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Chemical xenogenization of experimental tumors

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Abstract

Chemical xenogenization occurs when experimental tumors, treated *in vivo* or *in vitro* with selected chemicals, become immunogenic, i.e., able to induce a strong rejection response, immunological in nature, in the histocompatible hosts. Unlike modifications induced by haptens, changes in tumor cell immunogenicity associated with chemical xenogenization are heritable as a result of drug interference with the genetic code. Drugs endowed with potent mutagenic activity are known to be powerful xenogenizing agents, and their mechanism of action is traditionally regarded as involving changes in DNA nucleotide sequence. Triazene and nitrosoguanidine derivatives are among the best known examples of this type of compound, and a large body of information has been accumulated over the years regarding the immunogenic properties of the tumor variants obtained following treatment with those xenogenizing agents. The present paper reviews this information, and also discusses the therapeutic implications of xenogenization in experimental systems of tumor immunotherapy. Xenogenization of murine tumors has also been obtained by means of chemicals devoid of mutagenic activity but capable of affecting gene transcriptional activity. The characteristics of this 'new' type of xenogenization are also reviewed and compared to those of triazene xenogenization.

Introduction

The antigenic phenotype of a tumor cell population is not a static characteristic. Investigations of animal and human tumors document both heterogeneity and change in the antigenic make-up of cancer cells which may be explained on the basis of what Foulds first described as tumor progression [1]. The occurrence of tumor cell progression, especially in terms of selective advantage for cells with reduced immunogenicity, is one of the crucial factors underlying the failure of the host immune system to successfully combat tumor growth and spread [2].

The antigenic phenotype of tumor cells can also be experimentally modified through a variety of procedures [3–28] which either directly – and rather transiently, as a rule – affect the membrane structures of the cell or involve stable, often hereditary, changes in the cell biology (e.g., the genetic code, see Table 1). The term 'viral xenogenization' of tumor cells was first introduced by Kobayashi [6] to define the appearance of viral associated anti-

Table 1. Procedures known to artificially increase the immunogenicity of cancer cells.

1. Viral xenogenization (or 'heterogenization') (3–8)
2. Neuraminidase-induced cell surface modifications (9–13)
3. Chemical haptenization (14–16)
4. Chemical xenogenization by:
   a. Antineoplastic agents (18–21)
   b. Mutagens (22, 23)
   c. Gene activators (28)
5. Others (17, 24–27)

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gens on transplantable rat tumors exposed to Friend virus. In an elegant series of experiments, Kobayashi and associates showed that fibrosarcoma cells, persistently infected with the virus, had become antigenically 'foreign' to the host of origin which was thus able to resist challenge by the xenogenized tumor (for review, see [29]).

At about the same time, Bonmassar *et al.* [18] found that murine leukemia cells, on repeated *in vivo* exposure to the antitumor agent dacarbazine, would become increasingly more immunogenic, acquiring a degree of antigenic foreignness capable of conditioning the histocompatible host to resist the drug-treated tumor's challenge. Since that observation, a number of drugs or chemicals have been shown to be able to induce immunogenic changes in experimental tumors, a phenomenon which, on the analogy of the term introduced by Kobayashi, is often referred to as chemical xenogenization [30, 31].

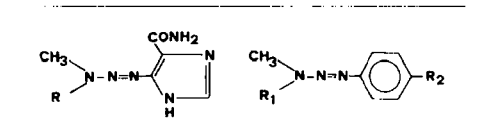
In this context, 'chemical xenogenization' indicates the induction of stable tumor variants with increased immunogenicity following exposure of the original ('parental') neoplasm to different chemicals.

We intend to review evidence that chemical xenogenization may represent a suitable way to increase the immunogenicity of tumor cells, and that this phenomenon can be successfully exploited in experimental models of tumor immunotherapy. It will be shown that drugs acting as mutagens and/or modulators of gene expression may indeed enhance the immunogenic strength of experimental tumors, most of which carry new transplantation antigens. We will also discuss the possible application of chemical xenogenization to treatment of human cancer.

Chemical xenogenization by triazene derivatives

Dacarbazine

Many synthesized triazene derivatives, with either an imidazole or an aryl moiety, possess both cytoreductive and xenogenizing properties [32] (see Fig. 1). The triazenyl-imidazole derivative



R	R_1	R_2	Drug
H			MIC
CH_3			DTIC
	CH_3	Cl	DM–Cl
	H	Cl	MM–Cl
	CH_3	NO_2	DM–NO_2
	H	NO_2	MM–NO_2
	CH_3	COOK	DM–COOK
	H	COOK	MM–COOK

Fig. 1. Chemical structure and abbreviations of the imidazole-(A) or aryl-(B) triazene derivatives cited in this study. MM, monomethyl; DM, dimethyl.

DTIC (or dacarbazine) was the first compound of this class to manifest a strong ability to induce immunogenic changes of murine lymphoma cells. The experimental model most suitable to reveal *in vivo* xenogenization by DTIC is the one originally described by Bonmassar *et al.* [18]. Typically, mice bearing a compatible leukemia are treated with DTIC for a number of days, and then the leukemic cells, recovered from the DTIC-treated donors, are re-injected into 2 groups of mice: A, untreated and B, animals to receive further treatment with DTIC. Group B will thus be used as donor-recipient of the DTIC-treated line for each successive transplant generation (TG). At TG_1 (i.e., using parental cells), the animals exposed to DTIC survive longer than untreated controls, an effect which is easily explained on the basis of the cytoreductive properties of the drug. At TG_3–TG_4 the survival times of groups A and B tend to be comparable, which indicates development of resistance to DTIC. From TG_5–TG_6 onwards, an apparently paradoxical phenomenon can be observed since the untreated (group A) mice survive longer than the hosts subject to drug treatment (group B). Eventually (at TG_n), group A animals will resist tumor challenge, and support lethal tumor growth only if immunodepressed prior to challenge. TG_n group B mice, which are constantly on DTIC treatment, die with generalized lymphoma. Fig. 2 illustrates a typical experiment in which DTIC medi-

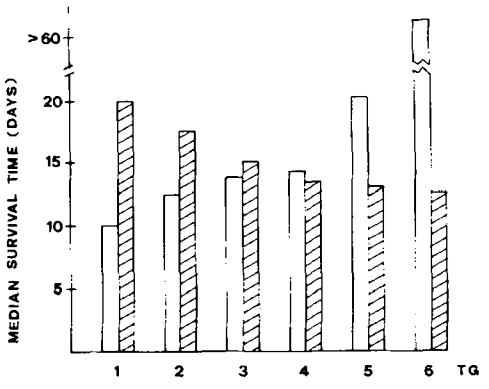


Fig. 2. Generation of an immunogenic tumor variant by DTIC treatment of LSTRA-bearing mice for 6 transplant generations. □, group A mice: ▨, group B mice (i.e., on continued DTIC chemotherapy, see text for details).

ates the antigenic transformation of Moloney virus-induced LSTRA lymphoma of BALB/c origin.

