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Chemical xenogenization of murine lymphoma cells with triazene derivatives: Immunotoxicological studies*

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Summary. *Equitoxic doses of 5-(3-3-dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC) and aryl-triazene derivatives (compounds all capable of inducing a marked increase in murine tumor cell immunogenicity) were studied for their effects on the host immune system. At different times after drug exposure the animals were tested for allograft responses, competence in producing lymphocytes active in lethal graft-versus-host disease, delayed-type hypersensitivity, humoral antibody production, and mitogen responsiveness. While some of the aryl-triazenes tested (DM-COOK DM-NO_2) showed a pattern of immunodepression similar to that of DTIC, others were less (MIC, MM-COOK, MM-Cl) or far less (DM-Cl, MM-NO_2) active than DTIC in impairing host immunocompetence, although all retained or even augmented their ability to induce chemical xenogenization.*

Introduction

The original observation, made in our laboratory [1, 2, 5], that the antitumor agent 5-(3-3-dimethyl-1-triazeno)-imidazole-4-carboxamide (DTIC) can considerably alter the antigenic makeup of tumor cells greatly stimulated the search for analog compounds with improved characteristics over those of DTIC, especially with a view to the possible application of chemical xenogenization (CX) in the immunotherapy of cancer [16]. Obviously, one major goal in this regard would be the finding of molecules with equivalent, or even enhanced, ability to induce immunogenic changes but lacking the dramatic suppressive effects DTIC exerts on the host immune system [7, 15]. There is indeed little doubt that the therapeutic exploitation of DTIC-mediated immunogenic changes in cancer cells is hampered by drug toxicity to the immune system, as this prevents the treated host from developing effective reactions against a DTIC-modified, potentially immunogenic tumor [2, 16].

We have recently reported that a series of aryl-triazenes, which are structural analogs of DTIC, are capable of increasing cellular immunogenicity in a similar way to DTIC [6], and in an extension of this study a monomethyl aryl-triazene derivative was identified that seemed to be extremely active in inducing immunogenic changes of murine lymphoma cells [14].

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The present study deals with the problem of the effects of these never compounds on the host immune system and compares them with those of DTIC. It was found that the pattern of immunodepression exerted by most aryl-triazenes can be considerably different from that of DTIC.

Materials and methods

Synthesis. The synthesis of monomethyl aryl-triazenes used in this investigation was performed according to the methods previously described [3], except in the case of MM-COOK, which was prepared from the corresponding methyl ester [3] by hydrolysis in methanolic potassium hydroxide and recrystallized from 90% methanol.

Dimethyltriazenes were also obtained according to previously reported procedures: DM-COOK [13], DM-C1 [11], and DM-NO_2 [4].

Animals. Mice of both sexes, 2–4 months old, of the C57Bl/6 *(H-2^d)* inbred strain and hybrids (DBA/2 Cr × BALB/cCr)F1 (CD2F1) *(H-2^d/H-2^d)* were obtained from the Mammalian Genetics and Animal Production Section, Division of Cancer Treatment, National Cancer Institute (NCI), National Institutes of Health (NIH), Bethesda, Md, USA.

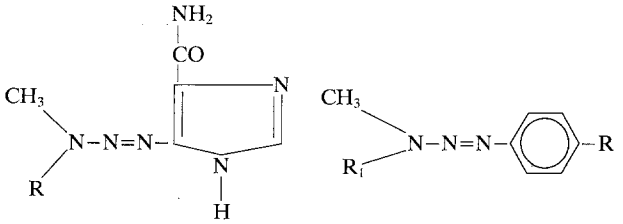
Drugs. DTIC was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, NCI, NIH, Bethesda, Md, USA. DTIC and the other triazene compounds were administered by IP injection at equitoxic doses (see Table 1), in suspension in peanut oil in a volume of 1.0 ml/10 g animal weight.

Tumors. EL-4, an ascitic leukemia of B6 origin [9], was maintained in histocompatible hosts by serial IP transplantation of neoplastic cells suspended in 0.2 ml medium 199.

Irradiation. Mice were irradiated at room temperature using a ^{60}Co-irradiator (Hot Spot MKIV, Harwell, England) delivering γ-rays at the rate of 12 Gy/min.

