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Changes of the Immunogenic Properties of K36 Lymphoma Treated *In Vivo* with 5(3,3-Dimethyl-1-Triazeno) Imidazole-4-Carboxamide (DTIC)*

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Abstract—*Mice of AKR strain bearing syngeneic K36 lymphoma were treated with DTIC for a number of transplant generations. The lymphoma line (K36/DTIC) became resistant to DTIC treatment and weakly immunogenic for AKR or (AKR × DBA/2)F_1 hosts. Previous findings, however, showed that DTIC-treated H-2^d or H-2^b lymphomas became DTIC-resistant as well, but acquired strong immunogenicity for histocompatible hosts. Transplantation resistance of allogeneic mice against K36 or K36/DTIC lines injected i.p. or i.v. was also investigated. Both lines inoculated i.p. were rejected by either H-2-incompatible recipients, or H-2-compatible mice incompatible for minor histocompatibility loci (MIH). When the tumors were injected intravenously, H-2-compatible MIH-incompatible mice were more susceptible than H-2-MIH-incompatible recipients to lymphoma challenge. Moreover K36/DTIC line elicited stronger transplantation resistance than K36 tumor. Unexpectedly H-2-MIH-incompatible mice homozygous for the H-2^q haplotype were partially susceptible to the i.v. challenge with K36 lymphoma cells. However, strong transplantation resistance was found in the same hosts against K36/DTIC line.*

In conclusion the limited increase of tumor cell immunogenicity obtained by treatment of K36 lymphoma with DTIC was detectable in syngeneic, hybrid and allogeneic mice.

INTRODUCTION

IMMUNOGENIC changes of murine lymphoma cells have been obtained following treatment of tumor-bearing mice with antineoplastic agents [1–5]. In particular DTIC was found to be highly active in inducing alteration of the antigenic makeup of lymphomas carrying the *H-2^d* or *H-2^b* haplotypes [2, 5]. On the other hand little or no information is presently available on the antigenic properties of DTIC-treated sublines of murine lymphomas, originated in mice homozygous for the *H-2^k* haplotype.

In the present studies the Gross-virus induced K36 lymphoma of AKR (*H-2^k*) origin [6] was treated with DTIC for a number of transplant generations. The DTIC-treated K36 subline was found to be slightly immunogenic for syngeneic hosts and induced presumably stronger transplantation resistance in a number of allogeneic mice than that elicited by the parental K36 lymphoma.

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MATERIALS AND METHODS

Animals

Inbred AKR (*H-2^k*) B10.BR (*H-2^k*) C3H (*H-2^k*) CBA (*H-2^k*) B10.A (*H-2^a*) SJL (*H-2^s*) C57Bl/6 (*H-2^b*) and hybrid (BALB/c Cr × DBA/2 Cr) F_1 (abbr. $CD2F_1$ *H-2^d/H-2^d*), (C57Bl/6 × DBA/2 Cr) F_1 (abbr. $BD2F_1$ *H-2^b/H-2^d*), (AKR × DBA/2 Cr) F_1 (abbr. $AKD2F_1$ *H-2^k/H-2^d*) mice of both sexes, 8–10 weeks old were obtained from the Mammalian Genetics and Animal Production Section of the National Cancer Institute, Bethesda, Md.

Tumors

Ascitic K36 leukemia of AKR origin was maintained by weekly passages i.p. in AKR mice. K36/DTIC, the DTIC-treated subline of parental K36 tumor, was obtained in AKR mice challenged with 10^6 leukemic cells i.p. and treated from days 1 to 7 with DTIC (100 mg/kg/day, i.p.). The subline was used after 16–25 transplant generations of DTIC treatment. Tumor cells were suspended in sterile medium 199 and inoculated i.p. or i.v. in 0.2 or 0.5 ml respectively. Mortality of mice was recorded for at least 60 days after leukemia challenge.

Drugs

DTIC was mixed with citric acid and mannitol in the proportion of 1:1:0.375 wt/wt and dissolved in distilled water. The solution was kept on ice and protected from light during manipulation. Cyclophosphamide (Cy) was dissolved in chilled 0.85% NaCl solution. The drugs were supplied by Dr. H. B. Wood, Jr., Drug Synthesis and Chemistry Branch; Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, Maryland.