A number of considerations and information gathered over the years suggest that this apparently paradoxical phenomenon has an immunological basis. Indeed, one major point to be noted is that, if normal recipients of a DTIC-treated line are immunodepressed with X-rays, cyclophosphamide or antilymphocytic serum prior to tumor challenge, there will be no long-term survivors. Interestingly, lethal growth also occurs in nude mice which lack T cell-mediated responses [33]. Thus, it appears that treatment of tumor cells with DTIC leads to a progressive increase in the immunogenicity of the tumor so that previously nonimmunogenic inocula become capable of evoking a strong antitumor response which creates a state of specific resistance. In DTIC-treated animals, however, progressive growth occurs since the highly immunodepressive activity of the drug prevents host antitumor responses without affecting significantly the growth of the xenogenized line that has become resistant to the cytoreductive activity of DTIC.

Aryl-triazenes

Studies of the relationship between chemical structure and cytoreductive and xenogenizing properties of triazene compounds led to the synthesis of a class of derivatives in which the imidazole ring present in DTIC was replaced by an aryl moiety [32, 34, 35] (see Fig. 1). Most of these aryl-containing compounds proved to be strong xenogenizing agents, thus suggesting that the imidazole ring is not mandatory for the activity [35]. Subsequent studies also showed that dimethyltriazenes, including DTIC, are not active *per se in vivo* but require metabolic activation which is carried out by liver microsomal enzymes and leads to generation of monomethyl species [35]. When a series of dimethyl aryl-triazenes and related monomethyl compounds were assayed for induction of xenogenization *in vitro*, it was found that the dimethyl derivatives required the presence of mouse liver microsomes whereas the corresponding monomethyl compounds did not [35]. Similar results had been obtained with DTIC and its related monomethyl derivative [35]. Table 2 provides a comparison of the antitumor, immunosuppressive and

Table 2. Properties of triazene derivatives.*

	Cytoreductive activity	Suppression of immunity		Xenogenizing activity
		humoral	cell-mediated	
DTIC	+	++	+−+	++
MIC	+	++	NT	++
DM-CI	++	+	+	+−+
MM-CI	+	+	−	+
DM-NO_2	+	+	+++	++
MM-NO_2	+	+++	+	−+
DM-COOK	±	+++	+++	+
MM-COOK	+÷++	++	NT	++++

* Information gathered from Refs. 32, 35, 65; NT, not tested.

xenogenizing properties of DTIC and aryl-triazene derivatives. It appears that the various compounds manifest different characteristics. For instance, among the aryl-triazenes, MM-COOK is very active in mediating xenogenization as judged by the strength of immunogenicity elicited *in vivo* by doses equitoxic with those of DTIC; this data is consistent with *in vitro* evidence showing that the drug is active at very low concentrations. MM-COOK has also advantageous properties over other triazene derivatives, such as hydrosolubility and no need for metabolic activation in order to induce xenogenization. MM-COOK is also characterized by a rather favourable immunotoxicology in comparison with other triazenes, as will be mentioned later.

A number of data are now available which point to the strict analogy between the activity of DTIC and that of aryl-triazenes [35], and even suggest that xenogenizing properties could be shared by antitumor drugs not belonging to the triazene class [36]. Although this discussion will be centered on DTIC, the subject of most studies on chemical xenogenization *in vivo*, the range of susceptible tumors and agents endowed with xenogenizing properties indicates that chemical xenogenization *in vivo* is not limited to selected experimental conditions but may have broad biological significance and considerable therapeutic implications.

Basic biology of DTIC tumor lines

According to the experimental design described above, DTIC xenogenization of murine tumor cell lines *in vivo* usually requires 4–6 cycles of daily exposures to the drug, which means that highly immunogenic ('xenogenized') tumor variants are obtained following 4–6 transplant generations on DTIC [18]. With other triazenes, or using *in vitro* conditions of exposure to the drug, the modalities for obtaining changes in immunogenicity may be different [35]. Once xenogenization has occurred and treatment with DTIC has been discontinued, an immunogenic tumor variant will be rejected by intact recipients after an apparent initial take [37]. The tumor, therefore, has to be maintained through serial passages in immunodepressed (cyclophosphamide, X-rays) syngeneic recipients [38]. For many DTIC tumor variants, this has been done for up to 100 passages [39] which shows that the changes induced by the drug are stable and heritable.

When injected into intact recipients, xenogenized tumor variants, even though from different parental tumors, display a common kinetic pattern of growth and rejection characterized by an initial phase of exponential growth, followed by a rapid tumor clearance which only takes place in immunocompetent hosts [37]. Moreover, when cultured *in vitro,* the growth rate of DTIC tumors is similar to that of parental lymphomas [40].

Nonimmunodepressed animals which have survived challenge by a xenogenized tumor variant are specifically resistant to subsequent inoculation with either the DTIC-treated or the nonxenogenized parental line [40, 41]. Live lymphoid cells from sensitized hosts can confer specific adoptive resistance to unresponsive hosts [42]. Cytotoxic T lymphocytes generated *in vitro* against a xenogenized tumor variant will also confer protection upon immunodepressed mice challenged *in vivo* with the same variant [43, 44] or the parental tumor [45].

A large body of evidence indicates that the rejection of DTIC tumor variants is a truly immunological phenomenon. In this regard, two aspects appear central to the problem of tumor xenogenization by triazenes: the first is the definition of the antigenic properties of the immunogenic tumor variants and the second is the elucidation of the mechanism(s) underlying the appearance of the novel immunogenicity.

Antigenic properties of DTIC tumors

Three major points relevant to antigenic characteristics emerge from resistance studies of the immunogenic tumor variants:

a – increased immunogenicity can be demonstrated after even a single exposure of tumor cells to a triazene derivative, although full immunogenicity is obtained after several transplant generations of

the line on the drug [46]. Indeed, an increase in the immunogenicity of lymphoma cells can be detected as early as TG_2 when synergism between the host response elicited by the drug-modified tumor cells and chemotherapy is exploited [46]. A representative experiment showing this approach for xenogenization of L1210Ha leukemia by the aryl-triazene MM-Cl is shown in Fig. 3. In this experiment, xenogenized tumor cells recovered from a number (1–6) of TGs on MM-Cl are injected into intact or immunodepressed hosts treated or not with cytoreductive chemotherapy (BCNU). The increased immunogenicity of TG_2 cells is clearly shown by the 30-day longer survival time of nonimmunodepressed, BCNU-treated mice as compared with their 400R-immunodepressed counterparts. It may also be noted that a majority of long-term survivors is found in mice challenged with TG_3 cells if synergism with BCNU chemotherapy is exploited, whereas similar proportions of cures are seen only with TG_5 cells if these are given to mice not receiving chemotherapy (Data not shown). It is still unclear whether the augmented immunizing capacity associated with cells of early TGs on triazene exposure is the result of novel antigenic specificities in low densities, as seen with fully immunogenic drug-modified cells (see below), or rather reflects an increased expression of pre-existing transplantation antigens already present on the parental line.

b – When full immunogenicity is obtained, even very large inocula of xenogenized cells are rejected whereas as few as one non-drug-exposed cell will produce lethal growth [35];

c – the immunogenic strength of xenogenized tumor lines is quantitatively similar to that associated with the products of the major histocompatibility complex [37]. Indeed, the kinetic pattern of growth and rejection of DTIC-xenogenized tumors is very similar to that of parental lymphomas grafted into *H-2* incompatible recipients [37].

