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Trophoblast Cell Line Resistance to NK Lysis Mainly Involves an HLA Class I-Independent Mechanism[1]

Tony Avril,[2]* Annie-Claude Jarousseau,[2]* Herve Watier,* Jose Boucraut,† Philippe Le Bouteiller,‡ Pierre Bardos,* and Gilles Thibault[3]*

The lack of classical HLA molecules on trophoblast prevents allorecognition by maternal T lymphocytes, but poses the problem of susceptibility to NK lysis. Expression of the nonclassical class I molecule, HLA-G, on cytotrophoblast may provide the protective effect. However, the class I-negative syncytiotrophoblast escapes NK lysis by maternal PBL. In addition, while HLA-G-expressing transfectants of LCL.721.221 cells are protected from lymphokine-activated killer lysis, extravillous cytotrophoblast cells and HLA-G-expressing choriocarcinoma cells (CC) are not. The aim of this work was therefore to clarify the role of HLA class I expression on trophoblast cell resistance to NK lysis and on their susceptibility to lymphokine-activated killer lysis. Our results showed that both JAR (HLA class I-negative) and JEG-3 (HLA-G- and HLA-Cw4-positive) cells were resistant to NK lysis by PBL and were equally lysed by IL-2-stimulated PBL isolated from a given donor. In agreement, down-regulating HLA class I expression on JEG-3 cells by acid treatment, masking these molecules or the putative HLA-G (or HLA-E) receptor CD94/NKG2 and the CD158a/p58.1 NKR with mAbs, and inducing self class I molecule expression on JAR cells did not affect NK or LAK lysis of CC. These results demonstrate that the resistance of CC to NK lysis mainly involves an HLA class I-independent mechanism(s). In addition, we show that the expression of a classical class I target molecule (HLA-B7) on JAR cells is insufficient to induce lysis by allospecific polyclonal CTL. ***The Journal of Immunology,* 1999, 162: 5902–5909.**

Pregnancy presents an intriguing problem for immunology because the fetus, a semiallograft by its genotype, survives without immunological rejection. One explanation for the maternal tolerance is that trophoblast cells, which in humans are in direct contact with the maternal immune system, lack expression of the classical HLA class I and class II molecules, except for HLA-C in the extravillous trophoblast during the first trimester of pregnancy (1, 2). This prevents allorecognition and lysis by maternal T lymphocytes. Nevertheless, it has been shown recently that lysis of HLA-A*0201-expressing transfectants of JEG-3 and JAR choriocarcinoma cell lines (CC),[4] which are derived from tumors of trophoblast origin, by allospecific cytotoxic T cells or by a peptide-specific cytotoxic T cell clone is very low, suggesting that in addition to the absence of expression of classical HLA class I and class II molecules, trophoblast cells may have a particular cell membrane structure that renders these cells resistant to lysis (3).

On the other hand, the lack of expression of classical HLA class I molecules is usually associated with an increased susceptibility of target cells to NK cytotoxicity (4–8). It has been proposed that the nonclassical HLA class I molecule, HLA-G, which is characterized by a limited polymorphism and preferentially expressed on extravillous trophoblast (9, 10), i.e., in contact with the abundant population of decidual NK cells (11–13), may provide the protective effect (14). Indeed, several studies have shown that the B lymphoblastoid cell line LCL.721.221 (15–21) and the erythroleukemia cell line K562 (22, 23) transfected with HLA-G exhibit decreased sensitivity to lysis by peripheral blood NK cells (22, 23), decidual leukocytes (16, 21), uterine blood mononuclear cells (22), NK cell lines (17, 22, 23), and peripheral blood and decidual NK clones (15, 17–21), as compared with untransfected cells. In all results, however, target protection was only partial. Results concerning the NK receptors (NKR) responsible for HLA-G recognition are more conflicting. Indeed, while the first studies suggested that CD158a/p58.1 and CD158b/p58.2 (17) and p70/NKAT3 (18) mediate HLA-G recognition, further studies indicated that the CD94/NKG2 heterodimers, which recognize a broad panel of HLA class I molecules (24–27) but not other known NKR, could be the predominant receptor for HLA-G on NK cells (19–21). The direct interaction of HLA-G with CD94/NKG2 is however still a matter of debate, due to the recent findings in several laboratories that CD94/NKG2 is a specific receptor for HLA-E with bound leader peptides derived from permissive HLA-A, -B, -C, and -G polypeptides (28–31).

Demonstration of the protective effect conferred by HLA-G expression has been established on nontrophoblast cells (15–23), whereas, to the best of our knowledge, the effect of HLA class I molecule expression on MHC-unrestricted lysis of trophoblast cells has been investigated in only two previous studies. In the first study, it was observed that IFN-γ treatment, which is known to increase HLA class I molecule expression, partially protected first trimester trophoblast cells as well as the HLA-G- and HLA-C-positive JEG-3 cell line, but not the HLA class I-negative JAR cell line from IL-2-stimulated decidual NK cells (32). In the second

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[4] Abbreviations used in this paper: CC, choriocarcinoma cells; β_2m, β_2-microglobulin; DL, decidual lymphocytes; LAK, lymphokine-activated killer; NKR, NK receptor.

