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Intratumor Heterogeneity of Cancer/Testis Antigens Expression in Human Cutaneous Melanoma Is Methylation-Regulated and Functionally Reverted by 5-Aza-2′-deoxycytidine

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ABSTRACT

Cancer/testis antigens (CTA) are suitable targets for immunotherapy of human malignancies, and clinical trials are mainly focusing on MAGE-A3. However, the heterogeneous intratumor expression of CTA may hamper the effectiveness of CTA-directed vaccination through the emergence of CTA-negative neoplastic clones. We investigated the intratumor heterogeneity of CTA in human melanoma and the underlying molecular mechanism(s) at clonal level using 14 single cell clones generated from the melanoma lesion Mel 313. Reverse transcription-PCR revealed a highly heterogeneous expression of MAGE-A1, -A2, -A3, -A4, -A6, GAGE 1–6, SSX 1–5, and PRAME among melanoma clones. Only nine clones expressed MAGE-A3 and competitive reverse transcription-PCR identified relative differences in the number of mRNA molecules of up to 130-fold between clones 5 and 14. This clonal heterogeneity of MAGE-A3 expression correlated with the methylation status of specific CpG dinucleotides in MAGE-A3 promoter: *i.e.*, hypomethylated CpG dinucleotides at positions −321, −151, −19, −16, −5, −2, +21, and +42 were found in clones expressing high but not low levels of MAGE-A3. Supporting the role of DNA methylation in generating the intratumor heterogeneity of CTA, the DNA hypomethylating agent 5-aza-2′-deoxycytidine (5-AZA-dCyd) invariably induced their expression in all CTA-negative clones. Furthermore, 5-AZA-dCyd–treatment reduced to 6 folds the differential expression of MAGE-A3 between clones 5 and 14, which became recognized to a similar extent by T cells specific for a MAGE-A–encoded peptide. These findings identify promoter methylation as directly responsible for the intratumoral heterogeneity of therapeutic CTA in melanoma and foresee the use of 5-AZA-dCyd to overcome the limitations set by their intratumor heterogeneous expression to CTA-based vaccine therapy.

INTRODUCTION

An increasing number of melanoma-associated antigens recognized by CTL in the context of specific HLA class I allospecificities are being identified (for review see ref. 1). Among melanoma-associated antigens, cancer/testis antigens (CTA), comprising the MAGE, NY-ESO, and SSX gene families and GAGE/PAGE/XAGE super-families (for review see ref. 2) are suitable therapeutic targets for active specific immunotherapy of melanoma patients, because of their limited expression in normal tissues and their *in vivo* immunogenicity (1, 3, 4).

However, immunohistochemical data have shown recently that the expression of different CTA (*i.e.*, SSX, NY-ESO-1, MAGE-A1) is for the most part heterogeneous within the tumor mass, focal expression being the most common finding (5–7). This *in vivo* pattern of CTA expression is likely to have a dramatic impact on the clinical outcome and on the design of more effective CTA-based immunotherapeutic approaches; in fact, CTA-negative neoplastic cells within the tumor mass, being unresponsive to CTA-specific immune recognition, can escape vaccination-induced or -up-regulated CTA-directed immune response, leading to the emergence of CTA-negative lesions, as reported previously for other melanoma-associated antigens (8).

Despite its obvious impact on the clinical outcome of CTA-based immunotherapies, to the best of our knowledge the molecular mechanism(s) responsible for the intratumor heterogeneity of CTA expression have not yet been elucidated. Indeed, their full identification is crucial to set up appropriate strategies to make all neoplastic cells within a given neoplastic lesion homogeneously susceptible to CTA-based immunotherapy.

In this study, we used 14 single cell clones, generated from a primary culture of the metastatic cutaneous melanoma lesion Mel 313, to (*a*) characterize at clonal level the heterogeneity of CTA expression within a distinct melanoma lesion; (*b*) unveil the molecular mechanisms underlying the observed CTA heterogeneity; (*c*) identify appropriate strategies to revert the constitutively heterogeneous intratumor expression of therapeutic CTA, allowing an efficient recognition of neoplastic cells by CTA-restricted CTL.

The results of our studies provide novel data showing that pharmacologic DNA hypomethylation is a suitable strategy to overcome the limitations set by the intratumoral CTA heterogeneity to CTA-based immunologic treatment of melanoma patients.