The complex of these data, generated in the early days of chemical xenogenization, provide compelling evidence that highly immunogenic lines can be obtained through exposure of nonimmunogenic tumors to DTIC, and that the effect of multiple treatments can be cumulative. Nevertheless, they do not provide an answer to the crucial question of whether the novel immunogenicity displayed by tumors repeatedly treated with DTIC is due to acquisition of novel foreign antigens or to an increase in the antigenicity of the existing tumor-associated transplantation antigens (TATA). An attempt to answer this question was made by resorting to tolerance experiments, which clarified two aspects particularly worth mentioning.

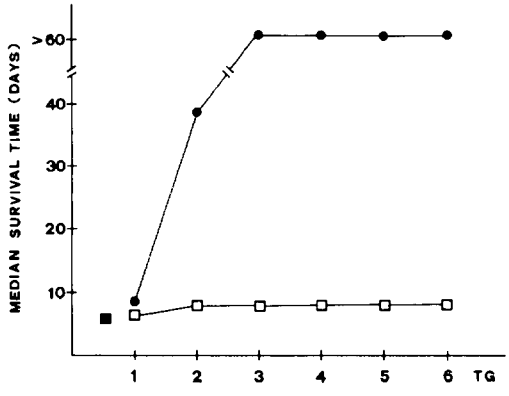


Fig. 3. Assessment of the immunogenicity degree in L1210Ha cells after a number of transplant generations on MM-Cl. Cells recovered from different TGs were injected into intact (●——●) or immunodepressed (□——□) mice further treated with BCNU. ■, parental cells into intact BCNU-treated recipients.

a – Mice rendered tolerant to parental lines possessing high levels of TATA still show strong transplantation resistance to their xenogenized variants [47]. This suggests that the highly immunogenic specificities expressed by the drug-treated tumors are qualitatively different from parental TATA to which the host has been made specifically tolerant.

b – Xenogenized tumors can induce both specific resistance [41] and tolerance [47] to parental tumors, which shows that the two kinds of tumors share common antigens.

These findings represent the first experimental evidence to support the concept that xenogenized tumors retain most of the parental TATA, but also express novel antigenic specificities which are distinct from the latter. Much evidence is now available that irrefutably demonstrates the existence of these drug-mediated tumor antigens or DMTA [48]. Table 3 reports the most relevant data in this

regard, largely derived from extensive *in vivo* experiments. Only more recently and mostly by exploiting *in vitro* methodologies has it been possible to elucidate the nature of DMTA, and provide answers to more specific questions about the repertoire of DMTA, the cross-reactivity among xenogenized variants from the same or different parental tumors, and the quantitative expression of both TATA and normal histocompatibility antigens on xenogenized tumors.

In vitro studies of DMTA

The characterization of DMTA has been made possible largely through two different approaches, the first relies on reactivity of cytotoxic T lymphocytes (CTL) and the second on humoral antibody production.

The presence of DMTA on DTIC-xenogenized tumors has been investigated in a number of *in vitro* studies in which specifically sensitized CTL were tested against ^{51}Cr-labelled target DTIC lymphomas. Effector CTL can be obtained in a primary *in vivo* response using, as a source of lymphocytes, spleens from animals that have rejected a DTIC tumor [49]. Similarly, specifically cytotoxic lymphocytes are generated *in vitro* in a primary response [48] or in secondary responses using *in vivo* presensitized responder cells [50]. The results of these studies are consistent with the hypothesis that DMTA are not detectable on the parental tumor [48], although, under selected experimental conditions, some reactivity against the nonxenogenized cells may apparently result from the sharing of common TATA [40]. The problem of the mechanism(s) by which otherwise poorly immunogenic TATA may become the target of cytotoxic responses in the context of the immunity elicited by DMTA will be dealt with later. The demonstration of specific T-cell-mediated killing of xenogenized tumors confirms the existence of DMTA as suggested by the *in vivo* protection and tolerance experiments. In addition, a number of other firm points can be summarized:

a) a DTIC tumor line, obtained through repeated *in vivo* exposure of parental cells to the drug, is a highly polyclonal cell population and contains a large number of variants with different antigens [51];

b) each monoclonal variant is, however, endowed with a unique set of DMTA [51].

c) All xenogenized variants of a tumor retain and share TATA that may pre-exist on parental cells [47–49].

d) No cross-reactivity between any pair of xenogenized variants can be detected except for that due to parental TATA [48].

e) Xenogenized variants do not display obvious changes in the qualitative or quantitative expression of normal histocompatibility (*H-2*) antigens [52].

The humoral antibody approach, also based on *in vitro* testing procedures, confirms the previous data gathered from studies of cell-mediated immunity. In addition, it has been possible to show that:

a) DTIC xenogenization of a murine lymphoma may induce determinants capable of eliciting specific antibodies in immunized mice, the levels of which progressively increase if the animals are exposed to repeated sensitizations. These antibodies are preponderantly IgG [53];

b) the novel antigenic determinants recognized by the anti-xenogenized tumor antibody are not spa-

Table 3. Evidence for the existence of DMTA

In vivo:
1. Transplantation resistance against xenogenized tumors (XT) in mice sensitized with XT and rendered incapable of primary graft responses by radiation (XT from poorly immunogenic parental tumors) (42)
2. Transplantation resistance against XT in hosts tolerant to parental tumors (47)
3. Specific protection by adoptive transfer of sensitized lymphocytes in mice rendered incapable of primary graft responses by radiation (42)
4. Production of strongly immunogenic XT from nonimmunogenic tumors (55)
In vitro:
1. Specific generation of secondary cytotoxic lymphocytes against XT (50)
2. Specific generation of primary cytotoxic lymphocytes against XT (48).

tially related to antigens shared with the parental tumor, which, in turn, react predominantly with the IgM present in the anti-xenogenized tumor hyperimmune sera [53]. Table 4 reports the reaction of both parental and xenogenized tumor cells with hyperimmune sera before and after absorption on parental cells; in this experiment, antibodies of both IgG and IgM classes are detected by means of flow microfluorometry analysis using fluorescein-conjugated antibody to mouse IgG or IgM as a reactant.

c) The novel determinants of the xenogenized tumor recognized by specific antibody are not expressed at detectable levels on normal cells of the same haplotype nor do they appear to be related to histocompatibility antigens [53].

d) Novel antigenic determinants on xenogenized lymphoma cells can also be detected by means of monoclonal antibodies. In a recent study, it was found that hybridomas derived from spleen cells of mice hyperimmunized against a xenogenized tumor variant may produce two kinds of antibodies: one is directed against determinant(s) shared by parental cells and nearly every xenogenized variant, while the second is specific for the immunizing variant and does not cross-react with either parental cells or other xenogenized variants of that tumor [54].

