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Anti-myeloma activity of natural killer lymphocytes

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Summary. Natural killer (NK) cells are assumed to contribute to a graft-versus-leukaemia effect. *In vitro* experiments have shown that many leukaemic cells are NK-cell sensitive. Nevertheless, no data concerning the influence of purified NK cells on malignant myeloma (MM) cells exist. We co-incubated NK cells with three different MM cell lines and fresh bone marrow samples of nine MM patients. The proportion of vital MM cells was determined before and after co-cultivation by a flow-cytometry-based assay. All MM cells tested, with the exception of one cell line (NCI H929), were susceptible to a NK-cell attack even without exogenous interleukin 2 (IL-2). The mean killing of the native MM samples was 23·1 ± 5·4% and 34·5 ± 6·5% at 10:1 and 20:1 effector:target ratio respectively. This corresponded to about 2/3 of those values obtained with the highly sensitive line K562. In contrast, CD34-positive haematopoietic stem cells as well as peripheral mononuclear cells were completely resistant under similar experimental conditions (1·3% killing). To elucidate the underlying triggering mechanisms, we measured human leucocyte antigen (HLA)-class I expression of the MM cells. No evidence for HLA loss, which could have explained the NK-cell recognition if it occurred, was demonstrated. These findings may contribute to the understanding of *in vivo* NK-cell activation and encourage clinical applications of NK cells for MM patients.

Keywords: natural killer cells, multiple myeloma, cellular cytotoxicity, HLA expression, GVL effect.

Multiple myeloma (MM) is a B-cell neoplasia characterized by a clonal expansion of malignant plasma cells in the bone marrow. Clinical observations have demonstrated that the immune system has a potential role in the regulation of MM (Tricot *et al*, 1996; Verdonck *et al*, 1996). This raises the question as to which type of immune cells can mediate such effects. Apart from T cells, which may possibly recognize the clonal immunoglobulin (Ig) and thus exert a graft-versus-myeloma effect (Titzer *et al*, 2000), NK cells might be important in this context. *In vivo*, they have a strong antitumour effect without triggering a graft-versus-host disease in a mouse leukaemia model (Zeis *et al*, 1995). *In vitro*, many tumour and leukaemia cell lines are destroyed by NK cells (Trinchieri, 1989; Cooley *et al*, 1999). Native leukaemia blasts are also sensitive to NK cells to a varying degree (Lotzova *et al*, 1987a); a very recent paper showed a strong antileukaemic effect of patient-derived *ex vivo*-expanded NK cells (Torelli *et al*, 2002). The particular situation in MM cells has never been examined until now.

The primary goal of our experiments was to determine the sensitivity of MM cells in continuous culture as well as fresh samples from bone marrow aspirates. For purposes of comparison, we analysed CD34-positive haematopoietic stem cells. The latter were chosen because an NK-cell activity against haematopoietic stem cells has been observed in allogenic and autologous situations (Cudkowicz & Bennett, 1971; Miller *et al*, 1991), and such activity could limit the clinical use of NK cells. In order to elucidate the underlying triggering mechanisms, we examined whether an observed activation is induced by a lack of human leucocyte antigen (HLA)-expression on the surface of the cells.

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MATERIALS AND METHODS

Cell lines. The following cell lines were obtained from the European Tissue Type Collection, Salisbury, UK: U266B1 [derived from a patient with an IgG myeloma (Nilsson, 1971)], RPMI-8226 (derived from a patient with IgG myeloma; Matsuoka *et al*, 1967), NCI H929 (derived from a patient with IgA plasmocytoma; Gazdar *et al*, 1986), K562, Daudi and HL60. All cell lines were routinely checked for mycoplasma contamination by polymerase chain reaction (PCR) (Stratagene, La Jolla, USA).

CD34-positive stem cells. CD34-positive cells with a purity greater than 90% were derived from apheresis products from granulocyte colony stimulating factor (G-CSF)-mobilized patients by positive selection on a magnetic-activated cell sorting (MACS) column (VS column; stem

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cell separation kit; Miltenyi, Bergisch-Gladbach, Germany), according to the manufacturers' recommendations.