MATERIALS AND METHODS

Cell Lines and Single Cell Clones. The primary culture of melanoma cells Mel 313 has been generated from a metastatic melanoma lesion surgically removed from a patient with no history of chemotherapy or immunotherapy, who has been admitted for surgery at the National Cancer Institute of Aviano (Italy), as described previously (9). Cells were grown in RPMI 1640 (Flow Laboratories, Inc., McLean, VA) supplemented with 10% heat-inactivated FCS and 2 mmol/L L-glutamine (Flow Laboratories, Inc.). Single cell clones were obtained diluting the Mel 313 melanoma cell population to 3 cells/mL and plating 100 μL of these cell suspensions in each well of 96-well plates (Corning Costar, Milan, Italy).

The HLA-B*3701-restricted cytotoxic T-cell line (TCL) 337 was isolated by stimulation of peripheral blood lymphocytes of a melanoma patient with autologous tumor cells expressing only the HLA-B37 allele. Target of TCL 337 is the peptide MAGE-A.127–136 (REPVTKAEML, hereafter referred to as M.127) endogenously processed from MAGE-A1, -A2, -A3, and -A6 products (10).

Reverse Transcription-PCR Analysis. Total RNA extraction and reverse transcription (RT)-PCR reactions were done as described previously (11). The oligonucleotide primer sequences and gene-specific PCR amplification programs used have been defined for MAGE-A1, -A2, -A3, -A4, -A6 (12), NY-ESO-1 (13), GAGE 1–6 (14), SSX 1–5 (5), PRAME (15), and tyrosinase (16). The integrity of each RNA and oligodeoxythymidylic acid-synthesized cDNA sample was confirmed by the amplification of the β-actin housekeeping

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gene (11). Ten μl of each RT-PCR sample were run on a 2% agarose gel and visualized by ethidium bromide staining.

We scored the level of expression of distinct antigens according to the intensity of the specific RT-PCR product, which was obtained by densitometric analysis of ethidium bromide-stained agarose gels using a Gel Doc 2000 documentation system and the QuantityOne densitometric analysis software (Bio-Rad, Milan, Italy). The intensity of RT-PCR products was compared with that of the reference human melanoma cell line Mel 142 (MAGE-A1–, -A2–, -A3–, -A4–, -A6–, GAGE 1–6–, SSX 1–5–, and PRAME–positive) or human fibrosarcoma cell line HT1080 (NY-ESO-1–positive). Samples were scored −, no RT-PCR product detectable; +, expression level <10% to that of the appropriate reference cell line; and ++, expression level >10% to that of the appropriate reference cell line. Competitive PCR for MAGE-A3 and β-actin was done as described previously (17).

Sodium Bisulfite Genomic DNA Modification and DNA Sequencing. The bisulfite reaction, converting all unmethylated but not methylated cytosines to uracil, was carried out as described previously (18). For the analysis of MAGE-A3 promoter methylation status, bisulfite-modified genomic DNA was amplified as reported previously (17). We extracted PCR products from agarose gel with the Concert Rapid Gel Extraction System (Life Technologies, Inc.), cloned into the pGEM-T plasmid vector (Promega, Milan, Italy), and sequenced in both orientations using T7 and SP6 primers and the ABI 377 system (Applied Biosystems, Monza, Italy). Results represent five independent DNA clones.

***In vitro* Treatment of Melanoma Cells with 5-AZA-dCyd.** Cultures of melanoma cells were treated with 1 μmol/L 5-AZA-dCyd (Sigma, Milan, Italy), as described previously (11). At the end of treatment, medium was replaced with fresh culture medium without 5-AZA-dCyd and after 48 hours of incubation cells were used for molecular analyses. Control cultures were grown under similar experimental conditions but without 5-AZA-dCyd.

Retroviral Vector-Mediated Gene Transfer of HLA-B*3701 into Mel-313 Clones. The retroviral vector B37-CSM, coding for the HLA-B*3701 molecule was constructed as described previously (10). It encodes the HLA-B*3701 molecule, under the control of the viral long terminal repeat, and the truncated form of the human low affinity nerve growth factor receptor (ΔLNGFR), which was driven by the SV40 promoter.