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study, treatment of first trimester cytotrophoblasts with the W6/32 mAb, which masks all HLA class I molecules including HLA-G, but not with the anti-HLA-A, -B, -C B1.23.2 mAb, was shown to induce partial lysis of these cells by NK cells from both maternal uterine blood and allogeneic peripheral blood (23). These studies thus supported the involvement of an HLA-G-dependent mechanism in the resistance of trophoblast cells to MHC-unrestricted lysis. However, the syncytiotrophoblast, which lines the placental villi, escapes lysis by maternal peripheral blood NK cells, although it is totally devoid of HLA class I molecule expression (10, 33, 34). In addition, while HLA-G-expressing transfectants of LCL.721.221 cells are protected from lysis by effector cells cultured in the presence of high concentrations of IL-2, i.e., exhibiting a LAK lysis activity (15, 17–21), the HLA-G-expressing cells BeWo, JEG-3, and first trimester trophoblasts are known to be sensitive to LAK lysis (35, 36).

The aim of this work was therefore to clarify the role of HLA class I expression, and particularly of HLA-G and HLA-C on trophoblast cell resistance to NK lysis and on their susceptibility to LAK lysis. This was done by using JAR and JEG-3 cell lines as target cells and unstimulated or IL-2-stimulated PBL as effector cells. Our results show that CC sensitivity to NK or LAK lysis is unrelated to HLA class I expression on these cells. In addition, we confirm that the expression of a classical HLA class I target molecule (HLA-B7) on JAR cells is insufficient to induce lysis by peripheral blood-allospecific polyclonal CTL.

Materials and Methods

Cytokines and mAbs

Human rIL-2 was obtained from Boehringer Mannheim (Mannheim, Germany; sp. act., 2×10^6 U/mg protein). 679.1 Mc7 (mouse IgG1) and U7.27 (mouse IgG2a) control irrelevant mAbs, B1G6 (mouse IgG2a) specific for β_2-microglobulin (β_2m), EB6 (mouse IgG1) specific for CD158a/p58.1, and HP-3B1 (mouse IgG2a) specific for CD94 were purchased from Immunotech (Marseille, France). W6/32 (mouse IgG2a) specific for HLA class I was obtained from Dako (Trappes, France).

Cell culture

HLA-negative wild-type JAR CC (37), HLA-G- and HLA-C-positive JEG-3 CC (38), 5-azacytidine-treated JAR CC (clones 3, 26, 106, 107) (39), JAR cells transfected by the *HLA-B7* gene (JAR-B7) (40), and C1R and JY cells (two EBV-transformed B lymphoblastoid cell lines) were cultured in 75-cm^2 tissue culture flasks (Falcon 3024; Becton Dickinson, Pont-de-Claix, France) or in 25-cm^2 tissue culture flasks (Falcon 3013) at 37°C in 5% CO_2 humidified air. Cells were grown in culture medium: RPMI 1640 (Life Technologies, Cergy Pontoise, France) supplemented with 10% heat-inactivated FCS (Life Technologies), 2 mM glutamine (Flow, Les Ulis, France), 1 mM sodium pyruvate (Flow), 50 U/ml penicillin and 50 μg/ml streptomycin (Flow), and 25 mM sodium bicarbonate (Flow). CC were removed from culture flasks using trypsin-EDTA (1–0.4 g/L; Life Technologies) and resuspended in culture medium at 1×10^6 cells/flask.

Acid treatment

JY and JEG-3 cells were cultured in 25-cm^2 tissue culture flasks to perform immunofluorescence assays or in 96-well plates (Falcon 3072) to perform cytotoxicity assays. Culture medium from flasks and from wells was replaced by 2.5 ml and 50 μl of an acid solution, respectively (0.263 M citric acid-0.123 M Na_2HPO_4 buffer, containing 1% (w/v) BSA, pH 3) at 4°C, as previously described (41). After 4 min, an excess of RPMI 1640 medium containing 20% FCS and 200 mM sodium bicarbonate was added and the cells were washed three times with culture medium. Cells were used immediately in the cytotoxicity assay and were used after an additional culture time (from 0–4 h) in the immunofluorescence assay.

Immunofluorescence assay

CC were removed from the tissue culture flasks by trypsin-EDTA treatment, then resuspended in PBS at 1×10^7 cells/ml. JY cells were adjusted to 1×10^7 cells/ml in PBS. Cells (1×10^6) were then incubated with optimal concentrations of control irrelevant mAb, W6/32 mAb, or B1G6 mAb for 30 min at 4°C. After washing in PBS, cells were incubated for an additional 30 min at 4°C with a FITC-conjugated goat anti-mouse IgG F(ab′)$_2$ (Immunotech). After washing in PBS, pellets were fixed in 0.5 ml of 0.5% paraformaldehyde PBS solution. Cell surface molecule expression was then analyzed by flow cytometry using a FACStar^{Plus} flow cytometer (Becton Dickinson), as previously described (42).

Isolation of PBMC and preparation of lymphoblasts

PBMC were isolated from the heparinized peripheral venous blood of healthy donors by Ficoll-Hypaque (MSL, Eurobio, Les Ulis, France) density-gradient centrifugation. The mononuclear cell-rich interface was collected, washed three times with RPMI 1640, and adjusted to 1×10^6 cells/ml in culture medium. PBMC were cultured in RPMI 1640 supplemented with antibiotics and 10% FCS in the presence of 1 μg/ml of PHA (Sigma, Saint-Quentin-Fallavier, France). After 3 days of culture (5% CO_2, 37°C), cells were recovered, washed twice, and then resuspended at 1×10^8 cells/ml in culture medium.

