Surface expression and function of p75/AIRM-1 or CD33 in acute myeloid leukemias: Engagement of CD33 induces apoptosis of leukemic cells

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p75/AIRM-1 is a recently identified inhibitory receptor expressed by natural killer and myeloid cells displaying high homology with CD33. Crosslinking of p75/AIRM-1 or CD33 has been shown to sharply inhibit the *in vitro* proliferation of both normal myeloid cells and chronic myeloid leukemias. In this study, we analyzed acute myeloid leukemic cells for the expression of p75/AIRM-1. p75/AIRM-1 marked the M5 (11/12) and M4 (2/2) but not the M1, M2, and M3 subtypes according to the French–American–British classification. Cell samples from 12 acute myeloid leukemias were cultured in the presence of granulocyte/macrophage colony-stimulating factor. Addition to these cultures of anti-CD33 antibody resulted in ≈70% inhibition of cell proliferation as assessed by [^{3}H]thymidine uptake or by the recovery of viable cells. Anti-p75/AIRM-1 antibody exerted a strong inhibitory effect only in two cases characterized by a high *in vitro* proliferation rate. After crosslinking of CD33 (but not of p75/AIRM-1), leukemic cells bound Annexin V and displayed changes in their light-scattering properties and nucleosomal DNA fragmentation, thus providing evidence for the occurrence of apoptotic cell death. Remarkably, when anti-CD33 antibody was used in combination with concentrations of etoposide insufficient to induce apoptosis when used alone, a synergistic effect could be detected in the induction of leukemic cell death. These studies provide the rationale for new therapeutic approaches in myeloid leukemias by using both chemotherapy and apoptosis-inducing mAbs.

During the past 10 years, many different receptors with inhibitory function have been discovered. A common feature of these novel molecules is the presence of immune tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tail (1). The majority of these receptors originally were identified in natural killer (NK) cells, in which they mediate functional inhibition on engagement with their ligands or with specific mAbs (2). In some instances, they also are expressed by T lymphocytes or cells belonging to the myeloid lineage (1–5). In this context, we recently have identified and cloned two receptors, termed IRp60 and p75/AIRM-1, that are expressed in both NK and myeloid cells. They are both members of the Ig superfamily and are characterized by different types of Ig-like domains in the extracellular portion. In addition, their cytoplasmic tails contain typical ITIMs (6, 7). Remarkably, p75/AIRM-1 displayed the highest degree of similarity with CD33, a major marker in the process of myeloid cell differentiation and in leukemic cell typing. However, limited information existed on the possible function of CD33. It has been proposed that, because it is a member of the sialoadhesin family, CD33 may be involved in the adhesion of myeloid cells at certain stages of their differentiation (8). However, the presence of typical functional ITIMs suggested that CD33 could function as an inhibitory receptor (9–11). On the basis of these data, we reinvestigated the role of CD33, together with that of p75/AIRM-1 and IRp60, in the *in vitro* proliferation/differentiation of normal myeloid cells as well as of chronic myeloid leukemias (CML). We found that the engagement of both CD33 and of p75/AIRM-1 (but not IRp60) had a strong inhibitory effect on the *in vitro* proliferation/differentiation of CD34$^+$ cell precursors toward the myelomonocytic cell lineage (12). In addition, the engagement of CD33 could efficiently prevent the maturation of dendritic cell from either CD34$^+$ cell precursors or peripheral monocytes (13). Perhaps more importantly, the engagement of p75/AIRM-1 or CD33 could efficiently inhibit the *in vitro* proliferation of CML cells (12).

In the present study, we analyzed a panel of acute myeloid leukemias (AML) belonging to different French–American–British (FAB) subtypes for the surface expression of p75/AIRM-1 in comparison with CD33 and IRp60. p75/AIRM-1 was expressed mostly by M4 and M5 AML. More importantly, we show that anti-CD33 mAb could block the *in vitro* proliferation and could induce apoptosis of all AML analyzed, whereas a variable inhibitory effect was detected on engagement of p75/AIRM-1.

Materials and Methods

mAbs and Reagents. QA79 (IgG1) mAb was obtained by immunizing a 5-week-old BALB/c mouse with the NK clone LM5 (surface phenotype: CD3$^-$, CD16$^+$, CD56$^+$, NKp46$^+$, NKp44$^+$, p140$^+$, CD94/NKG2A$^+$), as described previously (14). The following mAbs were produced in our lab: E59–126 (IgG1 anti-IRp60) (6, 7). mAb MY9 (anti-CD33 IgG2b) was purchased from Coulter. Purified mAb WM53 (IgG1 anti-CD33), sodium azide-free, and the fluorescein isothiocyanate- and phycoerythrin-conjugated antiisotype goat anti-mouse antibodies were purchased from Southern Biotechnology Associates. HPCA II (anti-CD34) IgG1 and leu73 (anti-CD14) IgG2b were purchased from Becton Dickinson. The affinity-purified anti-IgG (H + L) goat anti-mouse serum was purchased from ICN. Notably, the mAb-containing culture supernatants were endotoxin-free. In addition, the WM53 anti-CD33 mAb was supplied as endotoxin-free. Etoposide-VP16 (ETP) was purchased from Sigma.

