

SUPPRESSION OF CLASS I HUMAN HISTOCOMPATIBILITY LEUKOCYTE ANTIGEN BY *c-myc* IS LOCUS SPECIFIC

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Class I MHC antigens play a key role in the vertebrate immune defense system. They are transmembrane proteins expressed on nucleated cells of most tissues. In certain tumor types the expression of class I HLA (the human class I MHC) is often reduced or abrogated. This was observed for instance in melanomas, small cell lung carcinomas, neuroblastomas, and colon carcinomas (for review see reference 1). In some tumor types low class I HLA expression correlates with poor prognosis (2, 3). Involvement of class I HLA in human tumor progression is suggested by findings in rodent tumors that the level of class I MHC expression strongly influences the oncogenic and metastatic potential of tumor cells (reviewed in reference 4). Two different mechanisms with counteracting effects have been described.

First, tumor cells with low class I MHC expression could escape from destruction by cytotoxic T cells, as T cells only recognize specific antigens when they are presented on the cell surface by class I MHC molecules. In a number of murine tumor model systems, variant cells with a reduced class I MHC expression are more oncogenic and metastatic after inoculation in syngeneic animals than their counterparts with normal class I MHC expression (5-7). Re-establishment of class I MHC expression by gene transfer or treatment with IFN- γ reduces the malignancy of these tumor cells (8, 9).

Second, in other experimental tumor systems it was found that variant cells with a reduced class I MHC expression are in vitro more susceptible to lysis by NK cells than cells with a normal class I MHC expression (10, 11). In vivo these variant cells were found to be less malignant than the strongly class I-positive parental cells from which they were selected.

Recently, we have shown that in human melanoma cells the class I HLA expression is strongly downregulated by a high expression of the *c-myc* oncogene (12, and Versteeg, R., L. T. C. Peltenburg, A. C. Plomp, and P. I. Schrier, manuscript submitted for publication). This was demonstrated in a number of high *c-myc*-expressing melanoma cell lines as well as in melanoma lines with a low *c-myc* expression that we transfected with *c-myc* expression vectors. A similar effect was reported for the

These studies were supported by a grant (IKW 85-83) of the Netherlands Cancer Foundation (KWF).

N-*myc* gene in a rat neuroblastoma cell line (13). High expression of *myc* genes is found in many tumors. *c-myc* can contribute to cellular transformation and can enhance the metastatic potential of tumor cells (reviewed in reference 14). The finding that *c-myc* reduces class I HLA expression implies that this gene, primarily involved in oncogenesis, in addition may influence the immune recognition of the resultant tumor cells. The reduced class I HLA expression could lead to an escape from destruction by cytotoxic T cells, or, on the other hand, to an increased susceptibility to lysis by NK cells.

A complicating factor in the study of the downregulation of class I HLA by *c-myc* is the high complexity of the class I genes. At the three class I loci, A, B, and C, 20, 40, and 10 alleles, respectively, have been defined (15). Most individuals are heterozygous for each locus. For the class I HLA-A and -B loci, no explicit differences in expression or function have been described so far, which could be due to the fact that the >70 class I HLA alleles are highly homologous and difficult to discriminate. We wondered whether *c-myc* suppresses the HLA-A, -B, and -C loci to the same extent. Here, we report a highly differential regulation of class I HLA loci by *c-myc*, inducing a strong imbalance in the expression of HLA-A and HLA-B in human melanomas and melanoma cell lines. This mechanism has important consequences for the complex interactions between tumor cells and the immune system.

Materials and Methods

Tissue Culture. Melanoma cell lines (12) and mouse L cells (16, 17) were cultured in DMEM + 10% FCS. The EBV-immortalized B cell line was cultured in RPMI + 10% FCS. Treatment with rIFN- γ (Boehringer Ingelheim, Ingelheim am Rhein, BRD) was for 48 h with 500 U/ml.

Northern Blotting Procedures. Mouse L cells and melanoma cells were harvested by trypsinization and immediately quick frozen. Total RNA was isolated by the LiCl/ureum method (18). RNA (20 μ g/lane) was separated by electrophoresis through a 1% agarose-formaldehyde gel and transferred onto nitrocellulose. Hybridizations were performed with probes radiolabeled by the random primer method (19). The following probes were used: *c-myc*, a Cla I/Eco RI fragment of a human genomic clone, spanning most of exon 3 (20); GAPDH, a rat glyceraldehyde-3-phosphate dehydrogenase cDNA (21); γ -Actin, a mouse cDNA (gift of A. Bernards, MGH Cancer Center, Boston, MA); class I HLA-A locus, a 1.3-kb Pvu II/Xba I fragment of a genomic A2 clone, spanning the 3'-untranslated region (22); class I HLA-B locus, a 358-bp Pst I/Pvu II fragment of a B8 cDNA clone, spanning the 3'-untranslated region (22); class I HLA-C locus, a 432-bp Ssp I/Bam HI fragment of a Cw2 genomic clone, spanning the 3'-untranslated region (17). Hybridizations were performed in 50% formamide, 0.75 M NaCl, 50 mM NaH₂PO₄, 5 mM EDTA, 0.1% SDS, 0.1% BSA, 0.1% ficoll, 0.1% polyvinylpyrrolidone, and 200 μ g/ml salmon sperm DNA. The *c-myc*, GAPDH, and γ -actin probes were hybridized at 42°C. The class I HLA-A, -B, and -C probes were hybridized at 59°C, 61°C, and 61°C, respectively. After hybridization the filters were washed four times for 30 min in 2 \times SSC, 0.1% SDS at 50°C.

Antibodies. W6/32 (23) is an mAb recognizing all β_2 -microglobulin-bound class I HLA antigens. 4E is an mAb recognizing all class I HLA-B locus antigens and from the class I HLA-A locus the alleles A29 up to and including A33 (24). The mAb 4B recognizes exclusively the class I HLA-A2 and -A28 alleles (24). HC-A2 is an mAb recognizing mainly denatured H chains of class I HLA-A loci (Stam, N. J., Th. N. Vroom, P. J. Peters, E. B. Pastoors, and H. L. Ploegh, manuscript submitted for publication). The HC-10 mAb recognizes both native and denatured H chains of mainly class I HLA-B loci (25).