RESULTS

The mortality data of leukemic mice inoculated with K36 (transplant generation 0) or K36/DTIC line for 30 transplant generations are illustrated in Fig. 1. DTIC-treated mice carrying parental K36 leukemia survived longer than untreated controls. However rapid decline of sensitivity to DTIC was detected during subsequent transplant generations. No significant difference in survival times was found between DTIC-treated and untreated hosts from transplant generation 3 onward. Graded numbers of lymphoma cells of K36 leukemia or K36/DTIC subline at transplant generation 16 were inoculated i.p. into female AKR mice. The results, illustrated in Fig. 2, show that animals inoculated with DTIC subline lived longer than mice challenged with the same number of parental K36 cells. However AKR hosts immunodepressed with Cy administered 6 hr before tumor transplantation, died earlier than non-pretreated mice, injected with 10^3 K36/DTIC cells. On the other hand immunodepression did not influence the survival times of syngeneic hosts inoculated with parental K36 lymphoma (data not shown).

Transplantation resistance of hybrid or allogeneic mice against both K36 or K36/DTIC lines was tested using intraperitoneal or intravenous challenge of graded numbers of leukemic cells. The histocompatibility differences between the strain of origin of K36 tumor (AKR), and hybrid or allogeneic recipients, are illustrated in Table 1. Hybrid hosts included mice carrying the *H-2*^{k} haplotype ($AKD2F_1$) or animals histoincompatible with AKR strain for the *H-2* complexes of both parental chromosomes ($BD2F_1$, $CD2F_1$). Allogeneic hosts were: (a)

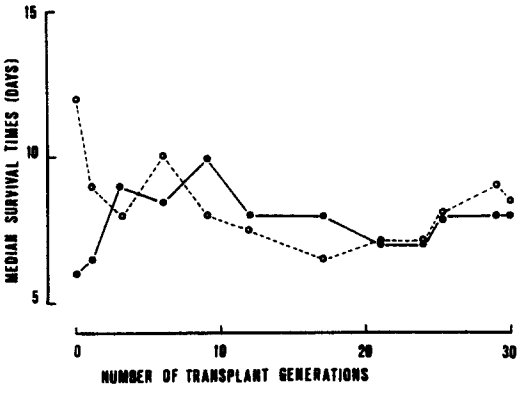

Fig. 1. Survival times of AKR male mice inoculated with K36 ascites lymphoma (transplant generation 0) or with lymphoma cells collected from DTIC-treated mice (K36/DTIC subline), for 30 transplant generations. ●——● control AKR mice inoculated with 10^6 lymphoma cells i.p.; ○---○ AKR mice, inoculated with 10^6 lymphoma cells and treated with DTIC (100 mg/kg/day i.p., days 1–7 after challenge).

At transplant generation 15, the median survival time of AKR mice non pretreated or immunodepressed with cyclophosphamide (180 mg/kg i.p. 6 hr before challenge) was 21 and 16 days respectively.

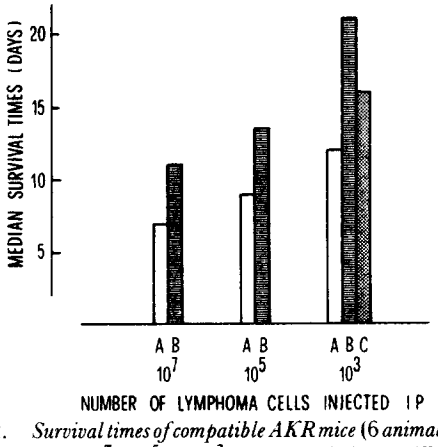

Fig. 2. Survival times of compatible AKR mice (6 animals/group) inoculated with 10^7, 10^5 or 10^3 cells i.p. of K36 or K36/DTIC lymphoma. A = mice inoculated with K36; B = mice inoculated with K36/DTIC; C = mice inoculated with K36/DTIC, pretreated with Cy (150 mg/kg i.p. given 5 hr before tumor challenge).

