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Modulation by cytokines of HLA antigens, intercellular adhesion molecule 1 and high molecular weight melanoma associated antigen expression and of immune lysis of clones derived from the melanoma cell line MeM 50-10*

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Summary. Clones were isolated from the cultured human melanoma cell line MeM 50-10, which metastasizes in nude mice with a pattern similar to that in patients with melanoma. Analysis with monoclonal antibodies detected heterogeneity among the clones in the expression of HLA class I antigens, HLA class II antigens, intercellular adhesion molecule 1 and high molecular weight melanoma associated antigen. The clones MeM A16 and MeM A18 were also shown to display differential susceptibility to modulation by immune interferon (IFN-γ) and/or tumor necrosis factor (TNF-α) of the expression of the four types of antigens analyzed. In spite of differences in the antigenic profile, the two clones did not differ in their susceptibility to lysis by lymphokine-activated killer (LAK) cells and by anti-HLA-A2 cytotoxic T cells. The increase in the expression of HLA class I antigens induced by IFN-γ and/or TNF-α on the two clones was associated with an increased susceptibility to lysis by anti-HLA-A2 cytotoxic T cells. Because of the metastasizing properties of cultured melanoma cells MeM 50-10, the clones we have isolated, with their distinct antigenic profile and differential susceptibility to modulation by cytokines, may represent useful models to investigate the role of distinct antigenic structures in the metastatic process.

Introduction

Human melanoma cells have been shown to be heterogeneous in a number of characteristics, including expression of HLA antigens and of melanoma-associated antigens (MAA), as well as in susceptibility to modulation of their antigenic profile by cytokines and to lysis by cytotoxic T cells [1–3, 6, 9, 15, 20–22, 26, 27]. Correlation of susceptibility to immune lysis of melanoma cells with expression and modulation of cell-surface markers may contribute to our understanding of their role in the escape of melanoma cells to immune lysis and of the molecular mechanisms underlying the metastatic process. Therefore, in the present study we have investigated this relationship utilizing two clones originated from the melanoma cell line MeM 50-10. The latter has been derived from the melanoma cell line MeWo by mutagenization with methylmethane sulfonate followed by selection for resistance to wheat germ agglutinin, and was utilized because it metastasizes in nude mice in a fashion similar in some respects to that in which melanoma cells metastasize in patients [16]. The cell-surface markers we have selected include HLA class I and class II antigens because of their role in the interaction of melanoma cells with the host's immune system (for review, see [9]), intercellular adhesion molecule 1 (ICAM-1) (31) because of its role in the generation of an immune response [7] and in T-cell-mediated cytotoxicity [23], and high molecular weight melanoma associated antigen (HMW-MAA) because of its use as a target for immunoscintigraphy with radiolabeled monoclonal antibodies and for active and passive immunotherapy (for review, see [10]). The cytokines we have utilized in our studies include immune interferon (IFN-γ) and tumor necrosis factor (TNF-α) because of their likely presence in melanoma lesions in patients and of their ability to modulate the cell-surface markers under investigation [20, 21, 22].

Materials and methods

Cells. The melanoma cell lines Colo 38 and MeM 50-10 and the clones derived from the latter cell line were grown in Dulbecco's modified Eagle medium (Gibco Laboratories, Grand Island, NY), supplemented with 10% fetal bovine serum (Gibco) and 2 mM L-glutamine in an atmosphere of 5% CO_2/95% air in a humidified incubator at 37° C. Adherent melanoma cell lines were harvested by incubation with 1 mM EDTA in phosphate-buffered saline (PBS). Mononuclear cells were isolated from heparinized blood by differential centrifugation on a Ficoll/Hypaque (Sigma Chemical Co., St. Louis, Mo) density gradient.

Recombinant cytokines. IFN-γ and interleukin-2 (IL-2) were obtained from Hoffman-LaRoche Inc., Nutley, NJ. TNF-α was obtained from Cetus Corporation, Emeryville, Calif.

Monoclonal antibodies and conventional antisera. The monoclonal antibody (mAb) W6/32 to a framework determinant of HLA class I antigens, the anti-β_2-microglobulin mAb NAMB-1, the mAbs Q5/6 and Q5/13 to distinct monomorphic determinants of HLA class II antigens, the

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mAbs CL413, SPV-L3 and B7/21 to monomorphic determinants of HLA-DR, HLA-DQ and HLA-DP antigens, respectively, the mAbs 149.53 and 225.28 to distinct determinants of HMW-MAA and the anti-96-kDa MAA mAb CL203.4 were developed and characterized as described [4, 11, 25, 28, 30, 36, 39]. Sequential immunoprecipitation experiments with the anti-ICAM-1 mAb RR1/1 (31) have shown that the mAb CL203.4 reacts with this molecule (unpublished results). Monoclonal antibodies were purified from ascitic fluid by caprylic acid precipitation [32]. Affinity-purified goat anti-mouse IgG antibodies and affinity-purified fluorescein-isothiocyanate-conjugated goat antibodies to $F(ab')_2$ fragments of murine IgG were purchased from Cooper Biomedical Inc., Malvern, Pa, and from Boehringer Mannheim Biochemicals, Indianapolis, Ind, respectively. Antibodies were radiolabeled with ^{125}I by the chloramine T method [13].

HLA cDNA probes. The cDNA probes for HLA-B7 antigen [35] and for HLA-DRβ, -DQβ and -DPβ chains [38] were obtained from Dr. S. M. Weissman, Department of Human Genetics, Yale University School of Medicine, New Haven, Conn, and from Dr. E. O. Long, National Institute of Allergy and Infectious Diseases, Bethesda, Md, respectively. Probes were labeled with ^{32}P by random priming to a specific activity of 50 μCi/μg [8].