Assay for lethal graft-versus-host disease (GVHD). Adult recipient mice were immunodepressed with sublethal doses of total-body irradiation (6 GY), and 6 h later the mice received allogeneic spleen cells (30×10^6 cells/mouse IV) in a total volume of 1 ml/mouse. Mortality of mice was recorded for at least 60 days after spleen cell transfer.

Table 1. Chemical structure, abbreviations and doses of the tested imidazole- or aryl-triazenes



R	Drug	Dose[a] (mg/kg)	R	R_1	Drug[b]	Dose (mg/kg)
H	MIC	70	Cl	CH_3	DM-Cl	91
CH_3	DTIC	270	Cl	H	MM-Cl	82
			NO_2	CH_3	DM-NO_2	365
			NO_2	H	MM-NO_2	82
			COOK	CH_3	DM-COOK	300
			COOK	H	MM-COOK	60

[a] The drugs were administered IP, suspended in peanut oil. The doses used are equitoxic, being the same fraction of the LD_{50} of each drug injected IP into mice
[b] MM, monomethyl; DM, dimethyl

Plaque-forming cells (PFC) test. Direct PFC were evaluated by the micromethod described by Jerne et al. [10].

Delayed-type hypersensitivity (DTH). Mice were sensitized SC with a single injection of 2×10^8 sheep red blood cells (SRBC) in 0.05 ml 0.85% NaCl solution mixed with an equal volume of Freund's complete adjuvant (FCA) (Gibco, New York, USA). Ten days later the animals were challenged with 10^8 SRBC in 0.05 ml 0.85% NaCl solution inoculated in the footpad. The DTH reaction was recorded 24 h later, when the footpad swelling was measured with a caliper [12].

Mitogen stimulation. Spleen cells (4×10^5 in 200 µl) were distributed in each well of culture microplates. Mitogens were added in a volume of 20 µl at concentrations previously shown to give maximum stimulation of DNA synthesis, and the final concentrations of concanavalin A (Miles-Yeda Ltd, Israel) and lipopolysaccharide were 16 and 5 µg/ml, respectively. After a 48-h incubation at 37° C in a 5% CO_2 incubator, the cultures were pulsed with ^{125}I-5-iodo-2′-deoxyuridine (^{125}IUdR, 0.1 µCi/well) along with 5-fluoro-2′-deoxyuridine (FUdR, 0.01 µg/well) to prevent endogenous thymidine synthesis. The cells were harvested 18 h later by a multiple suction-filtration apparatus on a fiberglass filter paper and the radioactivity associated with the paper disks was read on a γ-scintillation counter. Results were expressed as mean counts per minute of quadruplicate samples.

Results

1. Modulation of allograft responses

The chemical structures, abbreviations, and doses of the imidazole- and aryl-triazene derivatives used in this study are reported in Table 1. Equitoxic doses of imidazole- and aryl-triazene derivatives were given IP to CD2F1 mice a number of days (1, 5, 10, or 30) before challenge with 10^7 cells of allogeneic EL-4 lymphoma. The results (Fig. 1) show that nontreated CD2F1 mice rejected the allogeneic lymphoma, whereas the animals receiving drug treatment 1 day before tumor challenge succumbed with generalized lymphoma. The effects of MM-NO_2 and DM-Cl, however, were relatively short-lived (there were no lethal tumor takes after treatment on day − 5); MIC, MM-COOK, DTIC, DM-COOK, and DM-NO_2 also had dramatic suppressive effects when given 10 days earlier and, interestingly enough, the last three compounds still inhibited the anti-lymphoma graft rejection at 30 days after treatment.