Transduction of melanoma cells was done by cultivation with retrovirus-containing supernatant in the presence of 8 μg/mL Polybrene (Sigma). Three rounds of infection of at least 4 hours were done. Efficiency of infection was evaluated by immunofluorescence analysis with the LNGFR-specific monoclonal antibody 20.4 (American Type Culture Collection, Rockville, MD). Pure populations of infected cells were immunoselected for ΔLNGFR expression by magnetic beads (Dynabeads M-450, Dynal A.S., Oslo, Norway) coated with the LNGFR-specific monoclonal antibody 20.4.

Cytokine Release Assays. The Mel 313 clones expressing HLA-B*3701 (*i.e.*, 5-B37 and 14-B37) were treated with 1 μmol/L of 5-AZA-dCyd as described above and used to stimulate specific release of IFN-γ by TCL 337, as reported previously (10).

Briefly, 10^4 TCL 337 cells were incubated for 20 hours with 4×10^4 target cells in the presence of 50 units/mL IL-2. Cytokine release in 100 μL of supernatant was measured using an IFN-γ ELISA kit (Genzyme Corp., Cambridge, MA), according to the manufacturer's recommendations.

Statistical Analysis. Statistical significance was evaluated by the Student's *t* test for paired data.

RESULTS

Generation of Mel 313 Melanoma Clones and RT-PCR Analysis of CTA Expression. To evaluate the expression of CTA at clonal level within a distinct melanoma metastasis, 14 single cell clones were generated from a primary culture of the human cutaneous metastatic melanoma lesion Mel 313 and analyzed by RT-PCR for the expression of a panel of CTA. Results revealed a heterogeneous expression of MAGE-A1, -A2, -A3, -A4, A6, GAGE 1–6, SSX 1–5, and PRAME in investigated clones (Table 1). In particular, MAGE-A3 displayed a highly variable expression, being absent in five clones (1, 2, 3, 4, and 7), weak in six clones (5, 6, 9, 10, 12, and 13), and very strong in three clones (8, 11, and 14; Table 1).

Quantitative Analysis of MAGE-A3 Expression in Mel 313 Melanoma Clones. To further characterize the heterogeneity of MAGE-A3 expression among melanoma clones investigated, levels of MAGE-A3–specific mRNA were quantitated by competitive PCR in each melanoma clone (Fig. 1). Results showed a striking difference in the baseline levels of expression of MAGE-A3 among MAGE-A3–

Table 1 *RT-PCR analysis of CTA expressed by Mel 313 melanoma clones treated or not with 5-AZA-dCyd*

		CTA									
Clone no.	5-AZA-dCyd	MAGE-A1	MAGE-A2	MAGE-A3	MAGE-A4	MAGE-A6	GAGE1–6	NY-ESO-1	SSX1–5	PRAME	Tyrosinase
1	−	− *	−	−	−	+	+	−	−	−	++
	+	+	+	++	++	++	++	++	++	++	++
2	−	−	−	−	−	−	−	−	−	+	++
	+	+	+	++	++	+	++	++	++	++	++
3	−	−	−	−	−	−	+	−	−	+	++
	+	+	+	++	++	+	++	++	++	++	++
4	−	−	−	−	+	−	+	−	−	−	++
	+	+	+	++	++	+	++	++	++	++	++
5	−	−	−	+	−	++	+	−	+	−	++
	+	++	+	++	++	++	++	++	++	++	++
6	−	−	−	+	−	+	+	−	−	−	++
	+	+	+	++	++	++	++	++	++	++	++
7	−	−	−	−	−	+	−	−	+	−	++
	+	+	+	++	++	++	++	++	++	++	++
8	−	−	−	++	−	−	+	−	+	−	++
	+	+	+	++	++	+	++	++	++	++	++
9	−	−	−	+	−	++	+	−	+	−	++
	+	+	+	++	++	++	++	++	++	++	++
10	−	−	−	+	−	−	−	−	+	−	++
	+	+	+	++	++	+	++	++	++	++	++
11	−	−	+	++	−	++	++	−	+	−	++
	+	+	++	++	++	++	++	++	++	++	++
12	−	−	−	+	−	−	+	−	−	−	++
	+	+	+	++	++	+	++	++	++	++	++
13	−	−	−	+	−	++	+	−	+	−	++
	+	+	+	++	++	++	++	++	++	++	++
14	−	+	++	++	−	++	++	−	++	−	++
	+	++	++	++	++	++	++	++	++	++	++
Mel 313 †	−	−	−	+	−	−	+	−	+	−	++

* Intensity of RT-PCR products: −, not detectable; +, weak; ++, strong.
† Mel 313 parental primary culture of melanoma cells.

