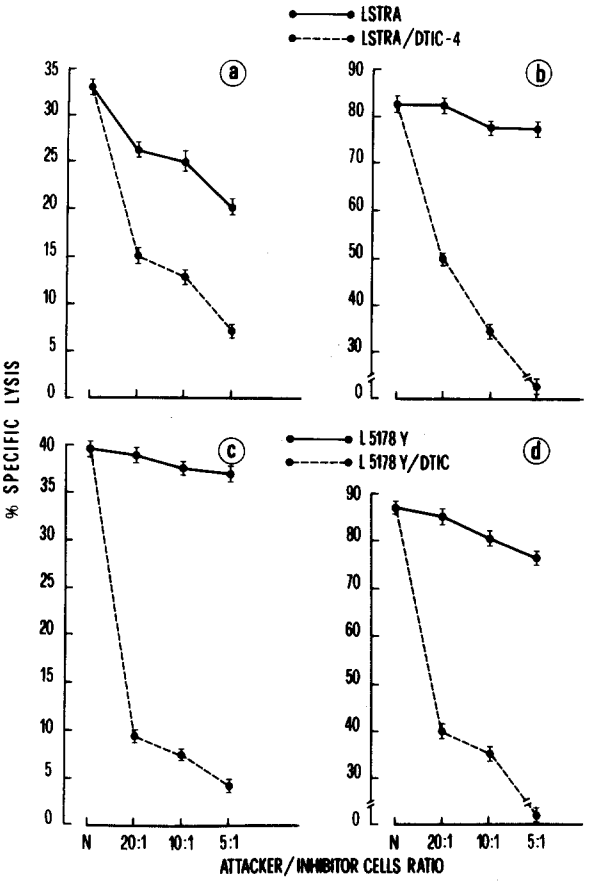

Fig. 2. Inhibition of primary and secondary cytotoxic responses against ^{51}Cr *labeled LSTRA/DTIC(a, b) and* L5178Y/*DTIC (c, d) cells be cold cells* in vitro. *(a) Effector cells = spleen cells of BALB/c mice immunized with* 10^7*cells i.p. of LSTRA/DTIC*-4, 10 days *before harvesting. (b) Effector cells = spleen cells of* $CD2F_1$ *mice* in vivo *primed with* 10^7 cells i.p. *of LSTRA/DTIC*-4 *lymphoma* (28 days *before harvesting) and cocultured* in vitro *with the same tumor cells* (R:S = 40:1). *(c) Effector cells = spleen cells of* $CD2F_1$ *mice immunized with* 10^7 cells *i.p. of* L5178Y/*DTIC*, 10 days *before harvesting. (d) Effector cells = spleen cells of* $CD2F_1$ *mice* in vivo *primed with* 10^7 cells *i.p. of* L5178Y/*DTIC lymphoma* (76 days *before harvesting) and cocultured* in vitro *with the same tumor cells* (R:S = 20/1). *N = no inhibitor cells.*

day 10 after sensitization and tested for cytotoxic activity *in vitro*. The results show that significant cytotoxic response was elicited by the tumors (Fig. 2a, c). The cold inhibition assay evidenced that marked inhibition of cytotoxicity was afforded by unlabeled DTIC-treated sublines, but not by parental lymphoma lines. Secondary cytotoxic response *in vitro* against LSTRA/DTIC-2 and LSTRA-DTIC-4 was obtained by coculturing irradiated cells of DTIC-subline with spleen cells of $CD2F_1$ mice, *in vitro* for 5 days, presensitized with 10^7 cells i.p. of the same tumor, 28 days before harvesting. As shown in Fig. 2(b), the anti-LSTRA/DTIC-4 cytotoxic response was higher than that obtained *in vivo*. Cold LSTRA/DTIC-4 cells were capable of inhibiting the lytic reaction, whereas no inhibition was detected when cold parental LSTRA cells were added. Similarly, when a secondary cytotoxic response was mounted *in vitro* against the L5178Y/DTIC subline, marked inhibition of cytotoxicity was only obtained by addition of cold L5178Y/DTIC tumor cells, but not of parental tumor cells (Fig. 2d). Comparable results were obtained with LSTRA/DTIC-2 cells (data not shown).