The culture medium was Iscove's modified Dulbecco's medium supplemented with 1% L-glutamine (GIBCO/BRL), an-

Abbreviations: CML, chronic myeloid leukemia; AML, acute myeloid leukemia; ITIM, immune tyrosine-based inhibitory motifs; FAB, French–American–British; ETP, etoposide; GM-CSF, granulocyte/macrophage colony-stimulating factor; NK, natural killer; PI, propidium iodide.

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tibiotic mixture (5 mg/ml penicillin and 5 mg/ml streptomycin; GIBCO), 10% FCS (Sigma), and human recombinant granulocyte/macrophage colony-stimulating factor (GM-CSF) at the final concentration of 50 ng/ml (PeproTech, Rocky Hill, NJ). Ficoll/Hypaque density gradient was purchased from Sigma.

Isolation and Culture of AML-Derived Myeloid Cells. Both frozen and fresh mononuclear cells derived from samples of peripheral blood of patients affected by AML, collected on informed consent, were analyzed. Frozen samples were collected at the Laboratoire d'Immunologie des Tumeurs, Institute Paoli–Calmettes, Marseille, France. Fresh peripheral blood samples were collected at San Martino Hospital, and mononuclear cells were isolated on Ficoll/Hypaque gradient.

The AML cells were plated at a final concentration of 5×10^5 ml/well in 24 flat-bottomed wells plates and cultured in the presence of GM-CSF at a final concentration of 50 ng/ml. Moreover, cells supplemented with GM-CSF also were plated at a different concentration (1.5 or 5 or 10×10^4 ml/well) in plates coated with goat anti-mouse (10 μg/ml). Cells were cultured with one or another of the following mAbs: QA79 (anti-p75/AIRM-1), MY9 (anti-CD33), or E59–126 (anti-IRp60). In titration experiments, they were used in the form of purified mAb at 1, 2.5, 5, or 10 μg/ml. In most subsequent experiments, the final concentration of 10 μg/ml has been used. QA79 and E59–126 also were used in the form of hybrid supernatant at a final concentration of 5–10 μg/ml. Cells also were cultured in the presence of ETP at different concentrations either alone or in combination with anti-CD33 mAb or anti-p75 mAb.

Proliferation Assay. AML-derived cells cultured as described above were pulsed with 1 μCi/well [^{3}H]thymidine (Amersham Pharmacia) after different culture intervals (2, 3, or 4 days), 16 h before harvesting. Cells were harvested by using a Titertek Cell Harvester 550 (Flow Laboratories). Radioactivity was measured in a scintillation β-counter (Beckman Coulter). All cultures were performed in triplicate.

Indirect Immunofluorescence and Cytofluorimetric Analysis. Before immunofluorescence analysis, fresh or cultured cells harvested at different time intervals were pretreated with human IgG (1 mg/ml) to block Fc receptors. Cells then were analyzed by single immunofluorescence as described previously (15). Briefly, 5×10^4 cells were stained with the corresponding mAb followed by FITC- and phycoerythrin (PE)-conjugated isotype-specific goat anti-mouse antibody second reagent. Samples were analyzed on a flow cytometer (FACSort, Becton Dickinson) equipped with an argon ion laser exciting FITC at 488 nm and PE at 514 nm. Results are expressed as log red fluorescence intensity (arbitrary units) vs. log green fluorescence intensity (arbitrary units) or vs. cell number.

Detection of Cell Death and Apoptosis. Annexin V-FITC (MBL Medical and Biological Laboratories, Naka-ku, Nagaya, Japan) was used to evaluate the number of cells undergoing apoptosis (16). Briefly, aliquots of cells in different culture conditions were collected, washed in PBS, resuspended in the binding buffer (10 mM Hepes/NaOH, pH 7.4/140 mM NaCl/2.5 mM $CaCl_2$), and mixed with 1 μl of Annexin V-FITC/10^5 cells and 1 μl of propidium iodide (PI) at a final concentration of 2 μg/ml. After 5 min of incubation in the dark at room temperature, cells were analyzed by using flow cytometry. The percentage of Annexin V-positive cells was calculated based on the PI-negative cell population. The criteria for cell death measured by flow cytometry were based on the following parameters: changes in light-scattering properties (forward side scattering and side scattering) of dead cells because of cell shrinkage and increased granularity (17, 18). For evaluation of apoptotic DNA fragmentation, a cell death-detection ELISA (Cell Death Detection ELISA^{PLUS}; Boehringer Mannheim) was used according to the manufacturer's instructions. After treatment with different stimuli, 15×10^3 cells were harvested at 18 and 40 h and analyzed. The principle of this test is based on the detection of mono- and oligonucleosomes in the cytoplasmic fractions of cell lysates by using biotinylated anti-histon- and peroxidase-coupled anti-DNA antibodies. The enrichment of mono- and oligonucleosomes released into the cytoplasm is calculated as absorbance of treated cells/absorbance of untreated cells. Enrichment factor was used as a parameter of apoptosis and shown on the *y* axis as mean of duplicates.

