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ORIGINAL ARTICLE

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Cell fusion: an approach to generating constitutively proliferating human tumor antigen-presenting cells

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Abstract Somatic cell hybrids of HLA-A2(+) EBV-transformed B- or dendritic cells (DC) and allogeneic HLA-A2(–) melanoma cell line Me15 were obtained by in vitro electrofusion using an electroporator. Before fusion, melanoma cells were stably transfected with green fluorescent marker protein (GFP) and neomycin resistance gene (neo^+). Stably growing hybrid antigen-presenting cells (HAPC) expressing HLA-DR and HLA-A2 (or HLA-A30/31), and melanoma-associated antigens (MART-1, gp100) were selected by a double strategy of immunomagnetic MACS and neomycin selection. Fusion efficiency ranged between 3% and 18% (mean: $8.0 \pm 4.7\%$) as defined by simultaneous GFP and HLA-A2 detection. Expression of melanoma-associated antigens (MART-1, gp100) in hybrid cells was determined by reverse transcription–polymerase chain reaction (RT–PCR). HLA-restricted antigen-specific presentation of melanoma antigens was demonstrated by killing of semi-allogenic HAPC by HLA-A2-restricted MART-1 or gp100-specific cytotoxic T lymphocyte (CTL) clones. HLA restriction and antigen specificity were confirmed by inhibition of specific cytotoxicity by anti-HLA antibodies and cold target inhibition. During long-term (42–70 days) neomycin selection of HAPC, a drastic loss of antigen-presenting cell (APC)-derived determinants (e.g. HLA-DR, HLA-A2) was observed which, however, could be "reversed" by repeated MACSorting (days 10, 21 and 49). Our method allows the generation of semi-allogenic HAPC that constitutively proliferate in vitro. This opens the possibility of establishing a number of tumor–APC hybrids expressing defined HLA haplotypes and tumor antigens, of investigating their specific properties (e.g. antigen processing), and testing their diagnostic or therapeutic potential.

Keywords CTL · DC · EBV · gp100 · Hybrid · MART-1 · Melanoma

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Introduction

Many human malignancies carry specific antigens [2, 3] which might constitute targets for the immune response. However, there is growing evidence that the failure of the immune system to control tumor growth may be due to defective antigen presentation by tumor cells. This may be caused by the down-regulation or loss of major histocompatibility complex (MHC) molecules [6, 17, 20, 33], alterations in antigen processing pathways, resulting in an inability to present tumor-specific antigens to T cells [19, 22, 23, 24], or the absence of costimulatory or adhesion molecules that are essential for the activation of the host immune system [5, 28].

Cell fusion vaccines represent one possibility to render tumor cells immunogenic and to provide an immunotherapeutic tool in an experimental [8, 10, 32] but also clinical [15, 16, 26, 27, 29] setting. The fusion of professional antigen-presenting cells (APC) and tumor cells results in hybrids with a high expression of HLA and costimulatory molecules, and functioning antigen processing machinery presenting tumor-specific peptides from tumor cells. Hybrid antigen-presenting cells (HAPC) have been "constructed" in various combinations of allogeneic and autologous APC and tumor cells, respectively [8, 10, 15, 26, 29, 32]. In most cases,

"transient" fusions were performed using HAPC immediately for vaccination [8, 10, 15, 26, 27, 29, 32, 34, 35].

In the present study, we tried to generate constitutively proliferating hybrids between HLA-A2(+) EBV-transformed B- or dendritic cells (DC) and allogenic, HLA-A2(–) melanoma cell lines [HLA-A1(+), or HLA-A3(+)]. The individual hybrid cell lines differ in their HLA molecule expression, antigen presentation and processing, but are able to present tumor antigenic epitopes in the context of respective HLA molecules to tumor-specific cytotoxic T lymphocytes (CTL).

Materials and methods

Cells and cell lines

Peripheral blood mononuclear leukocytes (PBL) were separated from the blood of healthy donors by Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) density centrifugation. HLA typing for HLA-A1, -A36, -A2, -A3, -A30, and -A31 was determined by fluorescence-activated cell sorter (FACS) analysis with biotin-conjugated monoclonal antibodies (mAb; One-Lambda, Canoga Park, Calif.) and R–PE–streptavidin (Southern Biotechnology Associates, Birmingham, Ala.). Flow cytometry was performed on a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.) using CellQuest software. EBV transformation was performed by incubating PBL in 96-well plates at a concentration of 1×10^6/ml in the presence of virus-containing supernatant of the cell line B95.8 (G. DeLibero, Basel, Switzerland) and cyclosporin A [7] in RPMI 1640 (Gibco, Life Technologies, Grand Island, N.Y.), 10% heat-inactivated fetal calf serum (FCS), 10 IU/ml penicillin, 10 μg/ml streptomycin, and 1% L-glutamine (RPMI–FCS). Cells were provided with RPMI 1640, 10% FCS, L-glutamine, penicillin, and streptomycin twice a week. Immortalized cells were expanded according to their growth and cell number, and finally characterized by FACS analysis using HLA class I (clone W6/32), HLA-DR (clone HB55) (G. DeLibero, Basel, Switzerland), and CD1a, CD3, CD11c, CD19, CD54, CD80, CD83 and CD86 (Pharmingen, San Diego, Calif.) specific mAb and R–PE-goat anti-mouse Ig (Southern Biotechnology Associates, Birmingham, Ala.). Binding specificity was determined using IgG1 (MOPC-21), IgG2a (UPC-10), or IgG2b (MOPC-141) isotype control mAb (Sigma-Aldrich Chemie, Buchs, Switzerland). Aliquots of immortalized B-lymphoblastoid cell lines (EBV) were frozen, and the remaining cells maintained in culture. DC were derived from highly purified circulating CD14(+) monocytes. The immunomagnetic purification of monocytes from PBL was performed according to the manufacturer's instructions using CD14 microbeads and MiniMACS high gradient magnetic separation columns (Miltenyi Biotech, Bergisch Gladbach, Germany). Isolated monocytes (5×10^5/ml) were cultured with human recombinant granulocyte–macrophage colony-stimulating factor (GM–CSF; 0.05 μg/ml) and IL-4 (0.025 μg/ml) (PromoCell, Heidelberg, Germany) in 6-well plates. Aliquots of the cells were reanalyzed by FACS with anti-CD14 mAb (Pharmingen, San Diego, Calif.). After 5–6 days, non-adherent cells were collected and DC purity was determined by FACS analysis.