Cross-reactivity between LSTRA/DTIC line and other DTIC-treated lymphomas

Male $CD2F_1$ mice were sensitized with 10^7 cells i.p. of L5178Y/DTIC, L1210Ha/DTIC or LSTRA/DTIC-4 lines and male B10(pd/cz) animals were sensitized with the same number of LBD-1 cells i.p. Ten days later spleen cells were collected from immunized donors or non-sensitized controls and tested in a 4-hr cytotoxicity assay against ^{51}Cr-labeled LSTRA/DTIC-4 cells or lymphoma cells of the same line used for sensitization. The results illustrated in Table 1 show

Table 1. In vitro *cytotoxic activity against* ^{51}Cr-*labeled LSTRA/DTIC*-4 *cells mediated by lymphocytes sensitized* in vivo *with different DTIC-treated sublines*

Spleen cell donor	Sensitized against*	% Specific ^{51}Cr-release from target cells of:	
		Homologous tumor†	LSTRA/DTIC-4
$CD2F_1$	L5178Y/DTIC	26.9 ± 0.7	1.9 ± 0.4
$CD2F_1$	L1210Ha/DTIC	24.0 ± 2.2	3.1 ± 0.3
B10(pd/cz)	LBD-1	18.0 ± 7.4	3.2 ± 0.6
$CD2F_1$	LSTRA/DTIC-4	‡	22.8 ± 0.4
$CD2F_1$	—	—	4.3 ± 0.2
B10(pd/cz)	—	—	3.2 ± 0.5

*10^7 viable cells given 10 days before spleen collection.
†^{51}Cr-labeled cells of the same line used for sensitization.
‡See under LSTRA/DTIC-4, which is the homologous tumor.

that significant cytotoxic activity can be found in presensitized mice against the DTIC-treated sublines used for immunization. However, when labeled LSTRA/DTIC-4 cells were used as target cells, the cytotoxic activity of lymphocytes immune against the other DTIC lymphomas was marginal and did not exceed that of non-immune splenocytes.

Further studies were conducted using secondary cytotoxic response *in vitro*. Spleen cells of male $CD2F_1$ mice presensitized *in vivo* with 10^7 cells of L5178Y/DTIC line were collected on day +28 after sensitization and cocultured *in vitro* with the same tumor for 5 days. Attacker lymphocytes were then tested against ^{51}Cr-labeled L5178Y/DTIC and graded amounts of unlabeled L5178Y/DTIC or LSTRA/DTIC-4 cells were added to the attacker–target mixture in the cytotoxicity assay. The results, illustrated in Fig. 3, show that marked 'cold' inhibition of the specific cytotoxicity was afforded by L5178Y/DTIC line whereas little and presumably non-specific inhibition was produced by graded numbers of cold LSTRA/DTIC-4 cells.

Expression of H-2^d *alloantigens in parental and DTIC-treated sublines*

The expression of *H-2*^{d} antigens in both LSTRA and its DTIC-treated sublines was tested using cytotoxic lymphocytes generated by primary sensitization *in vitro*.

In the first series of experiments, effector cells carrying the entire *H-2*^{b} haplotype (B10(pd/cz)) or *K-IA-IB* regions of it, B10.A(5R), were sensitized against alloantigens specified by the *H-2* complex, or by *K-IA-IB-IJ-IE* or *IC-S-G-D* regions of the 'd' allele. Immune lymphocytes were tested against ^{51}Cr-labeled. *H-2*^{d} LSTRA lymphoma cells. The cold inhibition assay performed with LSTRA or LSTRA/DTIC-1 lines showed that no substantial differences in lysis inhibition could have been detected when cold parental or DTIC-treated cells were used. On the other hand, the antigenically unrelated cold RBL-5 cells did not afford substantial inhibition of the immune cytolysis when the cell concentration did not exceed that corresponding to the attacker–inhibitor cell ratio of 4 : 1 (Fig. 4). Similar findings were obtained when effector lymphocytes carrying the *H-2*^{k} haplotype (C3H) or *K-IA-IB-IJ-IE* regions of it (B10.A), immune against antigens specified by K^b-IA^d-IB^d-IJ^d-IE^d or IC^d-S^d-G^d-D^d regions, were used (Fig. 5).