Isoelectric Focusing. Melanoma cells and B cells were labeled with ³⁵S-methionine and lysates were prepared as described (26). Samples with equal ³⁵S incorporation (10 μ Ci) were

precipitated with the mAb W6/32 bound to protein A-Sepharose beads. The protein A-Sepharose complexes were spun through a 30% sucrose cushion and the pellet was washed in neuraminidase buffer (50 mM NaAc, 1 mM CaCl₂, pH 5.0). Pellets were resuspended in neuraminidase buffer and neuraminidase was added (2 U/ml) (type VIII; Sigma Chemical Co., St. Louis, MO). Samples were shaken for 3 h at 37°C. The samples were centrifuged and the pellets resuspended in 30 μ l IEF buffer (9.5 M urea, 2% [vol/vol] ampholyte 3.5-10 [LKB Instruments, Inc., Gaithersburg, MD], 2% [vol/vol] β -ME) and subjected to IEF according to Neeffes et al. (27). Afterwards, gels were washed in fixative (7% HAc, 5% MeOH), soaked in Amplify (Amersham Corp., Arlington Heights, IL), and dried.

HLA Typing. EBV-immortalized B cell lines and PBL were typed for class I HLA-A, -B, and -C antigens, making use of alloantisera (derived from parous women) and complement lysis according to the standard National Institutes of Health technique (28). Melanoma cell lines were typed for class I HLA-A, -B, and -C antigens according to an adaptation of this method (29). Before typing, cell lines 136-2, 453A, and 603 were treated for 48 h with 500 U/ml IFN- γ . For a number of HLA antigens, we performed control experiments comparing IEF patterns of melanoma cell lines with those of well-typed control cell lines (Table I). Also, precipitations with antisera HC-10 and 4E (see above) followed by IEF served as an extra control for the identification of certain antigen specificities (Table I). The 603 cell line is positive for HLA-B53 or -B63, but we were unable to discriminate between these antigens as the used alloantisera are crossreactive and both HLA types have a nearly identical mobility on IEF gels. 136-2 cells gave only a weak reaction with alloantisera for A31, and therefore, the assignment of this antigen is based on the finding that the protein is precipitable with the 4E antibody, but not with the HC-10 antibody, indicating an A29 to A33 specificity. Final identification followed from comparison of the IEF pattern of 136-2 cells with that of control cells, which showed the identical mobility of the 136-2 band with the A31 protein (data not shown). The experiments enabling the HLA typing of the melanoma cell lines are summarized in Table I.

FACS Analysis. 5×10^5 cells were incubated for 30 min with a saturating concentration of the appropriate antibody. After washing, the cells were incubated with 1:10 dilution of FITC-labeled goat anti-mouse IgG (Nordic, Tilburg, The Netherlands). After washing, fluorescence of the cells was measured on a FACSTAR cytofluorimeter (Becton Dickinson & Co., Mountain View, CA). All incubations and wash steps were performed at 0°C.

Staining of Tissue Sections. Cryostat sections 4 μ m thick were cut from deep-frozen melanoma lesions. Consecutive sections were air dried, fixed in 100% acetone for 10 min, and stained with an indirect three-step immunoperoxidase procedure (30). In short, tissue sections were sequentially incubated with mAbs, with a rabbit anti-mouse Ig antiserum (Dako Corp., Santa Barbara, CA), and with a horseradish peroxidase-conjugated swine anti-rabbit Ig antiserum (Dako Corp.); 3-amino-9-ethylcarbazole was used as a substrate. Counterstaining was done with hematoxylin.

DNA Transfections and Vectors. Transfection of the 518A cell line is described in detail elsewhere (Versteeg, R., L. T. C. Peltenburg, A. C. Plomp, and P. I. Schrier, manuscript submitted for publication). Briefly, cotransfection was performed (31) with pSV₂neo and a derivative of pBR 322 containing *c-myc* exon 2 + 3 under control of the SV40 enhancer/promotor as well as the dihydrofolate reductase (DHFR) gene under control of the methallothionein promotor. Selection was performed with 800 μ g/ml G418. One G418-resistant clone was further cultured in DMEM + 10% dialyzed FCS and steadily increasing concentrations of methotrexate, resulting in a cell culture resistant to 200 nM methotrexate (518A-myc-200).

Results

In previous experiments we found an inverse relationship between the expression levels of class I HLA and the *c-myc* oncogene in a panel of 11 melanoma cell lines (12). To investigate whether the downmodulation of class I HLA by *c-myc* holds for all three class I HLA loci, we analyzed the expression of the individual loci in three representative melanoma cell lines with a high *c-myc* expression. We first measured

TABLE I
HLA Typing of Melanoma Cell Lines

Cell line	HLA antigens	Typed on tumor cells	Typed on PBL/B cells	IEF control	HC-10 reactive	4E reactive
518A	A1	+	+	+	-	ND
	A2	+	+	+	-	ND
	B8	+	+	ND	+	ND
IGR39	A1	+	ND	+	-	-
	B62	+	ND	+	+	+
453A	A2	+	ND	+	-	-
	B55	+	ND	ND	+	+
603	A24	+	±	+	-	-
	A30	+	+	ND	-	+
	B13	+	+	+	±	+
	B53/63	+	+	ND	+	+
136-2	A31	±	ND	+	-	+
	B56	+	ND	ND	+	+
	B62	+	ND	+	+	+

Summary of the experiments enabling the HLA typing of melanoma cell lines. +, positive reaction; -, negative reaction; ±, weak positive reaction.

the class I HLA expression at the mRNA level, using locus-specific probes derived of the 3'-untranslated region of the HLA-A2, -B8, and Cw2 genes, respectively. This region is strongly conserved within the different alleles of a particular locus, but shows a relatively high divergence between the three different loci. As a control for the specificity of the probes, we performed a Northern blotting analysis of total RNA isolated from three mouse L cell lines expressing transfected class I HLA-A2, -B7, and -Cw2, genes, respectively. Under high stringency conditions the three probes hybridize locus specifically (Fig. 1, lanes 10, 11, and 12). On the same filter we analyzed the mRNA of five melanoma cell lines. Two cell lines with low *c-myc* expression (IGR 39D and 518A; Fig. 1, lanes 8 and 9, respectively) express all three class I HLA loci. The three cell lines with a high *c-myc* expression (603, 136-2, and 453A; Fig. 1, lanes 2, 4, and 6, respectively) show hardly any hybridization with the B locus probe, but a good hybridization with the A locus probe. The C locus probe hybridizes with the RNA from cell lines 136-2 and 453A, but hardly with the RNA from cell line 603. From the patient from whom the melanoma cell line 603 was derived, we also established a B cell line (B 606) by immortalization with EBV. Comparison of the RNAs from the tumor cell line and the B cell line (Fig. 1, lanes 2 and 1) shows that the B and C loci are switched off in the tumor line, while the A locus expression is hardly affected.