Melanoma cell lines (Table 1), Me15 [14], HBL (kindly provided by Dr. Ghanem, Free University Brussels, Belgium), MZ2 [31], and MABI-9 (P. Jantscheff, unpublished data) were routinely passaged in RPMI 1640 (Gibco, Basel, Switzerland) supplemented with 10% FCS at 37°C with 5% CO_2 in a humidified atmosphere. Melanoma cell line Me15 was transfected with pEGFP-C3 plasmid (Clontech Lab, Palo Alto, Calif.) encoding green fluorescent protein (GFP) and neomycin (neo^+) resistance using lipofectin (Gibco, Basel, Switzerland) according to the manufacturer's instructions, and selected by culturing the cells in the presence of 600–800 μg/ml G418 (Gibco, Basel, Switzerland). Expression of GFP by neo^+ melanoma cells was confirmed by FACS analysis. High GFP expressing Me15 melanoma cells were established by repeated FACSorting of $FL1^{+++}$ cells.

Table 1. Melanoma cell characteristics

Cell line	HLA-A[a]	MART-1[b]	gp100	Tyr	MAGE-1	MAGE-3
HBL	HLA-A2	+	+	+	+	+
MZ2	HLA-A1	–	+	–	nt	+
Me15	HLA-A3	+	+	+	+	+
MABI-9	HLA-A3	+	+	+	+	+

[a]HLA-A subtypes were defined by mAb
[b]Expression of melanoma antigens was defined by RT–PCR

Preparation of RNA and cDNA from melanoma cell lines and HAPC

RNA was prepared using an RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions from samples of about 1×10^6 melanoma or hybrid cells. Single-strand cDNA was synthesized from 1–5 μg of total RNA, using a reverse transcription system (Promega, Madison, Wis.) with AMV reverse transcriptase and random primers.

Expression of melanoma antigen in tumor cell lines and HAPC

Expression of melanoma antigens in the tumor cell lines or HAPC was determined by specific RT–PCR amplification of MART-1, gp100, tyrosinase (Tyr), MAGE-1, or MAGE-3 in a final volume of 25 μl containing 10 mmol Tris–HCl (pH 8.1), 1.5 mmol $MgCl_2$, 50 mmol KCl, 0.1 μg gelatin, 2.5 mmol of each dNTP, 50 pmol primers and 2.5 IU AmpliTaq gold polymerase (Perkin–Elmer, Rotkreuz, Switzerland) together with defined amounts of cDNA prepared from the tumor or hybrid cell lines. The following primer pairs (Pharmacia Biotech, Uppsala, Sweden) were used: β-actin sense, 5′-CACCCACACTGTGCCCATC; anti-sense, 5′-CTAGAAGCATTGCGGTGGAC, amplifying a 650-bp gene fragment; MART-1 sense, 5′-AGATGCCAAGAGAAGATGCTC; anti-sense 5′-GCTCTTAAGGTGAATAAGGTGG, amplifying a 364-bp fragment; gp100 sense, 5′-CTGTGCCAGCCTGTGCTAC; anti-sense, 5′-CACCAATGGGACAAGAGCAG, amplifying a 334-bp fragment; tyrosinase-sense, 5′-TTGGCAGATTGTCTGTAGCC; anti-sense, 5′-AGGCATTGTGCATGCTGCTT, amplifying a 284-bp fragment; MAGE-1 sense, 5′-CGGCCGAAGGAACCTGACCCAG; anti-sense, 5′-GCTGGAACCCTCACTGGGTTGCC, amplifying a 421-bp fragment, and MAGE-3 sense, 5′-TGGAGGACCAGAGGCCCCC, anti-sense, 5′-GGACGATTATCAGGAGGCCTGC, amplifying a 725-bp fragment. The amplification was performed in a Perkin–Elmer Cetus DNA thermal cycler (cat. no. N801–0150) using the following cycle profiles: 20 s denaturation at 94°C, 20 s annealing at 58°C, and 40 s extension at 72°C for 34 cycles; or 1 min denaturation at 94°C followed by 2 min annealing at 68°C and 2 min extension at 72°C (MAGE-1, MAGE-3).

Cytotoxicity and inhibition assay

Tumor-specific CTL bulk lines against MART-1 or gp100 peptides were generated by stimulation of PBL from HLA-A2(+) healthy donors with irradiated tumor cell lines and EBV-transformed cells in the presence of 20 IU/ml IL-2. T-cell clones were generated by limiting dilution stimulation of CTL in the presence of PHA (1.5 μg/ml) and irradiated allogenic PBMC [13]. Specific killing of melanoma cell lines or HAPC by bulk-cultured HLA-A2-restricted CTL or CTL clones (UL2 and UL10: MART-1) was measured by the ^{51}Cr-release (Amersham, Little Chalfont, U.K.) technique [7,