Bone marrow specimens. Bone marrow aspirates were collected in heparinized containers, filtered through a 50 μm mesh and separated from erythrocytes by a Ficoll density gradient. After two wash steps, the cells were suspended in complete medium [Iscove's-modified Dulbecco's medium (IMDM) supplemented with 8% fetal calf serum (FCS) (Gibco, Karlsruhe, Germany), l-glutamine and antibiotics].

NK effector cells. Leucocytes from whole blood donations given by volunteer blood donors were separated from erythrocytes by a ficoll density gradient. After ficoll separation, we obtained 5×10^8 peripheral blood mononuclear cells (PBMC) from one donation which resulted in 5×10^7 NK cells, on average. Afterwards, NK cells were purified by depletion of all CD3/14/19-positive cells by immunomagnetic separation (MACS CS column, NK cell separation kit; Miltenyi). The contamination with T cells and monocytes was below 2%, and B cells ranged below 1%, as demonstrated by flow cytometric analysis (data not shown).

Cytotoxicity assay. The cytotoxicity was analysed flow cytometrically as described previously (Slezak & Horan, 1989; Höppner *et al*, 2002). The standard cytotoxicity assays (^{51}Cr-release, etc.) were not applicable, as cell killing in subpopulations of heterogeneous bone marrow was to be analysed (see below). The NK effector cells were membrane labelled with the fluorescence dye DIOC18 (Sigma, Deisenhofen, Germany). After incubating effector cells with target cells for the indicated periods of time, 1 μg/ml of DNA-stain 7-AAD (7-Amino-Actinomycin D) was added to detect dead cells. The membrane-stained effector cells were discriminated from the target cells in a forward scatter versus DIOC18 (FL-1) blot. In order to obtain 'percentage' kill values, the proportion of 7-AAD-positive cells was calculated in the target cell fraction. The percentage of target cells dying spontaneously (i.e. in the absence of effector cells, typically below 2%) was subtracted from the values obtained.

In primary bone marrow cultures, analysis was somewhat more complicated because only the MM or stem cell compartment of the bone marrow should be included, thus an additional phenotyping of target cells of interest had to be performed by staining with monoclonal antibodies (mAbs) against CD138 (MM marker, clone BB-4) or CD34 (stem-cell marker, clone HPCA-2) respectively. The membrane-stained effector cells were initially excluded from the analyses as described above. Within the non-effector cells, the proportion of 7-AAD-negative (vital) antigen-positive (CD138 or CD34 respectively) cells was determined. All fluorochrome-conjugated antibodies used were purchased from Pharmingen, Germany. All experiments were performed at three effector:target (E:T) ratios (5:1, 10:1 and 20:1); target and effector cells were diluted to 1×10^6 per ml and adjusted to the indicated E:T ratios (final volume 1 ml). Incubation was performed in 24-well flat bottom plates. All experiments with cell lines were performed in triplicates using three different NK-cell donors; concerning native myeloma cells, nine bone marrow samples were analysed.

Cell-surface HLA measurement. We measured semiquantitatively the β-microglobulin level on the cell surface as an overall indicator of HLA expression. Cells were incubated with a fluorescein isothiocyanate (FITC)-conjugated β2-microglobulin antibody (Coulter Electronics, Krefeld, Germany) for 10 min and analysed on the cytometer after a wash step to remove unbound antibody. In the bone marrow sample, a second antibody [BB-4, phycoerythrin (PE)-conjugated] was used to restrict the analysis on the plasmocytoma cells. In order to obtain semiquantitative antigen density values, a mixture of artificial beads with four calibrated numbers of antibody binding sites (Quantum simply cellular beads; Sigma, Steinheim, Germany) was used. These beads were used before and after every series of measurements. Based on the calibration curves obtained, the antigen densities of the plasmocytoma cells were calculated by a software programme (QUICKCAL PROGRAM; Sigma, Steinheim, Germany).

