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Drug-mediated Immunogenic Changes of Virus-induced Leukemia *in Vivo*

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SUMMARY

LSTRA and RBL-5 lymphomas induced by Moloney and Rauscher leukemia viruses, respectively, were used to determine whether antigenically altered tumors induced by 5-(3,3-dimethyl-1-triazeno)-imidazole-4-carboxamide *in vivo* would retain their original antigenic properties and/or have new antigenic properties. The tumors became highly immunogenic in the syngeneic hosts after 4 to 8 transplant generations with drug treatment. Syngeneic mice could be protected against challenge with the parental tumor by presensitization with the drug-altered sublines while unrelated tumor lines were incapable of protecting them. The drug-altered subline of LSTRA was used for treatment of the LSTRA in conjunction with chemotherapy, and this immunochemotherapy produced significant increases in number of survivors and increases in median survival time compared to either treatment alone. Tolerance studies indicated that there are novel antigens and parental tumor antigens associated with the drug-treated sublines.

INTRODUCTION

Increased immunogenicity of mouse lymphoma cells of DBA/2 or C57BL/10 origin was obtained following treatment with antitumor agents *in vivo* (2, 3, 5-7, 14, 17). It was found that one of the most active compounds was DIC.[2] Sublines of various tumors treated with DIC *in vivo* for a number of transplant generations in mice compatible with the parental tumor were totally rejected by nonimmunosuppressed hosts (2-4). Cell-mediated immune responses against DIC-treated tumor cells were demonstrated *in vitro* (14). The kinetics of peritoneal growth and rejection of DIC-treated leukemia sublines was similar to that detectable with *H-2*-incompatible tumors (9). In addition, studies conducted with adoptive transfer of immune lymphocytes indicated the appearance of new antigens on L1210 or L5178Y lymphoma following 6 to 8 transplant generations in DIC-treated recipient mice (15, 16).

Experiments performed in conventional or athymic (nude) mice indicated that highly immunogenic lymphoma sublines retained at least in part the TATA present in the tumor line of origin (7). In other studies transplantation resistance of various degrees was found in C57BL/6 and BALB/c mice against lymphomas induced by Friend leukemia virus, MLV, and RLV (8, 10, 11, 13). The current studies were performed to establish whether antigenic changes induced by drug *in vivo* would produce tumor lines retaining their original virus-dependent immunogenicity and exerting novel antigenic properties.

Two lymphomas induced by MLV and RLV of BALB/c and C57BL/6, respectively, were used. The results indicated that DIC treatment *in vivo* produced antigenic changes of tumor cells without abolishing their original TATA of virus origin.

MATERIALS AND METHODS

Animals. Inbred male C57BL/6 Cr *(H-2^b)*, BALB/c Cr *(H-2^d)*, B10.129 (5M) Cr *(H-2^b)*, and athymic BALB/c/A/Bom Cr (*H-2^d* nu/nu, "nude" strain on BALB/c background, hereafter referred to simply as nude) mice, and hybrid male BALB/c Cr × DBA/2 Cr F_1 (CD2F_1, *H-2^d/H-2^d*) mice, 8 to 10 weeks old, were supplied by the Animal Production Section, Drug Research and Development, Division of Cancer Treatment, National Cancer Institute.

Tumors. Three ascitic lymphoma lines, LSTRA induced by MLV in BALB/c mice (10), MBL-2 and RBL-5 induced by MLV and RLV, respectively, in C57BL/6 mice (13), and a splenic radiation-induced lymphoma L5MF-22 of B10.129 (5M) origin, were used. For tumor transplantation, ascites cells were diluted with Medium 199 at the desired concentration and injected i.p. in a volume of 0.2 ml, whereas spleen cells suspended in the same medium were injected i.v. in a volume of 0.5 ml. Mortality of mice was recorded for at least 60 days after transplantation and lymphoma growth was documented at gross autopsy.

