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Killer Cell Activity of Human Monoblastic Leukemia Cells as Detected With a Monocyte-Specific Target Cell

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Peripheral blood leukemia cells from patients with acute monoblastic leukemia (AMoL) were tested for killer cell activity against target cells that detected natural killer cell-mediated or monocyte-mediated spontaneous cytotoxicity. The fibrosarcoma cell line Wehi 164, pretreated with actinomycin D to induce susceptibility to lysis, specifically detects the activity of unstimulated human monocytes. In four of six cases of AMoL, high killer cell activity could be measured against this target. In three of these four cases, the killer cell activity could be assigned exclusively to the leukemic clone, based on the high leukocyte counts and the resultant dilution of normal cells, as evidenced by marker and by functional analysis. While leukemic cells with killer cell activity against Wehi 164 contained 34% to 45% cells that were positive for binding of the 63D3 monoclonal antibody, the two leukemic samples without killer cell activity contained only 1% and 12% 63D3-positive cells. Cell sorting of 63D3-positive and -negative cells from two leukemias with killer cell activity demonstrated that the killer cell activity was restricted to the 63D3-positive fraction of AMoL cells. These data demonstrate that monoblastic leukemia cells can be potent killer cells and that killing activity is linked to the 63D3-defined cell surface molecule.

CYTOTOXIC CELLS compose a major effector mechanism of the immune system. Because there is heterogeneity within the different types of killer cells, analysis of such cells is greatly facilitated by studies at the clonal level. This can be done by either cloning and expanding normal cells, using lymphokines such as interleukin 2 (IL-2),[1] or by studying malignant transformants of killer cells, either as fresh leukemic samples or as a cell line. Several reports have described killer cell activity mediated by leukemia cells and directed against the natural killer (NK) cell-sensitive target, K562.[2-4] The detailed analysis of such killer cells using monoclonal antibodies (MoAbs) provides insight into the role of the MoAb-defined determinants in normal cells; in one report, for instance,[3] it was demonstrated that such clonal NK cells can carry both T cell-associated and monocyte-associated antigens, a finding confirmed with heterogenous normal NK cells.[5]

Clonal representatives of human killer monocytes are fresh leukemia cells from patients with acute monoblastic leukemia (AMoL)[4,6,7] and cell lines that can be induced to differentiate and develop antibody-dependent cellular cytotoxicity (ADCC).[8,9] Some of the panel of recently developed MoAbs against monocytes[10-15] were used in studies with induced cell lines,[9] and induction of several antigens was observed, together with induction of ADCC activity.

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To our knowledge, fresh AMoL cells have not been analyzed yet for killer cell activity in conjunction with antimonocyte MoAbs. We therefore undertook a study in which we phenotyped the leukemic cells of patients with monocytic leukemia with several MoAbs, including the monocyte-specific 63D3,[10] and compared these data with the killer cell activity. The assay used detects the killing activity of unstimulated human monocytes against a fibrosarcoma cell line that is rendered susceptible to lysis by pretreatment with actinomycin D.[16] This analysis revealed high killing activity in several samples, with a correlation between binding of 63D3 and killing activity. Separation experiments could demonstrate further that only $63D3^+$ cells, but not the $63D3^-$ cells, of a given patient exerted high cytotoxic activity.

MATERIALS AND METHODS

Marker Analysis

Determination of Fc receptors with IgG-coated ox erythrocytes, determinations of phagocytosis with latex beads, and the indirect immunofluorescence test were performed according to procedures previously described.[17] In brief, immunofluorescence was done by incubation with MoAbs at saturating concentrations in phosphate-buffered saline (PBS), 2.5% fetal calf serum (FCS), and 0.02% NaN_3 (immunofluorescence [IF] buffer) for 30 minutes on ice, followed in the second step by incubation with goat anti-human Ig-FITC (Tago, Burlingame, Calif). Monoclonal antibodies used were OKT3, reactive with T cells (Ortho Pharmaceutical, Raritan, NJ), HNK-1 (Leu-7, Becton Dickinson, Rödermark, FRG), VEP13,[18] reactive with cells of the NK cell lineage, and M522[5] and 63D3,[10] which are reactive with monocytes. The MoAb 63D3 was derived from the supernatant of the respective hybrid, which was purchased from American Type Culture Collection (ATCC, Rockville, Md).