Table 1. Surface phenotype of acute myeloid leukemias

AML FAB	CD34	CD33	CD14	P75/AIRM1	IRp60
M1					
GT1	+	+	−	−	+
GT2	+	+	+/−	−	+
GT3	−	+	−	−	+
GT4	ND	+	+	+/−	+
AT34	−	+	−	−	−
AT202	+	+	−	−	−
M2					
GT5	−	+	−	−	+
GT6	−	+	−	−	+/−
GT7	−	+	−	+/−	+/−
AT113	+	−	−	−	+
AT205	+	+	+	−	+
M3					
GT8	+	+	−	−	+
AT258	+	+	−	−	+
AT154	+	+	−	−	+
AT33	−	+	−	−	−
M4					
AT18	+	+	+	+	+
AT21	+	+	+	+	+
M5					
GT9	−	+	+	+	+
GT10	−	+	+	+	+
GT11	+	+	+/−	+	+
2412	−	+	+	+	+
AT246	+	+	+	+	+
AT197	+	+	+	+	+
AT136	+	+	+	+	+
AT79	+	+	+	+	+
AT105	−	+	+	−	+
AT1105	−	+	+/−	+	+
AT5012	−	+	+	+	+
AT201	+	+	+	+	+

ND, not determined; +/−, a dull expression of the molecules.

Results

Surface Expression of p75/AIRM-1 in Human AML Cells. We have shown recently that the p75/AIRM-1 surface antigen is expressed by cells of the myelomonocytic cell lineage in which it appears at a later stage of differentiation than CD33 or IRp60 (12). To study the expression of these markers in AML belonging to different FAB subtypes, we analyzed a panel of 29 AML derived from either fresh or frozen samples (see Table 1). As revealed by cytofluorimetric analysis, 28 of 29 were CD33$^+$; 27 of 29 were IRp60$^+$, and a lower proportion (15/29) was p75/AIRM-1$^+$. Remarkably, p75/AIRM-1 was expressed by virtually all M5 (11/12) or M4 (2/2) AML, whereas only 2 of 11 M1 or M2 AML were weakly positive for this antigen. Moreover, none of the M3 AML analyzed (0/4) expressed p75/AIRM-1. Thus,

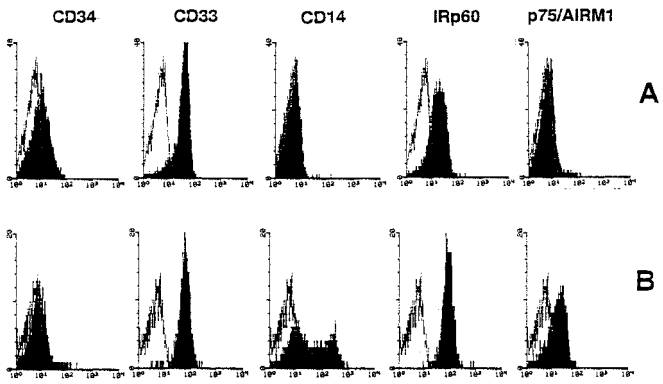

Fig. 1. Surface markers of two representative AML. AML cells were analyzed for the surface expression of the indicated molecules by indirect immunofluorescence and FACS analysis. Results are expressed as log green fluorescence intensity vs. arbitrary units. (*A*) GT1 belonging to the M1 FAB subtype. (*B*) 2412 belonging to the M5 FAB subtype.

p75/AIRM-1 was expressed selectively by monocytic (M5) and myelomonocytic (M4) AML cells, and its expression correlated with that of the monocytic cell marker CD14. Fig. 1 shows the surface expression of p75/AIRM-1 and IRp60 in comparison with informative markers, including CD33, CD34, and CD14, in two representative AML, namely GT1 (M1, Fig. 1*A*) and 2412 (M5, Fig. 1*B*).

Inhibitory Effect of mAb-Mediated Crosslinking of CD33 or p75/AIRM-1 in the *in Vitro* Proliferation of AML Cells. Because we showed previously that the engagement of CD33 or p75/AIRM-1 inhibited the *in vitro* cell proliferation of both myelomonocytic cell precursors and CML cells, we investigated whether a similar effect also could be exerted on AML cell proliferation (12). AML cells belonging to different FAB subtypes (see Table 1), isolated at diagnosis from chemotherapy-free patients, were cultured for 3 days in the presence of GM-CSF to allow a better cell survival. Cells underwent variable degrees of proliferation as assessed by evaluating cell recovery (not shown) or by measuring the uptake of [^{3}H]thymidine at different time intervals. We analyzed whether the addition of anti-CD33 or anti-p75/AIRM-1 or anti-IRp60 mAbs could affect cell proliferation. In titration experiments, anti-CD33- and anti-p75-AIRM1-purified antibodies were added at different concentrations (1, 2.5, 5, or 10 μg/ml). For both antibodies, the maximal inhibitory effect on cell proliferation was observed at a final concentration of 5 or 10 μg/ml; 10 μg/ml has been used in subsequent experiments. Alternatively, we used culture supernatants (5–10 μg/ml). Fig. 2*A* shows data collected from 12 different AML (all expressing the CD33$^+$, p75/AIRM-1$^+$, and IRp60$^+$ surface phenotype) cultured in the presence of different mAbs. Data represent the mean (±SE) of the percentages of cell proliferation detected in cultures containing different mAbs vs. control cultures with no mAb. It is evident that anti-CD33 mAb strongly inhibited cell proliferation. Notably, all 12 AML were inhibited by at least 60%, and, after 4 days, in cultures containing anti-CD33 mAb, virtually no viable cells could be detected (data not shown). On the other hand, no inhibitory effect occurred in cultures containing anti-IRp60 mAb. p75/AIRM-1 exerted a variable effect. Thus, only two of the AML analyzed were inhibited (>50%) by anti-p75/AIRM-1 mAb crosslinking. Fig. 2*B* shows data referred to the 2412 M5-AML (see also Fig. 1*B*), whose proliferation was inhibited not only by anti-CD33 but also by anti-p75/AIRM-1 mAb. Data represent the percentages of cell proliferation (at day 3) detected in cultures containing different mAbs vs. control cultures with no mAb. Note also that in this