MIH-incompatible, *H-2*-compatible (C3H, CBA, B10.BR); (b) *MIH*-incompatible and incompatible for regions of the *H-2* complex (B10.A); (c) *MIH*-incompatible and incompatible for the entire *H-2* complex (BALB/c, C57Bl/6, SJL, $BD2F_1$, $CD2F_1$).

Table 1. Histocompatibility pattern of AKR mice, host of origin of K36 line and of recipient hosts tested

Host	MIH*	H-2 haplotype	Regions of H-2 K	IA	IB	IJ	IE	IC	S	G	D
AKR	–	k	k	k	k	k	k	k	k	k	k
$AKD2F_1$	–	k/d	k/d	k/d	k/d	k/d	k/d	k/d	k/d	k/d	k/d
C3H	+	k	k	k	k	k	k	k	k	k	k
B10.BR	+	k	k	k	k	k	k	k	k	k	k
CBA	+	k	k	k	k	k	k	k	k	k	k
B10.A	+	a	k	k	k	k	k	d	d	d	d
BALB/c	+	d	d	d	d	d	d	d	d	d	d
$CD2F_1$	+	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d
C57B1/6	+	b	b	b	b	b	b	b	b	b	b
$BD2F_1$	+	b/d	b/d	b/d	b/d	b/d	b/d	b/d	b/d	b/d	b/d
SJL	+	s	s	s	s	s	s	s	s	s	s

*MIH, minor histocompatibility loci. This column shows the absence (–) or the occurrence (+) of graft responses against alloantigens specified by MIH of AKR strain. Congenic B10.BR and B10.A hosts share the same MIH, which are different from those of AKR strain.

The mortality data of recipient mice inoculated intraperitoneally with K36 or K36/DTIC cells are illustrated in Table 2. The results evidenced that: (a) weak transplantation resistance was elicited by K36/DTIC subline in AKR and $AKD2F_1$ mice compatible with K36 lymphoma. Such resistance was detectable in AKR mice inoculated with 10^2 cells, and in $AKD2F_1$ recipients challenged with 10^2, 10^4 and 10^6 cells, and was abrogated in AKR male hosts by pretreatment with Cy; (b) C3H and B10.BR mice, compatible for *H-2* but incompatible for *MIH*, rejected K36/DTIC lymphoma, and C3H recipients rejected parental K36 line, (c) all allogeneic hosts incompatible for the entire *H-2* complex or for the *IC-S-G-D* regions of it, rejected both parental and its DTIC-treated subline. Further studies were conducted in syngeneic or allogeneic mice challenged intravenously with K36 or K36/DTIC lymphoma cells (Table 3). Limited or no difference in survival times was found between syngeneic AKR and hybrid $AKD2F_1$ mice inoculated with the parental lymphoma. When K36 tumor was grafted into allogeneic hosts, the mortality data showed that: (a) moderate or no transplantation resistance was found in *H-2*-compatible, *MIH*-incompatible mice. The degree of resistance to tumor graft was in the order: CBA > C3H > B10.BR; (b) strong antilymphoma allograft responses were found in *H-2*-incompatible hosts carrying the $H\text{-}2^b$ (C57Bl/6) or $H\text{-}2^s$ (SJL) haplotypes. On the other hand inbred or hybrid mice homozygous for the $H\text{-}2^d$ haplotype (i.e., BALB/c and $CD2F_1$ recipients) were moderately susceptible to the challenge with 10^6 cells. However *H-2*-incompatible hybrid $BD2F_1$ recipients carrying the $H\text{-}2^b/H\text{-}2^d$ haplotypes, or inbred B10.A mice incompatible with the $H\text{-}2^k$ lymphoma for a portion of the *H-2* complex (i.e., the *IC-S-G-D* regions), showed marked transplantation resistance against the challenge with K36 cells.

The experiments carried out with K36/DTIC inoculated intravenously, showed that: (a) weak but significant transplantation resistance was detected in AKR and $AKD2F_1$ mice, and was abolished in AKR hosts by immunodepression with Cy; (b) in *H-2*-compatible, *MIH*-incompatible recipients, transplantation resistance was stronger than that elicited by parental K36 lymphoma, and was more pronounced in C3H or CBA mice than in B10.BR hosts; (c) strong transplantation resistance was detected in all recipient mice incompatible for the entire *H-2* complex (including hosts homozygous for the $H\text{-}2^d$ complex) or for *IC-S-G-D* regions of it.