12]. HLA-A2(+) HBL melanoma cells (Table 1) were used in some experiments to determine specific cytotoxic activity of CTL clones. Quadruplicates of 5.000 ^{51}Cr-labeled target cells in 100 µl RPMI 1640/FCS were incubated in the presence of an excess of 10, 5, 1, or 0.2 effector cells at 37°C for 4 h. Aliquots of the radioactive supernatants were then measured in a gamma counter, and cytotoxicity was calculated by the formula:

$$\% \text{ specific release} = \frac{\text{cpm (experimental)} - \text{cpm (spontaneous)}}{\text{cpm (maximal)} - \text{cpm (spontaneous)}} \times 100$$

where spontaneous release was determined without adding effector cells and maximal release was obtained by detergent lysis of target cells. Cold target inhibition was performed adding an excess of 20, 10, 2.5, or 1 times unlabeled inhibitor target cells to each well, comparing ^{51}Cr-release in the presence or absence of inhibitors. As control, HLA-unrelated melanoma cells (Me15) were added in the same amount to each well, giving similar results to those in the absence of competitor cells. Alternatively, labeled target cells were preincubated with an excess (100 µg/ml) of anti-HLA (W6/32) or isotype control mAb, washed twice with PBS and cultured with the effector cells at 37°C for 4 h. Inhibition was calculated according to the formula:

$$\% \text{ inhibition} = \left[1 - \left(\frac{\text{inhibitor}}{\text{control}}\right)\right] \times 100$$

Electrofusion parameters and HAPC selection (MACS)

Somatic cell fusion was achieved by electrofusion using a commercially available electroporator (EquiBio Easyject Plus electroporator; EquiBio, Kent, U.K.). EBV or DC were mixed with neo$^+$ melanoma cells at a 1:1 or 2:1 ratio to a final concentration of 1×10^8 cells in 200 µl RPMI 1640/FCS. Cells were added to 2-mm sterile electroporation cuvettes (Bio-Rad Laboratories, Hercules, Calif.), and were allowed to sediment onto basal electrode for 15 min by 1×G sedimentation at room temperature by switching cuvettes horizontally. Following this, the cuvettes were put into the electroporator, and cells were fused by a single pulse at an output voltage of 180 volts, capacitor at 1,200 µF and with parallel resistor in infinite position. After pulsing, the cells were removed immediately from the cuvettes with a Pasteur pipette, transferred into fresh medium and washed twice. Six fusions with EBV-HLA-A2 and two with DC-HLA-A2 were performed using an Me15-neo$^+$ melanoma cell line as common fusion partner. Two EBV-HLA-A2: Me15-neo$^+$ and one DC-HLA-A2:Me15-neo$^+$ hybrid were lost for technical reasons (contamination or cell death) 2 days, 7 days and 2 weeks after starting neomycin selection. Viability of the electroporated cells was 72.8 ± 13.4% as determined by trypan blue exclusion. Aliquots of fused cells were analyzed by flow cytometry. Fusion efficiency was determined by simultaneous GFP and HLA-DR expression. HAPC were selected from non-fused APC and transfected melanoma cells in two steps, combining MACS separation and neomycin selection (Fig. 1). After overnight growth, cells were labeled with anti HLA-DR (clone HB55) mAb (50 µg/ml) and immunomagnetically purified using goat anti-mouse IgG microbeads and MiniMACS high gradient magnetic separation columns. To select neomycin-resistant cells, isolated HAPC and non-fused APC were cultured (1×10^6/ml) in the presence of 600–800 µg/ml G418 (Gibco, Basel, Switzerland) in 6-well plates. Cells received RPMI 1640, 10% FCS, L-glutamine, penicillin, streptomycin, and 600–800 µg/ml G418 twice a week. Immunomagnetic purification of HLA-DR$^+$ cells was performed on days 10, 21, and 49 of neomycin selection.

Results

HAPC were generated in vitro by somatic electrofusion of HLA-A2(+) EBV-transformed B-cells (EBV-HLA-A2) or DC (DC-HLA-A2) and HLA-A2(–) melanoma cell line Me15 (HLA-A3$^+$) transfected with a marker protein (GFP) and neomycin resistance gene (neo$^+$). Fig. 2 shows surface marker expression of adhesion molecules (CD54), costimulatory molecules (HLA-DR, CD40, CD80, CD86), and lineage-specific molecules (CD1a, CD11c) expressed in EBV or DC but not (with the exception of CD54) in Me15 melanoma cells. Similar results were obtained with other melanoma cells (data not shown). Six fusions with EBV-HLA-A2 and two with DC-HLA-A2 were performed using the Me15-neo$^+$ melanoma cell line as common fusion partner. Fusion efficiency of APC and melanoma cells for the 8 electrofusions ranged between 3% and 18% (mean: 8.0 ± 4.7%) as determined by simultaneous GFP and HLA-DR expression (Fig. 3). Following overnight recovery from "electrofusion stress" culturing cells in RPMI/FCS medium, HLA-DR(+) cells i.e., HAPC and EBV-HLA-A2 or DC-HLA-A2 were isolated from non-fused melanoma cells by MACSeparation. Neomycin selection