DIC-treated sublines derived from LSTRA (LD-1 and LD-2) and RBL-5 (RD-1, RD-2) were obtained as follows. Mice were inoculated with 10^6 cells (LD-1, LD-2, RD-1) or 10^7 cells (RD-2) i.p. of the parental line and treated with DIC (100 mg/kg/day i.p.) from Day 1 through Day 5 (RD-1, RD-2) or through Day 7 [LD-1, LD-2 (generation 0)]. When ascites developed, tumor cells were collected and transplanted i.p. into 2 groups of mice: *(a)* untreated or *(b)* treated with DIC (generation 1). The same schedule was used for each transplant generation of DIC-treated lymphoma sublines, tumor cells always being collected from DIC-treated donor mice.

A highly immunogenic DIC-treated subline L5178Y/DIC, derived from L5178Y lymphoma (of DBA/2 origin) was obtained through the courtesy of Dr. A. Nicolin (Institute of

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[2] The abbreviations used are: DIC, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; TATA, tumor-associated transplantation antigens; MLV, Moloney leukemia virus; RLV, Rauscher leukemia virus; CY, cyclophosphamide; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea.

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Pharmacology, University of Milan, Italy) and maintained through serial transplantation in DIC-treated (100 mg/kg/day for 10 days) $CD2F_1$ mice.

Drugs. CY, DIC, and BCNU were provided by the Drug Research and Development Branch, National Cancer Institute, NIH. CY and BCNU were dissolved in 0.9% NaCl solution immediately before use and kept in an ice bath prior to injection. DIC:citric acid:mannitol [1:1:0.375 by weight (preparation for clinical use)] was dissolved in distilled water, kept at 4°, protected from light, and used within 3 days. The drug concentration was adjusted to inject the volume of 0.01 ml/g of body weight.

Irradiation. Tumor cells were exposed to X-rays *in vitro*, using a Westinghouse Quadrocondex X-ray machine, 200 kV, 15 ma, at a distance of 25 cm using 0.25-mm Cu and 0.55-mm Al filters, at a dose rate of 661 R/min.

RESULTS

Immunogenic Properties of DIC-treated Sublines. Chart 1 reports the median survival time and the percentage of long-term survivors of BALB/c mice inoculated with parental LSTRA, LD-1, or LD-2 DIC-treated sublines and C57BL/6 mice inoculated with parental RBL-5, RD-1, or RD-2 DIC-treated sublines over a series of transplant generations. Following 4 to 8 transplant generations of DIC treatment, all tumor lines were highly immunogenic for the host of origin since untreated conventional mice lived longer and showed a higher percentage of long-term survivors than did corresponding immunodepressed hosts (*i.e.*, DIC-treated or immunodepressed with CY (180 mg/kg) before tumor inoculation, or nude mice). These results were reproducible in other transplants. One exception was seen at transplant generation 12 of the LD-2 subline, where all BALB/c mice untreated or pretreated with CY survived beyond the 60-day observation period, whereas all DIC-treated animals succumbed with generalized leukemia. One subline derived from LSTRA (LD-2) and one from RBL-5 (RD-2) showed marked fluctuation in growth behavior and a decline of immunogenicity beginning at the 10th transplant generation.

To detect early changes of the immunogenic properties of DIC-treated cells, 2 additional groups of mice were used at various transplant generations of the LD-1 and LD-2 lines. One group was treated with BCNU (10 mg/kg i.p.) on Day 5