Although it is not clear whether the antigenic determinants recognized by specific antibodies are the same as those involved in cell-mediated immunity, the complex of the above data is largely confirmatory of the results obtained with CTL, and provides further insight into both nature and mechanism of induction of DMTA.

Table 4. Reaction of xenogenized and parental cells with anti-xenogenized tumor hyperimmune serum before and after absorption on parental cells (PC).

Tumor cells	First antibody[d]: absorption on PC	FICT antibody to[a]	
		IgG	IgM
Xenogenized[c]	–	9,340[b]	3,050
	+	7,530	93
Parental[c]	–	800	2,400
	+	450	13

[a] Procedure detailed in (53); FICT, fluorescein-isothiocyanate-conjugated; [b] Net linear fluorescence units; [c] Xenogenized, L5178Y/DTIC cells; Parental, L5178Y cells; [d] Serum from animals immunized four times with live L5178Y/DTIC cells.

Mechanisms of xenogenization by triazenes

The finding that immunogenic tumor variants are generated after exposure of parental cells to xenogenizing chemicals raises obvious questions as to the possible mechanisms underlying the phenomenon. It should be emphasized that several points are still very controversial in this respect; however, at least in the case of DTIC, the available evidence permits the exclusion of some potential mechanisms (see Table 5).

A decrease in the oncogenicity of xenogenized tumor cells as a major factor in the increased or indefinite survival of recipients of such tumors is ruled out by the finding that immunogenic variants grow and regress in intact hosts [37]; moreover, their growth rates are similar to those of parental lymphomas *in vitro* [40] and in immunodepressed hosts [37]. An increased density of pre-existing TATA as the main explanation for the appearance of strong immunogenicity is hardly supported by the tolerance experiments previously reported [47], or the occurrence of highly immunogenic variants from tumors not expressing detectable TATA [35]; moreover, the humoral antibody approach also provides results generally inconsistent with this hypothesis [53]. Similarly, increased immunosensitivity of xenogenized cells is unlikely since their susceptibility to *in vitro* lysis, mediated by alloreactive CTL, is comparable to that of non-xenogenized cells [56].

A popular hypothesis is that emergence of immunogenic tumor sublines might result from selection of pre-existing immunogenic clones. These would not be eliminated by immunologically incompetent hosts, as the DTIC-treated animals might be expected to be. Nevertheless, this possibility is rather unlikely, because parental lines serially transplanted in immunodepressed mice do not give rise to immunogenic variants [41]. Furthermore, and most importantly, DTIC xenogeniza-

tion can occur in the absence of drug-induced selection [30].

Viral activation and cell surface chemical haptenization are two other hypotheses worthy of consideration. Although the properties of DTIC-xenogenized tumors were not found to be transferrable with cell-free filtrates of drug-modified cells [38], possibly a more important factor against the hypothesis of viral activation is the lack of detectable cross-reactivity among variants of the same tumor. The idea of the drug acting as a hapten is refuted by the stability of the immunogenic properties of DTIC tumors.

An additional possibility, in line with recent data gathered from studies with other xenogenizing chemicals (see below), is that the triazene derivatives may activate the expression of 'silent' genes and thus condition the appearance of specificities coded for by the newly activated genes. In one such epigenetic model, the interference of xenogenizing compounds with DNA resides at the level of the enzyme that methylates the base cytosine. It is known that the extent of cytosine methylation regulates the expression of several gene functions [57, 58]. Immunogenic tumor variants might, therefore, have decreased levels of methylcytosine, which would increase the transcriptional activity of genes involved in the expression of immunogenicity. In this regard, Fig. 4 shows that xenogenization of a murine lymphoma *in vitro* by a triazene derivative is not accompanied by significant changes in DNA methylcytosine content, which tends to rule out this mechanism as a major factor in xenogenization by triazenes [59].

Not necessarily conflicting with some of the above mechanisms is the mutational hypothesis, which regards drug-induced somatic mutation as a major factor at work in chemical xenogenization by triazenes. Despite the fact that some inconsistency exists between the actual number of histocompatibility genes and that theoretically required in the mouse to account for the observed frequency of mutational events leading to immunogenic tumor variants, this hypothesis provides the rational basis for explaining most of the experimental data available, and is also supported by direct evidence [60].

Perhaps the mutational hypothesis, which also applies to xenogenizing mutagens other than the triazene derivatives, could be further elaborated so as to reconcile better the data on frequency of mutation with the number of histocompatibility genes. Along this line, it has been postulated that mutagens may cause DNA rearrangements in genes forming hypermutable regions which would

Table 5. Hypotheses concerning the decreased transplantability of DTIC-xenogenized cells in intact histocompatible mice.

	Hypothesis	Relevant data	Conclusion
1.	Decreased oncogenic potential	DTIC-lines grow and regress in intact mice (37). Growth rate of DTIC tumors is similar to that of parental lymphomas *in vitro* or in immunodepressed hosts (40).	Rejected
2.	Increased density of preexisting TATA on cell membrane	Mice rendered tolerant to the parental line still reject DTIC sublines (47). High immunogenicity is obtained in lymphomas not expressing detectable TATA (55). Specifically cytotoxic T lymphocytes do not lyse parental line in conventional assays (48, 50).	Rejected
3.	Increased immunosensitivity	Susceptibility to cell-mediated immunity against alloantigens of DTIC lines is similar to that of parental tumors (52).	Rejected
4.	Emergence of pre-existing highly immunogenic clones in immunodepressed hosts	Parental lines passaged in immuno-depressed hosts do not acquire strong antigenicity (41). DTIC xenogenization is demonstrable in the absence of drug-induced selection (30).	Rejected
5.	DTIC bound to cell membrane as a haptenic group	DTIC lines retain high immunogenicity following numerous passages in immunodepressed hosts not treated with DTIC (38).	Rejected
6.	Induction of novel antigenic specifics through interference with the genetic code	Most of the above data are compatible with this hypothesis.	Accepted