Table 2. Effect of aryl-triazene derivative treatment of donor mice on GVH produced by *H-2*-incompatible spleen cells

Donor mice			Recipient mice[b]	
Strain	Drug	Day[a]	MST[c]	D/T[d]
$CD2F_1$	−	−	−	0/6
C57Bl/6	−	−	8	6/6
C57Bl/6	DTIC	− 1	−	2/6
C57Bl/6	DTIC	− 5	−	0/5
C57Bl/6	DM-COOK	− 1	−	2/6
C57Bl/6	DM-COOK	− 5	−	0/6
C57Bl/6	DM-Cl	− 1	8	6/6
C57Bl/6	DM-Cl	− 5	13	4/4
C57Bl/6	DM-NO_2	− 1	9	6/6
C57Bl/6	DM-NO_2	− 5	11.5	6/6
C57Bl/6	MIC	− 1	16	5/5
C57Bl/6	MIC	− 5	12	5/5
C57Bl/6	MM-COOK	− 1	14	6/6
C57Bl/6	MM-COOK	− 5	12	4/5
C57Bl/6	MM-Cl	− 1	9	4/4
C57Bl/6	MM-Cl	− 5	8	4/4
C57Bl/6	MM-NO_2	− 1	14	6/6
C57Bl/6	MM-NO_2	− 5	16	4/4

[a] Day of drug treatment, before spleen cell collection for transfer into mice recipient mice
[b] $CD2F_1$ mice immunodepressed by 6 Gy 6 h before cell transfer
[c] Median survival time (days)
[d] Dead animals over total tested

2. Effects on GVHD

C57Bl/6 mice were treated with equitoxic doses of DTIC, MIC, or aryl-triazenes. Spleen cells were collected 1 or 5 days after treatment and transferred (30×10^6 cells/mouse, IP) into *H-2*-incompatible recipient mice given total-body irradiation (6 Gy) 6 h before the transfer. Lethal GVHD occurred in all mice immunodepressed by γ-rays and inoculated with allogeneic spleen cells from intact donors (Table 2). However, when donor mice had been treated with either DTIC or DM-COOK 1 or 5 days earlier (but not the other drugs) no lethal GVHD could be detected. Incidentally, and in line with previous data on DTIC [7], when total-body irradiation of the recipient mice was replaced by treatment with the drugs under investigation and the transfer was done with spleen cells from intact allogeneic donors, no GVHD occurred (data not shown).

3. Modulation of DTH

CD2F1 mice were sensitized SC with 2×10^8 SRBC, and 10 days later (day 0) challenged with 10^8 SRBC inoculated into

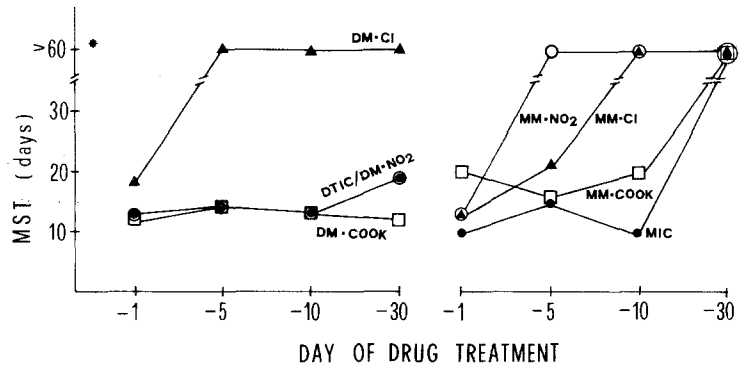


Fig. 1. Modulation of allograft responses: groups of six to eight CD2F1 mice either untreated (*) or given the triazene derivatives 1, 5, 10, or 30 days earlier were challenged with 10^7 allogeneic EL-4 lymphoma cells. MST, median survival time

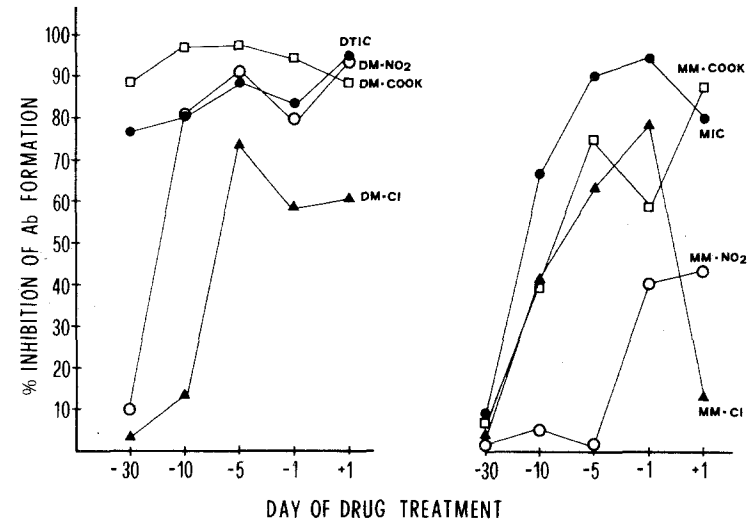


Fig. 2. Inhibition of Ab formation: groups of three to five CD2F1 mice were treated with triazene derivatives and tested at various numbers of days afterwards for humoral antibody formation