Serological HLA typing. A commercial kit containing 174 HLA-class I-specific test sera was used, based upon the complement-dependent cytotoxicity (National Institutes of Health) assay.

Genomic HLA typing. A commercial PCR sequence-specific primers (PCR-SSP) kit (HLA Class I low resolution kit; One lambda, Meerbusch-Osterath, Germany) was used according to the manufacturers' recommendations.

RESULTS

NK cell cytotoxicity on myeloma cells (Fig 1/Fig 2)

Our initial experiments were performed on three cell lines (U266 B1, RPMI 8226 and NCI H929), which were derived from MM patients and expressed typical MM markers (CD

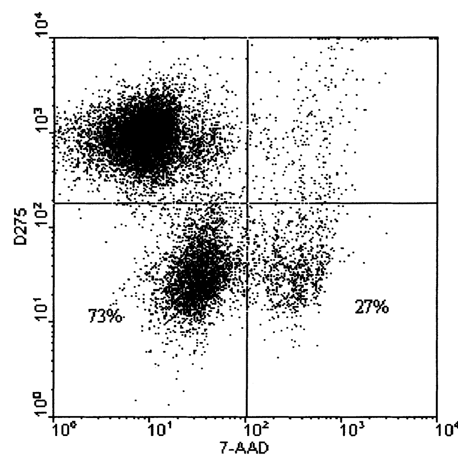

Fig 1. Flow-cytometric analysis of the killing of U266 MM cells after 4 h of co-incubation with unstimulated NK cells (E:T ratio 10:1). The NK effector cells are membrane stained with DIOC18 and appear, therefore, in the upper quadrants. Vital MM cells do not stain with 7-AAD and appear in the bottom left; dead MM cells are in the bottom right quadrant (one representative example).

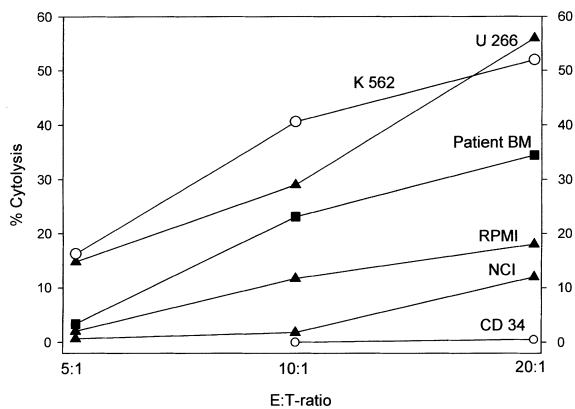

Fig 2. Percentage of killed cells after co-incubation with NK cells at different E:T ratios (mean of $n = 3$, patient BM: $n = 9$ independent experiments). The spontaneous cytolysis (typical below 2%) was subtracted from the values obtained. The killing level of U266 B1 MM and native MM cells (patient BM) almost reached the levels of the highly sensitive K562 cell line; the other MM lines (NCI and RPMI) were also constantly killed, albeit to a lower degree. CD34-positive stem cells, which were analysed simultaneously in the bone marrow samples, were not killed.

38^{++}, CD 138^{++}, CD 56^{+}). In all lines, intracytoplasmatic monoclonal Ig was demonstrated (data not shown). After incubation with NK cells for 4 h at an E:T ratio of 10:1, a substantial cytolysis was detected in U266 cells (29 ± 8·6%, mean ± SE). For comparison, the highly sensitive NK-target K562 cell line demonstrated a cytolysis of 40·6 ± 8·1%. NCI H929 did not show relevant cytolysis at this E:T ratio (1·8 ± 0·75%), RPMI ranged between those values (12 ± 4·6%). These results were confirmed by microscopic evaluation of cytospin preparations, which revealed effector-target aggregates and necrotic plasmocytoma cell cadaver.