Further studies were conducted using LSTRA and the other DTIC-treated sublines in the cold inhibition assay to compare their levels of histocompatibility antigens expressed by selected regions of this complex. Effector lymphocytes sensitized against K^d-IA^d-IB^d-IJ^d-IE^d or IC^d-S^dG^d-D^d alloantigens were tested against labeled *H-2*^{d} LSTRA. The cold inhibition assay performed with LSTRA, LSTRA/DTIC-2, LSTRA/DTIC-3 or LSTRA/DTIC-4 cells (Fig. 6) did not reveal substantial differences in lysis reduction afforded by parental or DTIC-treated lines.

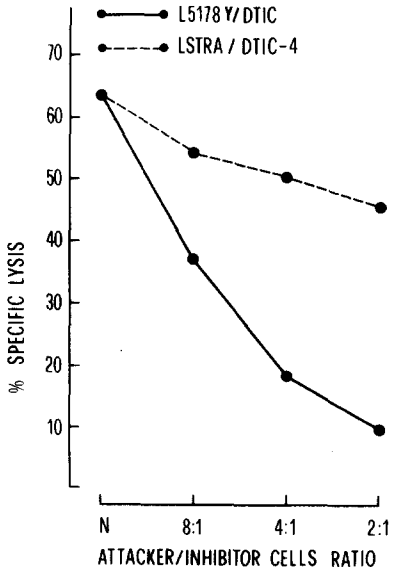

Fig. 3. Secondary cytotoxic response of $CD2F_1$ *lymphocytes against* L5178Y/*DTIC cells. Cold inhibition by graded numbers of non-labeled cells of the same lymphoma or LSTRA/DTIC-4 line. N=no inhibitor cells.*

Search for the appearance of foreign H-2 *specificities in a DTIC-treated subline*

The expression of foreign *H-2* determinants on tumor cell was investigated using both L5178Y and L5178Y/DTIC lymphoma cells. $CD2F_1$ effector cells (*H-2*^{d}) sensitized *in vitro* against alloantigens specified by the *H-2*^{b} (C57BL/10) or *H-2*^{k} (C3H) complex were tested against ^{51}Cr-labeled RBL-5 or 6C3HED tumor cells, respectively. The cold inhibition assay performed with L5178Y and L5178Y/DTIC lines is depicted in Fig. 7 (a and b). In no case did L5178Y or L5178Y/DTIC inhibitor cells reduce the specific lysis. Moreover, no cytotoxic effect was observed when the same effector lymphocytes were tested against ^{51}Cr-labeled L5178Y/DTIC target cells (Table 2). On the other hand, the lysability of the L5178Y/DTIC line was confirmed by the high specific immune lysis obtained when the labeled target