DISCUSSION

The results obtained in the present studies show that DTIC-treated K36 lymphoma cells elicited limited graft responses in inbred or hybrid mice compatible with the untreated K36 parental line. Previous investigations on murine lymphomas carrying the $H\text{-}2^d$ [2, 7] or $H\text{-}2^b$ [5] haplotypes, subjected to DTIC treatment for a number of transplant gene-

Table 2. Survival of various strains of mice inoculated intraperitoneally with graded numbers of tumor cells of K36 lymphoma or of its DTIC-treated K36/DTIC subline

Host*	H-2 haplotype	HTH†	Tumor challenge (day 0)	Number of tumor cells injected 10^2 MST‡	10^2 D/T§	10^2 P‖	10^4 MST	10^4 D/T	10^4 P	10^6 MST	10^6 D/T	10^6 P
AKR	k	no	K36	11	6/6	—	11	6/6	—	9	6/6	—
$AKD2F_1$	k/d	no	K36	13	6/6	C	10	6/6	C	8	6/6	C
C3H	k	MIH¶	K36	—	0/6	A	—	1/6	A	—	0/6	A
B10.A	a	MIH+IC-S-G-D**	K36	—	1/6	A	—	1/6	A	—	0/6	A
$CD2F_1$	d/d	MIH+H-2	K36	—	0/6	A	—	0/6	A	—	0/6	A
C57B1/6	b	MIH+H-2	K36	—	0/6	A	—	0/6	A	—	0/6	A
$BD2F_1$	b/d	MIH+H-2	K36	—	0/6	A	—	0/6	A	—	0/6	A
SJL	s	MIH+H-2	K36	—	0/6	A	—	0/6	A	—	0/6	A
AKR	k	no	Cy-K36/DTIC††	13	6/6	—	11	6/6	—	8	6/6	—
AKR	k	no	K36/DTIC	—	2/6	A	12	6/6	C	9	6/6	C
$AKD2F_1$	k/d	no	K36/DTIC	31	3/5	A	16	6/6	A	10	6/6	B
C3H	k	MIH	K36/DTIC	—	0/6	A	—	0/6	A	—	0/6	A
B10.BR	k	MIH	K36/DTIC	—	0/6	A	—	1/6	A	—	0/6	A
B10.A	a	MIH+IC-S-G-D	K36/DTIC	—	0/6	A	—	0/6	A	—	0/6	A
$CD2F_1$	d/d	MIH+H-2	K36/DTIC	—	0/6	A	—	0/6	A	—	0/6	A
C57B1/6	b	MIH+H-2	K36/DTIC	—	0/6	A	—	0/6	A	—	1/6	A
$BD2F_1$	b/d	MIH+H-2	K36/DTIC	—	0/6	A	—	0/6	A	—	0/6	A
SJL	s	MIH+H-2	K36/DTIC	—	0/6	A	—	0/6	A	—	0/6	A

*Mice, 8–10 weeks old.

†HTH, host–tumor histoincompatibility.

‡MST, median survival time.

§D/T, dead mice over total animals tested.

‖*P*, probability calculated according to the Mann–Whitney U test. A, $P<0.01$; B, $P<0.05$; C, $P>0.05$ (not significant). For K36 lymphoma, *P* was calculated according to AKR mortality, for K36/DTIC subline *P* was calculated according to mortality of AKR mice immunodepressed with Cy (see footnote††).

¶MIH, minor histocompatibility loci.

**IC-S-G-D regions of H-2.

††The mice were pretreated with cyclophosphamide (Cy, 180 mg/kg i.p.) 5–6 hr before challenge. K36/DTIC subline was used at transplant generation 24.