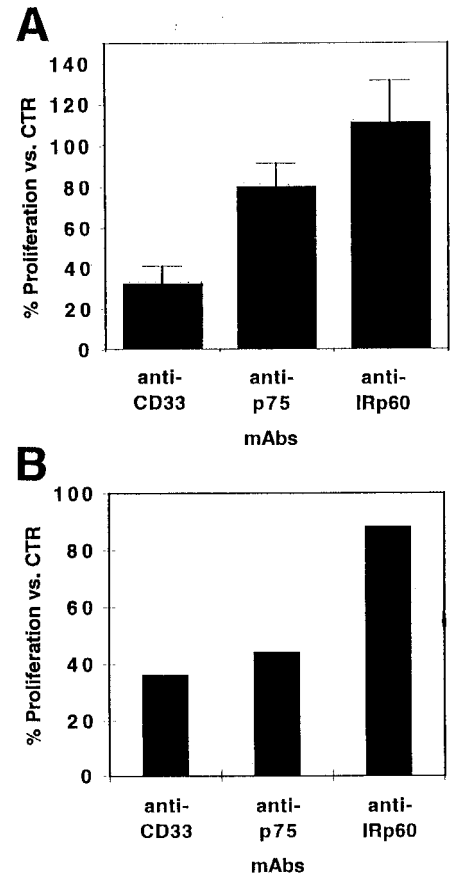

Fig. 2. The engagement of anti-CD33 or anti-p75/AIRM-1 inhibits the *in vitro* proliferation of AML cells. (*A*) AML cells expressing CD33, p75/AIRM1, and IRp60 isolated from 12 patients were cultured for 3 days in the presence of GM-CSF (50 ng/ml). Cell proliferation was assessed by measuring the uptake of [^{3}H]thymidine at the termination of the cultures. Data represent the mean (±SE) of the percentages of cell proliferation detected in cultures containing different mAbs vs. control cultures with no mAb. (*B*) Cell proliferation of the 2412 AML (M5 FAB subtype) cultured for 4 days in the presence of GM-CSF (50 ng/ml). Data represent the percentage of cell proliferation in cultures containing different mAbs vs. control cultures with no mAb. A representative experiment (of four) is shown.

leukemia, which expressed high levels of IRp60 (see Fig. 1), the crosslinking of this molecule had no inhibitory effect.

Anti-CD33 mAb Crosslinking Induces Apoptosis in AML Cells. In view of the marked inhibitory effect of CD33 engagement not only on cell proliferation but also on cell survival, we investigated further the mechanism(s) potentially responsible for cell death. To this end, we analyzed at different time intervals the surface binding of Annexin V, known as an early marker of apoptosis (16). Fig. 3*A* shows a representative experiment of 10 performed. In this experiment, cells derived from the 2412 M5-AML were cultured for 40 h with GM-CSF in the presence of anti-CD33, anti-p75/AIRM-1, anti-IRp60 mAbs, or ETP, a chemotherapeutic drug known to induce apoptosis, and analyzed for surface binding of Annexin V. Analysis was performed on PI-negative viable cells. A substantial fraction of cells treated with anti-CD33 mAb, but not of cells treated with anti-p75/AIRM-1 or anti-IRp60 mAb, bound Annexin V. Moreover, cells treated with ETP (68 μM/ml) displayed a similar proportion of Annexin V-binding cells. A similar pattern of mAb-induced apoptosis also was observed in cell cultures in the absence of GM-CSF; however, under these conditions, control cultures (i.e., containing no mAb) displayed