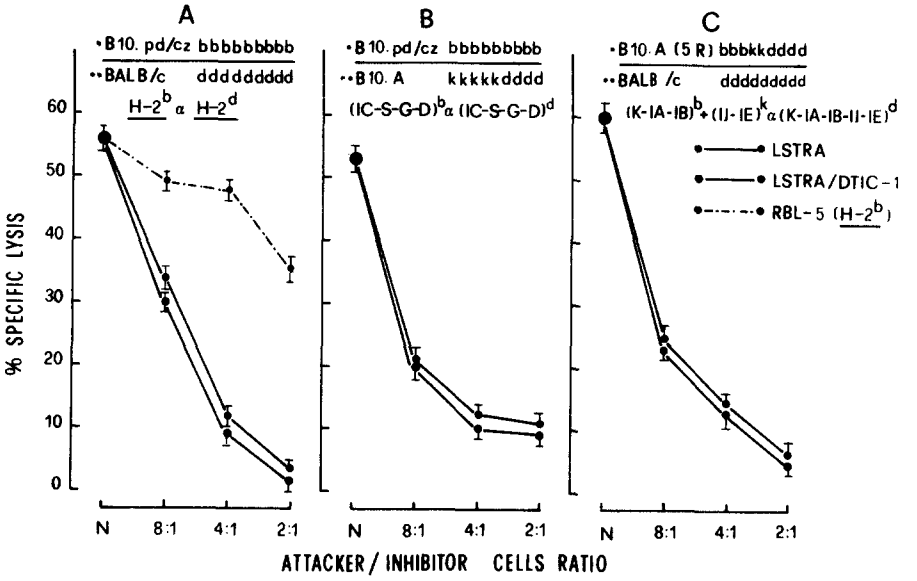

*Fig. 4. Cold cell inhibition of cytotoxicity of sensitized attacker cells against labeled LSTRA cells. Graded concentrations of cold inhibitory LSTRA, LSTRA/DTIC-1 or RBL-5 (H-2^b control) cells were used. (A) B10(pd/cz) attacker cells sensitized against BALB/c lymphocytes. Analysis of 'd' alloantigens recognized by 'b' responders for the entire H-2 complex. (B) B10(pd/cz) attacker cells sensitized against B10.A lymphocytes. Analysis of 'd' alloantigens recognized by 'b' responders for IC-S-G-D regions of H-2. (C) B10A(5R) attacker cells sensitized against BALB/c lymphocytes. Analysis of 'd' alloantigens recognized by 'b' responders for K-IA-IB regions of H-2, and 'k' responders for IJ-IE regions of H-2. * = responder cells; ** = sensitizing cells; N = no inhibitor cells.*

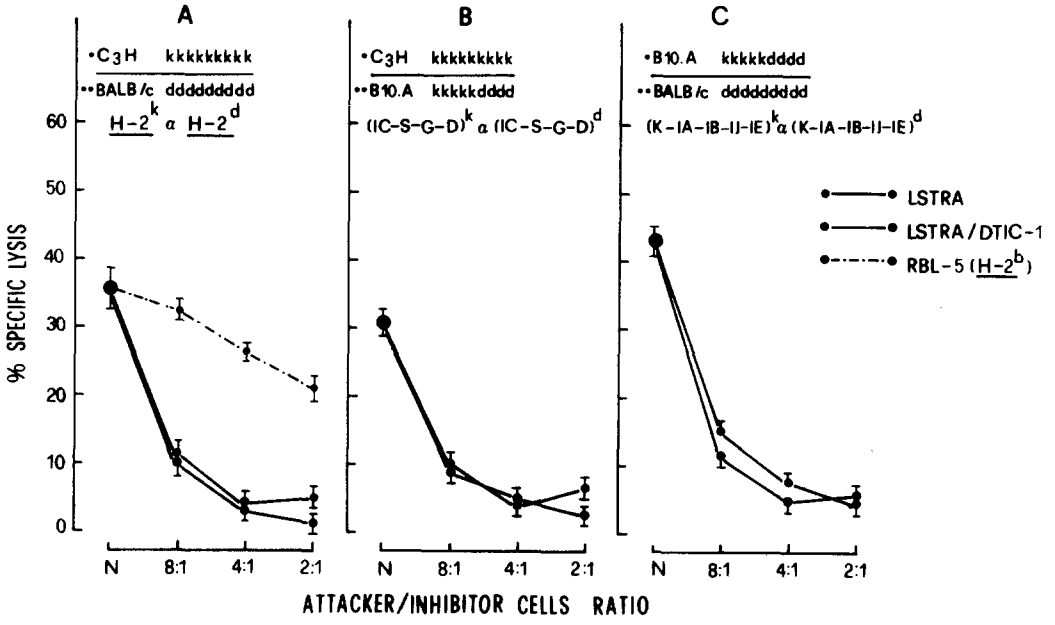

*Fig. 5. Cold cell inhibition of cytotoxity of sensitized attacker cells against labeled LSTRA cells. Graded doses of cold inhibitory LSTRA, LSTRA/DTIC-1 or RBL-5 (H-2^b control) cells were used. (A) C3H attacker cells sensitized against BALB/c lymphocytes. Analysis of 'd' alloantigens recognized by 'k' responders for the entire H-2 complex. (B) C3H attacker cells sensitized against B10.A lymphocytes. Analysis of 'd' alloantigens recognized by 'k' responders for IC-S-G-D regions of H-2. (C) B10.A attacker cells sensitized against BALB/c lymphocytes. Analysis of 'd' alloantigens recognized by 'k' responders for K-IA-IB-IJ-IE regions of H-2. * = responder cells; ** = sensitizing cells; N = no inhibitor cells.*