Leukemia Cells

Patients were diagnosed as having AMoL based on clinical presentation with high blood leukocyte counts, bone marrow infiltration, infection, bleeding, and rapid course, and on morphology,

cytochemistry, and surface marker analysis, as performed in the leukemia reference laboratory of one of the authors (E.T.). Morphologically, the leukemic blasts were of M5 type (FAB classification). They exhibited delicate lacy chromatin, one to three prominent vesicular nucleoli, and basophilic cytoplasm. The nucleus was round, indented, or cerebriform. The leukemia cells of all cases were negative for common acute lymphocytic leukemia antigen (CALLA) and T and B cell markers, but were positive for surface Ia antigens and naphthol-AS-acetate-esterase (NAS), which was NaF sensitive. Aliquots of liquid nitrogen-stored leukemic samples from these patients were used for all studies. These cells had been isolated from peripheral blood by density gradient separation using Ficoll-Hypaque (Pharmacia, Freiburg, FRG).[19] They were frozen under controlled conditions in the presence of 10% dimethyl sulfoxide (DMSO) and 20% FCS using a freezing machine (Messer-Griesheim, Krefeld, FRG). Immediately before use, cells were thawed rapidly in a 37 °C water bath and washed twice with RPMI 1640 with 10% FCS.

Cytochemistry

Cytochemical staining for acid phosphatase, NAS, and peroxidase was done according to routine laboratory procedures.

Target Cells

The NK cell-sensitive cell lines K562,[20] obtained from Dr P. Perlmann, University of Stockholm, and MOLT 4,[21] obtained from Dr L. Gürtler, University of Munich, were kept in continuous culture in 75 cm^2 plastic flasks (No. 3075, Costar, Cambridge, Mass). The mouse cell line Wehi 164,[22] a BALB/c fibrosarcoma, was cultured on plastic Petri dishes without surface treatment for tissue culture (Härtel, Munich). This strategy prevents adherence of the tumor cells and allows for growth in suspension. For use as targets, the Wehi 164 cells were pretreated with actinomycin D as described.[16] In brief, the cells were seeded at 5×10^4 cells/mL; on day 2, actinomycin D (Calbiochem-Behring, Giessen, FRG), at 1 μg/mL final concentration, was added. After two hours, cells were centrifuged, and the supernatant was decanted, leaving 200 μL in the tube. Twenty microliters (100 μCi) of $Na_2{}^{51}CrO_4$ (5 mCi/mL, New England Nuclear, Dreieich, FRG) was added. Thus, the Wehi 164 cells were exposed to actinomycin D for a total of three hours. The target cells, K562 and MOLT 4, were also labeled with 100 μCi for one hour. All targets were washed four times with PBS and 2.5% FCS and were adjusted to a concentration of 5×10^4/mL in complete medium.

Cytotoxic Assay

One hundred microliters of the effector cell populations were titrated in six twofold (K562, MOLT 4 targets) or six threefold (Wehi 164) serial dilutions in triplicate in round wells of microtiter plates (No. 3797, Costar). To these cells, 100 μL of the labeled target cells was added. Plates were spun for two minutes at 50 g and then incubated at 37 °C in a humidified 5% CO_2 atmosphere. After seven hours, 100 μL supernatant was collected and counted in a gamma counter. The percentage of specific release was calculated from experimental values, from spontaneous release (target cells in wells without effector cells), and from maximum release (activity in 2.5×10^3 target cells) using the formula:

$$\frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100.$$

Standard deviations of triplicates were always less than 5% specific release. For comparison of lytic activities after cell sorting, lytic units (LU) were determined by linear regression analysis, using a desk calculator as described,[16] with 1 LU defined as the number of effector cells required to give 20% specific release. Correlation coefficients for all curves were greater than .94.

Cell Sorting

Leukemic samples were stained in indirect immunofluorescence with 63D3 as above, except that NaN_3-free reagents were used. The 63D3-positive and -negative cells were sorted in a FACS-2 (Becton Dickinson, Mountain View, Calif) at a 488 nm exciting wavelength with the photo multiplier tube 1 at 650 to 800 V. Control cells were stained but not sorted. Cells were collected in tubes containing 1 mL of FCS with 100 IU heparin to prevent clumping. After two washes, cells were resuspended in complete medium and analyzed for killer cell activity as described.