Serological assays. Indirect immunofluorescence was performed by incubating cells (1×10^6) with an excess of monoclonal antibody at 4° C for 30 min. After three washings with PBS, pH 7.4, containing 1% bovine serum albumin and 0.02% sodium azide (PBS-BSA), cells were incubated with fluorescein-isothiocyanate-conjugated goat anti-mouse $F(ab')_2$ antibodies at 4° C for 30 min. After three washings with PBS-BSA, cells were resuspended in PBS-BSA and analyzed with an Ortho cytofluorograph model 50H attached to a 2150 Ortho computer, Ortho Pharmaceuticals, Raritan, NJ. Background fluorescence was assessed by incubating cells with the culture supernatant of myeloma cells P3X63.Ag8.653. Mean fluorescence intensity is given as channel number on a log scale from 1 to 1024. The indirect binding assay with radiolabeled antibodies was carried out in flexible microtiter plates by sequential incubation of target melanoma cells (1×10^5) with monoclonal antibodies and with ^{125}I-labeled goat anti-mouse IgG antibodies as described [20]. The double-determinant immunoassay to measure HLA class I antigens, HLA class II antigens and HMW-MAA in the culture supernatant of cell lines was performed as described [11, 33, 34] except that the incubation of spent medium with insolubilized anti-HLA class II monoclonal antibodies was prolonged to 48 h. The combinations of insolubilized mAb W6/32–^{125}I-mAb NAMB-1, insolubilized mAb Q5/13–^{125}I-mAb Q5/6 and insolubilized mAb 149.53–^{125}I-mAb 225.28 were used to measure HLA class I antigens, HLA class II antigens and HMW-MAA, respectively. Soluble ICAM-1 could not be measured since monoclonal antibodies with the appropriate specificity to develop a double-determinant immunoassay were not available to us.

Generation of lymphokine-activated killer (LAK) cells. Mononuclear cells isolated from a healthy donor were washed twice with Hanks' balanced salt solution (HBSS) (Gibco) and resuspended at the concentration of 1×10^6/ml in RPMI 1640 medium (Gibco) containing 10% heat-inactivated pooled human AB serum and IL-2 (1000 U/ml). Following a 3–4-day incubation at 37° C in a humidified CO_2 atmosphere, cells were harvested, washed twice with HBSS, resuspended at the concentration of 2×10^6/ml and used as effectors in the cytotoxicity assay.

Generation of anti-HLA class I cytotoxic T cells. Mononuclear cells isolated from the donor PBX (HLA-A1,A29/Bw51,-/DR2,) were mixed at the ratio of 1:1 with X-irradiated (3000 rad) mononuclear cells from the donor PBY (HLA-A2,A3/B13, Bw44/DR4,-). After a 10-day incubation at 37° C in RPMI 1640 medium supplemented with 10% human serum, blasts were restimulated with a mixture of stimulators and responders (feeder) at the ratio of 1:0.5:0.5. After a 3-day incubation at 37° C, cells were harvested, washed twice with HBSS, resuspended at the concentration of 2×10^6/ml and used as cytotoxic T cells.

Cell-mediated cytotoxicity assay. Target cells (1×10^6) in 0.5 ml RPMI 1640 medium supplemented with 10% fetal bovine serum were incubated with 100 μCi $Na_2\ ^{51}CrO_4$ (Amersham, Arlington Heights, Ill) for 1 h at 37° C with occasional gentle shaking. After extensive washing with HBSS, target cells (1×10^4) were incubated for 4 h at 37° C with effector cells in round-bottom microtiter plates. Supernatant (100 μl) was harvested from each well and counted in a gamma counter. The percentage of cytotoxicity was calculated as follows:

$$\frac{\text{release of } ^{51}Cr \text{ by targets in presence of effectors} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

where spontaneous release represents the radioactivity released by 1×10^4 ^{51}Cr-labeled target cells incubated in medium and the maximum release is the radioactivity released by 1×10^4 target cells lysed with NP-40.

Radiolabeling of cells, immunoprecipitation and sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS-PAGE). Melanoma cells were detached by incubation with PBS/1 mM EDTA, washed twice with HBSS and resuspended at the concentration of 2×10^6/ml in 10 ml methionine-free RPMI 1640 medium containing 1% fetal bovine serum and 250 μCi [^{35}S]methionine (Amersham). Following a 16-h incubation at 37° C, cells were washed three times with HBSS, solubilized with NP-40 and immunoprecipitated with monoclonal antibodies. Immune complexes were then isolated with protein-A-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden). One-dimensional SDS-PAGE was performed on 10% polyacrylamide slab gels under reducing conditions, utilizing the buffer system described by Laemmli [17]. Gels were fluorographed as described by Bonner and Laskey [5].

Northern blot analysis. Cytoplasmic RNA was extracted as described in Maniatis et al. [24]. Cytoplasmic RNA (10–15 μg) was size-fractionated by electrophoresis through 0.8% agarose gel containing 2.2 M formaldehyde, 20 mM 4-morpholinepropanesulfonic acid, pH 7.0, 5 mM sodium acetate, 1 mM EDTA [12] and transferred to nitrocellulose (Schleicher and Schüll, Keene, NH) using 20 × SSC (1 × SSC: 0.15 M sodium chloride, 0.015 sodium citrate, pH 7.0) as described by Thomas [37]. Blots were baked at 80° C under vacuum for 2 h and prehybridized at

42° C for 12–16 h using the prehybridization buffer. The latter consists of 50% formamide, 5 × SSC, 5 × Denhardt's reagent, 0.2 M sodium phosphate pH 7.0 and denatured salmon sperm DNA (250 μg/l). Filters were hybridized for 12–16 h at 42° C in the prehybridization buffer containing ^{32}P-labeled HLA-B7, -DRβ, -DQβ and -DPβ cDNA probes. Following three washings at room temperature for 5 min each time with 2 × SSC containing 0.1% SDS, blots were washed once at 42° C for 10 min with 2 × SSC containing 0.1% SDS. Then blots were dried and autoradiographed at −70° C for 5–7 days using XAR.5 X-ray film (Eastman Kodak, Rochester, NY) and an intensifying screen (Dupont Co., Wilmington, Del).