Table 3. Survival of various strains of mice inoculated intravenously with graded numbers of tumor cells of K36 or of its DTIC-treated K36/DTIC subline

Host	H-2 haplotype	HTH*	Tumor challenge (day 0)	Number of tumor cells injected								
				MST†	10^2 D/T‡	*P*§	MST	10^4 D/T	*P*	MST	10^6 D/T	*P*
AKR	k	no	K36	12.5	6/6	—	9	6/6	—	5	6/6	—
$AKD2F_1$	k/d	no	K36	15	6/6	A	10	6/6	A	5	6/6	C
C3H	k	MIH‖	K36	—	3/6	B	10	6/6	A	6	6/6	B
B10.BR	k	MIH	K36	13.5	4/4	C	9	4/4	C	6	4/4	C
CBA	k	MIH	K36	—	3/6	A	—	3/6	A	6	6/6	A
B10.A	a	MIH+IC-S-G-D¶	K36	—	0/3	A	—	0/6	A	—	0/6	A
BALB/c	d	MIH+H-2	K36	—	0/6	A	—	0/6	A	24	4/6	A
$CD2F_1$	d/d	MIH+H-2	K36	—	0/6	A	—	2/6	A	5	4/6	C
C57B1/6	b	MIH+H-2	K36	—	0/6	A	—	0/6	A	—	0/6	A
$BD2F_1$	b/d	MIH+H-2	K36	—	0/6	A	—	0/6	A	—	2/6	A
SJL	s	MIH+H-2	K36	—	0/6	A	—	0/6	A	—	0/6	A
AKR	k	no	Cy-K36/DTIC**	12	6/6	—	9.5	6/6	—	5	6/6	—
AKR	k	no	K36/DTIC	15	6/6	A	12	6/6	A	6	6/6	A
$AKD2F_1$	k	no	K36/DTIC	—	3/6	A	12	6/6	A	8	6/6	B
C3H	k	MIH	K36/DTIC	—	1/6	A	—	1/6	A	—	2/6	A
B10.BR	k	MIH	K36/DTIC	32	4/6	A	—	2/6	A	7	6/6	A
CBA	k	MIH	K36/DTIC	—	0/6	A	—	1/6	A	—	3/6	A
B10.A	a	MIH+IC-S-G-D	K36/DTIC	—	0/6	A	—	0/6	A	—	0/6	A
$CD2F_1$	d/d	MIH+H-2	K36/DTIC	—	1/6	A	—	1/6	A	—	1/6	A
C57B1/6	b	MIH+H-2	K36/DTIC	—	2/6	A	—	2/6	A	—	0/6	A
$BD2F_1$	b/d	MIH+H-2	K36/DTIC	—	0/6	A	—	0/6	A	—	1/6	A
SJL	s	MIH+H-2	K36/DTIC	—	0/6	A	—	0/6	A	—	0/6	A

*HTH, host–tumor histocompatibility.

†MST, median survival time.

‡D/T, dead mice over total animals tested.

§*P*, probability calculated according to the Mann–Whitney U test. A, $P<0.01$; B, $P<0.05$; C, $P>0.05$ (not significant). For K36 lymphoma, *P* was calculated according to AKR mortality; for K36/DTIC subline, *P* was calculated according to mortality of AKR mice immunodepressed with Cy (see footnote**).

‖MIH, minor histocompatibility loci.

¶IC-S-G-D, regions of H-2.

**The mice were pretreated with cyclophosphamide (Cy, 180 mg/kg i.p.) 5–6 hr before challenge. K36/DTIC subline was used at transplant generation 24.

rations, showed that: (a) lymphoma cells were initially susceptible to the drug; (b) the tumors became DTIC-resistant following 3–4 transplant generations; (c) drug-treated neoplastic cells acquired strong immunogenicity and were rejected in control mice not treated with DTIC. In contrast lethal tumor growth occurred in DTIC-treated hosts, immunodepressed by this antitumor agent [8].