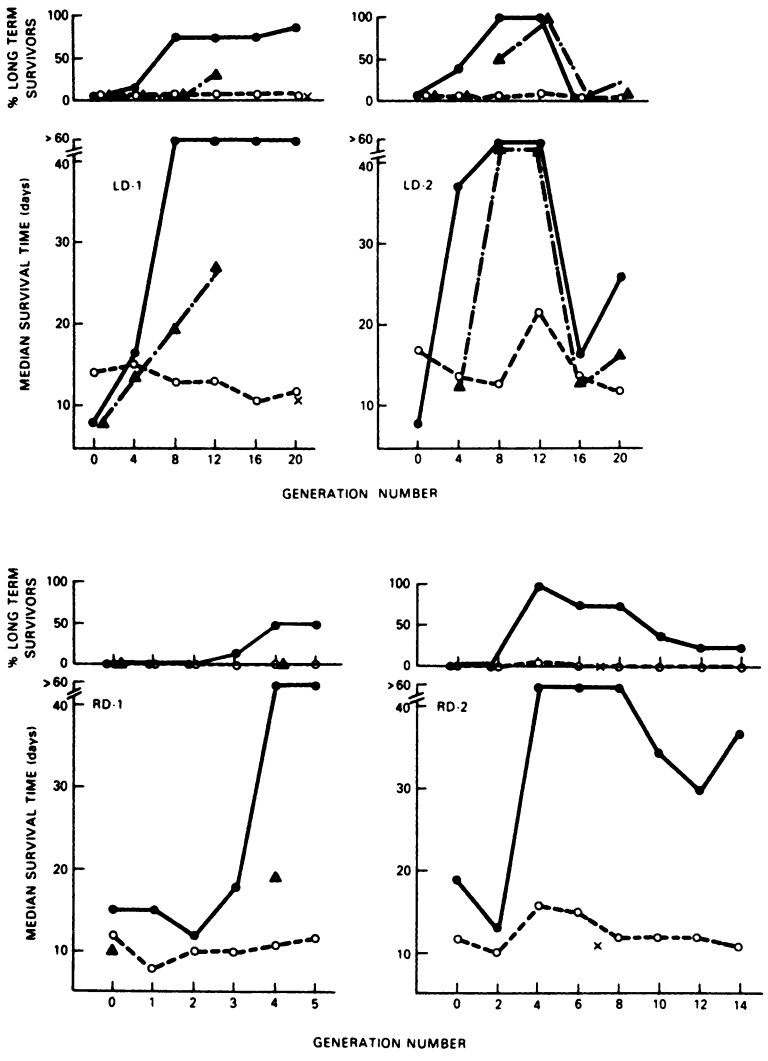

Chart 1. Median survival time and percentage of long-term survivors of animals receiving DIC-treated sublines of LSTRA (LD-1, LD-2) and RBL-5 (RD-1, RD-2) tumors. Mice were inoculated with 10^6 tumor cells from DIC-treated mice at each transplant generation. At generation 0 the mice were inoculated with the parental LSTRA or RBL-5 lymphomas. ●, untreated mice; ▲, CY (180 mg/kg) administered 5 hr prior to the tumor; ○, DIC (100 mg/kg/injection) administered Days 1 to 7 after the tumor; ×, nude mice.

after challenge and the other was subjected to the same treatment but was immunodepressed with CY (180 mg/kg i.p.) 6 hr before tumor challenge. This experimental design tends to reveal minor tumor-host histocompatibility differences (4) since a marked increase in therapeutic effectiveness occurs when BCNU treatment is associated with a host antitumor immune reaction. On the other hand, such an increase would be reduced when the immunological reactivity of the host is diminished by CY-induced immunodepression. As reported in Table 1, BCNU (10 mg/kg) produced only a 2-day increase in the median survival time of mice bearing the parental LSTRA lymphoma. BCNU was highly effective in both DIC-treated sublines even at the 1st transplant generation. In addtion BCNU treatment was more effective in nonpretreated than in immunodepressed recipients.

Cross-reactivity between DIC-treated Sublines and Parental Tumors. BALB/c, C57BL/6, and $CD2F_1$ mice were presensitized with 10^6 viable cells of allogeneic parental lymphomas or DIC-treated sublines derived from syngeneic or allogeneic tumor lines. After sensitization the mice were challenged with graded inocula of syngeneic parental tumor cells. The results (Table 2) indicate that: *(a)* the growth of LSTRA lymphoma in compatible mice was strongly inhibited by presensitization of recipient animals with LD-2 (DIC-treated subline of LSTRA) but not by presensitization with allogeneic RBL-5 lymphoma or DIC-treated subline of unrelated L5178Y tumor; *(b)* the growth of RBL-5 in C57BL/6 mice was modestly but significantly impaired by presensitization with the RBL-5-DIC-derived RD-2 subline, which inhibited also the growth of the cross-reactive MBL-2 (11, 13) in the same mice. Significant protection against RBL-5