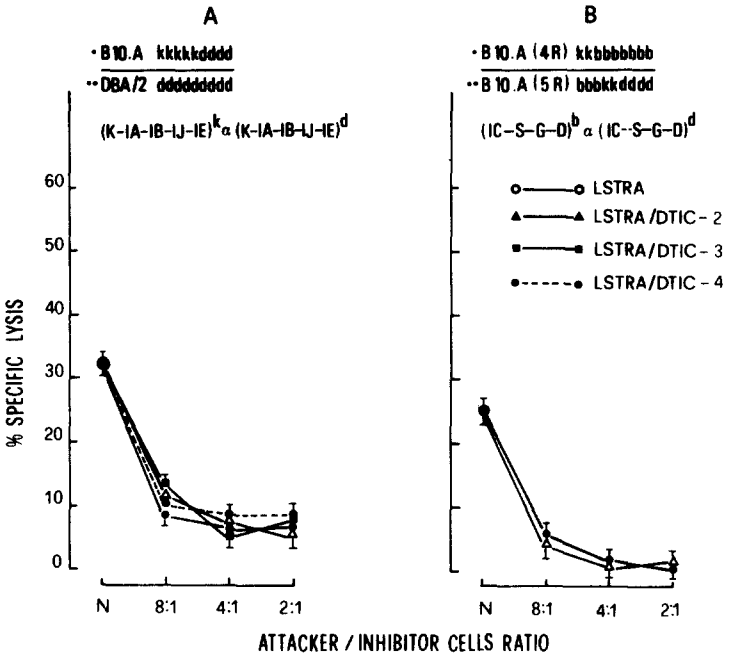

Fig. 6. Cold cell inhibition of cytotoxicity of sensitized attacker cells against labeled LSTRA cells. Graded concentration of cold inhibitory cells of LSTRA or DTIC-treated sublines were used. (A) B10.A *attacker cells sensitized against DBA/2 lymphocytes. Analysis of 'd' alloantigens recognized by 'k' responders for* K-IA-IB-IJ-IE *regions of* H-2. *(B)* B10.A (4R) *attacker cells sensitized against* B10.A(5R) *lymphocytes. Analysis of 'd' alloantigens recognized by 'b' responders for* IC-S-G-D *regions of* H-2. ** = responder cells; ** = sensitizing cells; N = no inhibitor cells.*

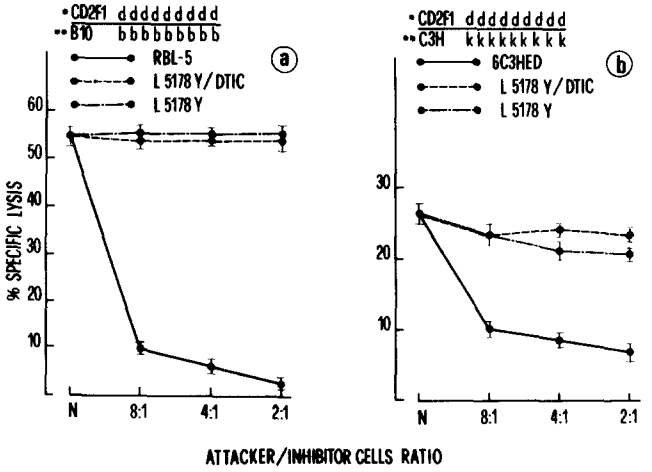

Fig. 7. Cold inhibition assay for the presence of foreign H-2 *alloantigens on* L5178Y/*DTIC lymphoma cells. (a)* $CD2F_1$ *attacker cells sensitized against* B10 *lymphocytes and tested against RBL-5* (H-2^b) *labeled target cells. (b)* $CD2F_1$ *attacker cells sensitized against C3H lymphocytes and tested against* 6C3HED (H-2^k) *labeled target cells. * = responder cells; ** = sensitizing cells; N = no inhibitor cells.*