In the present study the sensitivity of *H-2*^{k} K36 lymphoma to DTIC treatment was found to be similar to that reported for the lymphoma lines mentioned before. However following a number of transplant generations of DTIC treatment the K36 tumor became DTIC-resistant but not strongly immunogenic, since no difference in survival times was found between untreated or drug-treated hosts challenged with 10^6 K36/DTIC cells (Fig. 1). Nevertheless limited transplantation resistance against K36/DTIC lymphoma has been demonstrated in histocompatible AKR recipients, as evidenced by the data illustrated in Fig. 2 and Tables 2 and 3. In fact, non-immunodepressed AKR mice challenged with small inocula of K36/DTIC cells, survived longer than Cy-pretreated (i.e., immunodepressed) hosts. The lack of marked immunogenicity of K36/DTIC line for AKR hosts could have been the result of low responsiveness of this strain against strong DTIC-mediated transplantation antigens (DMTA, 7), possibly associated with K36/DTIC lymphoma. It was found that DBA/2 mice are good responders against DMTA of L1210/DTIC subline [2, 3]. If anti-DMTA responsiveness is genetically regulated, one would have expected that remarkable graft reaction might occur against the hypothesized DMTA of K36/DTIC line in F_1 hybrids between DBA/2 and AKR strains. Actually transplantation resistance against the DTIC-treated lymphoma was more pronounced in $AKD2F_1$ than AKR hosts (Tables 1 and 2). However graft response of hybrid recipients against the K36/DTIC subline was only slightly higher than that detectable in AKR mice (Tables 2 and 3). K36/DTIC cells might have elicited hybrid resistance [9] in $AKD2F_1$ hosts, specifically directed against lymphoma cells [10], and also detectable against K36 tumor (Table 3). In conclusion, no evidence was obtained indicating that DTIC treatment could mediate the appearance of strong DMTA in K36 lymphoma cells which became resistant to the drug. This finding seems to support the hypothesis that no direct relationship exists between DTIC-induced chemoresistance and the extent of DTIC-mediated immunogenicity.

Transplantation resistance against K36 and its DTIC-treated subline was studied also in allogeneic mice. Strong allograft responses were found in *H-2*-incompatible hosts and also in *H-2* compatible, *MIH* incompatible (C3H, B10.BR) recipients, challenged intraperitoneally with the two lymphomas (Table 2). These data seem to confirm the findings of Schultz *et al.* [11] showing that the strength of allograft reaction elicited by multiple non-*H-2* incompatibilities is similar to that detected in *H-2* incompatible recipients. More complex to interpret are the data obtained in allogeneic mice inoculated intravenously with the lymphoma lines studied. In general, transplantation resistance was weaker than that detectable in the same hosts inoculated intraperitoneally with tumors. Congenic-resistant B10.BR and B10.A strains were susceptible or strongly resistant respectively to the challenge with K36 lymphoma. It follows that differences restricted to the *IC-S-G-D* regions of *H-2* (Table 1) would confer resistance. On the other hand, BALB/c or $CD2F_1$ mice, homozygous for the *H-2*^{d} haplotype, differ from *H-2*^{k} K36 lymphoma cells for the entire *H-2* complex. Therefore one would have expected that BALB/c or $CD2F_1$ hosts were more resistant than *K-IA-IB-IJ-IE*-compatible B10.A recipient mice (Table 1). However this was not the case, since the *H-2*^{d} hosts were relatively susceptible to K36 challenge whereas all B10.A mice rejected the tumor. It could be suggested that the genetic background modulates the graft responses against transplantation antigens associated with K36 line. In particular the B10.A background would confer high resistance whereas the BALB/c and DBA/2 background would be responsible for partial susceptibility. Such susceptibility however is not detectable in hybrid $BD2F_1$ mice carrying the DBA/2 and C57Bl/6 genotypes.

The intravenous challenge of allogeneic mice with K36/DTIC cells evidenced that this line is more immunogenic than parental K36 lymphoma, in both *H-2* compatible, *MIH*-incompatible or *H-2–MIH*-incompatible recipients (Table 3). It can be hypothesized that K36/DTIC line would express higher levels of histocompatibility antigens, and/or other transplantation antigens, with respect to parental tumor cells. In conclusion DTIC treatment of K36 lymphoma for a number of transplant generations produced a limited increase of tumor-cell immunogenicity, detectable in syngeneic, hybrid or allogeneic hosts.

No data are presently available to determine whether such increase should be ascribed to higher density of tumor-associated antigens shared with K36 cells [12], or to novel DMTA not detectable in the parental line. In any case the DTIC-mediated increase of *H-2*^{k} lymphoma-cell immunogenicity is weaker than that detectable in murine lymphomas homozygous for the *H-2*^{d} or *H-2*^{b} haplotypes.

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