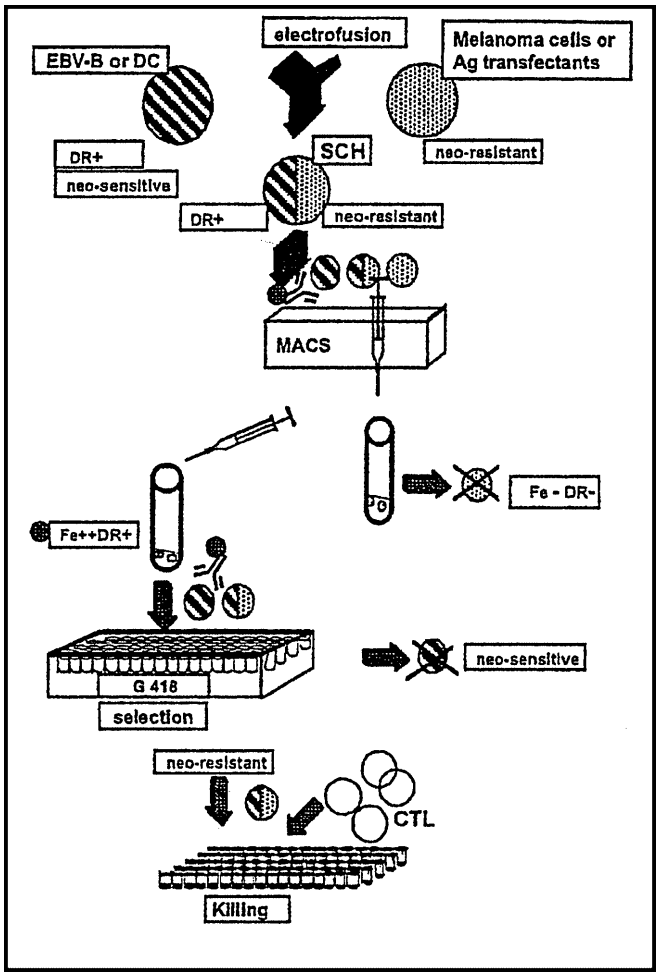

Fig. 1. Enhanced double selection strategy (MACS separation and G418 selection) of electrofused HAPC. Electrofused HLA-DR$^+$ and Me15 melanoma cells were separated on magnetic columns using anti-HLA-DR Ab and anti-mouse-Ig magnetic beads (Miltenyi Biotech, Germany). Hybrid APC were selected by G418 culture (600–800 µg/ml). MACS separation was repeated after 10, 21 and 49 days

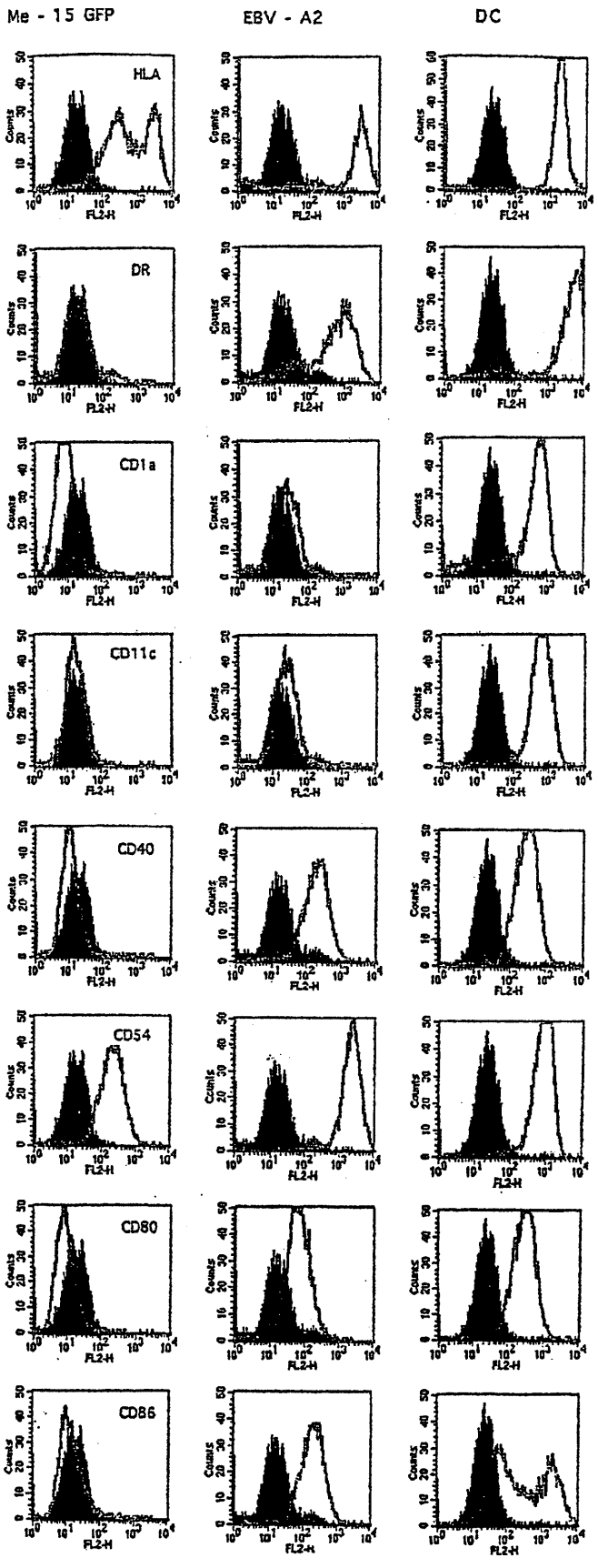

Fig. 2. Expression of HLA class I costimulatory, lineage-specific molecules in EBV-transformed B-cells, dendritic cells and Me15 melanoma cells. Expression of HLA-ABC, HLA-DR, CD1c, CD11c, CD40, CD54, CD80, CD86 molecules was tested by FACS analysis with anti-HLA class I (clone W6/32), -HLA-DR (clone HB55) (G. DeLibero, Basel, Switzerland), and -CD1a, -CD3, -CD11c, -CD19, -CD54, -CD80, -CD83 and -CD86 (Pharmingen, San Diego, Calif.) specific mAb and R–PE-goat anti-mouse Ig (Southern Biotechnology Associates, Birmingham, Ala.). Non-specific binding (*black histograms*) was determined using IgG1 (MOPC-21), IgG2a (UPC-10), or IgG2b (MOPC-141) isotype control mAb (Sigma-Aldrich Chemie, Buchs, Switzerland)