Table 1

Mortality data of BALB/c mice inoculated with 10^6 cells i.p. of parental LSTRA line or DIC-treated sublines, immunodepressed or not, subjected to DIC or BCNU treatment after challenge

Tumor line	Transplant generation	Untreated		Treated with DIC[a]		Treated with BCNU[b]			
						Nonpretreated		Pretreated with Cy[d]	
		MST[c]	D/T	MST	D/T	MST	D/T	MST	D/T
LSTRA		8	8/8	14	7/7	10	8/8	9	8/8
LD-1[e]	1	10	8/8	15	7/7	38.5	6/8	17	8/8
	3	12	8/8	11	5/5		3/8	28	8/8
	4	16.5	8/8	15	7/7		2/8	18.5	6/8
LD-2[e]	1	13	8/8	16.5	6/7		3/8	27	6/8
	3	19	7/7	17.5	6/6		4/8	48.5	6/8
	5		4/8	15	7/7		0/8	28	6/8

[a] 100 mg/kg/day i.p. from Day 1 through Day 7 after tumor inoculation.
[b] 10 mg/kg i.p. on Day 5 after challenge.
[c] MST, median survival time; D/T, dead mice/total tested.
[d] 180 mg/kg i.p. 6 hr before tumor inoculation, to induce immunodepression.
[e] DIC-treated subline of LSTRA lymphoma.

Table 2

Transplantation resistance induced by presensitization of recipient mice with 10^6 viable cells of allogeneic parental tumor lines or of DIC-treated sublines

Strain	Challenging tumor	Cell dose (i.p.)	Nonpresensitized		Presensitized with viable:[a] LSTRA		LD-2 (generation 8)		RBL-5		RD-2 (generation 8)		L5178Y/DIC	
			MST[b]	D/T[c]	MST	D/T	MST	D/T	MST	D/T	MST	D/T	MST	D/T
BALB/c	LSTRA	10^7	7	8/8	S		12	5/8[c]	7	10/10	NT		NT	
BALB/c	LSTRA	10^6	8	8/8	S		22.5	5/8[c]	9	9/10	NT		NT	
BALB/c	LSTRA	10^5	9	8/8	S		36	5/8	10	9/10	NT		9	7/7
$CD2F_1$	LSTRA	10^6	10	8/8	S			3/8[c]	NT		NT		9	8/8
C57BL/6	RBL-5	10^7 [d]	12.5	6/8	3/7[c]			0/8[c]	S		NT		NT	
C57BL/6	RBL-5	10^7 [d]	12	8/8	NT		NT		S		15	5/5[c]	NT	
C57BL/6	MBL-2	10^6	14.5	8/8	NT		NT		S		18	4/4[c]	NT	
C57BL/6	MBL-2	10^5	17	8/8	NT		NT		S		32	5/5[c]	NT	

[a] 10^6 cells 2 to 4 weeks before tumor challenge.
[b] MST, median survival time; D/T, dead mice/total tested; S, not tested since tumor is syngeneic and therefore lethal for the host; NT, not tested.
[c] Statistically significant difference in survival time compared with that of nonsensitized controls ($p < 0.01$, according to Mann-Whitney *U* test).
[d] Two different experiments.

was afforded also by presensitization with LSTRA and its DIC-treated subline, LD-2.

Other experiments (Ref. 7; D. P. Houchens and E. Bonmassar, unpublished data) indicate that LD-1 is also capable of sensitizing BALB/c mice and that the protection afforded by LD-1 and LD-2 against a subsequent challenge with graded inocula of LSTRA lymphoma cells, lasted longer with LD-5 (5 months) than with LD-1 (1 to 2 months).