Results

Cloning of MeM 50-10 melanoma cell line

Cells from the melanoma cell line MeM 50-10 were cloned by limiting dilution at 0.3 cells/well in Dulbecco's modified Eagle medium/fetal bovine serum and 2 mM L-glutamine in 96-well flat-bottom plates. After a 7-day incubation at 37° C in a 5% CO_2 atmosphere, 46 out of 600 seeded wells contained a single cluster of 3–4 cells. Primary colonies, obtained after 2 weeks of growth, were removed by incubation in PBS/1 mM EDTA and transferred to 24-well plates. Out of the 46 selected clones, 6, which grew well, were analyzed for cell-surface expression of HLA antigens and MAA when they reached semiconfluence.

Expression and modulation by TNF-α and/or IFN-γ of HLA antigens and MAA by melanoma cell line MeM 50-10

The melanoma cell line MeM 50-10 expresses HLA class I antigens, ICAM-1 and HMW-MAA, but lacks HLA class II antigens. Repeated testing by indirect immunofluorescence with the anti-HLA class I mAb W6/32, with the anti-HLA class II mAb Q5/13, with the anti-ICAM-1 mAb CL203.4 and with the anti-HMW-MAA mAb 225.28 over the course of 2 years showed that the phenotype of the cell line is stable.

Modulation by TNF-α and/or IFN-γ of HLA antigens and MAA by melanoma cell line MeM 50-10

TNF-α and IFN-γ, both individually and in combination, enhanced the expression of HLA class I antigens by MeM 50-10 cells. The increase induced by the combination of the two cytokines was higher than that induced by the individual ones, and that induced by IFN-γ was higher than that induced by TNF-α. While TNF-α did not induce the expression of HLA class II antigens on MeM 50-10 cells, IFN-γ both individually and in combination with TNF-α did. The increase induced by the combination of the two cytokines was similar to that induced by IFN-γ. TNF-α enhanced the expression of ICAM-1 by MeM 50-10 cells to a lower extent than IFN-γ and the combination of TNF-α and IFN-γ. TNF-α slightly increased the expression of HMW-MAA, while IFN-γ, both individually and in combination with TNF-α, had no detectable effect.

Expression of HLA antigens and MAA by clones derived from the melanoma cell line MeM 50-10

Indirect immunofluorescence staining of the six melanoma clones with monoclonal antibodies showed that most of them expressed a level of HLA class I and class II antigens similar to that of the parental cells. The percentage of cells stained by anti-ICAM-1 mAb CL203.4 and by anti-HMW-MAA mAb 225.28 in each clone was higher than that in the parental cell line. The intensity of staining by mAb CL203.4 of the clones was lower than that of the parental cell line, while that by mAb 225.28 was lower in three clones and higher in two. The six clones differed not only in the expression of MAA but also in that of HLA antigens, as measured by the percentage of cells stained and by the intensity of the staining (Table 1). The anti-HLA class I mAb W6/32 stained about 50% of cells of clone MeM A16 and between 89% and 98% of cells of the other five clones. The anti-HLA class II mAb Q5/13 stained less than 10% of cells of each clone. The anti-ICAM-1 mAb CL203.4 stained between a minimum of 39% of cells of clone MeM A18 and a maximum of 88% of cells of clone MeM A16. The anti-HMW-MAA mAb 225.28 stained between a minimum of 47% of cells of clone MeM A7 and a maximum of 85% of cells of clone MeM A17.

The clones MeM A16 and MeM A18 were selected for further characterization. The amount of radioactivity bound in the indirect binding assay with mAb W6/32 (Figs. 1 and 2) and the intensity of components immunoprecipitated by mAb W6/32 (Fig. 3) confirmed a higher expression of HLA class I antigens by clone MeM A18 than by clone MeM A16. HLA class II antigens were detected in neither clone with serological (Table 1, Figs. 1 and 2) and immunochemical (Fig. 4) assays. The percentage of cells stained by anti-ICAM-1 mAb CL203.4 was higher in clone MeM A16 than in clone MeM A18. On the other hand the intensity of fluorescence was higher in the latter than in the former (Table 1), suggesting that the level of expression of ICAM-1 on individual cells of clone MeM A16 is lower than on those of clone MeM A18. In the indirect binding assay the clone MeM A16 displayed a slightly higher reactivity with mAb CL203.4 than the clone MeM A18 (Figs. 1 and 2). The clone MeM A16 displayed a slightly higher reactivity than the clone MeM A18 with the anti-HMW-MAA mAb 225.28 both in indirect immunofluorescence (Table 1) and in the indirect binding assay (Figs. 1 and 2).

Table 1. Cell-surface expression of HLA, ICAM-1 and HMW-MAA by melanoma cell line MeM 50–10 and its clones[a]

Cells	HLA class I antigens	HLA class II antigens	ICAM-1 antigens	HMW-MAA antigens
MeM 50–10	94[b] (348)[c]	6 (57)	41 (147)	26 (100)
MeM A5	98 (280)	4 (41)	51 (96)	71 (97)
MeM A7	89 (498)	5 (39)	54 (107)	47 (85)
MeM A15	96 (381)	5 (57)	81 (98)	81 (91)
MeM A16	52 (210)	5 (45)	88 (70)	77 (179)
MeM A17	96 (415)	4 (39)	83 (79)	85 (157)
MeM A18	92 (365)	4 (52)	39 (87)	53 (101)

[a] Cells were sequentially incubated with monoclonal antibodies and with fluoresceine-isothiocyanate-conjugated anti-mouse F(ab')$_2$ xenoantibodies. After three washings cells were analyzed with a cytofluorograph