of HAPC (a total of 5% to 25% of the MACS-isolated cells) from non-fused EBV-HLA-A2 or DC-HLA-A2 was started by adding 600–800 μg/ml G418 to MACS-separated HLA-DR(+) cells. This dosage had been found to kill EBV cells and DC within 7 days. Therefore, expression of HLA-DR (MACS-selected APC antigen) and HLA-A haplotypes of parental cells was first analyzed on day 7 in aliquots of neomycin-selected hybrid cells (Fig. 4). Expression of APC-derived molecules, HLA-DR, HLA-A2 or HLA-A30/31 (expressed on EBV of two of the donors), was drastically reduced, and only $2.21 \pm 1.6\%$, $1.17 \pm 0.4\%$, and $1.25 \pm 0.4\%$ respectively of hybrid cells stained positive. HLA-A3 (Me15 melanoma) also showed variable expression (20.3–60.4% positive stained cells) at the beginning of selection and stabilized in about 80% of cells 21 days from the beginning of selection. Repeated enrichment of HLA-DR(+) HAPC on MiniMACS high-gradient magnetic separation columns (Fig. 4C; a: arrow, day 10)

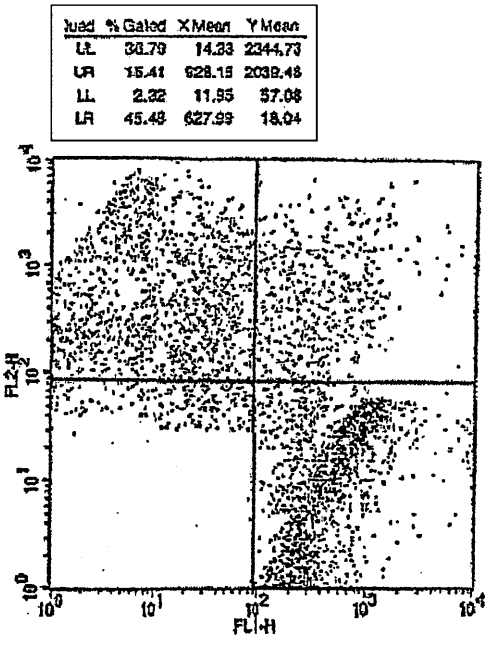

Fig. 3. FACS-analysis of fusion efficiency as determined by simultaneous expression of GFP (FL1) and HLA-DR (FL2) in HAPC. Data are shown from one representative fusion of EBV ($HLA\text{-}DR^+ = FL2$) and Me15 melanoma ($GFP^+ = FL1$) cells. About 15% of cells showed double fluorescence (*upper right quadrant*)