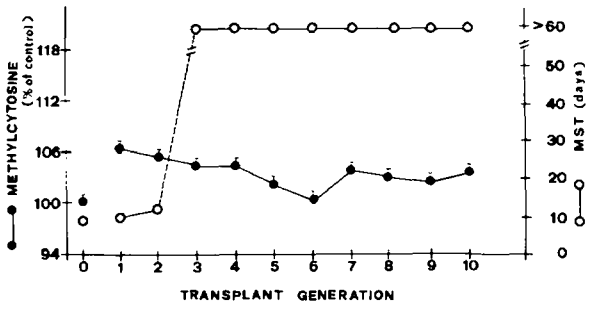


Fig. 4. Immunogenicity and methylation pattern of L1210Ha cells xenogenized by DM-Cl. Cells were treated *in vitro* for 1 hr with DM-Cl over the course of 10 transplant generations prior to each successive serial graft *in vivo*. At each TG, the degree of immunogenicity was tested by recovering the leukemic cells from the tumor-bearing mice (7–8 days after graft) and injecting them into intact or variously treated hosts ('immunogenicity test', see (47)). Results, as reported here, represent the median survival time (○——○) of the intact, tumor-challenged mice. A portion of the cells to be assayed *in vivo* for immunogenicity underwent quantitation of DNA 5-methylcytosine content by means of DNA labeling with tritiated uridine and HPLC fractionation. Results are given as methylcytosine content of the xenogenized cells at each TG (●——●, % of control). TG_0 indicates parental (control) cells. The entire procedure has been detailed elsewhere (59).

produce a wide variety of new antigens at very high frequencies [61]. Despite the lack of any direct evidence, this mechanism is the only one that justifies most of the experimental data on genetic interaction between triazene derivatives and tumor cells.

Mechanisms of protection of xenogenized against parental cells

One of the most intriguing problems related to chemical xenogenization is the elucidation of the mechanisms through which xenogenized tumor variants confer protection against parental poorly immunogenic cells. Much experimental evidence obtained so far indicates that:

a) protection against parental cells by DTIC-treated variants is clearly demonstrable when parental tumors do possess some degree of transplantation immunogenicity [47]. Thus, for instance, murine leukemias L1210, L5178Y and LSTRA, which have been the subject of most studies on triazene xenogenization, all elicit detectable transplantation immunity when injected as irradiated cells;

b) the corresponding DTIC variants confer strong anti-parental tumor protection when injected as irradiated or living cells [40, 41];

c) no protection is observed with DTIC variants of parental tumors for which no transplantation immunogenicity can be evidenced [55].

It appears that the presence of detectable TATA on parental cells, although not mandatory for the induction of DMTA [55], is nevertheless necessary for the xenogenized cells to immunize effectively against parental cells. This means that the presence of DMTA makes it possible for the host to develop an efficient anti-TATA immunity. One explanation for this phenomenon might be that immunization with parental tumor must necessarily be done with inactivated cells, whereas sensitization with xenogenized variants usually exploits living cells, which remain in the host in large numbers for a long time. Although this might be a working factor in the greater sensitizing ability of immunogenic variants over that of parental cells, much of the reviewed evidence suggests a direct role of DMTA in the development of anti-TATA immunity. Numerous mechanisms can be proposed [61], by analogy with those illustrated by Kobayashi for viral xenogenization [29, 62]. The first is that there might be quantitative and/or qualitative changes in the expression of TATA induced by xenogenization. Although much remains to be learned about TATA expression in cells xenogenized by triazene derivatives, both the cell-mediated and humoral antibody studies tend to rule out a major role for such changes in the immunogenicity of xenogenized variants [48–53]. Neither cell mediated nor humoral antibody studies suggest variations in the levels of major histocompatibility complex determinants [52], whose expression may affect the immunogenicity of TATA.

The second possible explanation as to why TATA of xenogenized tumor cells are more immunogenic may be that xenogenization causes changes in the mobility of tumor cell surface, so that TATA may be able to move more easily and

may actually cluster as a result of rearrangements which follow the appearance of DMTA. No data are presently available that either support or argue against this hypothesis.

The third possibility is associative recognition of the hapten-carrier-effect type (TATA conjugated with carrier DMTA), in which more specific cooperative effects would occur between the two kinds of structures through some degree of physical association. Triggering of immune reactivity might derive from cooperation of two lymphocytes that each recognize one determinant and thus come into close contact, or from association of the two determinants that combine to form an efficient immunogenic complex. Since, however, TATA and DMTA do not appear to be spatially related [53], this possibility is rather unlikely.

The final, and perhaps most likely, possibility is that there is an adjuvant effect of the xenogenized cells which results in the increased activity of accessory cells of the immune response, thus allowing development of a stronger anti-TATA response. A further elaboration of this hypothesis postulates that DMTA may serve as helper antigens for the anti-TATA response with involvement, for example, of factors released by lymphocytes and macrophages activated by the determinants induced by xenogenization on the same or different cells. Undoubtedly, further studies are needed to define more precisely the mechanisms that underlie the increase in anti-TATA immunity associated with chemical xenogenization of tumor cells.

Exploitation of DTIC xenogenization in immunotherapy models

The studies so far summarized clearly demonstrate that the appearance of DMTA is followed by the onset of specific antitumor cellular immunity. In models of tumor immunotherapy, this can be either actively induced or passively conferred through transfer of immune lymphocytes. Both kinds of immunity are primarily directed against DMTA (i.e., the immunizing variant) and, to a lesser extent, against TATA (i.e., the original tumor), as discussed earlier. However, as the strength of the reaction against DMTA is clearly greater than that against TATA, it is relatively easier to induce active or passive protection to a xenogenized variant than to the original tumor.

a) Actively induced protection: xenogenized tumor cells grow and regress in an immunologically intact host, conferring upon it a considerable degree of immunity to the parental tumor. Thus, xenogenized cells can be successfully exploited, in experimental models of immunotherapy of parental tumors, either prophylactically [47] or in combination with cytoreductive chemotherapy after they have been challenged with the original tumor [40]. Clearly, a more direct approach to exploitation of immunity associated with xenogenization would be treatment of the primary host with DTIC so as to generate levels of immunogenicity capable of evoking a strong response against the growing tumor. However, transplantation immunity is severely impaired in DTIC-treated mice because of the marked immunodepressant activity of the drug [63]. It follows that the DTIC-mediated increase of tumor immunogenicity cannot be of therapeutic value in the primary host in ordinary conditions. In one report [64], it was possible to restore, at least partially, the immunocompetence of lymphoma-bearing mice treated with DTIC by infusion of lymphoid cells from intact donors. This resulted in the development of antitumor immunity which acted synergistically with cytoreductive chemotherapy. Apart from this, however, a very important goal in studies of immunotherapy associated with xenogenization would be to find agents with a xenogenizing potential similar to that of DTIC, but devoid of any major immunotoxicity. Among the triazene derivatives presently under investigation in our laboratory, a few agents seem to provide encouraging results in this direction [65]. In this regard, table 2 shows a comparative analysis of the cytoreductive, xenogenizing and immunosuppressive properties of DTIC and the newly developed aryltriazenes.