Since cross-reactivity was found between DIC-treated sublines and their parental tumors, immunochemotherapy experiments were performed, injecting the LD-1 subline after LSTRA challenge in conjunction with chemotherapeutic treatment. The results of a typical experiment are reported in Chart 2. BALB/c mice were challenged with 10^3 cells of LSTRA lymphoma i.p. (Day 0). On Day 1 or Day 7 after challenge the mice received a single injection of 5×10^6 viable cells of the LD-1 subline or of the unrelated L5MF-22 allogeneic lymphoma. On Day 3 chemotherapeutic treatment was performed with a single injection of graded doses of BCNU i.p. Appropriate untreated controls or mice subjected to immunotherapy or chemotherapy alone were also included. While immunotherapy alone was ineffective, therapeutic synergism was observed when specific immunotherapy with LD-1 (given 1 day after challenge) was associated with BCNU chemotherapy, particularly in mice treated with BCNU (18 mg/kg; Chart 2, Group 1). No synergism occurred when LD-1 was given after BCNU therapy or when the unrelated allogeneic L5MF-22 lymphoma was administered before or after BCNU treatment. At 30 mg/kg, BCNU alone or preceded by specific or nonspecific immunotherapy was curative for the majority of mice. When immunotherapy followed treatment with BCNU (30 mg/kg), most of the mice succumbed with generalized lymphoma within 25 days after challenge.

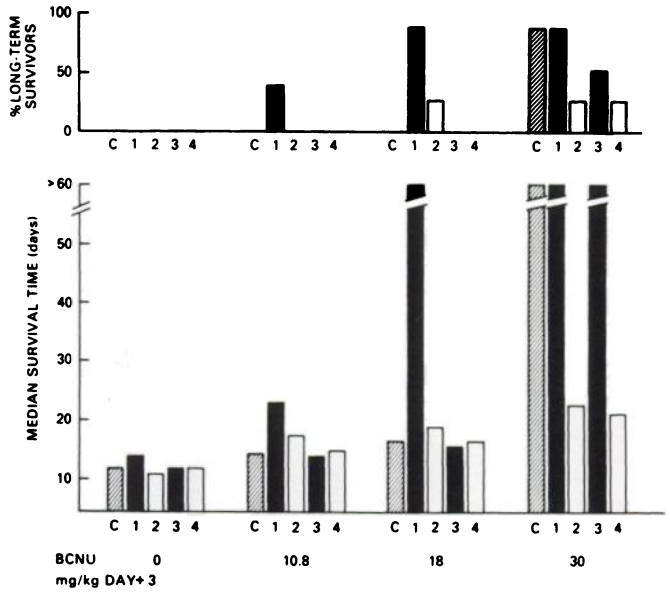

Chart 2. Immunochemotherapy of LSTRA lymphoma with a DIC-treated subline (LD-1) and BCNU. BALB/c mice were inoculated with 10^3 LSTRA cells on Day 0. *C*, control groups not receiving immunotherapy; *1*, groups inoculated with 10^6 LD-1 (generation 32) cells 1 day after LSTRA; *2*, groups inoculated with 10^6 LD-1 (generation 32) cells 7 days after LSTRA; *3*, groups inoculated with 10^6 L5MF-22 cells 1 day after LSTRA; *4*, groups inoculated with 10^6 L5MF-22 cells 7 days after LSTRA.