[b] Percentage of positive cells stained with monoclonal antibodies in indirect immunofluorescence

[c] Mean fluorescence intensity

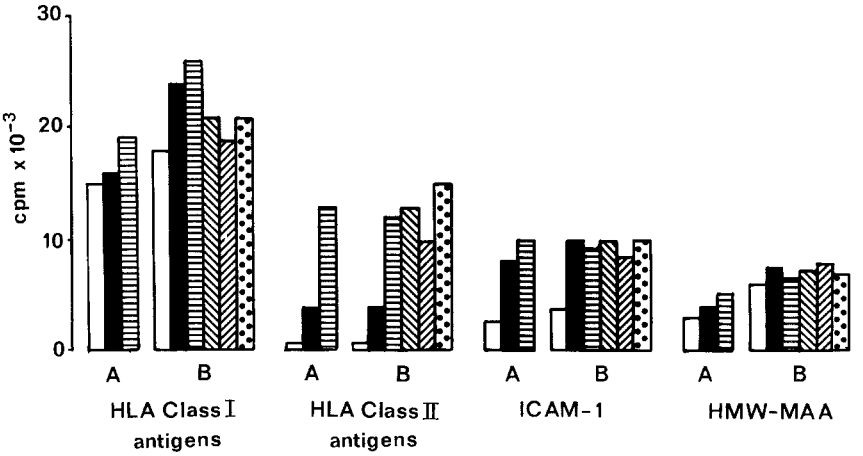

Fig. 1. Modulation by TNF-α and/or IFN-γ of the cell-surface expression of HLA antigens and MAA by melanoma cell clone MeM A16. Cells were incubated at 37° C with the individual cytokines TNF-α (final concentration 100 U/ml) (■) or IFN-γ (final concentration 1000 U/ml) (▤), with the combination of TNF-α (100 U/ml) and IFN-γ (1000 U/ml) (▧) or sequentially with TNF-α (100 U/ml) for 2 days and IFN-γ (1000 U/ml) for 2 days (▨) or with IFN-γ (1000 U/ml) for 2 days and TNF-α (100 U/ml) for 2 days (◪). The total incubation time was 2 (A) or 4 (B) days. Controls were incubated under the same experimental conditions, but were not exposed to cytokines (□). At the end of the incubation, cells were harvested, washed twice and tested in duplicate with anti-HLA class I mAb W6/32, anti-HLA class II mAb Q5/13, anti-ICAM-1 mAb CL203.4 and anti-HMW-MAA mAb 225.28 in the indirect binding assay

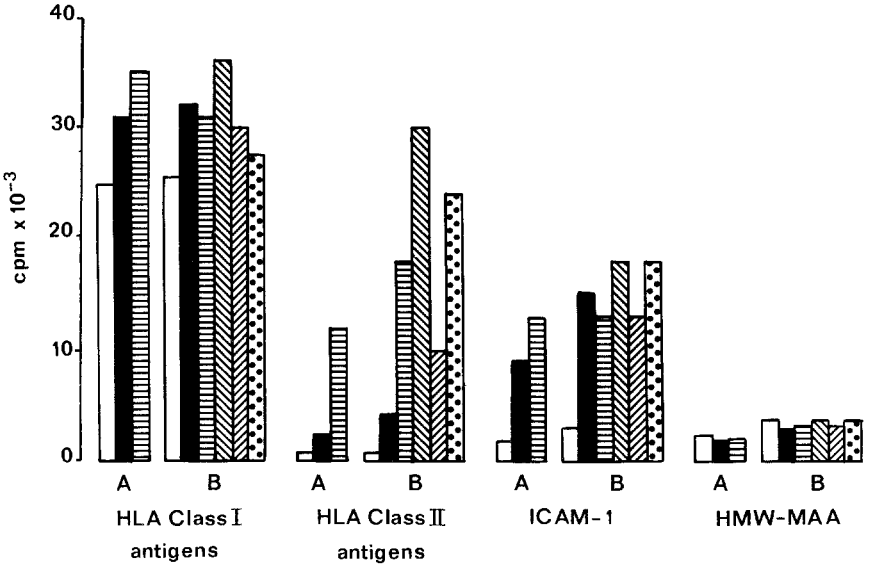

Fig. 2. Modulation by TNF-α and/or IFN-γ of the cell-surface expression of HLA antigens and MAA by melanoma cell clone MeM A18. Cells were incubated at 37° C with the individual cytokines TNF-α (final concentration 100 U/ml) (■) or IFN-γ (final concentration 1000 U/ml) (▤), with the combination of TNF-α (100 U/ml) and IFN-γ (1000 U/ml) (▧) or sequentially with TNF-α (100 U/ml) for 2 days and IFN-γ (1000 U/ml) for 2 days (▨) or with IFN-γ (1000 U/ml) for 2 days and TNF-α (100 U/ml) for 2 days (◪). The total incubation time was 2 (A) or 4 (B) days. Control cultures were incubated under the same experimental conditions, but were not exposed to cytokines (□). At the end of the incubation, cells were harvested, washed twice and tested in duplicate with anti-HLA class I mAb W6/32, anti-HLA class II mAb Q5/13, anti-ICAM-1 mAb CL203.4 and anti-HMW-MAA mAb 225.28 in the indirect binding assay

Modulation by TNF-α and/or IFN-γ of HLA antigens and MAA by clones MeM A16 and MeM A18