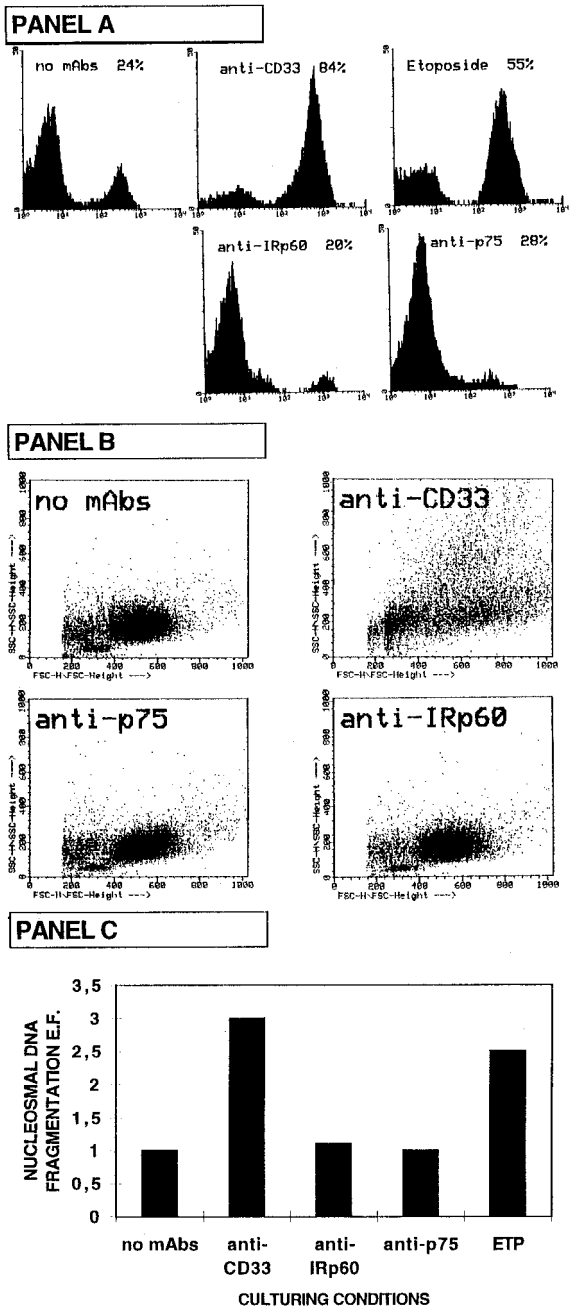

Fig. 3. Anti-CD33 mAb-induced apoptosis in AML cells. (*A*) The surface binding of Annexin V was measured at different time intervals by flow cytometric analysis. The experiment shown is representative of 10 independent experiments by using the 2412 M5 AML. Leukemic cells were cultured for 40 h with GM-CSF in the presence of anti-CD33 or anti-p75/AIRM-1 or anti-IRp60 mAbs or etoposide (68 μM). Samples were stained with anti-Annexin V FITC-conjugated mAb. Analysis was performed on PI-negative, viable cells. (*B*) Flow cytometric analysis to evaluate changes in light-scattering properties [forward side scattering (FSC) and side scattering (SSC)] was performed. It is evident that anti-CD33 mAb induced changes in light-scattering properties that are typical of apoptosis (reflecting cell shrinkage and increased granularity). (*C*) Analysis of DNA fragmentation. Nucleosomal DNA fragmentation has been measured by using an apoptosis-determination ELISA kit (Cell Death Detection ELISA^{plus}; Boehringer Mannheim). The enrichment of mono- and oligonucleosomes released into the cytoplasm has been calculated as absorbance of treated cells/absorbance of untreated cells. The enrichment factor (E.F.) used as a parameter of apoptosis is shown on the *y* axis as mean of duplicates. Data show the enrichment of nucleosomes in the cytoplasm of cells cultured for 40 h in the presence of GM-CSF (50 ng/ml) under different conditions.

a significantly high (30–50%) spontaneous cell death background. In view of this high background, the majority of the experiments aimed at the evaluation of the effect of mAbs to p75/AIRM1 or CD33 were performed in the presence of GM-CSF.

Fig. 3*B* shows changes in light-scattering properties [forward scatter (FSC) and side scatter (SSC)] of cells treated with anti-CD33 mAb. This phenomenon reflects cell shrinkage and increased granularity, typical of apoptosis; no changes could be detected in untreated cells or cells that have been treated with anti-p75/AIRM-1 or anti-IRp60 mAb.

To investigate whether DNA fragmentation had occurred, we measured nucleosomal DNA fragmentation by using an apoptosis-determination ELISA kit (see *Materials and Methods*). Fig. 3*C* shows the enrichment of nucleosomal DNA in the cytoplasm of cells cultured in different conditions [expressed as enrichment factor (E.F.)]. Cells cultured with anti-CD33 mAb displayed an E.F. comparable to that induced by ETP. In contrast, no DNA fragmentation could be observed in the presence of either anti-IRp60 or anti-p75/AIRM-1 mAbs.

Effect of the Combined Use of Anti-CD33 mAb and ETP on the Induction of Apoptosis in AML Cells. Because both anti-CD33 mAb and ETP induced apoptosis in AML cells, we investigated further a possible combined effect when used simultaneously. Fig. 4 shows 2412 AML cells cultured for 20 h in the presence or absence of anti-CD33 mAb and/or ETP. It can be seen that ETP, when used at optimal concentrations (68 μM/ml), induced an efficient binding of Annexin V (≈60%). On the other hand, in the presence of a suboptimal dose of ETP (34 μM/ml), no Annexin V binding over the control (i.e., cultures containing no drug) values could be detected. In the presence of anti-CD33 mAb (10 μg/ml alone), the Annexin V binding reached 40%. In cultures containing both ETP and anti-CD33 mAb, a markedly enhanced effect could be detected. Thus, in the presence of anti-CD33 mAb (10 μg/ml) and optimal concentration (68 μM/ml) of ETP, virtually all cells bound Annexin V, thus showing an additional effect. More importantly, when the anti-CD33 mAb was used with a suboptimal concentration of ETP (34 μM/ml), the proportions of apoptotic cells outnumbered those detected in the presence of ETP alone used at 68 μM/ml, thus showing a synergistic effect. It is also worth noting that the combined use of ETP and anti-CD33 mAb induced, after 20 h, an effect similar to that obtained after 40 h of culture with anti-CD33 mAb alone (see, for comparison, Figs. 3 and 4). In contrast, cultures containing combinations of anti-p75/AIRM1 and ETP did not result in any significant increase of binding of Annexin V over cultures containing ETP alone (not shown).