RESULTS

Marker Analysis

All patients studied had high leukocyte counts (range, 33,700 to 211,000), 81% to 99% of the cells having blast morphology (Table 1). Fc receptors for IgG ranged from 11% to 44%. The MoAb 63D3 specific for human monocytes showed binding to AMoL samples ranging from 0.8% to 45.0%. No correlation was seen between percentages of Fc receptor-positive cells and 63D3-positive cells. Fc receptor-mediated binding of the 63D3 MoAb was further excluded by control staining with a $F(ab)_2$ fragment of 63D3, giving staining similar to that with the intact 63D3. An irrelevant monoclonal IgG showed no binding, as assessed by FACS analysis. Comparing percentages of leukemic blasts according to morphology

Table 1. Marker Analysis of Leukemic Cells From Patients With AMoL

Donor	Leukocyte Count (Cells/μ^3)	Percentage of Cells Positive for						
		Blast Morphology	Fc Receptor	63D3	M522	OKT3	VEP13	HNK-1
Mu control	—*	—	—	12.0	—	77.0	16.0	9.0
Pi AMoL	103,000	96	11.0	43.3	59.1	1.5	0.0	0.0
Ec AMoL	33,700	92	26.5	45.0	57.0	1.9	0.0	0.0
Wa AMoL	96,000	97	44.0	34.0	75.0	0.0	0.0	0.0
Hu AMoL	—	81	17.0	39.0	58.0	7.0	0.0	2.7
Lo control	—	—	—	16.6	26.6	64.0	14.1	10.0
Ba AMoL	30,200	86	41.0	0.8	34.7	0.9	0.0	0.4
He AMoL	211,000	99	11.0	11.6	20.6	0.0	0.0	0.5

*Not determined.

and percentages of 63D3-positive cells, it is evident that in the first four patients in Table 1, the samples contained 63D3-positive and -negative leukemic cells. The samples from patient Pi, for instance, contained 96% leukemic blasts and 43% 63D3-positive cells. M522, a MoAb with a specificity similar to OKM1 stained higher percentages of cells in the AMoL samples, ranging from 27% to 75%.

Markers for nonleukemic lymphocytes showed less than 2% T3-positive cells in five cases and 7% T3-positive cells in patient Hu. VEP13, a MoAb reactive with active NK cells, stained no cells in the leukemic samples, while HNK-1, a marker for a portion of human NK cells, detected no cells or only up to 0.5% positive cells in five cases of AMoL and 2.7% positive cells in patient Hu.

Analysis of Killer Cell Activities

The killer cell activity of the AMoL cells of patient Pi against the monocyte-specific target Wehi 164 (actinomycin D treated) and against the NK cell target K562 are depicted in Fig 1 in comparison to the activity of a control donor. Lysis of Wehi 164 cells by the leukemic sample was as efficient as that of the control donor. In contrast, no killing of K562 by Pi AMoL cells could be detected, whereas peripheral blood mononuclear cells (PBMs) of the control donor exerted high killing activity. Cells from patient Pi and from five additional donors who had been tested initially in separate experiments (not shown) were reinvestigated in two combined experiments (Table 2), with results that were similar to those of the first