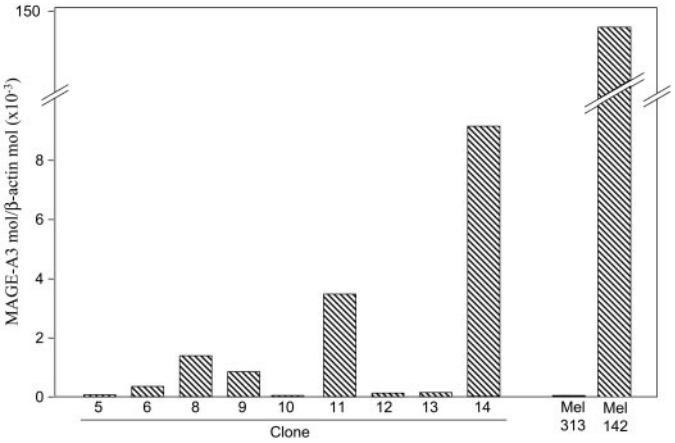

Fig. 1. Competitive PCR analysis of MAGE-A3 expression in Mel 313 melanoma clones. Total RNA was extracted from Mel 313 melanoma clones and subjected to RT. We carried out competitive PCR on 5 μL of cDNA mixed with increasing amounts of MAGE-A3 or β-actin competitors, using MAGE-A3– and β-actin–specific primer pairs. Ten μl of PCR products were size-fractionated on a 2% agarose gel, and competitor DNA and target cDNA amplification products were quantitated through densitometric scanning of the ethidium bromide-stained agarose gel. The number of MAGE-A3 cDNA molecules present in each sample was normalized to the number of β-actin molecules. The MAGE-A3–positive Mel 142 melanoma cells have been used as a reference.

positive clones, with clone 14 expressing 130 times the amount of MAGE-A3 mRNA expressed by clone 5 (Fig. 1).

Methylation Status of MAGE-A3 Promoter in Mel 313 Melanoma Clones. The methylation of CpG dinucleotides located in MAGE-A3 promoter (−363 to +119) was evaluated in investigated Mel 313 melanoma clones to assess whether a differential promoter methylation could account for the observed phenotypic heterogeneity of MAGE-A3 expression (Fig. 2). DNA methylation analysis done on clone 14 and clone 5, characterized by high and low levels of MAGE-A3 expression, respectively (Fig. 1), showed that cytosines at position −321, −151, −19, −16, −5, −2, +21, and +42 were rarely methylated in clone 14, whereas they were invariably methylated in clone 5 (Fig. 2). Furthermore, clone 1, which does not express MAGE-A3, showed a higher extent of CpG methylation in the region from −8 to +61, as compared with clone 5 (Fig. 2).

The presence of DNA populations displaying different patterns of CpG methylation in clone 1 and clone 14 is likely to result from the coamplification of MAGE-A6 promoter, which is indistinguishable from MAGE-A3 promoter. This could explain the presence of hypomethylated DNA molecules identified in clone 1, which does not express MAGE-A3, but expresses MAGE-A6 (Table 1). Supporting this assumption, Mel 142 melanoma cells, which express high levels of both MAGE-A3 and MAGE-A6, revealed a homogeneous population of completely unmethylated DNA molecules (Fig. 2).

Regulation of CTA Expression in Mel 313 Melanoma Clones by 5-AZA-dCyd. To define the possibility to revert the observed constitutive clonal heterogeneity of CTA expression, melanoma clones under study were treated *in vitro* with the DNA hypomethylating agent 5-AZA-dCyd. RT-PCR analyses revealed that 5-AZA-dCyd induced the expression of all investigated CTA in all CTA-negative clones (Table 1) and up-regulated it in clones expressing constitutively low levels of specific CTA (Table 1).

To further support RT-PCR data, clone 5 and clone 14, expressing low and high levels of MAGE-A3, respectively, were analyzed by MAGE-A3–specific competitive RT-PCR to measure levels of MAGE-A3 gene products before and after exposure to 5-AZA-dCyd. 5-AZA-dCyd treatment induced a significant ($P < 0.001$) 48-fold increase in the baseline levels of MAGE-A3 expressed by clone 5 (Fig. 3), in which it increased only 1.5-fold ($P = 0.006$) the levels of MAGE-A3 mRNA expressed by clone 14 (Fig. 3). Noteworthy, 5-AZA-dCyd–treatment drastically reduced the absolute difference in the constitutive levels of MAGE-A3 expression between clone 14 and clone 5; the latter being 130-fold and 6-fold before and after treatment, respectively (Fig. 3).