Discussion

In the present study, we provide evidence that the recently identified surface molecule p75/AIRM-1 may represent a useful marker for AML cell typing. More importantly, we show that the engagement of CD33 and, in some instances, p75/AIRM-1, can induce a substantial inhibition of proliferation and survival of AML cells *in vitro*.

Both p75/AIRM-1 and IRp60 represent two novel inhibitory receptors that were identified originally in human NK cells and subsequently found to be expressed on both normal myeloid cells and on CML cells (6, 7, 12). In view of the inhibitory effect on the proliferation of myeloid cells exerted by p75/AIRM-1 crosslinking, it appeared relevant to analyze its surface expression on AML cells and to study the possible effect on cell proliferation and survival. The analysis of a panel of AML samples belonging to different FAB subtypes revealed that p75/AIRM-1 was expressed selectively by AML belonging to M4 and M5 subtypes but not by M1, M2, and M3 subtypes. Thus, the expression of p75/AIRM-1 largely overlapped with that of

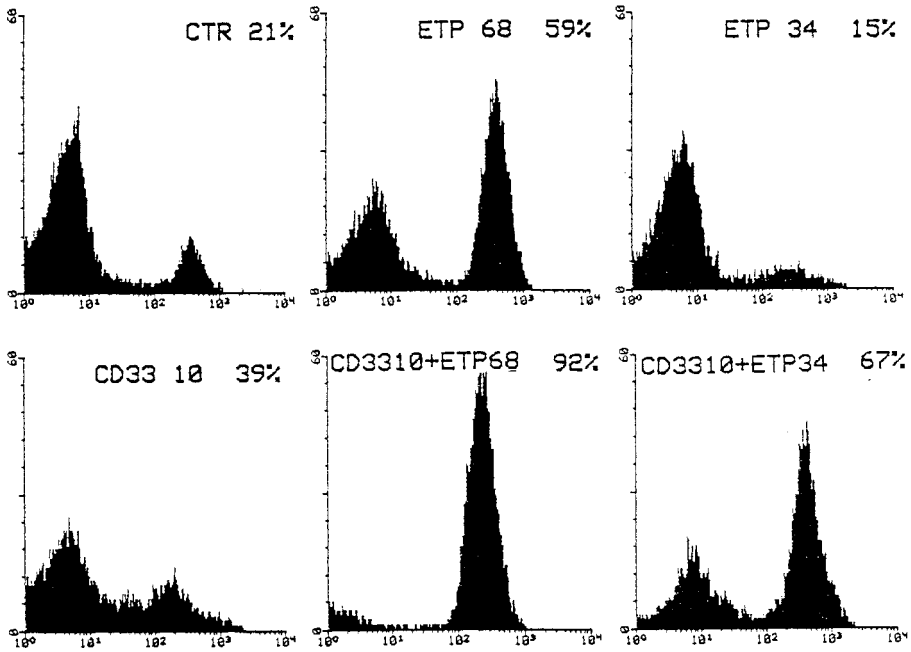

Fig. 4. The combined use of anti-CD33 mAb and etoposide displays a synergistic effect in binding of Annexin V. The surface binding of Annexin V to AML cells was measured after 20 h of culture with GM-CSF in the presence of anti-CD33 mAb (μg/ml), ETP (68 μM/ml), or ETP (34 μM/ml) used either alone or in combination. The experiment shown is representative of four independent experiments. Samples were stained with Annexin V FITC-conjugated mAb. Analysis was performed on PI-negative, viable cells.

CD14, i.e., a typical marker of the monocytic cell lineage. The only exceptions are represented by GT2, GT7, AT205, and AT105 AML (see Table 1). Because p75/AIRM-1 is not expressed by normal T and B lymphocytes or leukemic lymphoid cells, it may represent an additional marker for identifying M4 and M5 AML. In contrast, IRp60 was present in most AML analyzed, and its expression overlapped with that of CD33.

The recent identification and molecular cloning of p75/AIRM-1 revealed a novel member of the sialoadhesin family (also known as Siglec-7) displaying the highest homology with CD33 (Siglec-3), particularly in the IgV and the transmembrane region (6, 19). In addition, both molecules are characterized by the presence, in their cytoplasmic tail, of functional ITIMs that are known to recruit and activate SHP-1 and/or SHP-2 phosphatases (6, 9–11). Because the presence of ITIM is a typical feature of different inhibitory receptors, we reinvestigated the function of CD33, particularly with respect to a possible inhibitory role on hematopoietic cell proliferation and/or differentiation. The demonstration that the engagement of both CD33 and p75/AIRM-1 could strongly inhibit the proliferation of both normal myeloid and CML cells suggested a similar effect on AML cell proliferation (12). Indeed, the engagement of CD33 resulted in a sharp inhibition of cell proliferation and in cell death of all 12 AML cultured in the presence of GM-CSF. In contrast, the engagement of p75/AIRM-1 inhibited cell proliferation only in 2 of 12 cases analyzed. It should be stressed that both of these AML displayed the highest proliferation rate *in vitro*. Although both fresh and frozen samples of AML have been analyzed, it should be stressed that anti-CD33 mAb displayed a similar effect (i.e., induction of apoptosis) in both fresh and frozen AML samples. In addition, the AML 2412 (M5) could be analyzed both as a fresh sample and (for several times) after thawing. This AML also maintained a high proliferation rate after thawing and displayed a similar susceptibility to the effect of both anti-CD33 and anti-p75/AIRM1 mAb.