To analyze the susceptibility to modulation of HLA antigens and MAA by cytokines, the clones MeM A16 and MeM A18 were incubated for 2 and 4 days with TNF-α (final concentration 100 U/ml) and/or with IFN-γ (final concentration 1000 U/ml). Following a 2-day incubation TNF-α increased the expression of HLA class I antigens by MeM A18 cells, but did not affect that by MeM A16 cells. IFN-γ increased it on both clones, although to a larger extent on MeM A18 cells than on MeM A16 cells. Following a 4-day incubation, TNF-α enhanced the expression of HLA class I antigens on MeM A16 cells, but induced no additional increase on MeM A18 cells. Similarly, following a 4-day incubation with IFN-γ the level of HLA class I antigens displayed an additional increase on MeM A16 cells and a reduction on MeM A18 cells. HLA class I antigens, synthesized by MeM A16 and MeM A18 cells treated with TNF-α and/or IFN-γ, displayed the characteristic electrophoretic profile (Fig. 3). The combination of TNF-α and IFN-γ enhanced the level of HLA

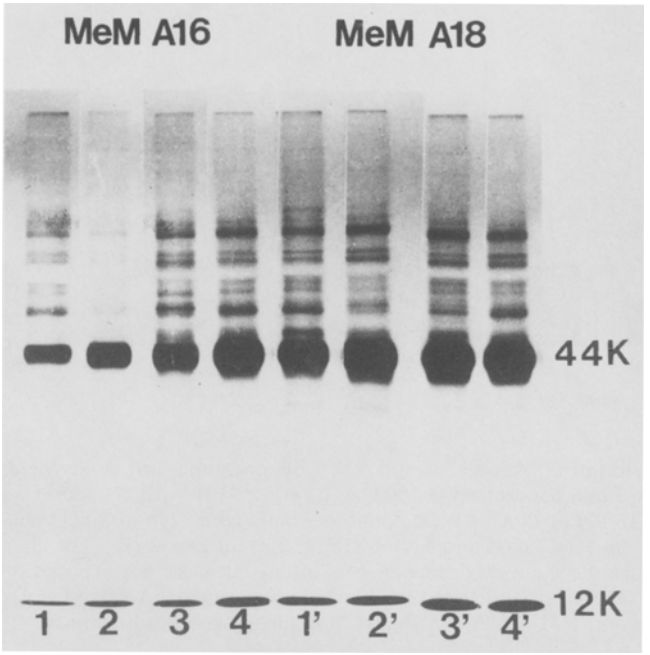

Fig. 3. SDS-PAGE analysis of antigens immunoprecipitated by anti-HLA class I mAb W6/32 from TNF-α- and/or IFN-γ-treated melanoma cell clones MeM A16 and MeM A18. Following a 72-h incubation at 37° C in medium containing TNF-α (final concentration 100 U/ml) (lanes 2 and 2′), IFN-γ (final concentration 1000 U/ml) (lanes 3 and 3′) or the combination of TNF-α (100 U/ml) and IFN-γ (1000 U/ml) (lanes 4 and 4′), cells were starved for 4 h in methionine-free medium and then incubated at 37° C for 16 h in medium containing [^{35}S]methionine (25 μCi/ml) and TNF-α (100 U/ml) and/or IFN-γ (1000 U/ml). Cells used as controls (lanes 1 and 1′) were cultured and radiolabeled under the same experimental conditions, but were not exposed to cytokines. At the end of the incubation cells were harvested, washed three times and solubilized with NP-40. After indirect immunoprecipitation with mAb W6/32, antigens were eluted from the immunoadsorbent and analyzed by SDS-PAGE in the presence of 2% β-mercaptoethanol

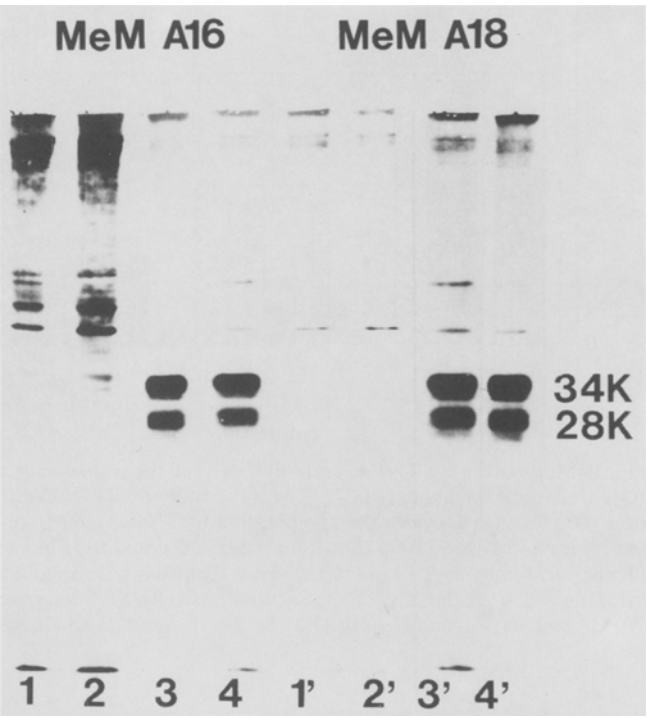

Fig. 4. SDS-PAGE analysis of antigens immunoprecipitated by anti-HLA class II mAb Q5/13 from TNF-α- and/or IFN-γ-treated melanoma cell clones MeM A16 and MeM A18. Following a 72-h incubation at 37° C in medium containing TNF-α (final concentration 100 U/ml) (lanes 2 and 2′), IFN-γ (final concentration 1000 U/ml) (lanes 3 and 3′) or the combination of TNF-α (100 U/ml) and IFN-γ (1000 U/ml) (lanes 4 and 4′), cells were starved for 4 h in methionine-free medium and then incubated at 37° C for 16 h in medium containing [^{35}S]methionine (25 μCi/ml) and TNF-α (100 U/ml) and/or IFN-γ (100 U/ml). Cells used as controls (lanes 1 and 1″) were cultured and radiolabeled under the same experimental conditions, but were not exposed to cytokines. At the end of the incubation cells were harvested, washed three times with HBSS and solubilized with NP-40. After indirect immunoprecipitation with mAb Q5/13, antigens were eluted from the immunoadsorbent and analyzed by SDS-PAGE in the presence of 2% β-mercaptoethanol

class I antigens to a greater extent than the individual cytokines on MeM A18 cells and to lesser extent on MeM A16 cells. The two cytokines, both individually and in combination, enhanced the shedding of HLA class I antigens from MeM A16 and MeM A18 cells to a similar extent (Table 2). Sequential incubation with the two cytokines for 2 days each, was less effective than incubation with each cytokine for 4 days in enhancing the level of HLA class I antigens on both MeM A16 and MeM A18 cells.