However, the results obtained with immortal cell lines may not represent the features of native cells. Thus, in the following set of experiments, we analysed fresh bone marrow samples from patients containing between 2% and 30% myeloma cells. The samples were analysed *in toto*, i.e. no separation of myeloma and non-myeloma cells was performed before the analysis. Therefore, manipulations which might lead to artificial results were essentially avoided in this *in vivo* like model. The grade of cytolysis of different cell populations was checked by simultaneous monitoring of effector cell-specific membrane dye, subpopulation-specific surface marker and DNA stain (7-AAD), the latter identifying dead cells. The specific cytolysis of myeloma cells was 23·1 ± 5·4% and 34·5 ± 6·5% at 10:1 and 20:1 E:T ratios, respectively, corresponding to 57·5% and 66·3% of the values obtained from K562. CD34-positive haematopoietic stem cells that were also analysed were not decreased in number or vitality after NK-cell co-incubation. This was the case concerning both the CD34-positive subpopulation in the analysed bone marrow samples and the purified homogenous stem cell samples. Allogenic fresh lymphocytes were not killed to a comparable degree in any of these experiments either (data not shown).

HLA-class I expression of myeloma cells (Fig 3, Table I)

The above-mentioned experiments posed the question as to which factors were responsible for the specific action against malignant myeloma cells in contrast to the normal cells examined. On the basis of current knowledge of NK-cell function, a lack of HLA-class I expression on the surface of the myeloma cells might be a reasonable explanation. In order to prove or reject this hypothesis, we analysed the total HLA expression of the cells by staining with the β2-microglobulin-specific antibody, W32. β2-microglobulin is a constitutive component of HLA class I, thus in most situations surface β2-microglobulin correlates with class I expression. As shown in Fig 3, all cell lines and one

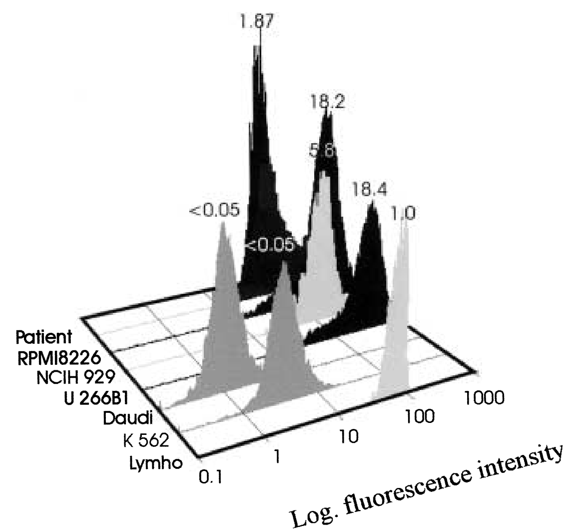

Fig 3. HLA expression of the MM cell lines compared with cells with reported HLA deficiency (K562 and Daudi) and normal HLA expression (lymphocytes, lymho), as analysed by flow-cytometric surface β2-microglobulin determination. All MM cell lines and one patient sample that could be included were strongly positive. The number above the peaks represents the relative antigen density with reference to lymphocytes.

Table I. HLA-class I typing of the MM cells.

Locus	HLA-A		HLA-B		HLA-C	
Level of typing	Geno	Sero	Geno	Sero	Geno	Sero
K562		– ; –		– ; –		– ; –
NCI H929	3; 24	3; 24	7; 18	7; 18	7; –	7; –
U 266 B1	2; 3	2; 3	7; 60	7; 60	7; –	7; –
RPMI 8226	30; 28	30; 28	39; 15	39; 15	2; 3	2; 3

A standard NIH cytotoxicity test was performed to analyse whether a partial loss of a particular HLA loci, especially HLA-C, might be responsible for the observed NK-cell sensitivity. As shown in the table, all cell lines expressed at least one allele of the analysed loci -A, -B and -C; genotyping confirmed the results obtained by serology.