Search for New TATA in DIC-treated Sublines, Using Mice Tolerant to the Parental Line. A single injection of CY given with antigenic stimulation renders the host specifically tolerant to the sensitizing antigen (1). Experiments were designed to determine whether mice rendered tolerant to TATA of the parental tumor would still reject its DIC-treated sublines, by virtue of newly expressed TATA not present in the parental cells. Hybrid $CD2F_1$ mice, less susceptible to the toxic effects of CY than inbred BALB/c mice, were used. $CD2F_1$ mice were subjected to a treatment schedule designed to induce tolerance to TATA of either the LSTRA tumor or the LD-1 subline. A single injection of 5×10^7 lethally irradiated (5000 R *in vitro*) cells of LSTRA or LD-1 lymphoma was administered and followed 1 day later by a single injection of CY (250 mg/kg i.p.). Mice pretreated with CY alone and untreated controls were also used. Ten days after CY treatment (Day 0), the animals were challenged with LSTRA or LD-1 tumor cells. In addition, tolerance to the parental LSTRA line was tested by sensitizing untreated mice or mice being tested for tolerance with 2.5×10^7 lethally irradiated (5000 R *in vitro*) LSTRA cells given on Day 0. On Day +14 these mice were challenged with 10^5 or 10^3 LSTRA lymphoma cells. The results (Table 3) show that: (*a*) LD-1 was rejected in untreated mice (Groups 2 to 3), in animals pretreated with CY alone (Groups 5 to 6), or with irradiated LSTRA cells and CY ("tolerant" to LSTRA, Groups 8 to 9); (*b*) lethal tumor growth occurred in mice tolerant to LD-1 and challenged with LD-1 (Groups 11 to 12); (*c*) mice challenged with LSTRA died with generalized lymphoma (Groups 1, 4, 7, 10); (*d*) tolerance to LSTRA was confirmed in mice sensitized with irradiated LSTRA cells on Day 0. Nontolerant mice rejected subsequent challenge with 10^5 or 10^3 cells of LSTRA lymphoma on Day +14 (Groups 15 to 16), whereas all mice tolerant to LSTRA (Groups 17 to 18) or to LD-1 (Groups 19 to 20) succumbed with generalized lymphoma with median survival times similar to those of nonsensitized controls (Groups 13 to 14). This would indicate a high probability of tolerance. Both LSTRA and LD-1 in conjunction with CY treatment induced tolerance to the LSTRA tumor line whereas LD-1 but not LSTRA was effective in inducing tolerance to the LD-1 subline (Groups 11 to 12 and 8 to 9, respectively).

DISCUSSION

This study has extended the analysis of the immunogenic changes induced by DIC to MLV- and RLV-induced tumors of *H-2*^{d} and *H-2*^{b} genotype, respectively. New observations have been made on the time of appearance of increased immunogenicity, on cross-reactivity between highly immunogenic sublines and parental lines which may lead to immunochemotherapeutic application, and on the presence of a novel transplantation antigen associated with a DIC subline of MLV-induced lymphoma, apparently not expressed in the parental line.

Following 1 cycle of treatment with DIC *in vivo*, MLV-induced LSTRA lymphoma cells became highly susceptible to BCNU treatment as evidenced by the survival times of recipient BALB/c mice given injections of BCNU (see Table 1, transplant generation 1 of LD-1 and LD-2). This result

suggests that lymphoma cells acquired higher immunogenicity, particularly since more extensive therapeutic response occurred in nonimmunosuppressed mice as compared with CY-pretreated animals (4, 17). This hypothesis is supported further by the evidence of progressive increase in immunogenicity following transplant generations of DIC treatment, leading to strong host-tumor incompatibility (Table 1; Chart 1). The possibility that early immunogenic changes may occur following 1 cycle of DIC treatment *in vivo* would appear to open new perspectives in using drug-mediated increase of immunogenicity for chemoimmunotherapy of hosts bearing parental tumors, possibly including spontaneous neoplasms.

Strong immunogenicity was associated with DIC lines of MLV- and RLV-derived tumors following 4 to 8 transplant generations in DIC-treated mice (Chart 1). Data not reported here showed that high inocula (10^7) of LD-1 subline administered i.v. were rejected by semisyngeneic $CD2F_1$ mice. In addition, studies (F. Campanile, unpublished observations) on *in vitro* cell-mediated immunity elicited by spleen cells of BALB/c mice that rejected the LD-1 line showed a specific release of approximately 25% of ^{51}Cr-labeled LD-1 cells. These observations along with data showing that athymic nude mice did not reject the DIC-treated sublines (Chart 1; Ref. 7) indicate that these lymphomas elicit a T cell-dependent immune response similar to that evoked by *H-2*-incompatible tumors (7, 9). Marked and heritable (2, 3) changes of the antigenic profile take place on the tumor cell membrane under the influence of DIC treatment *in vivo*. The mechanism of these changes remains unknown and also the decline of immunogenicity of 2 sublines following a number of transplant generations in DIC-treated recipient mice (Chart 1) has not been clarified.