Table 2. Lysis of L5178Y/*DTIC target lymphoma cells produced by allogeneic cytotoxic lymphocytes*

Responder cells	Sensitizer cells	% ^{51}Cr release*
$CD2F_1$	B10 spleen cells	1.7±0.8
$CD2F_1$	C3H spleen cells	0.8±0.3
B10†	L5178Y/DTIC tumor cells	47.2±1.2

*Attacker to target cell ratio was 40:1.
†Spleen cells of C57Bl/10 mice cocultured with X-irradiated (5.000 rad *in vitro* delivered by ^{60}Co irradiator) L5178Y/DTIC lymphoma cells at R:S ratio of 40:1.

cells were incubated with B10 (*H-2*^{b}) lymphocytes, sensitized *in vitro* for 5 days against the same tumor cells (R:S=40:1).

DISCUSSION

In the present study four immunogenic sublines were obtained by treating LSTRA lymphoma with DTIC for 4–8 transplant generations. Full and relatively stable immunogenicity was obtained after a limited number of transplant generations, as previously described for other LSTRA/DTIC sublines [11].

Direct cytotoxicity of immune anti-LSTRA/DTIC lymphocytes tested against parental or DTIC-treated subline or cold inhibition assay with both lines (Fig. 2) suggested that LSTRA/DTIC cells express antigenic specificity(ies) not detectable in the parental LSTRA lymphoma. This is consistent with previous findings *in vivo* showing that DMTA can be demonstrated in DTIC-treated LSTRA [11]. Expression of DMTA has been also found in the present study (Fig. 2) in a highly immunogenic DTIC-treated subline of L5178Y lymphoma, thus confirming previous results obtained in *in vitro* cytotoxicity experiments performed with this line [20]. Moreover, further studies conducted with the same DTIC-treated lymphoma subline showed that the immunogenic tumor cell population is composed of leukemic clones possessing distinct antigenic specificities [7].

The preliminary studies illustrated in the present report add the finding that antigenic cross-reactivity cannot be easily detected also among DMTA of DTIC-treated lines generated from different parental lymphomas carrying either the *H-2*^{d} or the *H-2*^{b} haplotypes.

The presence of DMTA on tumor cell membrane might interact with normal antigens in several ways. The new antigenic specficity(ies) could be associated or placed close to normal determinants leading to changes of their reactivity. Moreover, the emergence of new antigens on the cell membrane could be balanced by a decrease of the amount of the other normal surface antigens [19]. In addition, the presence of tumor-associated transplantation antigens in carcinogen-induced neoplastic cells has been shown to be associated with the expression of foreign *H-2* specificities in a mouse model [8].

The major histocompatibility complex specifies a discrete portion of membrane antigens and can be subjected to detailed antigenic analysis. Therefore, the study on the possible changes in the expression of the *H-2* antigens in tumor cells carrying DMTA was considered of potential interest. In particular, it has been suggested that the appearance of DMTA would result from DTIC-induced somatic mutation(s), leading to novel cell surface antigens [22]. If the mutation affected the *H-2* gene(s) responsible for the expression of strong transplantation antigens, significant changes in the reactivity of one or more determinants of the *H-2* antigenic makeup could be expected to take place in DTIC-treated lymphoma cells. However, the results of the antigenic analysis carried out with cell-mediated immunity *in vitro* did not evidence substantial differences of the expression of *H-2* antigens between parental and DTIC-treated LSTRA sublines.

In the first set of experiments the lysis of *H-2*^{d} lymphoma cells produced *in vitro* by *H-2*^{b} or *H-2*^{k} allogeneic lymphocytes sensitized against the entire *H-2*^{d} complex was equally inhibited by parental or DTIC sublines, but not by the genetically unrelated *H-2*^{b} RBL-5 cells (Figs. 4a and 5a). This would indicate that the lymphomas tested carry the same density of *H-2*^{d} antigens on cell membrane, recognized by *H-2*^{d} or *H-2*^{k} lymphocytes as

suggested by previous reports [6, 23]. However, it was considered that changes of the amount of antigens specified by one region of the *H-2*^{d} complex could have been balanced by opposite changes of the same complex. More detailed analysis concerning the levels of antigenicity associated with *K-IA-IB*, *IC-S-D* or *D* regions of *H-2*^{d} was performed on LSTRA and DTIC-treated sublines. Again, marginal or no differences were detected according to the experiments illustrated in Figs. 4, 5 and 6.