Preparation of PBL and stimulation of PBL by IL-2

PBMC were incubated twice for 45 min at 37°C, 5% CO_2 in plastic tissue culture flask (Falcon 3024) to remove monocytes. Nonadherent PBL were collected by gentle washing with RPMI 1640 and adjusted to 1×10^6 cells/ml. Less than 0.2% of cells stained with FITC RMO52 mAb (anti-CD14) (Immunotech), as determined by flow cytometry. PBL were cultured for 4 days (5% CO_2, 37°C) in culture medium in the presence of 200 U/ml of IL-2, recovered, washed twice, and resuspended at 1×10^7 cells/ml in culture medium.

Preparation of HLA-B7-specific CTL

PBMC (1×10^6) isolated from HLA-A2-positive and HLA-B7-negative donors were cultured in RPMI 1640 supplemented with antibiotics and 10% human AB serum in the presence (HLA-B7-specific CTL) or absence (control PBMC) of 1×10^6 irradiated (50 Gy) JY cells, which are homozygous for the *HLA-A2* and *HLA-B7* alleles. After 8 days of culture (5% CO_2, 37°C), cells were recovered, washed twice, and then resuspended at 1×10^8 cells/ml in culture medium.

Preparation of target cells

JAR and JEG-3 cells were removed from the tissue culture flasks by trypsin-EDTA treatment, then adjusted to 2×10^5 cells/ml in culture medium. They were then cultured in 96-well plates (2×10^4 cells/well) and labeled overnight with 1 μCi/well of $Na_2{}^{51}CrO_4$ (DuPont-NEN, Les Ulis, France) at 37°C in 5% CO_2, washed three times in culture medium, and incubated for 1 h to allow spontaneous release. JEG-3 cells were then exposed or not to the acid solution, as described above. Finally, chromium-labeled cells were washed twice in culture medium, and 100 μl/well of culture medium was added.

5-Azacytidine-treated JAR clones and JAR-B7 cells (and wild-type JAR cells used as a control) were removed from the tissue culture flasks by trypsin-EDTA treatment, then centrifuged and resuspended at 1×10^8 cells/ml. JY cells, C1R cells, or lymphoblasts were centrifuged and resuspended at 1×10^8 cells/ml. Cells (1×10^7) were labeled for 90 min with 0.1 mCi of $Na_2{}^{51}CrO_4$, then washed three times in culture medium and incubated for 1 h to allow spontaneous release. JY cells were then exposed or not to the acid solution, as described above. Finally, chromium-labeled cells were washed twice in culture medium and resuspended at 2×10^5 cells/ml, and 2×10^4 cells/well were added to 96-well plates.

Cytotoxicity assay

Effector cells were added to each well to achieve a total volume of 200 μl. The E:T ratios were different for each experiment. The plates were incubated for 4 h at 37°C in 5% CO_2, and 25 μl of supernatant was collected from each well and counted in a gamma counter (TopCount, Packard, Rungis, France). Spontaneous ^{51}Cr release (spont. cpm) was measured in wells containing only labeled target cells. Maximum ^{51}Cr-release (max. cpm) was determined by the addition of 100 μl of 1% Triton X-100 (Sigma) to the wells containing labeled target cells. Each assay was set up in triplicate, and the results were expressed as the percentage of specific lysis: (experimental cpm − spont. cpm) × 100/(max. cpm − spont. cpm). In experiments in which mAbs were used to block HLA class I-NKR interactions, PBL were incubated with HP-3B1, EB6, or control mAb, and CC were incubated with W6/32 or control mAb 10 min before adding the effector cells. The mAb was present throughout the cytotoxicity assay. We verified that the addition of a F(ab′)$_2$ goat anti-mouse IgG Ab (Immunotech) to prevent Ab-dependent cell cytotoxicity by interaction of the mAb

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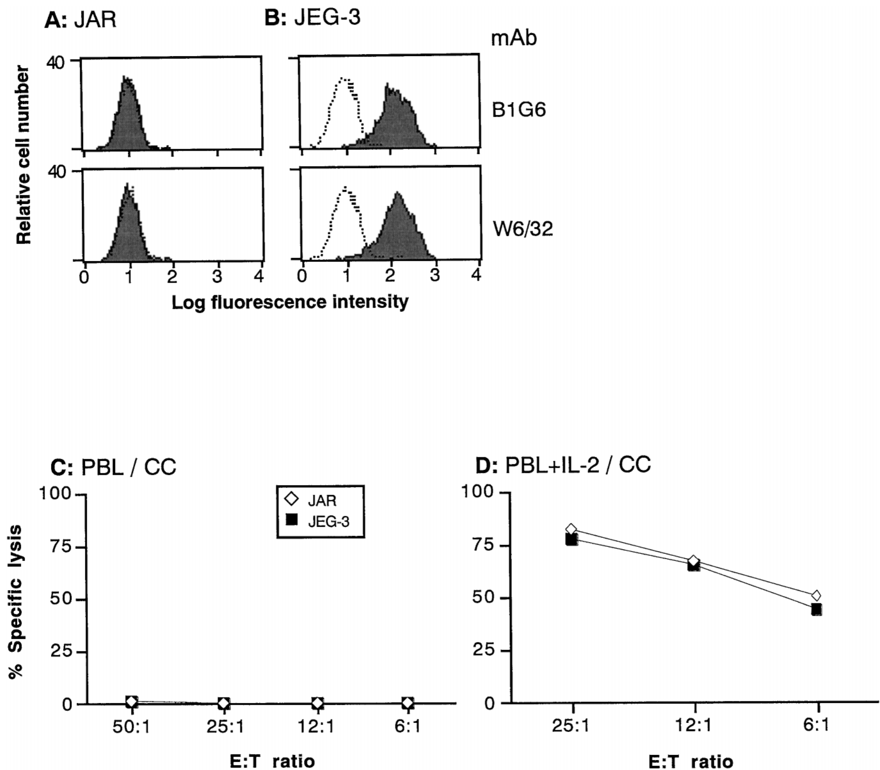