We also analyzed RNAs isolated after IFN- γ treatment of the three high *c-myc*-expressing melanoma lines (Fig. 1, lanes 3, 5, and 7). In all cases a strong hybridization is seen with the B and C probes. This shows first that the B and C alleles of these patients are well recognized by our probes, and second, that the suppression of these alleles is a regulatory phenomenon that can be overruled by IFN- γ treatment. The combined results show that in the high *c-myc*-expressing cell lines, the HLA-B locus is switched off, while the HLA-A locus expression is hardly affected.

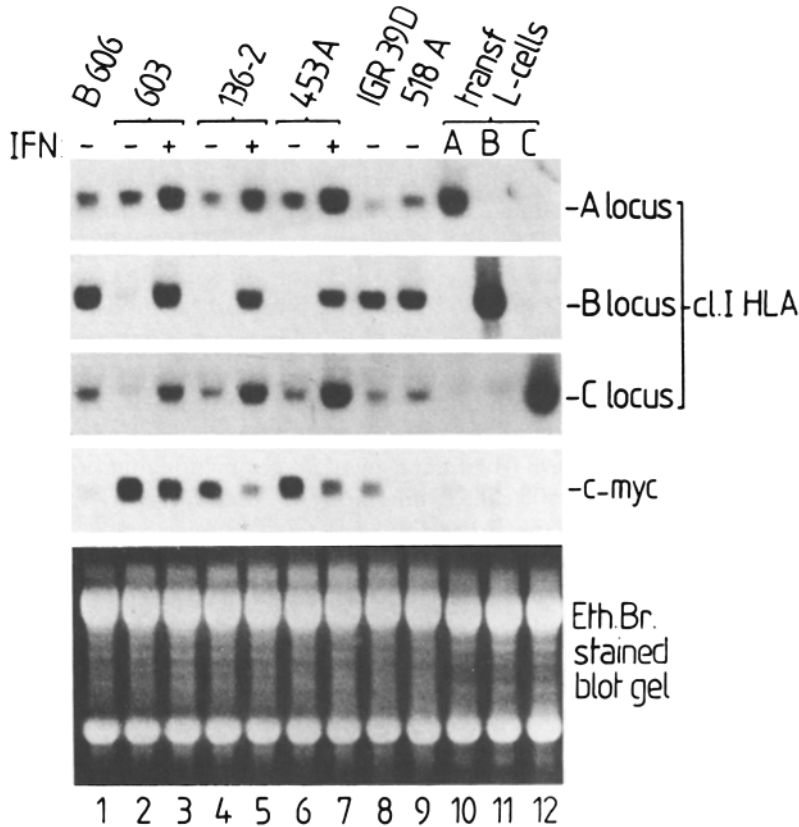


FIGURE 1. mRNA expression of class I HLA-A, -B, and -C loci in five melanoma cell lines assayed by Northern blotting. Lanes 10, 11, and 12 were loaded with RNAs from mouse L cells expressing transfected class I HLA-A2, -B7, and -Cw2 genes, respectively. Lanes 2, 4, and 6, melanoma cell lines 603, 136-2 and 453A; lanes 3, 5, and 7, melanoma cell lines 603, 136-2, and 453A treated for 48 h with IFN- γ ; lanes 8 and 9, the melanoma cell lines IGR 39D and 518A; lane 1, the B cell line B 606. The filter was sequentially hybridized with probes as indicated in the figure. As a control for the quantification, the ethidium bromide-stained blot gel is shown.

The situation with respect to the C locus is not clear, as two cell lines with high *c-myc* have normal expression (453A and 136-2) and one (603) seems to have down-regulated HLA-C expression.

Exclusive Expression of HLA-A Alleles. As the three class I HLA loci are multi-allelic systems, we further investigated whether the differential regulation of A and B loci holds for all A and all B alleles present in our cell lines. Class I HLA proteins of different alleles can be separated by IEF (27). Combined with the identification of the class I HLA alleles by classical HLA typing using alloantisera, the IEF technique enabled us to analyze the expression of individual class I alleles (the data resulting in the HLA typing of the melanoma cell lines are given in Materials and Methods). IFN- γ -treated and untreated cells of the melanoma line 603 and the B cell line B 606 (derived from the same patient) were labeled with ^{35}S -methionine. Cell lysates were precipitated with the mAb W6/32, which recognizes all class I HLA proteins associated with β_2 -microglobulin (23). Some C locus products are poorly precipi-

tated as they do not associate effectively with β_2 -microbulin (32). The immune precipitates were subjected to IEF. Both the IFN- γ -treated melanoma line 603 and the B cell line B 606 show, besides the β_2 -microglobulin band, the same four predominating bands (Fig. 2 A, lanes 2 and 3). A fifth band is nonspecifically immunoprecipitated, as it is also found in a control precipitation of B 606 with pre-immune serum (Fig. 2 A, lane 4). Two of the four specifically precipitated bands are not found in the untreated tumor cell line (Fig. 2 A, lane 1), even after a longer exposure of the filter (Fig. 2 A, lane 1'). The IFN- γ -treated tumor cell line 603 and the B cell line B 606 were typed as HLA-A24, -A30, -B13, and -B53 or -B63 (we were unable to discriminate between B53 and B63; see Materials and Methods). This enabled us to assign the bands as indicated in Fig. 2 A. Clearly, no B antigens are precipitated from the tumor cells with W6/32. HLA-B antigens were also not found after precipitation with the HC-10 antibody recognizing free HLA-B chains (25), thus excluding the possibility that these antigens are present in the cells in a free, not β_2 -microglobulin-bound, form (data not shown). We conclude that both HLA-B alleles are switched off in the 603 cells, while the HLA-A antigens are well expressed, albeit less strongly than in the B cell line.