b) Passively conferred protection: this kind of immunity relies on the adoptive transfer of specifically (anti-xenogenized tumor) immune lymphocytes, which confer protection when injected into immunodeficient mice challenged with the same

variant [43–45, 66]. In models designed to detect protective activity against an immunogenic variant, mice have to be immunodepressed prior to tumor challenge, i.e., rendered incapable of an autonomous antitumor cytotoxic response. In our laboratory the intracranial model, in which lymphoma cells are injected into the cerebral parenchyma by means of a microsyringe, has received much attention. This model was chosen on the basis of different considerations, among which was the small tumor inocula needed, and small inocula allow for the exploitation of a range of lymphocyte to tumor cell ratios. Moreover, the brain is traditionally considered an immunologically privileged site (an anatomical district largely incapable of autonomous immune responses), and the intracranial model allows better analysis of the contribution of adoptively transferred immunity. Using this model, we have been able to demonstrate that CTL raised *in vivo* or *in vitro* (in a primary culture) against a xenogenized tumor variant exerts considerable protection against the same variant when injected locally (i.e., intracerebrally) [66] or systemically [43]. The protection is mediated by lymphocytes, most of which express the Lyt-1^+2^+ phenotype, and requires the collaboration of host macrophages through involvement of helper lymphocytes also present in the transferred population [44]. In addition, recent data in our laboratory show that a CTL population sensitized against a xenogenized variant and systemically injected may be capable of inhibitory effects on the growth of the parental lymphoma grafted intracerebrally. The tumor-suppressive effects are specific and conditioned by dose, time and route of lymphocytes administration according to a pattern similar to that previously observed for the inhibition of the xenogenized lymphoma [45].

The possibility of impairing the growth of a parental tumor through adoptive transfer of lymphocytes sensitized *in vitro* against a xenogenized variant represents a major goal in the field of tumor immunotherapy. Further studies are needed toward this end along this direction, with a view to the possible application of chemical xenogenization to the treatment of human cancer.

Chemical xenogenization by mutagens

In vitro treatment, followed by cloning of murine tumor cells with chemicals traditionally regarded as potent mutagens, results in a high frequency of monoclonal variants that are rejected by intact histocompatible recipients [61, 67]. These variants have been designated 'tum$^-$' to distinguish them from the original tumorigenic 'tum$^+$' tumor lines [22, 68]. Most of these variants appear to express new antigens, and share many characteristics with the immunogenic tumor lines induced by DTIC *in vivo*. The failure of tum$^-$ variants to give rise to lethal growth in histocompatible hosts seems to be the result of an immune reaction, which can be demonstrated both *in vivo* and *in vitro* by means of CTL.

We will review evidence of similar xenogenization caused by mutagens (in particular nitrosoguanidine derivatives) and by triazenes.

Nitrosoguanidine derivatives

The literature indicates that *in vitro* exposure of experimental tumors to mutagens may lead to decreased tumorigenicity in histocompatible recipients (for review, see [61]). With the intent of obtaining variants with altered differentiation properties, Boon *et al.* [69] exposed a malignant mouse teratocarcinoma cell line to mutagen treatment. Using the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), the authors obtain a high frequency of stable tum$^-$ variants which form tumors only in immunodepressed animals. The tum$^-$ clones remain nontumorigenic in intact hosts even after months of continuous culture. The tum$^-$ and tum$^+$ clones are similar in karyotype and *in vitro* growth rate and, in animals exposed to 600 rad whole body irradiation, tum$^-$ is as tumorigenic as tum$^+$. Interestingly, the tum$^-$ clones are able to immunize actively against the original non-drug-exposed tumor cells.

Characteristics of tum⁻ variants

The results obtained with teratocarcinoma could be extended to other tumors [23, 70–72], and to spontaneous carcinoma and leukemia cells with little, if any, initial immunogenicity [72, 73]. In general, induced immunogenicity by MNNG has the following characteristics:

a) with a dose of MNNG that allows approximately 0.1% survival of the treated population, the frequency of immunogenic variants among surviving cells is in the 1–20% range [67], but can be increased by additional rounds of mutagenesis [74]. The resulting tum⁻ clones form progressive tumors only in irradiated or nude [22, 23, 71] mice, and give rise to a pattern of growth and rejection in intact recipients which is very similar to that of DTIC-xenogenized tumors. Experiments demonstrate that P815 tum⁻ cells multiply exponentially for at least 10 days when injected into the peritoneal cavity of immunocompetent hosts, but are completely eliminated by about day 20 after injection [71], at a time when the peritoneal cavity is considerably infiltrated by lymphocytes and macrophages [71].

b) An immune memory can be demonstrated in mice that reject a tum⁻ variant, since sublethal irradiation does not abrogate resistance to subsequent challenge with the same variant [22, 70, 71]. Moreover, adoptive transfer of splenic T lymphocytes from mice that have rejected a tum⁻ clone protects immunosuppressed recipients from challenge with the same variant [22].

c) When cross-protection experiments are performed in irradiated hosts, using different tum⁻ variants from a single parental tumor, some protection can often be detected, but a preferential degree of protection against challenge with the same tumor is the most general finding [67]. This again raises the question of which antigens (parental TATA or tum⁻ determinants) are involved in the cross-protection, i.e., are shared by the tum⁻ variants of a given parental tumor. Answers to this, as well as to other crucial problems of MNNG mutagenization, are provided -as in the case of DTIC xenogenization- by an *in vitro* approach exploiting CTL.

Analysis of tum⁻ antigens by means of CTL

In the P815 tum⁻ system, CTL are generated *in vitro* in a secondary response using *in vivo* presensitized responder cells, and cytotoxic activity is measured against ^{51}Cr-labelled target cells represented by the sensitizing or other tum⁻ clones [75]. A systematic analysis of numerous independently isolated P815 tum⁻ variants leads to important conclusions that have been recently summarized by Boon [61].

a) Most tum⁻ variants generate a high variant-specific response. Each clone expresses new individual antigens.

b) No two variants appear to express the same variant-specific antigen.

c) All tum⁻ variants share a common antigen with the parental cells. This tum⁺ antigen is responsible for the limited cross-reactivity detectable between pairs of tum⁻ variants.

d) No new antigens are found on mutagen-treated clones that remain tumorigenic, although some variants, for which no tum⁻ antigen can be defined by CTL, are nevertheless highly immunogenic.

The recent availability of variant-specific long-term CTL clones has allowed further analysis of the antigens expressed on tum⁻ variants. By using CTL clones directed against tum⁻ antigens of P815, it has been possible to dissect these antigens into several components that can be lost independently of each other. Upon incubation of some tum⁻ variants in the presence of the appropriate anti-tum⁻ CTL clone, stable secondary variants are obtained that appear to have lost the tum⁻ antigen recognized by CTL. By means of this immunoselection of secondary antigen-loss variants, it is possible to define residual variant antigenic determinants, and thus establish that two components of the tum⁻ antigen can be selected independently of each other [76]. The occurrence of antigen-loss secondary variants will certainly be most useful to the biochemical characterization of the tum⁻ antigens.