FIGURE 1. Expression of HLA class I molecules and comparison of JAR and JEG-3 cell sensitivity to NK and LAK lysis. JAR (*A*) and JEG-3 (*B*) cells were stained with an isotype irrelevant mAb (dotted histograms), W6/32 mAb (anti-HLA class I), or B1G6 mAb (anti-human β_2m) (shaded histograms), followed by FITC-conjugated goat anti-mouse IgG F(ab′)$_2$, and analyzed by flow cytometry. Fluorescence intensity is displayed on the *x*-axis (in log scale) and cell number on the *y*-axis. ^{51}Cr-labeled JAR (◇) and JEG-3 (■) cells were incubated for 4 h at 37°C with PBL (*C*) or PBL grown for 4 days in the presence of 200 U/ml of IL-2 (*D*). Cytotoxicity against CC is expressed as the mean percentage of specific lysis of each triplicate (one experiment representative of four).

with NK cell FcR did not modify the results of cytotoxicity assays in our experimental conditions.

Results

Comparison of sensitivity of JEG-3 and JAR cells to NK and LAK lysis

JAR and JEG-3 cells were analyzed by flow cytometry after labeling them with the anti-HLA class I W6/32 mAb and with the anti-human-β_2m B1G6 mAb. As expected, fluorescence histograms showed that JEG-3 cells expressed high levels of HLA class I molecules, whereas no expression could be detected on JAR cells (Fig. 1, *A* and *B*).

JAR and JEG-3 CC were then used as targets in the cytotoxicity assay. As shown in Fig. 1*C*, both cells were resistant to freshly isolated unstimulated PBL (NK lysis). By contrast, both cells were lysed efficiently (Fig. 1*D*) by PBL stimulated for 4 days with 200 U/ml of IL-2 (LAK lysis). More importantly, the same effector cells, i.e., IL-2-stimulated PBL isolated from a given donor, killed JAR and JEG-3 cells to the same extent, whatever the E/T ratio.

Effect of down-regulating expression of HLA class I molecules by acid treatment on sensitivity of JEG-3 cells to NK and LAK lysis

JEG-3 cells and JY cells used as controls were or were not treated for 4 min with a citrate-phosphate, pH 3 acid buffer, and then analyzed by flow cytometry after labeling the cells with W6/32 mAb and with B1G6 mAb. Labeling was performed immediately and 4 h after acid treatment to follow the expression of class I molecules during the period corresponding to the duration of the cytotoxicity assay. The fluorescence histograms showed a considerable decrease in the level of expression of HLA class I molecules in both cells immediately after acid treatment (Fig. 2, *A* and *B*). Expression increased substantially after 4 h, although it did not reach the pretreatment level.

Acid-treated and untreated JY and JEG-3 cells were then tested for NK and LAK lysis. Acid treatment did not induce NK lysis of JAR cells used as negative controls (data not shown), but it induced substantial NK lysis of JY cells used as positive controls (Fig. 2*C*). By contrast, it neither induced detectable NK lysis nor increased LAK lysis of JEG-3 cells (Fig. 2, *D* and *E*).

Effect of masking HLA class I molecules, CD94/NKG2 and CD158a/p58.1 with mAbs on sensitivity of JEG-3 cells to NK and LAK lysis

JEG-3 cells were tested for NK and LAK lysis in the presence of saturating concentrations of W6/32 mAb to mask HLA class I molecules on target cells. As expected, the addition of W6/32 to target cells 10 min before the beginning of the cytotoxicity assay induced very strong NK lysis of JY cells used as positive controls (Fig. 3*A*). However, W6/32 mAb treatment neither induced NK lysis nor increased LAK lysis of JEG-3 cells by PBL (Fig. 3, *B* and *C*).

Since CD94/NKG2 is the putative receptor for the HLA-G molecule (19–21), we tested the effect of masking this molecule with the anti-CD94 HP-3B1 mAb on the susceptibility of JEG-3 cells to NK and LAK lysis. We took advantage of the fact that JY cells are homozygous for the *HLA-B7* allele to verify the efficacy of the HP-3B1 mAb treatment in the cytotoxicity assay. Indeed, CD94/NKG2 was initially described as an inhibitory receptor for the HLA-B7 molecule (43). As expected, the addition of HP-3B1 mAb to effector cells 10 min before the beginning of the cytotoxicity assay induced substantial NK lysis of JY cells (Fig. 3*D*). By contrast, the addition of HP-3B1 mAb alone (Fig. 3*E*) or in combination with W6/32 mAb (data not shown) did not induce detectable NK lysis of JEG-3 cells by PBL. Furthermore, LAK lysis of JEG-3 cells was not increased in the presence of HP-3B1 mAb (Fig. 3*F*).