Following a 2-day incubation with TNF-α or with IFN-γ, both MeM A16 and MeM A18 cells acquired reactivity with anti-HLA class II mAb Q5/13. The level of HLA class II antigens induced by IFN-γ was markedly higher than that induced by TNF-α on both cell lines. Prolongation of the incubation to 4 days caused an additional increase in the expression of HLA class II antigens by MeM A18 cells, but did not affect that by MeM A16 cells. The combination of TNF-α and IFN-γ enhanced the expression of HLA class II antigens by MeM A18 cells to a larger extent than the individual cytokines; the effect of the combination of TNF-α and IFN-γ on MeM A16 cells was similar to that of IFN-γ. Components with the characteristic electrophoretic mobility of the two subunits of HLA class II antigens were detected in the immunoprecipitates with mAb Q5/13 from MeM A16 and MeM A18 cells that had been incubated with IFN-γ or with the combination of TNF-α and IFN-γ, but not in those from cells which had been incubated with TNF-α (Fig. 4). The latter finding may reflect the level of HLA class II antigens below the sensitivity of the method. Only IFN-γ and the combination of IFN-γ and TNF-α induced an increase in the level of HLA class II antigens in the medium from MeM A16 and MeM A18 cells (Table 2). Sequential incubation with TNF-α for 2 days and with IFN-γ for 2 days induced an increase of HLA class II antigens on MeM A16 and MeM A18 cells, which was lower than that induced by incubation with IFN-γ for 2 and 4 days and higher than that induced by incubation with TNF-α for 2 and 4 days. Sequential incubation with IFN-γ for 2 days and with TNF-α for 2 days enhanced HLA class II antigens on MeM A18 cells more than the individual cytokines but less than the combination of TNF-α and IFN-γ. The increase on MeM A16 cells was higher than that induced by the other types of treatments.

Table 2. Modulation by TNF-α and IFN-γ of the shedding of HLA antigens and HMW-MAA by melanoma cell clones MeM A16 and MeM A18[a]

Cells	TNF-α	IFN-γ	HLA class I antigens	HLA class II antigens	HMW-MAA
MeM A16	−	−	1350[b]	374	1972
MeM A16	+	−	2655	445	2295
MeM A16	−	+	2740	1420	1983
MeM A16	+	+	2634	1400	2131
MeM A18	−	−	1430	932	881
MeM A18	+	−	2781	1339	889
MeM A18	−	+	2800	2042	1626
MeM A18	+	+	2833	2025	1878

[a] Cells were incubated for 72 h at 37° C with TNF-α (final concentration 100 U/ml), IFN-γ (final concentration 1000 U/ml) or the combination of TNF-α (100 U/ml) and IFN-γ (1000 U/ml). Control cultures were incubated under the same experimental conditions, but were not exposed to cytokines. At the end of the incubation, medium was harvested and tested for its content of HLA class I antigens, HLA class II antigens and HMW-MAA utilizing a double-determinant immunoassay

[b] cpm

To determine the effect of TNF-α and/or IFN-γ on the gene products of the HLA-D region, MeM A16 and MeM A18 cells were incubated with the cytokines and then tested with the anti-HLA-DR mAb CL413, anti-HLA-DQ mAb SPV-L3 and anti-HLA-DP mAb B7/21. The activity of the monoclonal antibody preparations used was monitored by testing with control and IFN-γ treated melanoma cells Colo 38. Following incubation with the individual cytokines or their combination both MeM A16 and MeM A18 cells acquired reactivity only with anti-HLA-DR mAb CL413. Its modulation was similar to that with the anti-HLA class II mAb Q5/13 (Fig. 5).

To analyze the mechanism(s) underlying the differential susceptibility to induction by TNF-α and/or IFN-γ of the gene products of the HLA-D region on MeM A16 and MeM A18 cells, the steady-state levels of mRNA for HLA-DR, -DQ and -DP antigens were analyzed by Northern blot hybridization in the two clones following incubation with TNF-α, IFN-γ and their combination for 96 h. Both clones contain RNA molecules that hybridize to HLA-DRβ,-DQβ and -DPβ gene probes. Incubation with TNF-α caused no detectable change in the level of mRNA specific for HLA-DR, -DQ and -DP antigens in both clones. IFN-γ and the combination of IFN-γ and TNF-α induced a marked increase in the level of mRNA specific for HLA-DR and -DQ antigens, but caused no detectable change in the level of mRNA specific for HLA-DP antigens in both clones. The level of mRNA specific for HLA class I in both clones increased following incubation with IFN-γ and the combination of IFN-γ and TNF-α, but did not change following incubation with TNF-α (Fig. 6).