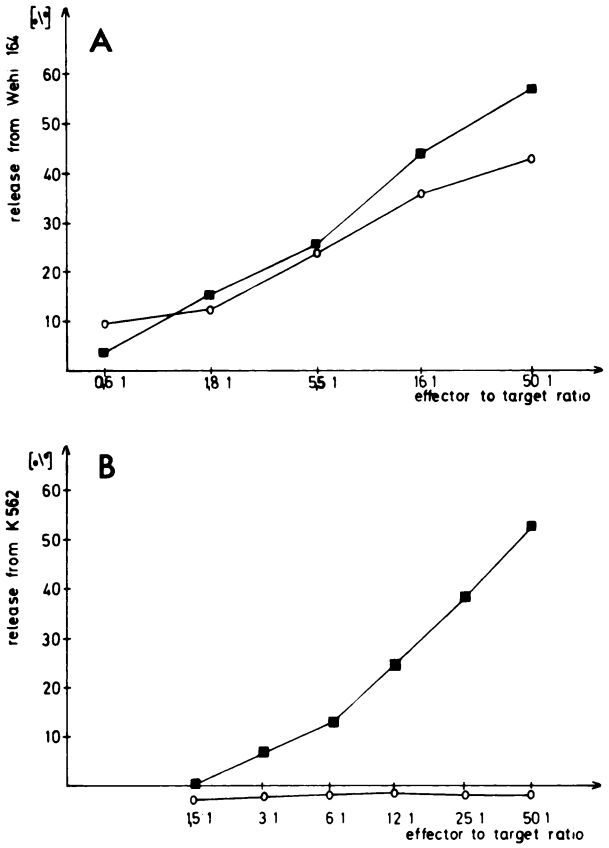

Fig 1. Killer cell activity of AMoL cells against Wehi 164 and K562 target cells. Cell from patient Pi (○) and from a control donor (■) were tested in parallel against (A) Wehi 164 fibrosarcoma cells, which, after pretreatment with actinomycin D, are exclusively lysed by monocyte effector cells, and against (B) K562 target cells that are lysed by NK cells.

Table 2. Killer Cell Activity of Leukemic Cells From Patients With AMoL

	Specific Release (%) From					
Exp. Donor	E:T	Wehi 164 (Actinomycin D-Treated)	E:T	K562	E:T	MOLT 4
1. Mu control	30:1	48.8	100:1	49.3	100:1	56.0
	10:1	42.9	50:1	39.3	50:1	48.9
Pi AMoL	30:1	39.5	100:1	1.2	100:1	14.4
	10:1	31.7	50:1	0.4	50:1	12.9
Ec AMoL	30:1	43.8	100:1	1.2	100:1	12.0
	10:1	36.9	50:1	0.9	50:1	11.3
Wa AMoL	30:1	38.5	100:1	−1.0	100:1	11.6
	10:1	35.5	50:1	−0.8	50:1	10.1
Hu AMoL	30:1	56.5	100:1	8.8	100:1	29.9
	10:1	61.0	50:1	5.8	50:1	25.6
2. Lo control	30:1	63.7	100:1	19.8	100:1	46.6
	10:1	56.0	50:1	14.2	50:1	38.7
Ba AMoL	30:1	12.8	100:1	−1.1	100:1	11.2
	10:1	5.4	50:1	−0.1	50:1	9.8
He AMoL	30:1	5.4	100:1	1.5	100:1	1.7
	10:1	2.8	50:1	0.4	50:1	6.0
Wa AMoL	30:1	59.3	100:1	1.6	100:1	12.5
	10:1	53.0	50:1	1.0	50:1	11.6

E:T, effector to target ratio.

experiment. As obvious in experiment 1, four patients, including Pi, exhibited a high killing of Wehi 164 (actinomycin D treated) that was comparable to the activity of the control donor. At the same time, lysis of K562 was absent in three donors, while a low level of lysis was detected with effector cells from patient Hu. The absence or presence of lytic activity against K562 goes along with the absence or presence of HNK-1 cells in these samples (Table 1). A low level of cytolysis was seen with the MOLT 4 target in all four leukemic samples in experiment 1.

In experiment 2 (Table 2), two additional cases of AMoL (Ba, He) were tested. The findings with respect to the NK cell targets K562 and MOLT 4 were similar to those of experiment 1. Lysis of the monocyte-specific target Wehi 164 (actinomycin D treated), however, was marginal with these two effector populations. Ba AMoL and He AMoL were low in binding of the 63D3 MoAb in the marker analysis (Table 1). This finding indicates that there exists a correlation between the 63D3-defined cell surface marker and cytotoxic activity.

Cell Sorting of 63D3$^{+/-}$ Fractions of AMoL

In order to confirm this assumption, we performed cell sorting experiments on 63D3-positive and -negative cells in two samples (Pi and Wa). In Fig 2, data from FACS analysis and sorting of cells from patient Pi are depicted. Staining with 63D3 showed a continuous spectrum overlapping with the weak autofluorescence of the leukemia cells (Fig 2A), which is in contrast to the pattern seen with PBMs, in which a clear gap between positive and negative cells is seen (not shown). Sorting of AMoL cells was performed with gates set as indicated on the dot plot (Fig 2B). For