Since JEG-3 cells also express the HLA-C-locus product, HLA-Cw*0401 (44), which is recognized by CD158a/p58.1 NKR (45),

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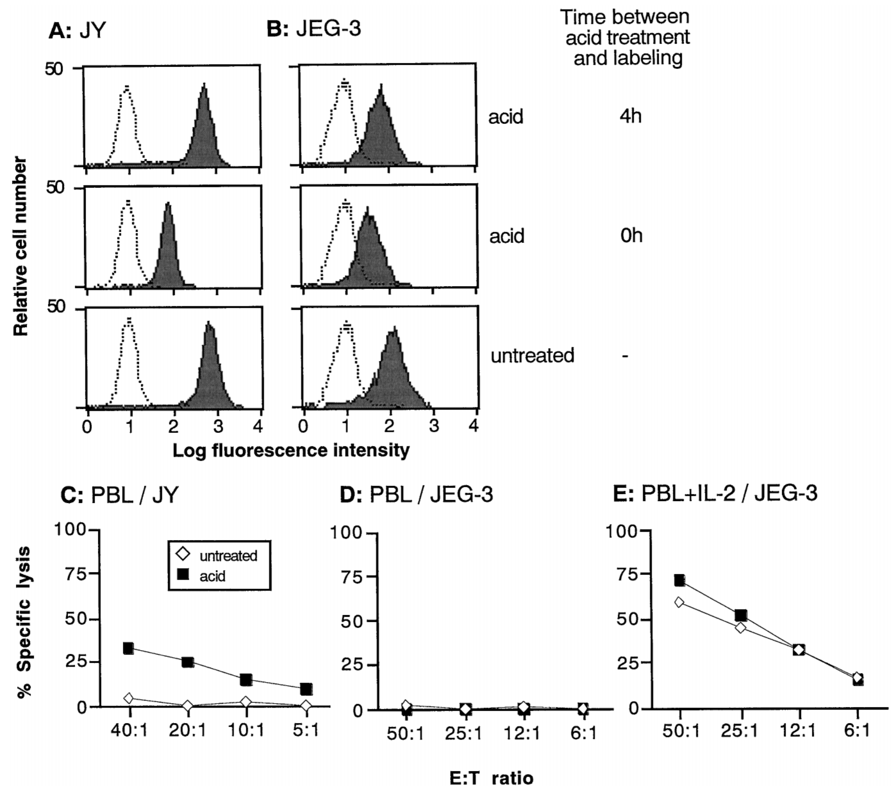

FIGURE 2. Expression of HLA class I molecules and sensitivity to NK and LAK lysis of acid-treated JEG-3 cells. Untreated JY (*A*) and JEG-3 cells (*B*) or cells exposed to a citric acid solution with pH 3 for 4 min were stained with irrelevant mAb (dotted histograms), W6/32 mAb, or B1G6 mAb (shaded histograms), as described in Fig. 1, immediately or 4 h after the acid treatment. Untreated (◇) ^{51}Cr-labeled JY (*C*) and JEG-3 (*D* and *E*) cells or cells exposed for 4 min to a citric acid solution with pH 3 (■) were used immediately as targets in the NK (*C* and *D*) and LAK (*E*) cytotoxicity assays, as described in Fig. 1. Cytotoxicity is expressed as the mean percentage of specific lysis of each triplicate (one experiment representative of five).

we tested the effect of masking the CD158a/p58.1 NKR with the EB6 mAb on the susceptibility of JEG-3 cells to NK and LAK lysis. C1R cells, which also express HLA-Cw*0401 product (45), were used to verify the efficacy of the EB6 mAb treatment in the cytotoxicity assay. As expected, the addition of EB6 mAb to effector cells 10 min before the beginning of the cytotoxicity assay increased NK lysis of C1R cells (Fig. 3*G*). However, the addition of EB6 mAb neither induced NK lysis nor increased LAK lysis of JEG-3 cells by PBL (Fig. 3, *H* and *I*). Finally, NK lysis of JEG-3 cells was still undetectable in the presence of a combination of EB6 and HP-3B1 mAbs (Table I).

Sensitivity to LAK lysis of untreated and 5-azacytidine-treated JAR cells

Four clones of JAR cells (3, 26, 106, and 107), which reexpress HLA class I molecules after treatment with the demethylating agent 5-azacytidine (39), and untreated JAR cells were analyzed by flow cytometry after labeling them with W6/32 mAb and with B1G6 mAb. Fluorescence histograms showed that the expression of HLA class I molecules increased in clones 3, 106, 26, and 107 (Fig. 4, *A* and *B*).

Wild-type JAR cells and the four clones were then used for NK and LAK lysis. All cells were resistant to NK lysis (data not shown), but were lysed in a similar manner by PBL isolated from a given donor and stimulated for 4 days with 200 U/ml of IL-2 (Fig. 4*C*).

Sensitivity of JAR and JAR-B7 cells to CTL lysis

Control PBMC and HLA-B7-specific CTL were obtained respectively by incubating PBMC isolated from HLA-A2-positive and HLA-B7-negative donors over 8 days in the presence or absence of irradiated JY cells, which are homozygous for the *HLA-A2* and *HLA-B7* alleles. The cytotoxicity of control PBMC and HLA-B7-specific CTL was then tested against JY cells, HLA-B7-positive lymphoblasts, wild-type JAR cells, and JAR cells transfected by the *HLA-B7* gene (JAR-B7) (40). As expected, HLA-B7-specific CTL lysed JY cells (Fig. 5*A*) or HLA-B7-positive lymphoblasts (data not shown), whereas control PBMC did not. By contrast, HLA-B7-specific CTL lysed neither JAR cells nor JAR-B7 cells, although the latter cells strongly express HLA-B7 (Fig. 5, *B* and *C*).