Modulation of MAGE-A3–Specific CTL-Recognition of Melanoma Clones by 5-AZA-dCyd. To evaluate the functional role of the up-regulated expression of MAGE-A3 induced in melanoma clones by 5-AZA-dCyd, the HLA-B37–positive clones 5 (clone 5-B37) and 14 (clone 14-B37), expressing low and high baseline levels of MAGE-A3, respectively, were treated with 5-AZA-dCyd and challenged with the HLA-B37–restricted TCL 337 recognizing the M.127 peptide encoded by MAGE-A3 (10). Consistent with their baseline expression of MAGE-A3, TCL 337 efficiently recognized untreated cells from clone 14-B37 whereas it poorly recognized untreated cells from clone 5-B37 (Fig. 4). In contrast, both melanoma clones were efficiently recognized by TCL 337, and to a similar extent, after exposure to 5-AZA-dCyd (Fig. 4). In particular, levels of IFN-γ released by TCL 337 rose from 8 to 251 pg/mL and from 125 to 218 pg/mL when cytotoxic cells were challenged with untreated or 5-AZA-dCyd—treated melanoma cells from clone 5-B37 and clone 14-B37, respectively (Fig. 4).

DISCUSSION

In this study, using human cutaneous melanoma as a model, we provide the first evidence that promoter methylation is the molecular mechanism directly responsible for the highly heterogeneous intratumor expression of CTA. On the basis of these findings, we also showed that the DNA hypomethylating agent 5-AZA-dCyd reverts

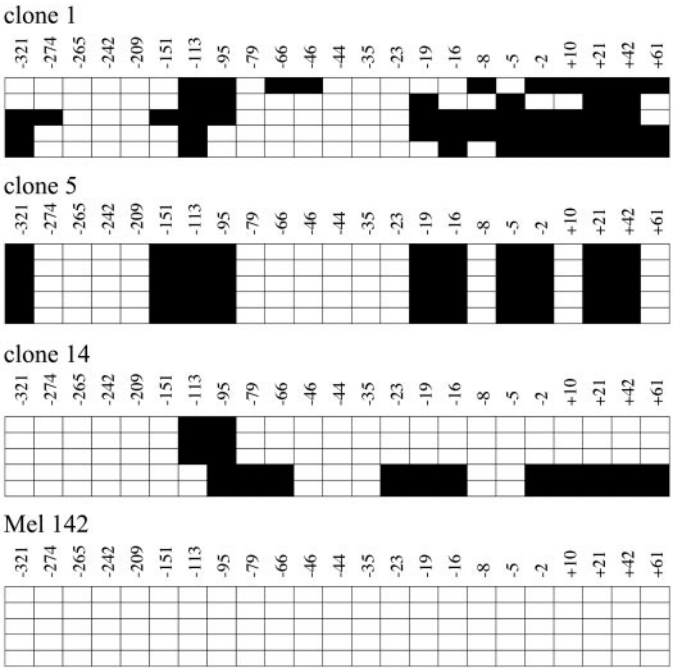

Fig. 2. Analysis of the methylation status of MAGE-A3 promoter in Mel 313 melanoma clones. Genomic DNA extracted from Mel 313 melanoma clone 1 (no expression of MAGE-A3), 5 (low expression of MAGE-A3), and 14 (high expression of MAGE-A3) and from the MAGE-A3–positive Mel 142 melanoma cells was subjected to modification by sodium bisulfite, followed by sequence analysis of PCR-amplified MAGE-A3 promoter. Results shown for each clone represent five independent DNA molecules, with *black rectangles* representing methylated cytosines and *empty rectangles* representing unmethylated cytosines. The position of the CpG dinucleotides is reported with respect to the transcription start site (+1).