The possibility that DTIC-mediated somatic mutation would have affected the pattern of the *H-2* complex of lymphoma cells, in terms of generation of alien *H-2* specificities, has been tested with the L5178Y/DTIC line. The data of Fig. 7 and Table 2 show that no foreign *H-2*^{b} or *H-2*^{k} alloantigens recognizable by cytotoxic lymphocytes could be demonstrated in the L5178Y/DTIC line. Although no exhaustive analysis of foreign *H-2* specificities has been carried out, these data do not support the hypothesis that DMTA could be identified with alien histocompatibility antigens. Moreover, the lack of obvious antigenic cross-reactivity among different DTIC-treated sublines (Table 1, Fig. 3) speaks against the possibility that common foreign *H-2* specificity(ies) could be generated by DTIC treatment either in *H-2*^{d} (i.e., L5178Y/DTIC, L1210Ha/DTIC) or *H-2*^{b} (i.e., LBD-1) lymphoma lines. Therefore, these findings seem to provide further support for the hypothesis that DTIC treatment *in vivo* does not affect the genetic profile of the *H-2* complex.

In conclusion, the results of the studies described in the present communication showed that the presence of DMTA on DTIC-treated lymphoma cells did not interfere with the reactivity and the specificity of surface antigens specified by selected regions of *H-2*^{d} complex and recognized by cytotoxic lymphocytes. This finding suggests also that no interaction between DMTA and *H-2* alloantigens would occur on the tumor cell surface. It follows that the present study does not support the hypothesis that possible DTIC-mediated somatic mutation(s) would take place within the *H-2* region, altering profoundly the structure of normal determinants of strong transplantation alloantigens.

However, further studies on the *H-2*^{d} specificities detectable by specific alloantisera and by allogeneic cytotoxic lymphocytes carrying *H-2* haplotypes other than *H-2*^{b} or *H-2*^{k} would be required in order to provide a possible additional support to the present suggestion.

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REFERENCES

1. E. Bonmassar, A. Bonmassar, S. Vadlamudi and A. Goldin, Immunological alteration of leukemia cells *in vitro* after treatment with an antitumor drug. *Proc. Nat. Acad. Sci.* (*Wash.*) **66,** 1089 (1970).
2. M. C. Fioretti, Immunopharmacology of 5-(3, 3'-dimethyl-1-triazeno)-imidazole-4-carboxamide (DTIC). *Pharm. Res. Commun.* **7,** 481 (1975).
3. A. Nicolin, A. Bini, E. Coronetti and A. Goldin, Cellular immune response to a drug-treated L5178Y subline. *Nature* (*Lond.*) **251,** 654 (1974).
4. M. C. Fioretti, L. Romani, D. Taramelli and A. Circolo, Risposta immune citotossica del topo verso un linfoma di origine virale, alterato antigenicamente mediante trattamento chemioterapico *in vivo*. *Ann. Med.* (*Perugia*) **67,** 35 (1976).
5. A. Santoni, Y. Kinney and A. Goldin, Secondary cytotoxic response *in vitro* against Moloney lymphoma cells antigenically altered by drug treatment *in vivo*. *J. nat. Cancer Inst.* **60,** 109 (1978).
6. D. Taramelli, L. Romani, M. C. Fioretti, E. Bonmassar and A. Goldin, Normal and drug-mediated transplantation antigens (DMTA) of drug-treated murine lymphomas. *Proc. Amer. Ass. Cancer Res.* **19,** 147 (1978).
7. M. C. Fioretti, L. Romani, D. Taramelli and A. Goldin, Antigenic properties of lymphoma sublines derived from a drug treated immunogenic L5178Y leukemia. *Transplantation* **26,** 449 (1978).