Relation of tum⁻ antigens to TATA

As in the case of DTMA associated with triazene

xenogenization, it seems probable that tum⁻ antigens are physically distinct from pre-existing TATA and that both structures co-exist in chemically xenogenized tumor cells. Induction of DMTA and tum⁻ antigens may be in some way connected with the appearance of TATA in tumors induced by chemical carcinogens [77]. Such tumors, indeed, appear to invariably express TATA, the relation of which to the transformation process has long been the matter of considerable debate. Since most chemical carcinogens are potent mutagens and since mutagens like the triazene [35] and nitrosoguanidine derivatives induce immunogenic tumor variants at high frequency, one might hypothesize that appearance of TATA in cells transformed by carcinogens is an independent effect of these agents, rather than directly related to the transforming event.

Whatever the case, *in vitro* analysis with CTL reveals that cells mutagenized by MNNG retain TATA that might pre-exist on parental tumor. *In vivo* studies of cross-protection with parental cells, besides further supporting this contention [70, 71, 78, 79], also indicate that apparently nonimmunogenic tumors may nevertheless carry TATA which can be revealed by tum⁻ variants [72, 73].

If the capacity of tum⁻ variants to induce a protective response against tum⁺ cells could be applied to human tumors, which express little, if any, antigenicity, this would undoubtedly provide new opportunities in cancer immunotherapy.

Mechanisms of xenogenization by MNNG

The studies so far reviewed are compatible with the hypothesis that the immunogenic variants obtained by triazene and nitrosoguanidine derivatives are examples of closely related phenomena, and also suggest that xenogenizing properties may be shared by a number of mutagenic compounds. However, the tum⁻ variant system apparently represents a genetic paradox, the complexity of which has recently been illustrated in detail [61]. Tum⁻ variants are indeed obtained at frequencies several orders of magnitude higher than those at which metabolic mutants are obtained after similar mutagen treatment. This implies that either the genomic domain that determines the expression of tum⁻ antigens is enormous or that it is hypermutable or hyper-rearrangeable. In the latter case, one could hypothesize that a wide variety of new antigens may result from DNA rearrangements of a relatively low number of genes, according to a mechanism similar to that involved in the formation of immunoglobulin sequences. Not mutually exclusive with this hypothesis, is the possibility that a set of genes may exist that are normally silent and, once activated by mutagens, may produce new antigens [28]. This mechanism, although not grossly operative in xenogenization by triazene derivatives, will nevertheless be shown to account for the appearance of novel immunogenicity in tumors altered by another group of xenogenizing chemicals. Increased *H-2* antigen expression has recently been described in murine melanoma cells treated with MNNG [80], and biochemical evidence has been provided by a recent study for MNNG-induced DNA hypomethylation associated with amplification of selected surface components in mouse lymphoma cells [81].

Undoubtedly, a major step toward the clarification of the mechanisms of xenogenization could be represented by the recent availability of specific anti-DTIC-tumor antibodies. In the case of the tum⁻ variant system, where the expression of tum⁻ antigens is dominant on somatic hybrids between tum⁻ and tum⁺ cells [82], a gene transfection approach leading to cloning of the relevant genes might also be crucial to the elucidation of the mechanisms of xenogenization by MNNG.

Protection of tum⁻ variants against parental cells

One issue central to both comprehension and exploitation of xenogenization by MNNG is represented by the analysis of the protective effects of tum⁻ variants against parental cells. We have already mentioned that sensitization of the compatible host with tum⁻ cells confers upon the latter a long-lasting but weak radioresistant specific protection against tum⁺ cells [72, 78].

a) protection against parental cells can be demon-

strated not only in the case of tumors that elicit transplantation resistance or have at least detectable TATA, but also in the case of parental cells for which no immunogenicity can otherwise be demonstrated [78];
b) with the only exception of one P815 tum$^-$ variant [71], the corresponding immunogenic variants effectively immunize only when injected as living (not irradiated) cells [70, 78, 79].

By immunizing with tum$^-$ variants, an immune response is triggered against parental TATA which may be present in such low amounts as to remain undetected in conventional assays of transplantation immunity. As in the case of DMTA, it appears that the presence of tum$^-$ antigens enables the host to develop an efficient anti-TATA immunity. Although in the tum$^-$ variant system the proliferation of the sensitizing cells appears to be a more crucial factor in conferring immunity, it seems plausible that the mechanisms of anti-TATA protection in that system may be similar to those described for triazene xenogenization.

As will be discussed later, the tum$^-$ variant system and triazene xenogenization also share a number of unanswered questions ranging from those relating to the molecular nature of tum$^-$ and DTIC-mediated tumor antigens, to the mechanism(s) through which nitrosoguanidine and triazene derivatives induce new transplantation antigens at such high frequency, and to the possibility of successfully applying chemical xenogenization to human cancer. It is obvious that more work is needed to elucidate these points.

Xenogenization by gene activators

Gene expression is under the control of a number of partially understood biological mechanisms. Evidence accumulated over the years indicates that enzymatic methylation of cytosine to 5-methylcytosine is an important gene silencing factor in both cellular and viral genes, a contention substantiated by the fact that demethylation of DNA, as obtained by replacement of cytosine with 5-azacytidine (5-Aza), may result in the expression of otherwise silent genes in a number of biological systems [57, 58, 82]. The exposure of experimental tumors to hypomethylating agents ('gene activators') may induce the appearance of variants displaying many new phenotypic characteristics whose *in vivo* behaviour can not be thoroughly predicted *a priori* [83]. When DNA hypomethylation enhances biochemical pathways whose expression is associated with successful organ colonization [84, 85] nonmetastatic clones of murine tumors can become highly metastatic. But, on the other hand, 5-Aza may also turn an originally metastatic tumor into a nonmetastatic one [84], or even reverse the induction of the nonmetastatic phenotype which follows mutagen treatment [86]. Furthermore, when the 5-Aza-induced gene activation involves functions related to expression of immunogenicity, an apparently poorly metastatic phenotype may result from selection of clones with increased immunogenicity [87].