It is possible that the engagement of p75/AIRM-1 may impair cell cycle progression and that only cells characterized by a high proliferation rate may be susceptible to the p75/AIRM-1-mediated inhibitory effect. Experiments aimed to investigate this issue are in progress. In agreement with previous data both in normal myeloid cells and in CMLs, IRp60 failed to exert any inhibitory effect on cell proliferation/survival. Note that anti-IRp60 mAb was isotype-matched (IgG1) with both anti-p75/AIRM1 and anti-CD33 mAbs.

Different from p75/AIRM-1, the engagement of CD33 appears to activate a process leading to apoptotic cell death. Indeed, treatment of AML cells with anti-CD33 mAb led to binding of Annexin V and to oligonucleosomal DNA fragmentation. Importantly, experiments in which anti-CD33 mAb was used in combination with ETP revealed an additive and even synergistic effect. In particular, a suboptimal dosage of ETP that, when used alone, did not induce binding of Annexin V over the control values displayed a marked effect when used in combination with anti-CD33 mAb. Preliminary experiments showed a similar synergistic effect of anti-CD33 mAb used in combination with cytosine-arabinoside, a widely used drug in the therapy of AML. These findings may have relevant implications for the treatment of AML, as well as for a more efficient *ex vivo* bone marrow purging (20). Notably, even in AML samples in which anti-p75/AIRM-1 mAb induced a strong inhibition of cell proliferation, no apoptotic cell death could be detected. Thus, despite a similar molecular structure and a similar ability to recruit SHP-1, CD33 and p75/AIRM-1 appear to act via different mechanisms, possibly reflecting their coupling to different downstream pathways.

Regarding the possible role of SHP-1 in the negative regulation of cell proliferation and survival, it has been shown recently that the myelomonocytic cell line U937 transfected with an enzymatically inactive mutant (dominant negative) of SHP-1 revealed an increased cell proliferation and a diminished rate of apoptosis as compared with untransfected U937 cells (21). The role of SHP-1 in the regulation of proliferation and function of myeloid cells also is highlighted by the motheaten (me/me) and motheaten-viable (me^v/me^v) murine models. These mice, in

which the activity of the protein tyrosine phosphatases (thus, also including SHP-1) is virtually abrogated, display an enormous myelomonocytic cell expansion (22–24). The molecular mechanism(s) by which SHP-1 may induce apoptotic cell death after CD33 engagement remains to be defined.

Regarding the inhibitory effect on cell proliferation mediated by the engagement of p75/AIRM-1, it is possible that SHP-1 may act by modulating the function of growth promoting receptors. Indeed, SHP-1 has been implicated in the negative regulation of tyrosine kinases, including c-Kit (25, 26), CSF-1 receptor (27), and epidermal growth factor receptor (28), as well as cytokine receptors, including IL-3-R (25) and erythropoietin-R (29, 30). It has been shown that SHP-1 dephosphorylates the Janus kinases associated to the noncatalytic subunits of cytokine receptors (30, 31).

Whatever the mechanisms of CD33- or p75/AIRM-1-mediated inhibition would be, it is relevant that the crosslinking of these molecules leads to a strong antileukemic effect, at least *in vitro*. Along this line, it is noteworthy that unconjugated anti-CD33 mAb has been used for the treatment of AML refractory to chemotherapy. It is possible that this effect on leukemic cells in patients may result not only from their killing via phagocytosis or complement activation or antibody-dependent cell-mediated cytotoxicity, as suggested previously (32–34), but also by a direct effect of anti-CD33 mAb resulting in the induction of apoptosis in leukemic cells.