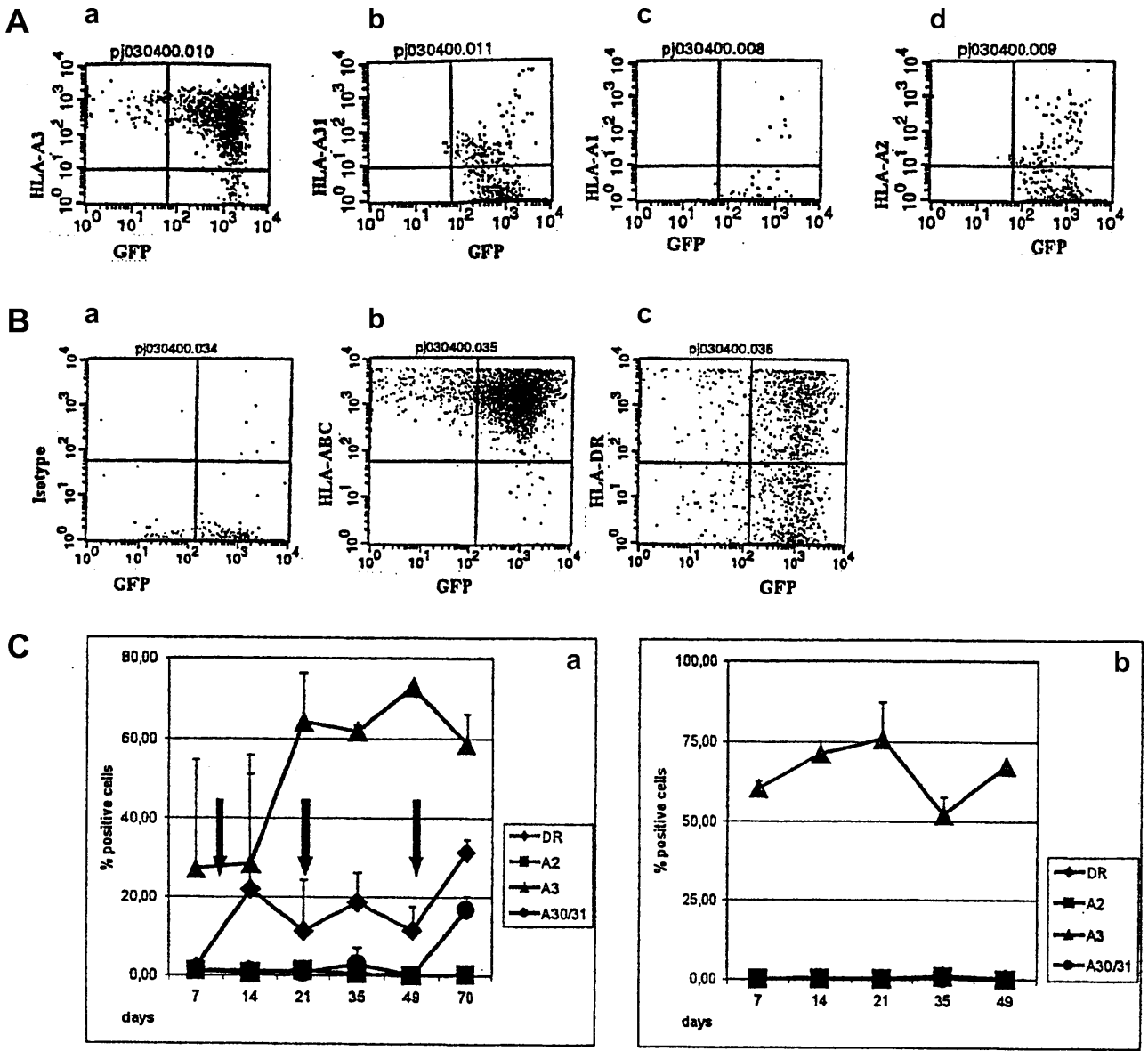

Fig. 4A–C. Expression of parental antigens in primary and long-term selected HAPC cultures before and after MACS selection: HLA-A subtypes, HLA-DR and GFP expression. Data are shown from one representative donor (AR). **A** Day 35: **a** biotin-conjugated anti-HLA-A3, **b** anti-HLA-A31 or **d** anti-HLA-A2 mAb stained about 62%, 3%, or 0.5% of GFP-fluorescent HAPC [EBV-B (HLA-A2, A31)×Me-15 (HLA-A3)], whereas **c** isotype control mAb (HLA-A1) were completely negative. Antibody binding was determined by PE-labeled streptavidin. **B** day 35: **a** no cells are double positive with isotype control mAb, whereas **b** mAb W6/32 (HLA-ABC) or **c** HB55 (HLA-DR) stained 94% and 23% of GFP-fluorescent HAPC [EBV-B (HLA-A2, -A31)×Me-15 (HLA-A3)], respectively. Antibody binding was determined by PE-labeled anti-mouse Ig antibodies. **C** Time course of HLA-DR, HLA-A3, -A2, and -A31 expression in HAPC during HLA-DR/neomycin selection procedure; **a** data show mean values ± SD of individual antigens in HLA-DR(+) selected HAPC from 4 somatic cell fusions of EBV-HLA-A2 and Me15. *Arrows* indicate date of MACS selection. Days 21, 49: FACS analysis was performed before MACS selection. **b** mean values ± SD of individual antigens in HLA-DR(–) selected HAPC which were grown from only two fusions. All cells were cultured under permanent G418 selection (600–800 μg/ml)

led to significantly enhanced expression of HLA-DR in cultured HAPC (day 14) which, however, was again reduced during further culture (Fig. 4C; a). Additional MACS separation (days 21, 49) stabilized and finally enhanced HLA-DR expression. Unexpectedly, this did not simultaneously lead to a stronger expression of HLA-A2 (or HLA-A30/31 in two of the donors). While most cells expressed HLA-A3 (Fig. 4A; a), only about 0.5% and 3% of HAPC respectively showed a weak expression of HLA-A2 (Fig. 4A; d) or HLA-A30/31 (Fig. 4A; b) but about 20% of these cells strongly expressed HLA-DR (Fig. 4B; c) in 35-day cultures. Similar results were obtained by fusing DC-HLA-A2 and Me15 melanoma cells (one donor) and culturing Me15-DC-HLA-A2 HAPC in the presence of G418 (6 weeks). These cells, however, grew much more slowly, and therefore considerably fewer data points were available (not shown). Expression of tumor antigens (MART-1, gp100, Tyr) in HAPC, as demonstrated by

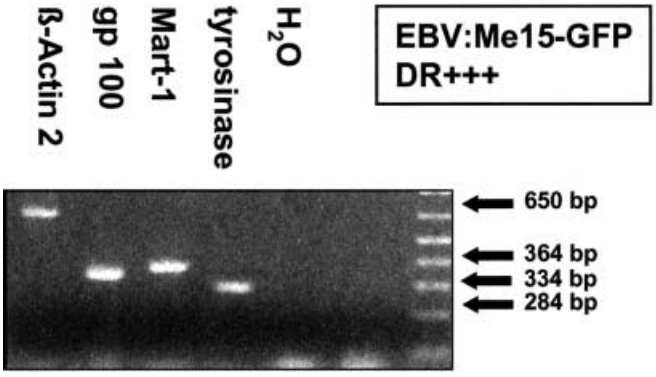

Fig. 5. Expression of melanoma antigens in long-term selected HAPC cultures (day 35) as determined by PCR. Expression of melanoma antigens in the tumor cell lines or HAPC was determined by specific RT–PCR amplification of MART-1, gp100, or Tyr using the following primer pairs: β-actin sense, 5′-CACCCACACTGTGCCCATC; anti-sense, 5′-CTAGAAGCATTGCGGTGGAC, amplifying a 650-bp gene fragment; MART-1 sense, 5′-AGATGCCAAGAGAAGATGCTC; anti-sense 5′-GCTCTTAAGGTGAATAAGGTGG, amplifying a 364-bp fragment; gp100 sense, 5′-CTGTGCCAGCCTGTGCTAC; anti-sense, 5′-CACCAATGGGACAAGAGCAG, amplifying a 334-bp fragment; Tyr sense, 5′-TTGGCAGATTGTCTGTAGCC; anti-sense, 5′-AGGCATTGTGCATGCTGCTT, amplifying a 284-bp fragment. PCR products of gp100, MART-1, and Tyr were demonstrated in parental Me15, HBL and MABI-9 melanoma cells whereas in MZ2 melanoma cells only significant expression of gp100 was detected (Table 1). None of the antigens was detected in EBV cells or DC (data not shown)