In this study cross-reacting antigen(s) eliciting varying degree of transplantation resistance were found between DIC-treated sublines and parental tumors. In particular 3 different tests provided evidence for cross-reactivity between LSTRA-derived DIC lines and LSTRA: (*a*) LD-1 and LD-2 sensitized the hosts against a subsequent challenge with the parental line (Table 2; Ref. 7); (*b*) LD-1 given after challenge with the parental line increased the efficacy of BCNU treatment, presumably through eliciting an anti-LSTRA immune response in mice (Chart 2); (*c*) $CD2F_1$ mice rendered tolerant to the LD-1 line were also tolerant to the parental LSTRA lymphoma (Table 3). No protection was afforded by an unrelated DIC subline (L5178Y/DIC) against a subsequent challenge with LSTRA tumor (Table 2) indicating that anti-LSTRA sensitization required specifically LSTRA-derived DIC-treated cells. Furthermore, ^{51}Cr release tests *in vitro* did not show cross-reactivity between L5178Y/DIC and LD-1, both highly immunogenic DIC-treated sublines of 2 different *H-2*^{d} lymphomas (F. Campanile, unpublished observations). The data concerning transplantation resistance in C57BL/6 mice (Table 2) evidence cross-reactivity between MLV- and RLV-derived lymphomas (8, 10–13) both of parental and DIC-treated lines. DIC-treated sublines of virus-induced lymphomas retain at least part of the virus-dependent transplantation antigens.

The results of the immunochemotherapy studies (Chart 2) and of other experiments not reported here lead to the

Table 3
Rejection of LD-1 line by $CD2F_1$ mice tolerant to LSTRA tumor cells

Groups	Pretreatment: Day −11	Pretreatment: Day −10	Challenge (Day 0 i.p.): LSTRA, 10^5 cells: MST[a]	LSTRA, 10^5 cells: D/T	LD-1 (generation 26): 10^7 cells: MST	LD-1 (generation 26): 10^7 cells: D/T	LD-1 (generation 26): 10^6 cells: MST	LD-1 (generation 26): 10^6 cells: D/T	Sensitization (Day 0 i.p.)	Challenge (Day +14 i.p.), LSTRA: 10^5 cells: MST	10^5 cells: D/T	10^3 cells: MST	10^3 cells: D/T
1–2–3			10	8/8		0/8		0/8					
4–5–6		CY[b]	9	8/8		0/8		2/7					
7–8–9	LSTRA/R[c]	CY	10	8/8		0/7		0/6					
10–11–12	LD-1/R[c]	CY	10	7/7	18	6/7	26	4/6					
13–14										10	8/8	12	8/8
15–16									LSTRA/R[d]		3/8		0/8
17–18	LSTRA/R[c]	CY							LSTRA/R[d]	11.5	8/8	13	8/8
19–20	LD-1/R[c]	CY							LSTRA/R[d]	11.5	8/8	13	8/8

[a] MST, median survival time; D/T, dead mice/total tested.
[b] 250 mg/kg i.p.
[c] 5×10^7 irradiated (5000 R *in vitro*) cells i.p.
[d] 2.5×10^7 irradiated (5000 R *in vitro*) cells i.p.

conclusion that DIC-treated sublines could be suitable for specific immunotherapy even when essentially inactive *per se* but capable of increasing considerably the antitumor effectiveness of chemotherapy. No definitive adjuvant effect was obtained when LD-1 subline was given after BCNU therapy (Chart 2). Moreover, the early deaths occurring with this schedule of BCNU (30 mg/kg) may have resulted from lethal growth of the LD-1 line in BCNU-immunodepressed recipient mice. Unrelated L5MF-22 lymphoma did not afford any protection, whereas the same harmful effects were seen when the line was injected after BCNU (30 mg/kg).

The results of the tolerance experiments are consistent with the hypothesis that novel TATA are associated with LD-1, not evidenced in the parental LSTRA cells. Transplantation resistance (2, 3, 5-7), cell-mediated immunity *in vitro* (14), and specific adoptive transfer of immunocompetent cells (15, 16) seem to confirm the view that new antigenic properties are associated with DIC-treated sublines not detectable in parental cells.

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