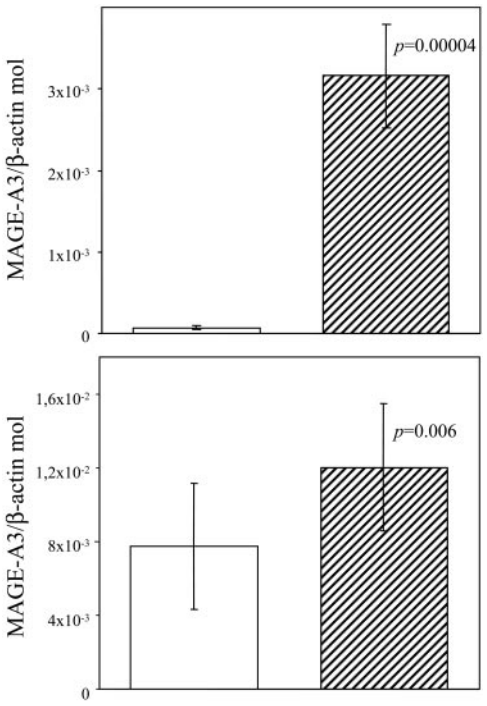

Fig. 3. Up-regulation of MAGE-A3 expression in Mel 313 melanoma clones by 5-AZA-dCyd. Total RNA was extracted from Mel 313 melanoma clones 5 (*top*) and 14 (*bottom*) treated (▨) or not (□) with 5-AZA-dCyd, and subjected to RT. We carried out competitive PCR on 5 μL of cDNA mixed with increasing amounts of MAGE-A3 or β-actin competitors, using MAGE-A3– and β-actin–specific primer pairs. Ten μl of PCR products were size-fractionated on a 2% agarose gel, and competitor DNA and target cDNA amplification products were quantitated through densitometric scanning of the ethidium bromide-stained agarose gel. The number of MAGE-A3 cDNA molecules in each sample was normalized to the number of β-actin molecules and is reported as mean value ± SD of MAGE-A3 mol/β-actin mol obtained in three independent experiments.

this phenotypic heterogeneity, leading to an efficient and comparable recognition of neoplastic cells by CTA-specific CTL.

The cloning of a primary culture of neoplastic cells from a melanoma lesion metastasized to the lymph-node has allowed for the generation of a panel of melanoma clones that were found to be highly heterogeneous for the expression of several CTA. In particular, MAGE-A3, which is being used widely as target for immunotherapeutic intervention in cancer patients, was not expressed in 5 of 14 clones (Table 1), whereas positive clones displayed highly heterogeneous levels of the antigen (Fig. 1). This variable distribution of MAGE-A3 identified at clonal level is consistent with recent immunohistochemical data reporting a prevalent focal expression of different CTA investigated, including NY-ESO-1 (19), MAGE-A1 (6), and SSX (5) in neoplastic lesions of different histologic origin.

At molecular level, the intratumoral heterogeneity observed both for presence and levels of CTA expression is clearly dependent on the extent of methylation of their promoter (Fig. 2). In fact, melanoma clones expressing high levels of MAGE-A3 displayed a hypomethylated status of specific CpG dinucleotides within the region of MAGE-A3 promoter investigated, as compared with clones expressing no or low levels of MAGE-A3 (Fig. 2). This finding is further supported by our recent results showing the requirement of a hypomethylated promoter for MAGE-A2, -A3, and -A4 genes to be expressed in metastatic melanoma lesions (17). In addition, the demonstrated ability of the DNA hypomethylating agent 5-AZA-dCyd to induce a *de novo* expression of all investigated CTA in all melanoma clones analyzed (Table 1) rules out the potential role of structural defects in the generation of the observed clonal heterogeneity of CTA expression and further supports its dependence on the epigenetic changes occurring during cell transformation and tumor progression (20).

5-AZA-dCyd up-regulated CTA expression among distinct melanoma clones to a much different extent, depending on their constitutive levels of antigen expression (Fig. 3). Although interesting, this finding was not surprising considering our initial data and when the relationship between CTA expression, CTA promoter hypomethylation, and the DNA-hypomethylating ability of 5-AZA-dCyd are taken into account. In fact, no major up-regulation of CTA expression should be expected in clones expressing constitutively high levels of CTA because of their heavily hypomethylated promoter, whereas a strong up-regulation should be anticipated in clones expressing low baseline levels of CTA as the result of an extensively hypermethylated CTA promoter. Remarkably, however, exposure to 5-AZA-dCyd led to comparably high levels of CTA expression among investigated melanoma clones (Fig. 3, data not shown). This finding is particularly relevant from a therapeutic point of view. In fact, a homogeneous expression of the therapeutic target among neoplastic cells is an essential prerequisite to efficiently target neoplastic cells within a distinct neoplastic lesion by vaccination-induced CTA-directed immune response, allowing the clinical outcome of CTA-specific vaccination strategies to be optimized (8). Strong experimental support to this notion is provided by the demonstration that the differential up-regulation of MAGE-A3 induced by 5-AZA-dCyd in melanoma clones 5 and 14, expressing constitutively different levels of the antigen, resulted in a similar recognition of investigated clones by the TCL 337 specific for the M.127 peptide encoded by MAGE-A3 (Fig. 4). This finding shows the potential of 5-AZA-dCyd to generate a population of neoplastic cells with a similar susceptibility to CTA-specific CTL recognition and foresees its clinical use in combination with CTA-based vaccines in cancer patients.