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1. Long, E. O. (1999) *Annu. Rev. Immunol.* **17,** 875–904.
2. Moretta, A., Bottino, C., Vitale, M., Pende, D., Biassoni, R., Mingari, M. C. & Moretta, L. (1996) *Annu. Rev. Immunol.* **14,** 619–648.
3. Cella, M., Dohring, C., Samaridis, J., Dessing, M., Brockhaus, M., Lanzavecchia, A. & Colonna, M. (1997) *J. Exp. Med*. **185,** 1743–1751.
4. Cosman, D., Fanger, N., Borges, L., Kubin, M., Chin, W., Peterson, L. & Hsu, M.-L. (1997) *Immunity* **7,** 273–282.
5. Mingari, M. C., Vitale, C., Cambiaggi, A., Schiavetti, F., Melioli, G., Ferrini, S. & Poggi, A. (1995) *Int. Immunol.* **7,** 697–703.
6. Falco, M., Biassoni, R., Bottino, C., Vitale, M., Sivori, S., Augugliaro, R., Moretta, L. & Moretta, A. (1999) *J. Exp. Med.* **190,** 793–801.
7. Cantoni, C., Bottino, C., Augugliaro, R., Morelli, L., Marcenaro, E., Castriconi, R., Vitale, M., Pende, D., Sivori, S., Mills, R., *et al.* (1999) *Eur. J. Immunol.* **29,** 3148–3159.
8. Freeman, S. D., Kelm, S., Barber, K. E. & Crocker, P. R. (1995) *Blood* **85,** 2005–2012.
9. Taylor, V. C., Buckley, C. D., Douglas, M., Cody, A. J., Simmons, D. L. & Freeman, S. D. (1999) *J. Biol. Chem.* **274,** 11505–11512.
10. Ulyanova, T., Blasioli, J., Woodford-Thomas, T. A. & Thomas, M. L. (1999) *Eur. J. Immunol.* **29,** 3440–3449.
11. Sujatha, P. P., Taylor, L. S., Stransbury, E. K. & McVicar, D. V. (2000) *Blood* **96,** 483–490.
12. Vitale, C., Romagnani, C., Falco, M., Ponte, M., Vitale, M., Moretta, A., Bacigalupo, A., Moretta, L. & Mingari, M. C. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 15091–15096.
13. Ferlazzo, G., Spaggiari, G. M., Semino, C., Melioli, G. & Moretta, L. (2000) *Eur. J. Immunol.* **30,** 827–833.
14. Moretta, A., Bottino, C., Pende, D., Tripodi, G., Tambussi, G., Viale, O., Orengo, A. M., Barbaresi, M., Merli, A., Ciccone, E., *et al.* (1990) *J. Exp. Med.* **172,** 1589–1598.
15. Mingari, M. C., Poggi, A., Biassoni, R., Bellomo, R., Ciccone, E., Pella, N., Morelli, L., Verdiani, S., Moretta, A. & Moretta, L. (1991) *J. Exp. Med.* **174,** 21–26.
16. Martin, S. J., Reutelinsperger, C. P., McGahon, A. J., Rader, J. A., Van Schie, R. C., LaFace, D. M. & Green, D. R. (1995) *J. Exp. Med.* **182,** 1545–1556.
17. Cotter, T. G., Lennon, S. V., Glynn, J. M. & Green, D. R. (1992) *Cancer Res.* **52,** 997–1005.
18. McGahon, A., Bissonette, R. P., Schimitt, M., Cotter, K., Green, D. R. & Cotter, T. G. (1994) *Blood* **83,** 1179–1187.
19. Nicoll, G., Ni, J., Liu, D., Klenerman, P., Munday, J., Dubock, S., Mattei, M.-G. & Crocker, P. R. (1999) *J. Biol. Chem.* **274,** 34089–34095.
20. Stiff, P. J., Schulz, W. C., Bishop, M. & Marks, L. (1991) *Blood* **77,** 355–362.
21. Dong, K., Siminovitch, K. A., Fialkow, L., Fukushima, T. & Downey, G. P. (1999) *J. Immunol.* **162,** 3220–3230.
22. Tsui, H. W., Siminovitch, K. A., de Souza, L. & Tsui, F. W. (1993) *Nat. Genet.* **4,** 124–129.
23. Kozlowski, M., Mlinaric-Rascan, I., Feng, G. S., Shen, R., Pawson, T. & Siminovitch, K. A. (1993) *J. Exp. Med.* **178,** 2157–2163.
24. Bignon, J. S. & Siminovitch, K. A. (1994) *Clin. Immunol. Immunopathol.* **73,** 168–179.
25. Yi, T. & Ihle, J. (1993) *Mol. Cell. Biol.* **13,** 3350–3358.
26. Paulson, R. F., Vesely, S., Siminovitch, K. A. & Bernstain, A. (1996) *Nat. Genet.* **13,** 309–315.
27. Chen, H. E., Chang, S., Trub, T. & Neel, B. (1996) *J. Mol. Cell. Biol.* **16,** 3685–3697.
28. Tomic, S., Greise, R. U., Lammers, R., Kharitonenkov, A., Imyanitov, E., Ylrich, A. & Bohmer, F. (1995) *J. Biol. Chem.* **270,** 21277–21284.
29. David, M., Chen, H. E., Goelz, S., Larner, A. C. & Neel, B. G. (1995) *Mol. Cell. Biol.* **15,** 7050–7058.
30. Klingmuller, U., Lorenz, U., Cantley, L. C., Neel, B. G. & Lodish, H. F. (1995) *Cell* **80,** 729–738.
31. Klinmuller, U. (1997) *Eur. J. Biochem.* **249,** 637–647.
32. Jurcic, J. G., Caron, P. C., De Blasio, A., Dumont, L., Warrell, R. P. & Scheinberg, D. A. (1995) *Blood* **86,** (Suppl.), 517a.
33. Xu, Y. & Scheinberg, D. A. (1995) *Clin. Cancer Res.* **1,** 1179–1187.
34. Caron, P. C., Dumont, L. & Scheinberg, D. A. (1998) *Clin. Cancer Res.* **4,** 1421–1428.