A similar IEF gel made with precipitates of melanoma cell line 136-2 shows besides the β_2 -microglobulin band only one major band (Fig. 2 B lane 1) that can be assigned to the HLA-A31 antigen (see Materials and Methods). After IFN- γ treatment a strong and a weak extra band appear, which based on HLA-typing data could be identified as B56 and B62 proteins, respectively (Fig. 2 B, lane 2). As both HLA-B bands are not found with W6/32, nor with the HC-10 antibody (data not shown),

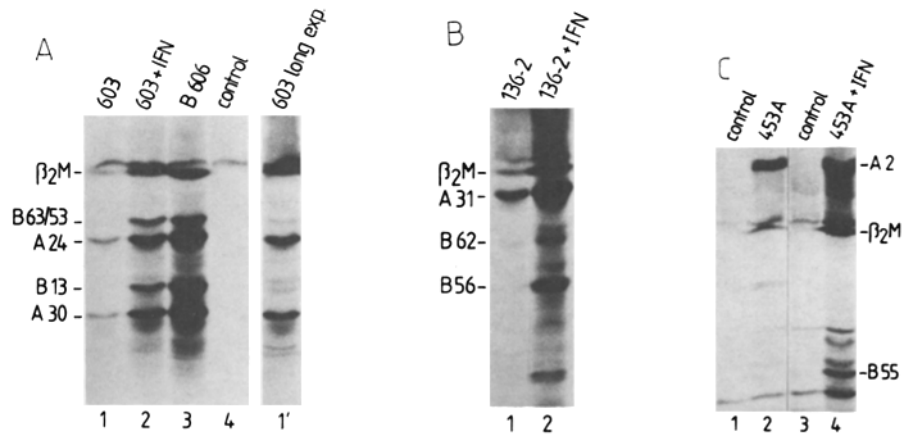


FIGURE 2. Analysis of the expression of individual class I HLA alleles in three melanoma cell lines. Untreated or IFN- γ -treated (48 h, 500 U/ml) cells were labeled with ^{35}S -methionine and lysates of equal ^{35}S incorporation were precipitated with W6/32 and subjected to IEF. Ascription of the indicated class I HLA alleles is based on data described in Materials and Methods. (A) Precipitations from the 603 melanoma cell line and the B cell line B 606 derived from the same patient. Lane 1, 603 cells; lane 2, 603, after IFN- γ treatment; lane 3, B cell line B 606; lane 4, control precipitation of B 606 with pre-immune serum; lane 1', long exposure of lane 1. (B) Precipitations from the 136-2 melanoma cell line. Lane 1, 136-2; lane 2, 136-2, after IFN- γ treatment. (C) Precipitations of the 453A cell line. Lane 1, control precipitation of 453A with pre-immune serum; lane 2, 453A; lane 3, control precipitation with pre-immune serum of 453A treated with IFN- γ ; lane 4, 453A cells after treatment with IFN- γ .

we conclude that in 136-2 cells, unless treated with IFN- γ , only the HLA-A31 allele is expressed.

The IEF pattern of 453A cells shows, besides a nonspecific band also precipitated with pre-immune serum (Fig. 2 C, lane 1), only the bands of HLA-A2 and β_2 -microglobulin (Fig. 2 C, lane 2). IFN- γ treatment results in the appearance of a new band (Fig. 2 C, lane 4), which we identified as HLA-B55 (see Materials and Methods). Other faint bands seen in Fig. 2 C, lane 4 are probably HLA-C products. As only one A and one B allele are expressed, we guess that 453A cells are homozygous for HLA-A and -B or have lost the class I HLA region of one chromosome 6. In 453A cells the B55 band is not found with the W6/32 antibody, nor with the HC-10 antibody (data not shown). We conclude that in this cell line also, only the HLA-A locus is expressed.

These IEF data show that in three melanoma cell lines with high *c-myc* expression, the A alleles are well expressed, while all B alleles are largely switched off. This favors the idea that in melanoma cell lines the suppression of class I HLA is locus specific and not just allele specific.

Only HLA-A Locus Products Are Expressed on the Cell Surface. For the biological function of class I HLA proteins, only the expression on the cell membrane is relevant. We therefore used flow cytometry to assay for cell surface expression of class I HLA. With the antibody W6/32 (recognizing, as well, HLA-A and -B) a high amount of cell surface labeling could be detected on the high *c-myc*-expressing melanoma cell line 453A (Fig. 3 A). The same result was obtained with the mAb 4B, specific for HLA-A2 (24), which is in agreement with the protein chemistry data, i.e., 453A being of the HLA-A2 specificity (Fig. 3 B). Strikingly, antibody 4E, which recognizes all HLA-B antigens but not HLA-A2 (24), reacts only very weakly with the 453A cell line (Fig. 3 C). After IFN- γ treatment this antibody gives a strong signal (Fig. 3 D). This shows that in cell line 453A, hardly any HLA-B antigens are expressed on the cell surface, except after IFN- γ treatment.

*Preferential Downmodulation of the HLA-B Locus by Transfected *c-myc* Genes.* Previously we have shown that *c-myc* suppresses the class I HLA mRNA and protein expression (12). The antibody used in those studies (B1.23.2; reference 33) happened to have a strong preference for B locus products. For the hybridization of the Northern blots we used at that time a cDNA probe of the B7 allele. It is generally assumed that this probe recognizes all class I HLA transcripts, but we recently found that it hybridizes mainly with B and C locus transcripts, even under low stringency conditions (data not shown). Our detailed analysis of the class I HLA expression in three high *c-myc*-expressing melanoma cell lines suggests that *c-myc* down modulates mainly B locus expression. To test this, we investigated the downregulation of the HLA-A and -B loci in *c-myc* transfectants of the IGR 39D and 518A melanoma cell lines.

First, cell line IGR 39D (low *c-myc*, high class I HLA) and the three *c-myc*-transfected clones, 6, 7, and 3, which we described before (12), were analyzed. Flow cytometry after labeling with the HLA-B-specific mAb 4E of IGR 39D and clones 6, 7, and 3 shows a relative fluorescence of 100%, 46%, 34%, and 14%, respectively (data not shown). Lysates of these cells after labeling with ^{35}S -methionine were precipitated with W6/32 and subjected to IEF. IGR 39D was typed as HLA-A1 and HLA-B62, and both antigens are well expressed (Fig. 4 A). In the *c-myc* transfectants, the bands of the B62 antigen are strongly reduced, while the A1 antigen expression is only