Effect of drug-induced hypomethylation on immunogenicity

The effect of drug-induced hypomethylation on the expression of antigenicity and immunogenicity on tumor cells is the high frequency appearance of clones with increased immunogenicity. The increased immunogenicity generally prompts rejection by the compatible host. Frost *et al.* [28] first showed that 5-Aza treatment induces strongly immunogenic tumor variants at high frequency, and the underlying mechanism possibly involves an increased expression of *H-2* products [88], which are known to play a crucial role in the recognition of cell surface antigens by immune T-lymphocytes. Like mutagen-induced tum$^-$ variants, those obtained after 5-Aza treatment usually generate strong CTL responses *in vitro*, and give rise to lethal growth only in immunodeficient mice [28]. Phenotypic alterations induced by 5-Aza are heritable but not necessarily permanent, a finding that, combined with the extraordinarily high frequency of their appearance, strongly supports the contention that epigenetic rather than mutational mechanisms are responsible for the observed changes [28]. Ethylmethanesulfonate (EMS), a

xenogenizing chemical which, unlike 5-Aza possesses strong mutagenic activity, shows a hypomethylating pattern of activity virtually identical with that of 5-Aza [28]. This again raises the question of the relative contributions of mutagenesis and DNA hypomethylation to the xenogenizing activity of mutagens.

Induction of a 5-Aza tumor line

We have already mentioned that repeated exposure of a polyclonal tumor cell population to a triazene derivative leads to establishment of a tumor subline whose immunogenicity progressively increases as the extent of DNA methylation appears to be unimpaired, thus ruling out any major contribution of hypomethylation to the xenogenizing activity of the drug (Fig. 4). In a recent study [59, 89], we addressed the problem of both DNA methylating activity and induction of novel immunogenicity in murine lymphoma cells xenogenized by 5-Aza according to the repeated 'mutagenesis' protocol which has long been used in our laboratory to study the effects of most triazene derivatives. We thus developed an L1210Ha/5-Aza tumor subline in much the same way as that described for induction of the L1210Ha/DM-Cl subline (see Fig. 4), the only exception being that exposure of cells to 5-Aza *in vitro* prior to each successive *in vivo* transplantation was carried out for 24 h. At each transplant generation (i.e., following each round of drug exposure), the cells were assayed for immunogenicity and DNA methylating activity (Fig. 5). If one compares the results of Fig. 4 with those in Fig. 5, it stands immediately to reason that, while the stable, highly immunogenic phenotype was independent of DNA methylating activity, both the induction and reversion of a weak immunogenicity by 5-Aza correlated well with the levels of DNA methylation. In particular, it should be noted that the L1210Ha/5-Aza tumor subline never reached the levels of immunogenicity associated with triazene xenogenization, as those cells were only rejected by histocompatible recipients also receiving cytoreductive chemotherapy. No such chemo-immune collaborative activity was required by the

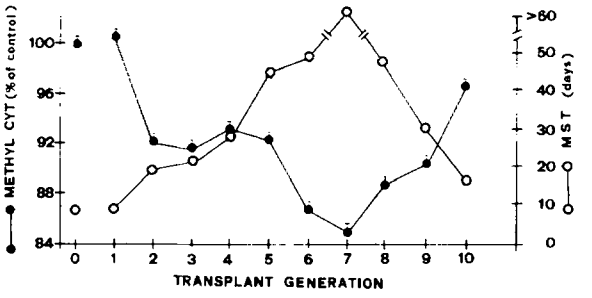


Fig. 5. Immunogenicity and methylation pattern of L1210Ha cells during xenogenization by 5-Azacytidine. The experimental procedure is the same as that described in legend to Fig. 4, the only difference being that the cells were exposed *in vitro* to 5-Aza (3 μM) for 24 hr. An immunogenicity test was performed at each transplant generation, and the results are given as median survival time (○——○) of the tumor-challenged animals on BCNU chemotherapy (which amplifies the effects of the newly acquired immunogenicity, see (47)). In the DNA methylating activity assay, results are expressed as methylcytosine content (●——●, % of control, see (59)). TG_0 means control parental cells.

L1210Ha/DM-Cl tumor subline in order to manifest fully its immunogenic potential. Therefore, it seems reasonable to conclude that the mechanisms of xenogenization by 5-Aza may be different from those involved in the activity of triazene derivatives, although the contribution of DNA hypomethylation to the xenogenizing activity of other mutagens still remains an open question.

Conclusions

Chemical xenogenization appears to be induced by agents not necessarily acting through a unitary mechanism: mutagenesis and gene activation are perhaps the most common mechanisms, and it is possible that, in some instances, they may act synergistically.

Whatever the mechanisms of chemical xenogenization, the finding that the antigenic structure of tumor cells can be altered *in vivo* by employing appropriate treatments with antineoplastic agents or resorting to *in vitro* exposure to selected chemicals could be of relevance both for a more effective use of cytoreductive drugs and in designing new approaches to cancer immunotherapy.

On one hand, in the choice of drugs for combination chemotherapy, xenogenizing drugs, or effectively xenogenizing agents might be included in the regime.

On the other hand, entirely new immunotherapeutic approaches could be developed similar to those successfully attempted in experimental models of tumor immunotherapy [43, 44, 64, 66]. New approaches might include:

a) Tumor-bearing hosts treated with xenogenizing drugs in order to increase the immunogenic potential of the malignancy.

b) The host treated with immunogenic tumor variants obtained *in vitro* through exposure of the original tumor to xenogenizing chemicals.

c) The host adoptively transferred with cytotoxic lymphocytes raised *in vitro* against immunogenic variants of the original tumor.

Key unanswered questions

As Thierry Boon recently pointed out, chemical xenogenization studies 'still provide us with more questions than answers' [77], and the list of unanswered questions must be very long. However, if one focuses on the mechanism(s) of xenogenization and the possible application of this approach to human cancer, the following appear to be the most crucial questions.

a)Are DMTA and tum$^-$ antigens a family of molecules coded for by a hypermutable or hyperrearrangeable genomic domain or are they carried by an array of completely unrelated genes? If the latter is the case, how can frequency of mutation be reconciled with the current knowledge on histocompatibility genes?

b) Do CTL and specific antibodies recognize the same antigenic determinants? If so, can the antibodies be used in immunoprecipitation studies aimed at defining the nature of DMTA as well as the mechanisms of their induction?

c) What is the molecular mechanism by which quinacrine, an 'antimutagenic compound', is capable of inhibiting xenogenization induced by triazene derivatives? Also: What is the contribution of epigenetic mechanisms to the xenogenizing compounds traditionally regarded as potent mutagens?

d) Can human cancer cells undergo immunogenic changes by xenogenizing chemicals? And: Can CTL raised against xenogenized variants of such tumors exert cytotoxic activity against parental cells *in vitro* and possibly protection *in vivo*?

e) Or, if *in vivo* xenogenization is to be used as the immunotherapeutic approach, will it be possible to find molecules with potent xenogenizing activity but devoid of any major immunotoxicity?

Thus, although the finding that tumor immunogenicity can be artificially manipulated could be of interest in the development of novel chemoimmunotherapeutic approaches in cancer treatment, the last remark must necessarily be an indication of the amount of effort to be expended on this type of immunological engineering before firm conclusions about its practical therapeutic potential can be advanced.

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