examined patient sample had very high levels of HLA. The β2-microglobulin-negative line K562 and the TAP-deficient line Daudi did not stain with the antibody as expected. Therefore, the reported sensitivity of K562 and Daudi may be due to the HLA deficiency, but the sensitivity of the plasmocytoma cells as demonstrated here is not. Whether or not the HLA-C epitopes on the myeloma cells were matching the respective epitopes in the NK-cell donors was irrelevant in our experiments. To rule out the possibility that a partial loss of single HLA molecules may trigger the NK cells, we performed a serotyping for specific HLA molecules of the A- B- and C-locus on the cells (Table I). In addition, genotyping by PCR of the HLA genes confirmed the results obtained by serotyping. In summary, all of these cells had clearly detectable antigens on all three loci; the antigens found serologically were confirmed by analysis of the genetic background. HLA-C, in particular, which is of special relevance for deactivation of NK cells, was clearly detectable on all plasmocytoma cells. On the β2-microglobulin-negative cell line K562, no HLA antigens were found as expected.

DISCUSSION

NK cells are known to kill a broad range of leukaemic cells (Lotzova *et al*, 1987b; Uharek *et al*, 1996; Torelli *et al*, 2002), therefore, they have been considered as potential mediators of the clinically observable graft-versus-leukaemia effect, and have been transfused adoptively as a therapeutic modality in solid tumours and leukaemia (Kolb *et al*, 1995; Frohn *et al*, 2000). A graft-versus-myeloma effect, induced by unknown cells has been only rarely examined but was clearly established at least in three cases (Tricot *et al*, 1996). These clinical observations are in accordance with experiments demonstrating an anti-myeloma activity in IL-2 stimulated lymphocytes (Shimazaki *et al*, 1988; Gottlieb *et al*, 1990). The results presented here make it reasonable to assume that NK cells essentially contribute to these phenomena. In contrast to the MM cells, haematopoietic stem cells, which have been assumed *a priori* to be of potential sensitivity to NK cells as concluded from basic experiments demonstrating a particular NK-cell influence on bone marrow stem cells (Cudkowicz & Bennett, 1971; Miller *et al*, 1991), are not killed directly by allogenous NK cells.

In a further set of experiments, we attempted to elucidate the underlying trigger mechanisms that are responsible for the observed specific action against the myeloma cells. After the detection of HLA-recognizing killer-cell inhibitory receptors (Long *et al*, 1996), the 'search for missing self', i.e. search for lack of HLA, is believed to be a major trigger mechanism for NK cells (Gumperz & Parham, 1995). However, it is not yet clear whether this mechanism is of predominant importance also in situations apart from the initial experimental settings (Sentman *et al*, 1994; Frohn *et al*, 1997; Multhoff *et al*, 1997). We wondered if this mechanism might explain the NK-cell behaviour in our situation, i.e. myeloma cells might have partially or completely lost their set of HLA molecules, thereby triggering the NK cells. Using an antibody that reacts with all HLA-class I molecules, we did not demonstrate any correlation between HLA expression and NK-cell killing; the examined plasmocytoma cells were strongly class I positive. These observations are in accordance with previously published results, which also demonstrated a unimpaired antigen-processing machinery in several plasmocytoma cell lines (Crucian *et al*, 1997).

We further examined whether an allele-specific loss of particular HLA-class I molecules was demonstrable, as this might also hypothetically explain the NK cell sensitivity, and is observed in some tumours (Garrido *et al*, 1993). In this context, HLA-C is of special relevance because the HLA-C-specific killer-cell inhibitory receptors are likely to predominate the others (Young *et al*, 1998), especially HLA-B-specific ones. We serologically detected HLA molecules of all three classical class I loci (-A, -B, -C), where the reactions with the HLA-C-specific sera were of particular clarity. At that point, the question arises as to which alternative pathways were used to enable the recognition of the plasmocytoma cells. One possible family of candidate molecules which might trigger an MM-specific NK-cell response are the heat shock proteins (Multhoff *et al*, 1997). In conclusion, these or other molecules might activate the NK cells by triggering the finely balanced set of activating receptors that are expressed on NK cells (Barao & Ascensao, 1998).

In summary, NK cells recognize and kill myeloma cells to a varying degree by underlying trigger mechanisms that need to be elucidated in the future. These results illustrate that the use of NK cells may offer an opportunity for the development of novel therapeutic regimens in the treatment of patients with malignant myeloma.

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