Discussion

To investigate the possible role of HLA class I expression on trophoblast cell line susceptibility to MHC-unrestricted cell cytotoxicity, we used HLA-G- and HLA-C-positive JEG-3 and HLA class I-negative JAR cell lines as target cells and unstimulated or IL-2-stimulated PBL as effector cells. CC rather than isolated trophoblast cells were used because of the need to control HLA class I expression on a pure cell population. On the other hand, the use of PBL rather than decidual lymphocytes (DL) was justified by the fact that 1) the majority of NK cells isolated from blood or from decidua express the putative HLA-G receptor CD94/NKG2 (23, 46), 2) both PBL and DL are sensitive to the HLA-G-protective effect (16, 21–23), and 3) PBL are in close contact with the syncytiotrophoblast during pregnancy. Our results demonstrate that trophoblast cell line susceptibility to MHC-unrestricted cell cytotoxicity by PBL is mainly independent of HLA class I expression.

It has been shown previously that IFN-γ treatment of normal trophoblast cells and of JEG-3 cells partially protects these cells from killing by IL-2-stimulated DL (32). This observation has led to the proposal that the protective effect may be due to IFN-γ-induced up-regulation of trophoblast HLA class I molecules (32). However, our results showed that JAR and JEG-3 cells were lysed equally by IL-2-stimulated PBL isolated from a given donor, and that down-regulating HLA class I expression on JEG-3 cells or masking these molecules with mAb, masking the CD94/NKG2 and the CD158a/p58.1 NKRs on effector cells, or inducing self HLA class I molecule expression on JAR cells did not affect LAK lysis of CC. These results strongly support the conclusion that HLA class I expression on CC does not regulate their susceptibility to

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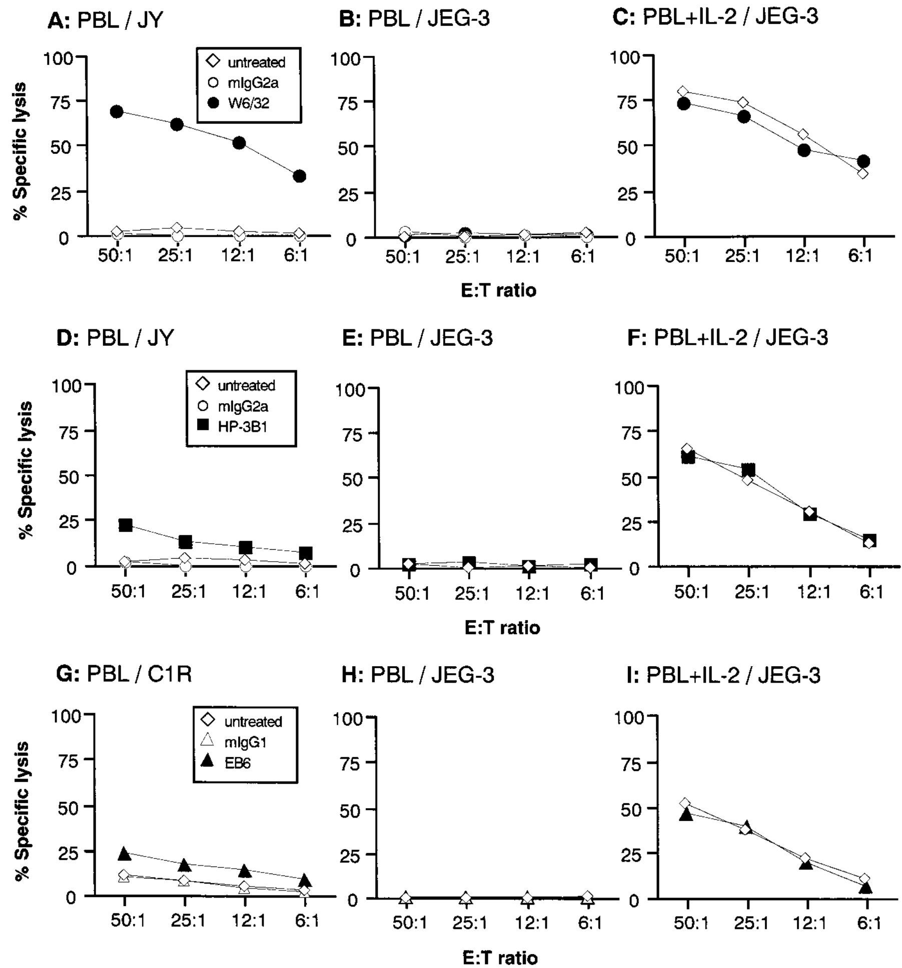

FIGURE 3. Sensitivity to NK and LAK lysis of JEG-3 cells in the presence of anti-HLA class I-, anti-CD94-, and anti-CD158a/p58.1-blocking mAbs. ^{51}Cr-labeled JY (*A* and *D*), JEG-3 cells (*B*, *C*, *E*, *F*, *H*, and *I*), and C1R (*G*) were used as targets in the NK (*A*, *B*, *D*, *E*, *G*, and *H*) and LAK (*C*, *F*, and *I*) cytotoxicity assays, as described in Fig. 1, in the absence (◇) or in the presence of 10 μg/ml of irrevelant mouse IgG2a (*A*, *B*, *D*, and *E*) (○), irrevelant mouse IgG1 (*G* and *H*) (△), W6/32 mAb (*A–C*) (●), HP-3B1 mAb (anti-CD94) (*D–F*) (■), and EB6 mAb (anti-CD158a/p58.1) (*G–I*) (▲). Cytotoxicity is expressed as the mean percentage of specific lysis of each triplicate (one experiment representative of five).

LAK lysis. Finally, this conclusion agrees with the early observation that JEG-3, BeWo, and freshly isolated trophoblast cells are lysed efficiently by IL-2-stimulated DL and PBL (35, 36).