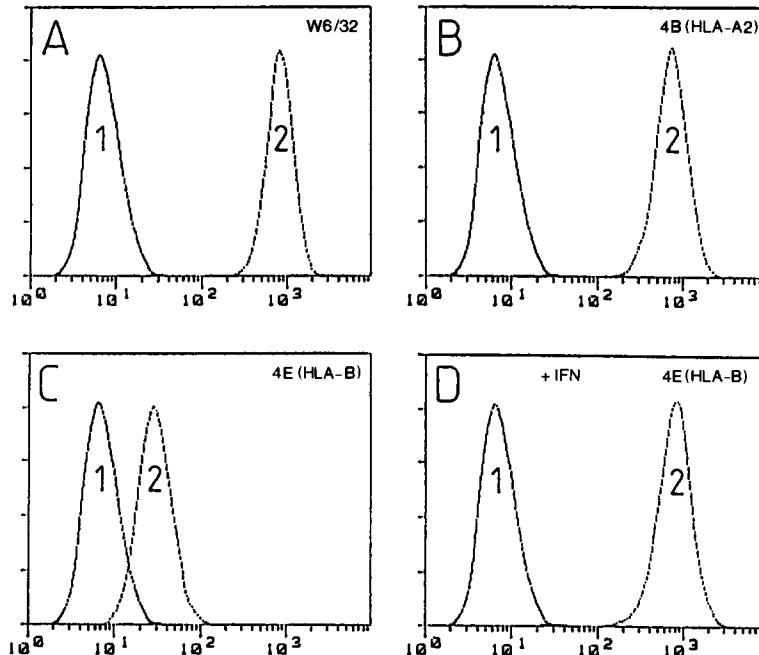


FIGURE 3. Cell surface expression of class I HLA-A and -B antigens on 453A melanoma cells as measured by FACS analysis. The *y*-axis gives the relative cell number; the *x*-axis gives the relative fluorescence intensity in a logarithmic scale. Cells were incubated with specific mAbs followed by FITC-labeled goat anti-mouse IgG (curves 2). Negative controls were stained omitting the first antibody (curves 1). (A) staining with the mAb W6/32, recognizing both HLA-A and HLA-B antigens; (B) staining with mAb 4B, specific for the HLA-A2 allele; (C) staining with the mAb 4E, recognizing all HLA-B alleles; (D) same as C, after IFN- γ treatment of the 453A cells.

slightly downmodulated (Fig. 4 A). The difference is strongest in clone 3, which has the highest *c-myc* expression (12). In this clone, the A locus expression is not completely unaffected, which suggests the possibility that at even higher *c-myc* concentrations further suppression of the A locus might occur.

We have also transfected a *c-myc* construct to melanoma cell line 518A (Versteeg, R., L. T. C. Peltenburg, A. C. Plomp, and P. I. Schrier, manuscript submitted for publication). To test for locus-specific downregulation, we analyzed 518A (low *c-myc*, high class I HLA), 518A-neo (a pSV2neo-transfected control), and 518A-myc-200, a clone selected for high expression of the transfected *c-myc* gene (see Materials and Methods). PBL of the patient from whom the 518A melanoma cell line was derived were typed as HLA-A1, -A2, and (homozygous) -B8. The 518A and 518A-neo cell lines show a strong expression of all three antigens (Fig. 4 B, lane 1 and 2). However, in the 518A-myc-200 transfectant, the expression of the B8 antigen is strongly reduced, while the expression of the A1 and A2 antigens remained unaltered (Fig. 4 B, lane 3).

From these transfection experiments we conclude that *c-myc* suppresses the B locus

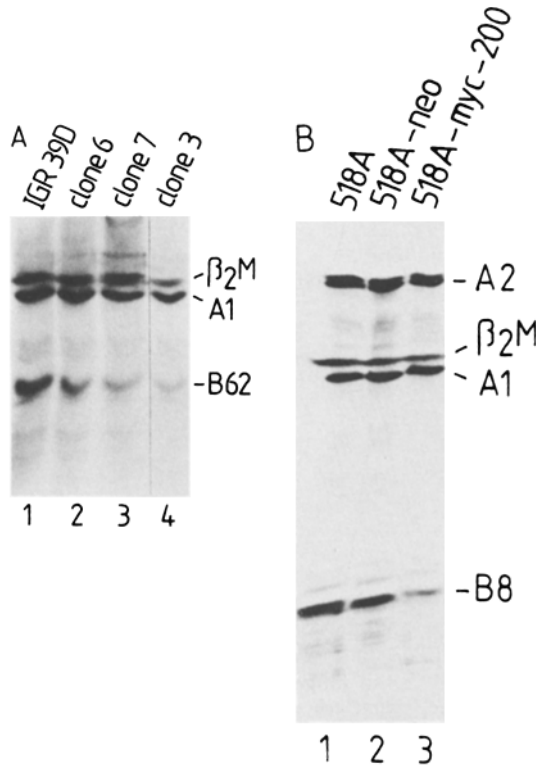


FIGURE 4. Expression of class I HLA alleles in *c-myc*-transfected melanoma cell lines. Cells were labeled with ³⁵S-methionine and lysates were precipitated with W6/32 and subjected to IEF. Assignment of the indicated class I HLA antigens is based on HLA typing data described in Materials and Methods. (A) IEF of precipitates from IGR 39D and three *c-myc*-transfected derivatives. Clones 6, 7, and 3 have the same numbering as described earlier (12) and are shown here in order of decreasing class I HLA-B expression as determined by FACS analysis (relative to IGR 39D, respectively, 46%, 34%, and 14%). (B) Precipitations from 518A and *c-myc*-transfected derivatives. Lane 1, 518A; lane 2, 518A-neo, a pSV₂neo-transfected control of 518A; lane 3, 518A-myc-200, a clone of 518A cotransfected with pSV₂neo and a *c-myc*/DHFR construct, which was selected for a highly increased *c-myc* expression (see Materials and Methods).

alleles much more strongly than the A locus alleles, which is in agreement with the results found in the three melanoma cell lines with high *c-myc* expression.

Melanoma Tumor Lesions also Lack HLA-B Expression. For the interpretation of the biological function of our present findings, it is highly relevant to establish whether locus-specific downregulation is also found in vivo. Therefore, we determined class I HLA expression in the primary tumor from which we established cell line 453A. Successive tissue sections were stained with the antibodies HC-A2, preferentially recognizing A locus products (Stam, N. J., Th. N. Vroom, P. J. Peters, E. B. Pastoors, and H. L. Ploegh, manuscript submitted for publication) and HC-10, preferentially recognizing B locus products (25). The sections stained with HC-A2 (Fig. 5 A) have a homogeneous positive reaction pattern, but in the sections stained with HC-10 (Fig. 5 B), only a few areas are positive, showing that the vast majority of the tumor cells has no B locus expression. In all cells, however, the A locus is expressed. Also, the metastatic tumor lesion from which the 603 melanoma cell line was established (a subcutaneous metastasis) was investigated. Staining of successive tissue sections with the HC-A2 and HC-10 antibodies revealed in this tumor, as well, an exclusive expression of HLA-A (data not shown).