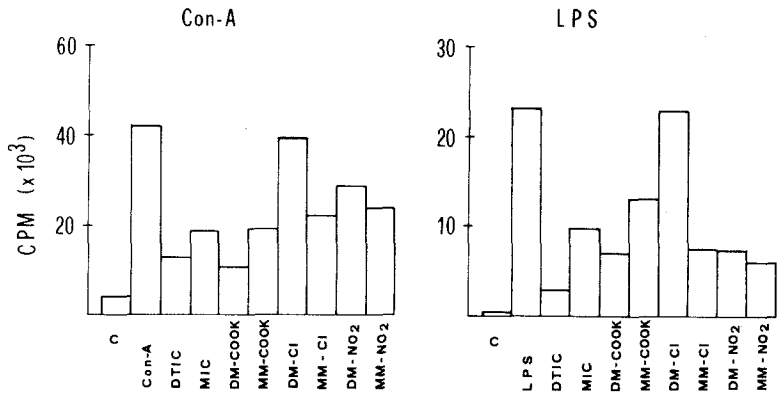


Fig. 3. Inhibition of responsiveness to mitogens: spleen cells from groups of three to five mice treated with equitoxic doses of triazene derivatives were cultured in the presence of either ConA or LPS. C, no mitogens added to the cultures

the footpad. Selected groups had been pretreated on day − 1 or − 5 with equitoxic doses of imidazole- or aryl-triazene derivatives. The DTH reaction was recorded 24 h later by measuring footpad swelling. Treatment with any drug under investigation failed to significantly affect the expression of this response (data not shown).

4. Effects on humoral antibody formation

CD2F1 mice were inoculated IP with single equitoxic doses of the drugs under investigation a number of days (30, 10, 5 or 1) before, or 1 day after, sensitization with 4×10^8 SRBC. Evaluation of primary direct hemolytic PFC response was performed on day +4 after SRBC sensitization. Figure 2 shows that marked inhibition of Ab formation occurred in mice pretreated with any of the drugs given 1 day before SRBC. All drugs, except for MM-NO_2, retained their depressive activity when administered on day −5. Ten days after treatment, DM-NO_2, MM-Cl, MIC, MM-COOK, DTIC, and DM-COOK were still active, and the last two compounds significantly inhibited Ab formation at 30 days after treatment.

5. Effects of drugs on spleen cell responsiveness to mitogens

CD2F1 mice were treated with equitoxic doses of the imidazole- or aryl-triazene derivatives and, 1 day after the

treatment, spleen cells were collected and cultured for 72 h with Con A or LPS. Mitogen responsiveness was measured in terms of radiolabel uptake. The results (Fig. 3) show that the drugs considerably inhibited responsiveness to both mitogens, apparently with the sole exception of DM-Cl. Once again, DTIC and DM-COOK were the most potent inhibitors of this function.

Discussion

Much experimental evidence is now available indicating that in vivo or in vitro treatment of murine lymphoma cells with triazene derivatives carrying an imidazole moiety or a phenyl ring [6] may result in increased immunogenicity of the tumor. This alteration of the antigenic makeup, known as chemical xenogenization (CX) and first described for DTIC (1), has suggested a new approach in cancer immunotherapy, which would exploit the novel antigenic targets artificially created on tumor cells [8]. The strong immunosuppressive activity of DTIC, however, represents a major obstacle to the triggering of effective responses in the host against a DTIC-modified, potentially immunogenic tumor [2]. The recent availability of DTIC analogs endowed with CX-inducing properties raises the problem of the effects of these newer compounds on the host immune functions. In the present study we attempted to draw preliminary immunopharmacological profiles of these triazene derivatives.