We also showed that the treatment of JEG-3 cells with the W6/32 mAb did not induce detectable NK lysis of these cells by PBL. This result was unexpected because the treatment of first trimester cytotrophoblasts with W6/32 induces partial lysis of these cells by NK cells (23). The discrepancy may be due to the nature of target cells (trophoblast cell lines versus first trimester enriched cytotrophoblast cells). Nevertheless, in agreement with the lack of effect of W6/32 treatment, we found that NK lysis of JEG-3 cells by PBL was still undetectable after down-regulation of HLA class I expression on these cells and when the putative HLA-G receptor CD94/NKG2 was masked with HP-3B1 mAb. These results strongly suggest that JEG-3 cell resistance to NK lysis involves an HLA-G-independent protective effect. Several recent studies have, however, indicated that HLA-E is recognized by the CD94/NKG2A-inhibitory receptor and not HLA-G (28–31). In fact, HLA-G recognition is probably limited to a peptide derived from HLA-G leader sequence that stabilizes the expression of HLA-E. In these conditions, JEG-3 cells probably express HLA-E. However, the fact that the anti-CD94 HP-3B1 mAb, which is known to block HLA-E-mediated protection (28), was ineffective on JEG-3 cells excludes the possibility that HLA-E

Table I. *Sensitivity to NK lysis of JEG-3 cells in the presence of a combination of anti-CD94 and anti-CD158a/p58.1 blocking mAbs*[a]

	E:T Ratio					
	50:1			6:1		
mAb	0	mIgG2a + mIgG1	HP-3B1 + EB6	0	mIgG2a + mIgG1	HP-3B1 + EB6
Specific lysis						
PBL 1	0	0	0	0	2	0
PBL 2	0	4	1	0	4	0
PBL 3	4	4	2	4	4	3

[a] ^{51}Cr-labeled JEG-3 cells were used as targets in the cytotoxicity assays as described in Fig. 1, in the absence or in the presence of 10 μg/ml of irrevelant mouse IgG2a and 10 μg/ml of irrevelant mouse IgG1, or in the presence of 10 μg/ml of HP-3B1 mAb and 10 μg/ml of EB6 mAb. Cytotoxicity is expressed as the mean percentage of specific lysis of each triplicate. Experiments were performed with PBL from three different blood donors.

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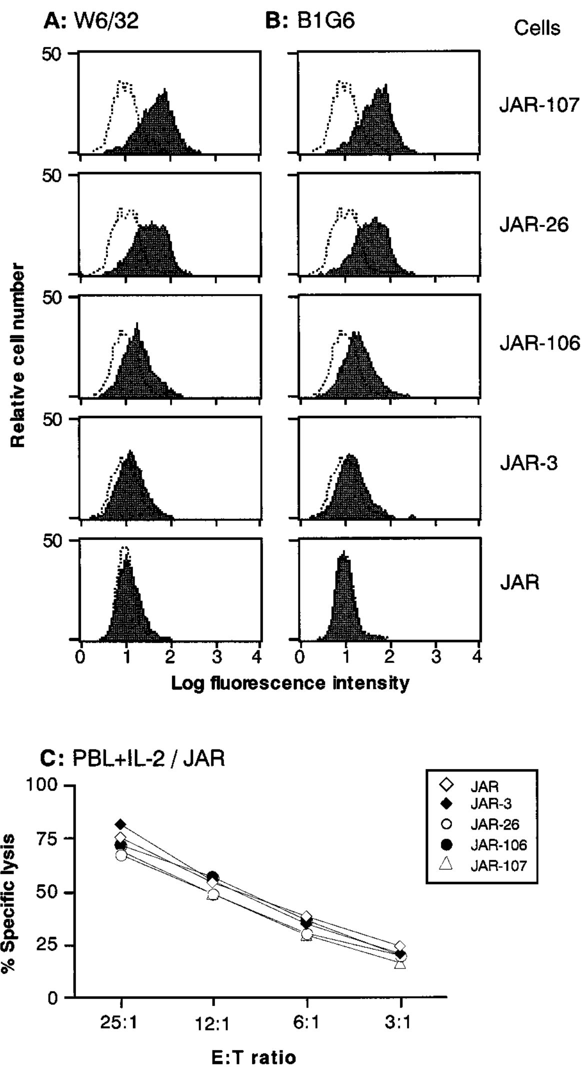

FIGURE 4. Expression of HLA class I molecules and sensitivity to LAK lysis of 5-azacytidine-treated JAR cells. Wild-type JAR cells and 5-azacytidine-treated JAR cells: clones JAR-3, JAR-26, JAR-106, and JAR-107 were stained with irrelevant mAb (dotted histograms), W6/32 mAb (*A*), or B1G6 mAb (*B*) (shaded histograms), as described in Fig. 1. Wild-type JAR cells (◇) and clones JAR-3 (◆), JAR-26 (○), JAR-106 (●), and JAR-107 (△) were ^{51}Cr labeled and used as targets in the LAK cytotoxicity assay (*C*), as described in Fig. 1. Cytotoxicity against CC is expressed as the mean percentage of specific lysis of each triplicate (one experiment representative of three).