Both MeM A16 and MeM A18 cells displayed an enhanced reactivity with anti-ICAM-1 mAb CL203.4 following a 2-day incubation with TNF-α or with IFN-γ, the increase induced by the latter being greater than that by the former. While TNF-α induced a similar increase on both clones, IFN-γ induced a higher increase on MeM A18 cells than on MeM A16 cells. Prolongation of the incubation to 4 days was associated with an additional increase

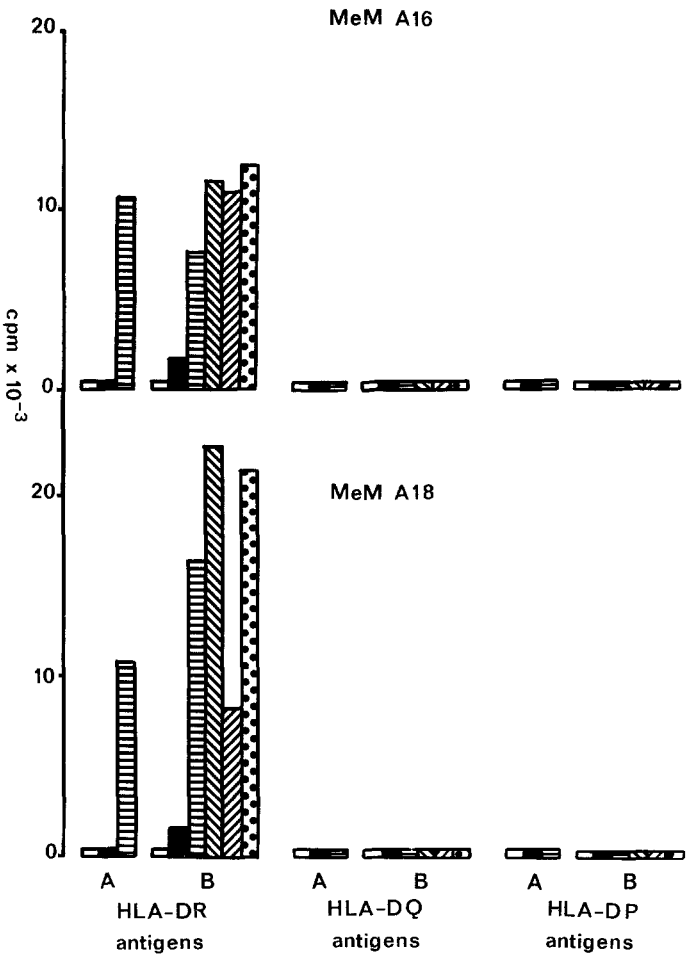

Fig. 5. Differential susceptibility to modulation by TNF-α and/or IFN-γ of the expression of HLA-DR, -DQ and -DP antigens by melanoma cell clones MeM A16 and MeM A18. Cells were incubated at 37° C with the individual cytokines TNF-α (final concentration 100 U/ml) (■) or IFN-γ (final concentration 1000 U/ml) (▤), with the combination of TNF-α (100 U/ml) and IFN-γ (1000 U/ml) (▧) or sequentially with TNF-α (100 U/ml) for 2 days and IFN-γ (100 U/ml) for 2 days (▨) or with IFN-γ (1000 U/ml) for 2 days and TNF-α (100 U/ml) for 2 days (▩). The total incubation time was 2 (A) or 4 (B) days. Control cultures were incubated under the same experimental conditions, but were not exposed to cytokines (□). At the end of the incubation, cells were harvested, washed twice and tested in duplicate with anti-HLA-DR mAb CL413, anti-HLA-DQ mAb SPV-L3 and anti-HLA-DP mAb B7/21 in the indirect binding assay

of the reactivity of mAb CL203.4 with MeM A16 cells and even more with MeM A18 cells incubated with TNF-α. No change in the reactivity of cells incubated with IFN-γ was detected. The combination of TNF-α and IFN-γ and the sequential incubation with TNF-α and IFN-γ enhanced the expression of ICAM-1 by MeM A16 cells to an extent similar to that induced by the individual cytokines. On the other hand, the combination of TNF-α and IFN-γ and the sequential incubation with IFN-γ and TNF-α enhanced the expression of ICAM-1 by MeM A18 cells more than the individual cytokines and the sequential incubation with TNF-α and IFN-γ (Figs. 1 and 2).

TNF-α and IFN-γ, both individually and in combination, caused no marked change in the expression of HMW-MAA by MeM A16 and MeM A18 cells (Figs. 1 and 2). Only IFN-γ and the combination of IFN-γ and

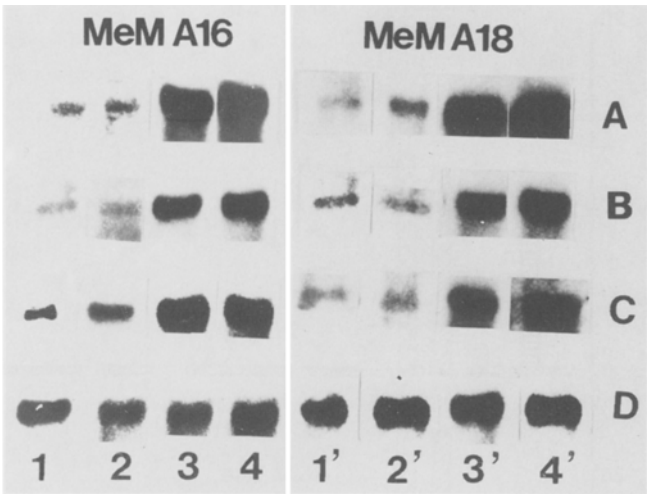

Fig. 6. Northern blot analysis of HLA-specific mRNA in TNF-α- and/or IFN-γ-treated melanoma cell clones MeM A16 and MeM A18. Cells were incubated for 72 h at 37° C with TNF-α (final concentration 100 U/ml) (lanes 2 and 2'), IFN-γ (final concentration 1000 U/ml) (lanes 3 and 3') or the combination of TNF-α (100 U/ml) and IFN-γ (1000 U/ml) (lanes 4 and 4'). Control cultures were incubated under the same experimental conditions, but were not exposed to cytokines (lanes 1 and 1'). At the end of the incubation, cells were harvested and washed twice with PBS. Then total cytoplasmic RNA was extracted and hybridized with a probe specific for HLA-B7 (lane A), HLA-DRβ (lane B), HLA-DQβ (lane C) and HLA-DPβ (lane D)