8. G. Parmiani, A. Meschini, G. Invernizzi and G. Carbone, Tumor-associated transplantation antigens distinct from $H\text{-}2^k$-like antigens on a BALB/c ($H\text{-}2^d$) fibrosarcoma. *J. nat. Cancer Inst.* **61,** 1229 (1978).
9. P. Alexander, D. I. Connel and Z. B. Mikulska, Treatment of a murine leukemia with spleen cells on sera from allogeneic mice immunized against the tumor. *Cancer Res.* **26,** 1508 (1966).
10. J. P. Glynn, A. R. Bianco and A. Goldin, Studies on induced resistance against iso-transplants of virus-induced leukemia. *Cancer Res.* **24,** 502 (1964).
11. D. P. Houchens, E. Bonmassar, M. R. Gaston, M. Kende and A. Goldin, Drug-mediated immunogenic changes of tumor cells *in vivo*: studies with virus-induced leukemias. *Cancer Res.* **36,** 1347 (1976).
12. A. Nicolin, F. Spreafico, E. Bonmassar and A. Goldin, Antigenic changes of L5178Y lymphoma after treatment with 5-(3, 3'-dimethyl-1-triazeno)-imidazole-4-carboxamide *in vivo*. *J. nat. Cancer Inst.* **56,** 89 (1976).
13. E. Bonmassar, A. Bonmassar, S. Vadlamudi and A. Goldin, Antigenic changes of L1210 leukemia in mice treated with 5-(3, 3'-dimethyl-1-triazeno)-imidazole-4-carboxamide. *Cancer Res.* **32,** 1445 (1972).
14. M. C. Fioretti, A. Santoni, L. Romani, D. Taramelli, M. C. Caprino, A. Goldin and E. Bonmassar, Transplantation resistance of syngeneic mice against a drug-treated lymphoma derived from non-immunogenic tumor line. *Fed. Proc.* **36,** 1260 (1977).
15. R. B. Herberman, T. Aoki, M. Nunn, D. H. Lavrin, N. Soares, A. Gazdar, H. Holden and K. S. S. Chang, Specificity of ^{51}Cr-release cytotoxicity of lymphocytes immune to murine sarcoma virus. *J. nat. Cancer Inst.* **53,** 1103 (1974).
16. W. V. Garner, A. Kirschbaum and L. C. Strong, Lymphoid tumors in mice receiving estrogens. *Arch Path.* **29,** 1 (1940).
17. T. I. Mishell and R. W. Dutton, Immunization of dissociated spleen cell cultures from normal mice. *J. exp. Med.* **126,** 423 (1967).
18. P. W. Wright, M. Ortiz de Landazuri and R. B. Herberman, Immune response to Gross virus-induced lymphoma: comparison of two *in vitro* assays of cell-mediated immunity. *J. nat. Cancer Inst.* **50,** 943 (1973).
19. M. Ortiz de Landazuri and R. B. Herberman, Specificity of cellular immune reactivity to virus-induced tumors. *Nature New Biol.* **238,** 18 (1972).
20. L. Romani, M. C. Fioretti and E. Bonmassar, *In vitro* generation of primary cytotoxic lymphocytes against L5178Y leukemia antigenically altered by 5-(3, 3'-dimethyl-1-triazeno)-imidazole-4-carboxamide *in vivo*. *Transplantation* **28,** 218 (1979).
21. M. Cikes, S. Friberg Jr. and G. Klein, Progressive loss of *H-2* antigens with concomitant increase of cell-surface antigen(s) determined by Moloney leukemia virus in culture murine lymphomas. *J. nat. Cancer Inst.* **50,** 347 (1973).
22. A. Giampietri, M. C. Fioretti, A. Goldin and E. Bonmassar, Antigenic changes of murine leukemia cells following treatment with 5-(3, 3'-dimethyl-1-triazeno)-imidazole-4-carboxamide (DTIC) *in vivo*: antagonistic effects of quinacrine, an antimutagenic compound. *J. nat. Cancer Inst.* **64,** 321 (1980).
23. A. Nicolin, P. Franco, C. Testorelli and A. Goldin, Immunosensitivity and histocompatibility antigens in drug-altered leukemia cells. *Cancer Res.* **36,** 222 (1976).