A comparative analysis of various immunological parameters was carried out. Most immune functions investigated, such as tumor allograft reactions and humoral antibody formation, appeared to be depressed by the aryl-triazene derivatives as well as by DTIC and its related monomethyl compound (MIC). However, marked differences were found among the various drugs, especially with regard to duration and extent of the drug-induced impairment of immune responsiveness.

The strong inhibitory effects of DTIC, DM-COOK, and DM-NO_2 on antilymphoma allograft responses confirmed previous findings that lethal tumor growth occurs in CD2F1 mice treated with DTIC up to 2 months before challenge with *H-2*-incompatible lymphomas [7]. However, the results obtained in the present study point out that a much earlier recovery of allograft responsiveness followed exposure to equitoxic doses of MM-NO_2, DM-Cl and, to a lesser extent, MIC and MM-COOK. Similarly, the ability of allogeneic *H-2*-incompatible spleen cells to produce GVHD when inoculated into immunosuppressed recipient mice was significantly inhibited by pretreatment of donor mice with DTIC or DM-COOK but not with the other drugs under investigation. Thus, it appeared that DTIC and DM-COOK belonged to a category of triazene derivatives (mostly dimethyl compounds) with dramatic and long-lasting immunosuppressive activity, which could be separated from the group of drugs with intermediate or relatively low immunotoxicity (largely represented by monomethyl derivatives). Further experiments helped clarify this point.

Humoral antibody formation was depressed by DTIC and DM-COOK given up to 1 month earlier; DM-NO_2, MM-COOK, MM-Cl, and MIC were still capable of depression when given 10 days before SRBC. All drugs inhibited antibody formation if administered on day −5, the only exception being MM-NO_2. Interestingly, MM-NO_2, and DM-Cl were the least active in impairing allograft reactions, and DM-Cl was the only drug with no significant effect on mitogen responsiveness.

Although many factors may be involved in determining the relative immunotoxicity of each compound, it is possible that a role is also played by the different pharmacokinetic behaviour of the various molecules. We have already shown that the dimethyl derivatives require enzymatic *N*-demethylation as an essential step in the generation of the metabolite(s) responsible for CX activity. On the other hand, the monomethyl compounds appear to be active per se [14] and undergo rapid spontaneous decomposition in aqueous solutions [3]. Our study shows that the monomethyl derivatives are, as a rule, less immunotoxic than the related dimethyl compounds. Among the latter, DM-Cl is the least active and also behaves as an optimal substrate for enzymatic *N*-demethylation; DTIC and DM-COOK, on the other hand, are the poorest substrates (Fioretti et al., manuscript in preparation). It is therefore possible that *N*-demethylation of the dimethyl derivatives and the spontaneous decomposition of the related monomethyl compounds both condition the in vivo half-life of the immunotoxic species. In this respect, an inverse relationship could exist between immunotoxicity of the dimethyl derivatives and their susceptibility to enzymatic *N*-demethylation. The persistence of adequate levels of immunotoxic species in the blood might condition interaction with lymphoid cell populations of different turnover and thus determine the outcome (i.e., duration and extent) of immunosuppression.

Taken together, the data in the present paper demonstrate that the pattern of immunodepression exerted by triazene derivatives is not uniform. DTIC can be regarded as the prototype of heavily immunodepressive compounds; on the other hand, MM-NO_2 and DM-Cl exhibit relatively low immunotoxicity. A third category, containing most of the tested drugs, displays intermediate properties: the depression induced by these compounds is barely detectable by 10 days after exposure and is usually milder than that of DTIC. It should be noted that MM-COOK falls into this category. MM-COOK is indeed one of the most promising DTIC analogs, since it exhibits enhanced CX-inducing and cytoreductive properties [14]. Moreover, this molecule has some advantageous properties over those of other triazene derivatives, such as hydrosolubility and the ability to produce CX without metabolic activation. MM-COOK and the other compounds with reduced immunotoxicity might prove to be a valuable means of developing successful immunochemotherapy approaches based on chemical xenogenization of cancer cells. A wider range of doses of each drug should be investigated to define more complete immunopharmacological profiles of these newer compounds.

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