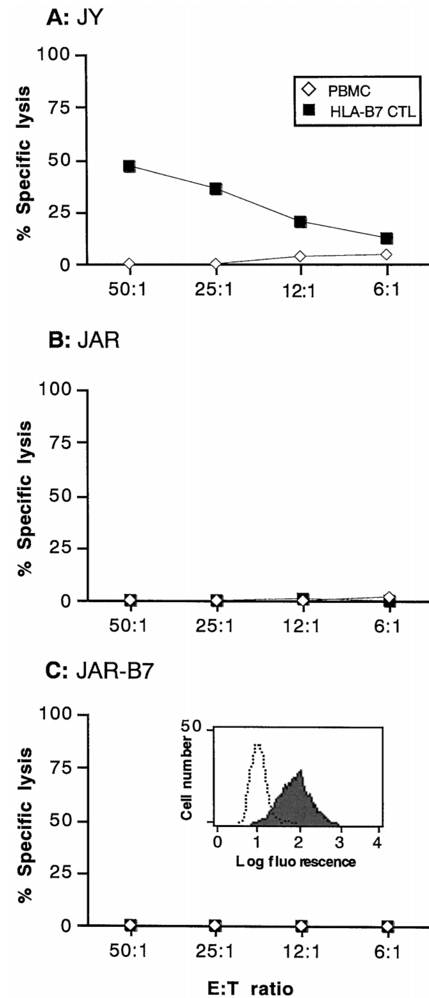

FIGURE 5. Sensitivity to HLA-B7-specific CTL lysis of JAR-B7 cells. JY (*A*), JAR (*B*), and JAR-B7 (*C*) cells were ^{51}Cr labeled and incubated for 4 h at 37°C with PBMC (◇) or HLA-B7-specific CTL (■) obtained by incubating PBMC isolated from HLA-A2-positive and HLA-B7-negative donors without or with irradiated HLA-A2- and HLA-B7-positive JY cells, respectively. Cytotoxicity is expressed as the mean percentage of specific lysis of each triplicate (one experiment representative of three). JAR-B7 cells were stained with irrelevant mAb (dotted histogram) or W6/32 (shaded histogram), as described in Fig. 1.

protects these cells from NK lysis. On the other hand, JEG-3 cells express the product of *HLACw*0401* (44). This molecule could be recognized by CD158a/p58.1 NKR (45), and thus inhibit NK cells. This is unlikely because masking CD158a/p58.1 NKR with EB6 mAb did not induce NK lysis of JEG-3 cells. Taken together, our results thus support the conclusion that JEG-3 cell resistance to NK lysis mainly involves an HLA-G-, -E-, and -C-independent protective effect. Finally, this conclusion agrees with the fact that JAR cells, which did not express detectable HLA class I heavy chain or β_2m, were resistant to NK lysis. Although the protective role exerted by class I molecules toward NK lysis has been largely demonstrated, some contradictions have been reported, especially with models using nonlymphoid targets such as solid mouse tumor lines (47–50) and human lines (51–53). This observation led Litwin et al. to propose that NK cells may possess cytotoxic mechanisms that are both MHC dependent and MHC independent depending on the nature of the target cell (53). Our study demonstrates that CC resistance to NK lysis mainly involves an HLA class I-independent mechanism(s).

The reason that the HLA-G-dependent protective effect that is effective in LCL.721.221 (15–21) and K562 (22, 23) cells is apparently not present in JEG-3 cells is unknown. It can be postulated that the HLA-G-independent protective effect is predominant in JEG-3 cells and thus masks the HLA-G-dependent effect. In this context, it is of note that JAR cells transfected by the *HLA-B7* gene were as resistant to lysis by allospecific polyclonal CTL as untransfected cells. In agreement with previous findings (3), this result suggests that, besides the absence of classical HLA class I molecule expression, CC can make use of additional mechanism(s) to escape CTL lysis (3). It is possible that this additional mechanism(s) is, at least in part, that used by CC to resist NK lysis.

Given that NK cell functions depend on a balance between activatory signals mediated by ill-defined triggering receptors and inhibitory signals mainly mediated by HLA-specific NKR, several nonexclusive mechanisms may be proposed to explain the HLA

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class I-independent protective effect demonstrated in CC. The first, which was postulated earlier for isolated trophoblast cells (36, 54), is that CC lack target structures and are thus not recognized by triggering receptors on NK cells. Nevertheless, the fact that CC were efficiently lysed by IL-2-stimulated effectors, which are essentially cells of the NK lineage, suggests that at least some target structures are expressed on CC. On the other hand, the resistance of classical HLA class I-expressing transfectants of CC to lysis by allospecific CTL (this study, 3) suggests that the expression of an adequate target molecule on CC may be insufficient to induce efficient lysis by effector cells. A second possible mechanism might be that one or several non-MHC ligands expressed on CC could bind to NKR, unrelated to CD94/NKG2 and CD158a/p58.1, and give an inhibitory signal to NK cells. Although such a possibility cannot be excluded, it seems very speculative and has not yet been documented. A further possibility is that the adhesion of effector cells to CC is defective (3, 32). Nevertheless, our previous results (55) strongly suggest that a defect in the adhesion phase is probably not involved. Another plausible mechanism might be that soluble factors released by CC can inhibit NK cell function. Work is in progress in this laboratory to test these putative mechanisms.

In conclusion, it is tempting to speculate that during pregnancy, an HLA class I-independent mechanism of resistance to NK lysis analogous to that observed with CC in this study might be involved at the interface between the syncytiotrophoblast and PBL, in addition to an HLA-G-dependent mechanism that could take place at the interface between extravillous trophoblast and DL. Finally, the fact that the resistance of trophoblast cells is circumvented when polyclonal effector cells are stimulated by IL-2 (this study, 35, 36) implies that cytokine secretion, in particular by Th1 lymphocytes, is perfectly regulated at the feto-maternal interface.

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