Along this line is the observation that 5-AZA-dCyd, through its ability to induce a *de novo* expression of CTA, allows an efficient recognition of melanoma clones otherwise refractory to CTA-specific CTL recognition (data not shown). This observation extends at clonal level previous data obtained with tumor cell lines of different histology, reporting a *de novo* lysis of CTA-negative neoplastic cells by CTA-specific CTL after 5-AZA-dCyd treatment (21, 22), and suggests that 5-AZA-dCyd might prevent the emergence of CTA-negative neoplastic clones able to escape treatment-induced immune surveillance, as shown previously for other tumor-associated antigen (8). Thus, it can be comprehensively suggested that systemic administra-

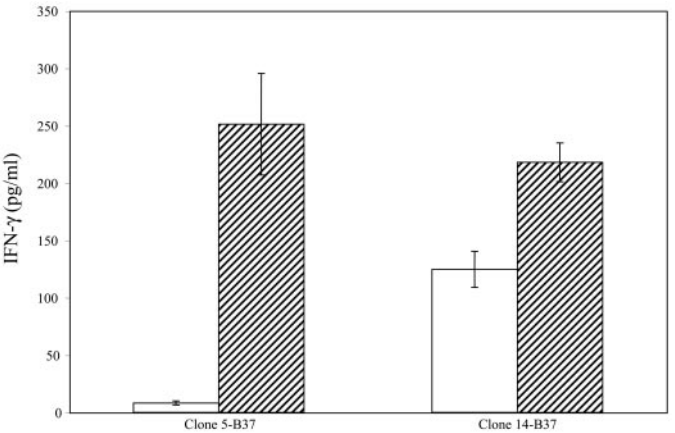

Fig. 4. CTL recognition of 5-B37 and 14-B37 melanoma clones treated with 5-AZA-dCyd. The HLA-B37–transduced Mel 313 melanoma clones 5 (5-B37) and 14 (14-B37) untreated (□) or treated with 1 μmol/L 5-AZA-dCyd (▨) were used as stimulators for the M.127-specific HLA-B37–restricted TCL 337. Recognition of melanoma clones by TCL 337 was assessed by ELISA determination of IFN-γ–release.

tion of 5-AZA-dCyd to melanoma patients could revert the CTA-negative and weakly-positive phenotype of distinct melanoma cells within neoplastic lesions, generating a population of neoplastic cells homogeneously expressing therapeutic CTA to be targeted by CTA-specific CTL.

Exposure to 5-AZA-dCyd concomitantly induced the expression of all investigated CTA in all CTA-negative melanoma clones used in this study (Table 1). Therefore, systemic administration of 5-AZA-dCyd could also allow a concomitant vaccination against multiple therapeutic targets and could widen the biological eligibility of CTA-directed immunotherapy to virtually all melanoma patients. These perspective therapeutic properties of 5-AZA-dCyd are further strengthened by its long-lasting effects on the antigenic profile of tumor cells, as shown previously for *de novo* expression of MAGE-A1 induced by 5-AZA-dCyd in melanoma cells, which was still detectable 2 to 5 months after the end of treatment (11, 23).

In addition to the immunologic rationale identified in this study, the clinical use of 5-AZA-dCyd is corroborated by its current use as a cytotoxic, differentiating, and/or proapoptotic agent in ongoing phase II/III clinical trials in hemopoietic malignancies (24–27). Thus, according to the complex of its immunobiological properties, 5-AZA-dCyd represents an appropriate pharmacologic agent to design new chemo-immunotherapeutic regimens to improve the therapeutic efficacy of multivalent CTA-directed vaccines in metastatic melanoma patients.

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