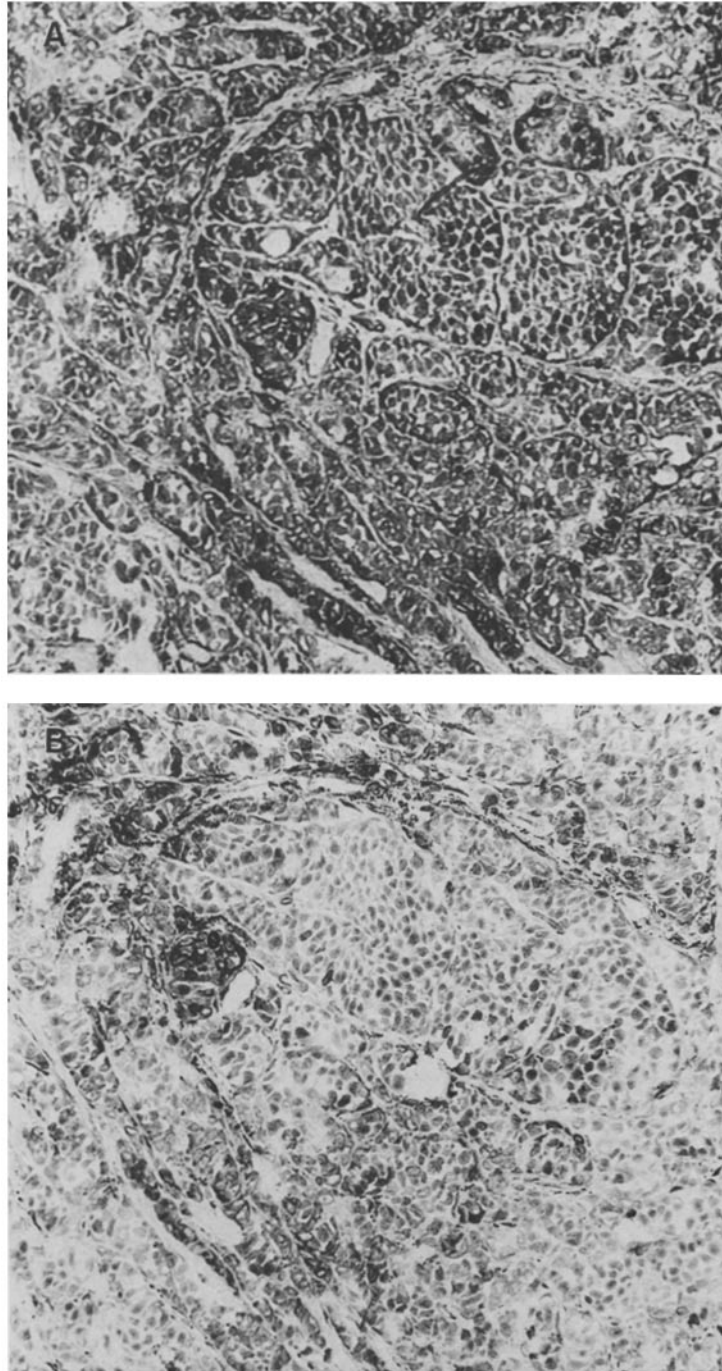


FIGURE 5. Immunohistochemical analysis of the HLA-A and HLA-B expression on the primary cutaneous melanoma from which cell line 453A was derived. Consecutive frozen sections were stained with the anti HLA-A antibody HC-A2 (*A*) or the anti HLA-B antibody HC-10 (*B*). Note the marked staining of a single nest of tumor cells in *B*. Sections were counterstained with hematoxylin to visualize the nuclei.

Discussion

In this work we show, for five melanoma cell lines with a high expression of endogenous or transfected *c-myc* genes, that certain class I HLA alleles are preferentially switched off. This observation was made for the B8, B13, B53/63, B55, B56, and B62 alleles. Alleles that are not or hardly switched off were identified as A1, A2, A24, A30, and A31. Suppression of the C locus was found by comparing the melanoma cell line 603 and a B cell line of the same patient, but was not evident in other cell lines (see Fig. 1). We conclude that suppression of class I HLA by *c-myc* is restricted mainly to the B locus and possibly the C locus. Comparison of melanoma cell lines expressing increasing amounts of transfected *c-myc* genes suggests that the A locus does not stay completely unaffected, but its suppression is limited relative to the suppression of the B locus. In three melanoma cell lines with high *c-myc* expression, this preferential downregulation results in the virtually complete absence of B locus products, while the A locus is strongly expressed. The same differential expression pattern was found in two surgically removed melanoma lesions, suggesting that the differential suppression of the class I loci by *c-myc* can have biological relevance for the functioning of the class I HLA system in human melanomas.

Because low class I HLA expression or high *c-myc* expression are found in a large variety of human tumors, it is fairly likely that a differential expression of class I HLA loci occurs in more tumor types than melanomas. In many studies the class I HLA expression on tumor lesions was quantified, but only in a few tumor types was a correlation between low class I HLA expression and malignancy or differentiation grade found (2, 3). However, such studies usually did not discriminate between the three class I HLA loci. As illustrated by our experiments, the W6/32 antibody may give a strong signal, while HLA-B expression was absent. Therefore, tumors with reduced expression of only one class I locus are probably overlooked so far. The use of locus-specific antibodies could give a better insight in the relation between class I HLA expression and malignancy of tumors. Interestingly, two examples of allele-specific downregulation of class I HLA in tumors have been described very recently. In some Burkitt lymphomas, suppression of HLA-A11 was described (34), and in some colon carcinomas, absence of HLA-A2 was found (35). A differential expression of HLA-A and -B transcripts was reported for the Molt-4 cell line (36). Whether *c-myc* is involved in these processes is presently unknown.

Melanoma-specific cytotoxic T cells, which are class I HLA restricted, have been isolated and cloned from human melanoma lesions (37, 38). In these studies it was not investigated whether the tumors had high *c-myc* expression, nor was it established by which class I locus products the tumor antigens were presented to cytotoxic T cells. Our results predict that melanoma cells with tumor antigens presented by HLA-A are, irrespective of their *c-myc* expression, good targets for cytotoxic T cells. However, we expect that HLA-B-restricted cytotoxic T cells will be unable to kill high *c-myc*-expressing melanoma cells. There is evidence that class I MHC-restricted cytotoxic T cells are involved in the strong antitumor effects of LAK therapy (39). Recent studies by Rosenberg et al. (40) suggest that the population of melanoma-infiltrating lymphocytes used in immunotherapy largely consists of class I HLA-restricted cytotoxic T cells. A thorough analysis of class I HLA expression on tumor cells, as described here, should reveal whether suppression of particular class I HLA loci correlates with a poor response to immunotherapy with tumor-infiltrating lymphocytes.