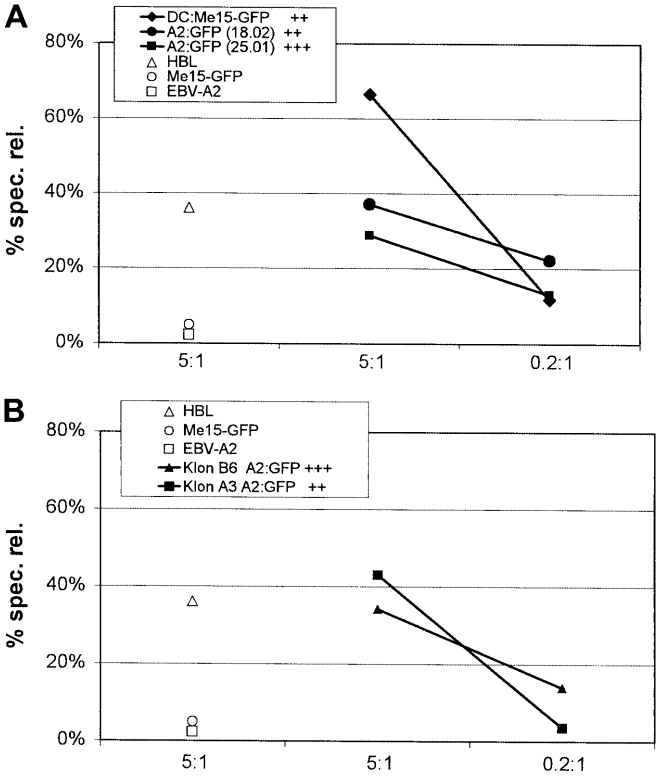

Fig. 6A, B. Killing of primary or long-term selected HAPC cultures by anti-MART-1 CTL clone UL-2. Killing of HAPC, HLA-A2(+) HBL melanoma cells, Me15 melanoma (HLA-A3) or EBV-transformed HLA-A2(+) B-cells by HLA-A2-restricted, MART-1 specific CTL clone UL-2 was determined by ^{51}Cr-release at different E: T ratios (5:1 or 0.2:1). **A** Primary (days 7–10) HAPC cultures (DC:Me15-GFP, A2:GFP); or **B** long-term selected (1–3 additional MACS selections) HAPC cultures (Klon B6, Klon A3) and HLA-A2(+) HBL melanoma cells (L) showed similar sensitivity to killing by MART-specific CTL, whereas HLA-A3(+) Me-15 melanoma cells (±) or HLA-A2(+) EBV-transformed B-cells (–) were not killed

RT–PCR (Fig. 5), was found to be similar to parental melanoma cells in all HAPC tested.

Antigen processing and presentation by primary or long-term selected HAPC was tested using MART-1-specific CTL clones (Fig. 6) or bulk cultures and gp100-specific cytotoxic T-cells, respectively (not shown). All HAPC cells tested were killed, and we did not find any significant difference in killing in primary (days 7–10) HAPC cultures (Fig. 6A) or in long-term selected (1–3 additional MACS selections) HAPC cultures (Fig. 6B). In contrast, parental Me15 melanoma (HLA-A3) or EBV-HLA-A2 were not killed by CTL (Fig. 6A, B). Antigen-specificity and HLA-restricted presentation was demonstrated by cold target and antibody-mediated inhibition of melanoma antigen-specific killing (Fig. 7A, B). Addition of HLA-A2 melanoma cells (HBL) or HAPC (E4:F4) but not of HLA-A3 melanoma cells (Me15) or EBV-HLA-A2 cells inhibited the killing of HBL and HAPC (Fig. 7B), respectively. Furthermore, the addition of an excess of anti-HLA mAb (W6/32) led to a marked inhibition of the killing of melanoma or HAPC cells (Fig. 7B), thus confirming HLA restriction.

Discussion

The aim of our study was to establish a method to generate somatic cell hybrids of APC and malignant cells expressing specific tumor antigens. HAPC might be used to present tumor antigens in an HLA-restricted manner, to monitor clinically relevant tumor-specific immune responses, to define new TAA epitopes or to vaccinate tumor patients [8, 10, 15, 26, 27, 29, 32, 34, 35]. Here we have demonstrated that somatic cell fusion is a useful method to establish long-term growing HAPC. Sufficient fusion efficiency was obtained by electrofusion using a commercially available electroporator, whereas the efficiency of "classical" polyethylene glycol (PEG) fusion (5 fusions) was too low, and ranged between 0.5% and 4.5% of fused cells (data not shown). Hybrid cells were able to specifically present HLA-restricted tumor antigens which might be targets for the immune response [2, 3]. There is growing evidence that malignant transformation [17, 20, 22] may cause down-regulation or loss of major histocompatibility complex (MHC) molecules [6, 17, 20, 33], alteration of antigen processing pathways, resulting in an inability to present tumor-specific antigens to T cells [19, 22, 23, 24], or in the absence of costimulatory or adhesion molecules that are essential for activation of the host immune system [5, 28].