Table 3. Susceptibility of control and TNF-α- and/or IFN-γ-treated melanoma cell clones MeM A16 and MeM A18 to lysis by LAK cells and anti-HLA-A2 cytotoxic T cells[a]

Cells	TNF-α	IFN-γ	^{51}Cr release (%) by	
			LAK cell lysis	anti-HLA-A2 T cell lysis
MeM A16	−	−	43	40
MeM A16	+	−	45	50
MeM A16	−	+	46	56
MeM A16	+	+	42	56
MeM A18	−	−	43	40
MeM A18	+	−	49	54
MeM A18	−	+	48	60
MeM A18	+	+	49	52

[a] Melanoma cells were incubated for 72 h at 37°C with TNF-α (final concentration 100 U/ml), IFN-γ (final concentration 1000 U/ml) or the combination of TNF-α (100 U/ml) and IFN-γ (1000 U/ml). Control cultures were incubated under the same experimental conditions, but were not exposed to cytokines. At the end of the incubation, cells were harvested, washed twice, labeled with ^{51}Cr and tested for susceptibility to lysis by LAK cells and by anti-HLA-A2 cytotoxic T cells

TNF-α enhanced the shedding of HMW-MAA by MeM A18 cells (Table 2).

Modulation by TNF-α and/or IFN-γ of lysis of clones MeM A16 and MeM A18 by LAK cells and anti-HLA-A2 cytotoxic T cells

To analyze the potential functional role of the differences in the expression and modulation by cytokines of HLA antigens and MAA between MeM A16 and MeM A18 cells, they were compared in their susceptibility to lysis by LAK cells and by anti-HLA-A2 cytotoxic T cells. No difference was detected in the susceptibility to lysis between the two clones, even following incubation with TNF-α and/or IFN-γ. The increase in the expression of HLA class I antigens induced by the two cytokines on MeM A16 and MeM A18 cells was associated with an enhanced susceptibility to lysis by anti-HLA-A2 cytotoxic T cells. The two cytokines did not affect susceptibility of MeM A16 and MeM A18 cells to lysis by LAK cells (Table 3).

Discussion

This study has shown differences in the expression or/and susceptibility to modulation by IFN-γ and/or TNF-α of HLA antigens, ICAM-1 and HMW-MAA by clones MeM A16 and MeM A18 derived from the melanoma cell line MeM 50-10. The degree of difference between the two clones varies among the antigens tested, being more marked for HLA class I antigens than for ICAM-1 and HMW-MAA. HLA class II antigens were detected on neither clone. The clone MeM A18 displayed a higher susceptibility of HLA class II antigens and ICAM-1 to modulation by cytokines than the clone MeM A16. Furthermore, differences were found between the parental cell line and the two clones in terms of antigenic phenotype and susceptibility to modulation by cytokines. The clone MeM A16 expressed a lower level of HLA class I antigens and a higher level of ICAM-1 and both clones expressed a higher level of HMW-MAA than the parental cell line. The latter results parallel those of Cillo et al. [4], who have described the isolation of HLA class-II-positive clones from a melanoma cell line without detectable HLA class II antigens. These findings are consistent with the observation that tumor cell subpopulations behave differently when isolated or when growing together [29]. Furthermore, the two clones acquired HLA class II antigens following incubation with TNF-α, while the parental cell line did not. Similar results were obtained by Houghton et al. [15], who found minimal changes of differentiation during induction of parental melanoma cell lines with either cholera toxin or phorbol myristate acetate, but definite shifts in differentiation in selected cloned subpopulations.

IFN-γ and TNF-α displayed different effects on the various types of antigens analyzed: IFN-γ enhanced the expression of HLA class II antigens much more than TNF-α, but enhanced that of HLA class I antigens and of ICAM-1 as much as TNF-α. The combination of IFN-γ and TNF-α enhanced the level of ICAM-1, HLA class I antigens and especially of HLA class II antigens expressed by MeM A18 cells to a greater extent than the individual cytokines, but did not differ from them in modulating the antigenic profile of MeM A16 cells. The differential sensitivity of the two melanoma cell lines to the combination of IFN-γ and TNF-α parallels results obtained with endothelial cells: while Lapierre et al. [18] did not detect any effect of TNF-α on the IFN-γ-mediated HLA class II induction on endothelial cells. Leeuwenberg et al. [19] described its modulation by TNF-α. Like the latter investigators, we found that the time of addition of the cytokines influenced the modulatory activity of the combination of IFN-γ and TNF-α. However, in our system simultaneous addition of the two cytokines enhanced the expression of HLA class II antigens more than the individual cytokines, while

Leeuwenberg et al. [19] reported that simultaneous addition of TNF-α and IFN-γ to endothelial cells inhibited the induction of HLA class II antigens. TNF-α enhanced the expression of HLA class II antigens only when added 24 h after IFN-γ. If not caused by technical factors, these different results suggest differences among cells in the regulatory pathways which control the expression of HLA class II antigens.

In spite of differences in the expression and susceptibility to modulation of the antigens analyzed, no difference was detected between MeM A16 and MeM A18 clones in their susceptibility to cell-mediated lysis. The lack of relationship between the level of expression of the antigens analyzed and susceptibility to lysis by LAK cells of control and cytokine-treated MeM A16 and MeM A18 cells may reflect the fact that either the antigens analyzed do not play a role in the lytic phenomenon, or the differences in antigen expression are below the sensitivity of the assay and/or variables other than antigen expression determine the outcome of the lytic process. The latter two variables may also account for the lack of differences in the extent of lysis of MeM A16 and MeM A18 cells by anti-HLA-A2 cytotoxic T cells.

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