Selective downmodulation of specific class I loci was not observed previously in human tissues and, therefore, it is generally supposed that the class I loci are coregulated and exert identical biological functions. Assuming that the preferential suppression of HLA-B by *c-myc* has a biological function, we wondered whether for some function of class I HLA the A and B loci play a differential role.

Of the different functions proposed for class I HLA, the one generally accepted is the presentation of antigens to cytotoxic T cells. Although our findings predict that *c-myc*-activated melanomas could escape from destruction by HLA-B-restricted cytotoxic T cells, this mechanism seems somewhat paradoxical. Such paralysis of the T cell surveillance would favor the unrestrained dissemination of the most dangerous category of *myc*-activated tumor cells. As it is unlikely that this bears any selective advantage to the organism, we think that the reason for *c-myc* suppressing HLA-B must not be sought in the fact that this interferes with a possible T cell-immune reaction. Another proposed function for class I HLA is modulation of NK susceptibility of tumor cells: class I MHC-negative variants of tumor cells were found to be more prone to NK lysis than the original tumor cells with normal class I MHC expression (10, 11, 41). We recently found that *c-myc*-transfected melanoma cells with an abrogated HLA-B expression are more sensitive to NK lysis than the parental cell lines (Versteeg, R., L. T. C. Peltenburg, A. C. Plomp, and P. I. Schrier, manuscript submitted for publication). This suggests that the suppression of HLA-B by *c-myc* could function as a mechanism by which cells with an aberrant *c-myc* expression are recognized and destroyed by NK cells.

Summary

The *c-myc* oncogene downregulates class I HLA expression in human melanoma. The major class I HLA antigens are encoded by three loci, A, B, and C, and we investigated whether these loci are suppressed equally by *c-myc*. In three melanoma cell lines with high *c-myc* expression, we analyzed mRNA, protein, and cell surface expression of the class I HLA antigens. Whereas the HLA-B locus expression was found to be strongly reduced, the HLA-A locus was expressed normally. Analysis of *c-myc*-transfected clones of two melanoma cell lines confirmed that *c-myc* preferentially suppresses the class I HLA-B locus. Immunohistochemical analysis of fresh melanoma lesions also showed that in the tumors the HLA-A loci are expressed normally, while on the majority of tumor cells no HLA-B antigen expression was found. This downregulation may have consequences for the recognition of malignant cells by tumor-infiltrating lymphocytes. Our results predict that HLA-B-restricted cytotoxic T cells will be unable to kill high *c-myc*-expressing melanoma cells.

We thank Maarten van der Keur for expert operation of the flow cytometer, Lia Schalkwijk for excellent assistance in the immunohistological procedures, and Simone van Bree for HLA typing.

Received for publication 10 April 1989.

References

1. Hämmerling, G. J., D. Klar, F. Momburg, and G. Moldenhauer. 1987. The influence of major histocompatibility complex class I antigens on tumor growth and metastasis.

- Biochim. Biophys. Acta.* 907:245.
2. Momburg, F., T. Degener, E. Bacchus, G. Moldenhauer, G. J. Hämmerling, and P. Möller. 1986. Loss of HLA-A,B,C and de novo expression of HLA-D in colorectal cancer. *Int. J. Cancer.* 37:179.
 3. van Duinen, S. G., D. J. Ruiter, E.-B. Bröcker, A. E. van der Velde, C. Sorg, K. Welvaart, and S. Ferrone. 1988. Level of HLA antigens in loco-regional metastases and clinical course of the disease in patients with melanoma. *Cancer Res.* 48:1019.
 4. Goodenow, R. S., J. M. Vogel, and R. L. Linsk. 1985. Histocompatibility antigens on murine tumors. *Science (Wash. DC).* 230:777.
 5. Schrier, P. I., R. Bernards, R. T. M. J. Vaessen, A. Houweling, and A. J. van der Eb. 1983. Expression of class I major histocompatibility antigens switched off by highly oncogenic adenovirus 12 in transformed rat cells. *Nature (Lond.).* 305:771.
 6. Hui, K., F. Grosveld, and H. Festenstein. 1984. Rejection of transplantable AKR leukaemia following MHC DNA-mediated cell transformation. *Nature (Lond.).* 311:750.
 7. Wallich, R., N. Bulbuc, G. J. Hämmerling, S. Katzav, S. Segal, and M. Feldman. 1985. Abrogation of metastatic properties of tumour cells by de novo expression of H-2K antigens following H-2 gene transfection. *Nature (Lond.).* 315:301.
 8. Tanaka, K., K. J. Isselbacher, G. Khoury, and G. Jay. 1985. Reversal of oncogenesis by the expression of a major histocompatibility complex class I gene. *Science (Lond.).* 228:26.
 9. Hayashi, H., K. Tanaka, F. Jay, G. Khoury, and G. Jay. 1985. Modulation of the tumorigenicity of human Adenovirus-12-transformed cells by interferon. *Cell.* 43:263.
 10. Taniguchi, K., K. Kärre, and G. Klein. 1985. Lung colonization and metastasis by disseminated B16 melanoma cells: H-2 associated control at the level of the host and tumor cell. *Int. J. Cancer.* 36:503.
 11. Kärre, K., H. G. Ljunggren, G. Piontek, and R. Kiesling. 1986. Selective rejection of H-2 deficient lymphoma variants suggests alternative immune defence strategy. *Nature (Lond.).* 319:675.
 12. Versteeg, R., I. A. Noordermeer, M. Krüse-Wolters, D. J. Ruiter, and P. I. Schrier. 1988. *c-myc* down-regulates class I HLA expression in human melanomas. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:1023.
 13. Bernards, R., S. K. Dessain, and R. A. Weinberg. 1986. N-myc amplification causes down-modulation of MHC class I antigen expression in neuroblastoma. *Cell.* 47:667.
 14. Cole, M. D. 1986. The myc oncogene: its role in transformation and differentiation. *Annu. Rev. Genet.* 20:361.
 15. Bodmer, W. F., E. Albert, J. G. Bodmer, B. Dupont, B. Mach, W. Mayr, T. Sasazuki, G. M. Th. Schreuder, A. Svejgaard, and P. I. Terasaki. 1988. Nomenclature for factors of the HLA system. 1987. *Vox Sang.* 55:119.
 16. Rein, R. S., G. H. A. Seeman, J. J. Neeffes, F. M. H. Hochstenbach, N. J. Stam, and H. L. Ploegh. 1987. Association with β_2 -microglobulin controls the expression of transfected human class I genes. *J. Immunol.* 138:1178.
 17. Güssow, D., R. Rein, I. Meijer, W. de Hoog, G. H. A. Seemann, F. M. Hochstenbach, and H. L. Ploegh. 1987. Isolation, expression and primary structure of HLA-Cw1 and HLA-Cw2 genes: evolutionary aspects. *Immunogenetics.* 25:313.
 18. Auffray, C., and F. Rougeon. 1980. Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. *Eur. J. Biochem.* 107:303.
 19. Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6.
 20. Colby, W. W., E. Y. Chen, D. H. Smith, and A. D. Levinson. 1983. Identification and nucleotide sequence of a human locus homologous to the v-myc oncogene of avian myelocytomatosis virus MC 29. *Nature (Lond.).* 301:722.
 21. Fort, Ph., L. Marty, M. Piechaczyk, S. El Sabrouy, C. Dani, P. Jeanteur, and J. M.