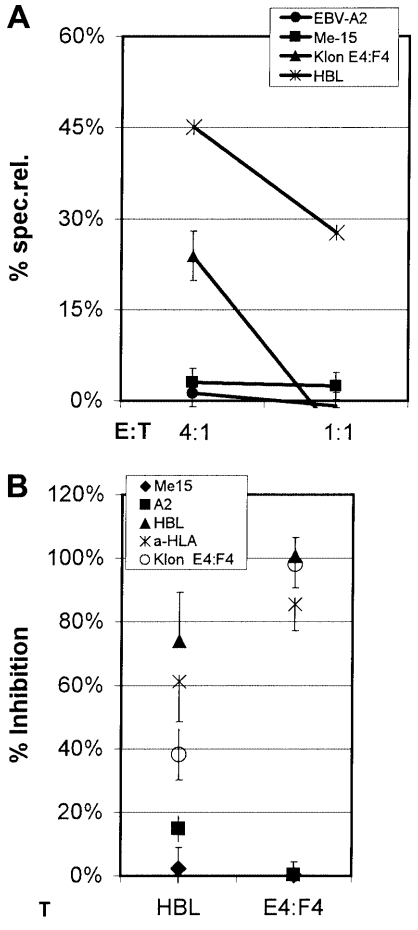

Fig. 7A, B. Cold target and antibody mediated inhibition of melanoma antigen-specific killing. **A** Killing of HAPC (E4:F4), HLA-A2(+) HBL melanoma cells, Me15 melanoma (HLA-A3) or EBV-transformed HLA-A2(+) B-cells by HLA-A2-restricted, MART-1 specific CTL clone UL-2 was determined by ^{51}Cr-release at different E: T ratios (4:1 and 1:1). **B** Inhibition of ^{51}Cr-release was determined adding HLA-A2(+) HBL melanoma cells, HAPC (E4:F4), HLA-A3(+) Me15 melanoma cells or EBV-transformed HLA-A2(+) B-cells at an E:T ratio of 20:1 (4:1). To test the inhibition of killing by anti-HLA class I Ab, target cells were preincubated with a W6/32 Ab (100 μg/ml) for 30 min on ice and washed twice before use in a ^{51}Cr-release assay. HLA-A2(+) HBL melanoma cells or HAPC (E4:F4) were used as targets in the inhibition assay

These circumstances may lead to deficient antigen presentation by the tumor cells and insufficient activation of the immune system, thereby preventing the immunological control of tumor growth. Various approaches have been chosen to induce an efficient anti-tumor response. One way is to modify tumor cells, e.g. by transfection with cytokine genes, genes of costimulatory molecules or HLA-related molecules and genes which inhibit expression of suppressive substances produced by tumor cells, respectively [11]. Another strategy is to manipulate professional APC to induce an effective anti-tumor response. This was attempted with professional APC (EBV, DC) pulsed with antigenic peptides [18, 21], or transfected with distinct human tumor antigens [1, 4]. Cell fusion has been shown to be another way to establish effective tumor APC. HAPC have been "constructed" in various combinations of allogeneic and autologous APC or tumor cells and cell lines, respectively [8, 10, 15, 62, 29, 32]. In most cases, "transient" fusions have been performed using HAPC immediately for testing tumor antigen presentation or for vaccination in experimental and clinical applications [8, 10, 15, 26, 27, 29, 32, 34, 35].

Cell fusion has a number of advantages as compared to other methods. First, it is not necessary to isolate, characterize or process tumor antigens since professional APC using their functioning antigen-processing machinery are able to process and present tumor-specific peptides from tumor cells [22, 32]. Second, APC express high amounts of HLA and costimulatory molecules that are necessary to trigger primary and costimulatory signals critical for the T-cell response [9, 25]. A third advantage is that tumor/APC hybrids not only present one peptide or antigen [18, 21] but the complete panel of tumor antigens available in the respective tumor cells.

Establishment of constitutively growing HAPC would offer some additional advantages. It would allow characterization of the properties of such cells in more detail. With our method we were able to generate semi-allogenic HAPC growing stably in vitro (42–70 days). These cells processed tumor antigens (MART-1, gp100) and presented tumor-specific peptides in the context of known APC-derived HLA-class I antigens (HLA-A2), but showed a drastic loss of APC-derived cell surface molecules and a shift to a more melanoma-derived phenotype. This shift was not unexpected, since neomycin treatment preferentially selects the phenotype of the stably (GFP and neomycin) transfected melanoma fusion partner. Although only low amounts of APC-derived HLA class I antigens (in contrast to tumor cell-derived HLA) were detected on HAPC, the cells were effectively killed by tumor-specific CTL. This confirms data of others that only small numbers of peptide-MHC complexes are necessary to induce specific killing [30]. However, the reduced amount of APC-derived HLA-A2, HLA-DR (and other costimulatory) molecule expression might render the HAPC weak stimulators of tumor-specific CTL induction. This was not tested here, since strong expression of melanoma-derived, allogeneic HLA-A3(+) antigens by HAPC might induce a strong anti-allogeneic CTL response in HLA-A2(+) responder cells which makes it difficult to discriminate between strong allogeneic and much weaker tumor-specific response in vitro. Furthermore, LAK cells are induced during allogeneic stimulation, which could kill respective HLA-A2(+) melanoma cell lines (e.g. HBL). With our method of repeated MACS selection of growing hybrid cells, however, we were able to stabilize expression of individual costimulatory molecules (e.g. HLA-DR) on HAPC. This might be a prerequisite for establishing HAPC, which can also be used for vaccination studies in tumor patients [9, 25], since it has been demonstrated in other studies that T lymphocytes can also be activated by

such antigen-presenting cells displaying only very small numbers of peptide–MHC complexes on their surface [30]. Taken together, our data indicate that semi-allogenic HAPC show "correct" tumor antigen processing, express APC-derived HLA and costimulatory molecules, display APC-derived HLA-restricted tumor antigen presentation, express a number of tumor antigens, grow in a stable manner and can be expanded. This opens up the possibility of establishing a number of tumor–APC hybrids expressing defined HLA haplotypes and tumor antigens, to investigate their specific properties (e.g. antigen processing), and to test their diagnostic or therapeutic potential. Thus, HAPC cells might be useful tools for testing anti-tumor reactivity or for detecting new tumor antigens. Furthermore, if expanded in sufficiently high amounts, HAPC could be applied – if they were to display sufficient stimulatory activity– to various HLA-matched patients as a more general vaccine allowing better standardization of vaccination protocols.

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