- Blanchard. 1985. Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. *Nucleic Acids Res.* 13:1431.
22. Koller, B. H., B. Sidwell, R. DeMars, and H. T. Orr. 1984. Isolation of HLA locus-specific DNA probes from the 3'-untranslated region. *Proc. Natl. Acad. Sci. USA.* 81:5175.
23. Barnstable, C. J., W. F. Bodmer, G. Brown, G. Galfrè, C. Milstein, A. F. Williams, and A. Ziegler. 1978. Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens: new tools for genetic analysis. *Cell.* 14:9.
24. Yang, S. Y., Y. Morishima, N. H. Collins, T. Alton, M. S. Pollack, E. J. Yunis, and B. Dupont. 1984. Comparison of one-dimensional IEF patterns for serologically detectable HLA-A and B allotypes. *Immunogenetics.* 19:217.
25. Stam, N. J., H. Spits, and H. L. Ploegh. 1986. Monoclonal antibodies raised against denatured HLA-B locus heavy chains permit biochemical characterization of certain HLA-C locus products. *J. Immunol.* 137:2299.
26. Bernards, R., A. Houweling, P. I. Schrier, J. L. Bos, and A. J. van der Eb. 1982. Characterization of cells transformed by Ad 5/Ad 12 hybrid early region I plasmids. *Virology.* 120:422.
27. Neeffes, J. J., I. Doxiadis, N. Stam, C. J. Beckers, and H. Ploegh. 1986. An analysis of class I antigens of man and other species by one-dimensional IEF and immunoblotting. *Immunogenetics.* 23:164.
28. Mittal, K. K., M. R. Mickey, D. P. Singal, and P. I. Terasaki. 1968. Serotyping for homotransplantation. XVIII. Refinement of microdroplet lymphocyte cytotoxicity test. *Transplantation (Baltimore).* 6:913.
29. Bruning, J. W., F. H. J. Claas, M. J. Kardol, A. M. Naipal, J. G. S. Niterink, J. H. Parlevliet, and H. J. Tanke. 1987. Automated reading of HLA-A, -B, -C, and -Dr typing and screening. In *Cellular, Molecular and Genetic Approaches to Immunodiagnosis and Immunotherapy*. K. Kano, S. Mori, T. Sugisaki, and M. Torisu, editors. University of Tokyo Press, Tokyo. 171-174.
30. DeLellis, R. A., L. A. Sternberger, R. B. Mann, P. M. Banks, and P. K. Nakane. 1979. Immunoperoxidase technics in diagnostic pathology. *Am. J. Clin. Pathol.* 71:483.
31. van der Eb, A. J., and F. L. Graham. 1980. Assay of transforming activity of tumor virus DNA. *Methods Enzymol.* 65:826.
32. Neeffes, J. J., and L. Ploegh. 1988. Allele and locus-specific difference in cell surface expression and the association of HLA class I heavy chain with β_2 -microglobulin: differential effects of inhibition of glycosylation on class I subunit association. *Eur. J. Immunol.* 18:801.
33. Rebaï, N., and B. Malissen. 1983. Structural and genetic analysis of HLA class I molecules using monoclonal xenoantibodies. *Tissue Antigens.* 22:107.
34. Masucci, M. G., S. Torsteindottir, J. Colombani, C. Brautbar, E. Klein, and G. Klein. 1987. Down-regulation class I antigens and of the Epstein-Barr virus-encoded latent membrane protein in Burkitt lymphoma lines. *Proc. Natl. Acad. Sci. USA.* 84:4567.
35. Smith, M. E. F., W. F. Bodmer, and J. G. Bodmer. 1988. Selective loss of HLA-A, B, C, locus products in colorectal adenocarcinoma. *Lancet.* i:823.
36. Girdlestone, J., and C. Milstein. 1988. Differential expression and interferon response of HLA class I genes in thymocyte lines and response variants. *Eur. J. Immunol.* 18:139.
37. Anichini, A., G. Fossati, and G. Parmiani. 1985. Clonal analysis of cytotoxic T lymphocyte response to autologous human metastatic melanoma. *Int. J. Cancer.* 35:683.
38. Hersey, P., M. MacDonald, S. Schibeci, and C. Burns. 1986. Clonal analysis of cytotoxic T lymphocytes (CTL) against autologous melanoma. *Cancer Immunol. Immunother.* 22:15.
39. Weber, J. S., G. Jay, K. Tanaka, and S. A. Rosenberg. 1987. Immunotherapy of a murine tumor with interleukin 2. Increased sensitivity after MHC class I gene transfection. *J. Exp. Med.* 166:1716.

40. Rosenberg, S. A., M. S. Packard, P. M. Aebersold, D. Solomon, S. L. Topalian, S. T. Toy, P. Simon, M. T. Lotze, J. C. Yang, C. A. Seipp, C. Simpson, C. Carter, S. Bock, D. Schwartzentruber, J. P. Wei, and D. E. White. 1988. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. *N. Engl. J. Med.* 319:1676.
41. Quillet, A., F. Presse, C. Marchiol-Fournigault, A. Harel-Bellan, M. Benbunan, H. Ploegh, and D. Fradelizi. 1988. Increased resistance to non-MHC-restricted cytotoxicity related to HLA A, B expression. *J. Immunol.* 141:17.