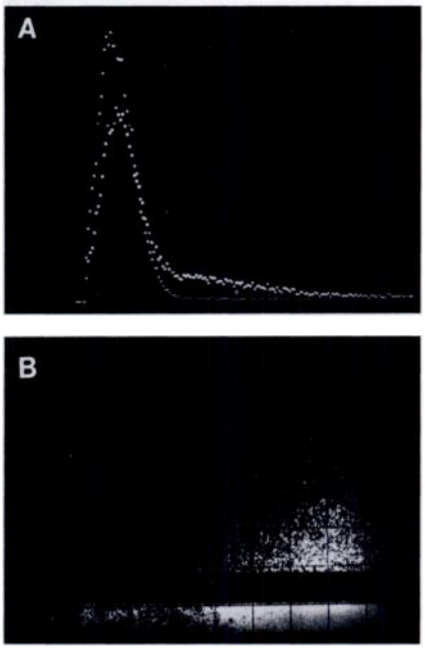

Fig 2. FACS analysis of 63D3-stained AMoL cells. AMoL cells (patient Pi) were stained with 63D3 in direct immunofluorescence and analyzed in a FACS-2. (A) Fluorescence intensity on the x-axis and cell number on the y-axis. The left curve represents the control stained cells, the right curve the 63D3-stained cells. (B) Forward angle light scatter on the x-axis and fluorescence intensity on the y-axis. The dark bar indicates the gates set for sorting of positive and negative cells. Scales are linear.

both donors, 63D3-positive cells were over 90% pure, and the negative fraction contained less than 2% 63D3-positive cells (Table 3). The sorted cells were analyzed for killer cell activity against actinomycin D-treated Wehi 164. Data in Table 3 demonstrate that 63D3-negative cells were depleted and 63D3-positive cells were enriched in killer cell activity against the

Table 3. Killer Cell Activities of AMoL Cells Separated Into 63D3-Positive and -Negative Cells

Donor	Cell Population	63D3-Positive Cells (%)	E:T†	Specific Release From Wehi 164 (Actinomycin D-Treated)	LU/10^6 Cells*
Pi AMoL	Unsorted	25.7	30:1	33.0	19.8
			10:1	15.0	
	63D3$^+$	94.1	30:1	58.8	143.8
			10.1	41.7	
	63D3$^-$	0.9	30:1	14.7	4.5
			10:1	4.2	
Wa AMoL	Unsorted	24.8	30:1	52.5	107.3
			10:1	40.5	
	63D3$^+$	91.2	30:1	65.0	551.9
			10:1	49.0	
	63D3$^-$	1.7	30:1	19.5	5.9
			10:1	15.3	

Cells were stained in indirect immunofluorescence with 63D3 and either left unsorted or were sorted into positive and negative cells using a FACS-2.

*LU, lytic unit, with 1 LU defined as the number of cells required for 20% specific release. LUs were calculated from a titration curve, consisting of six titration steps.

Table 4. Cytochemistry of AMoL Cells Separated Into 63D3-Positive and -Negative Cells

Cell Population*	Cells Positive for (%)		
	ACP†	NAS	POX
63D3$^+$	67.5 ± 12.1	76.0 ± 27.2	7.5 ± 2.3
63D3$^-$	45.0 ± 8.3	31.5 ± 21.1	18.5 ± 7.2

The mean values ± SD of the four cases of AMoL are given.

*Cells from four cases of AMoL (Pi, Ec, Wa, Hu) were stained in indirect immunofluorescence with 63D3 and sorted into positive and negative cells using a FACS-2. Purities of all preparations were greater than 90%.

†Cytochemistry was performed according to routine laboratory procedures. ACP, acid phosphatase; NAS, naphthol-AS-acetate-esterase; POX, peroxidase.

monocyte-specific target. For instance, for donor Pi, at 30:1 effector to target (E:T) ratio, unsorted cells had a cytotoxicity of 33% specific release, the 63D3-positive cells achieved 58.8%, and the negative cells had only 14%. In terms of lytic units, the 63D3-positive cells were 32 times more active compared to the 63D3-negative cells (Table 3). Similar data were obtained with sorting of cells from donor Wa, showing a 94-fold enrichment. Cytologic analysis of cytospin preparations did not reveal a clear difference in morphology (eg, monoblastic *v* monocytic) between 63D3-positive and 63D3-negative cells. Cytochemistry of the sorted cells revealed that enzymes associated with mature monocytes, such as acid phosphatase and NAS, are enriched in the 63D3-positive cells, while the myeloid marker, peroxidase, was enriched in the 63D3-negative fraction (Table 4).

DISCUSSION

The marker analysis on the AMoL samples demonstrated that only a portion of the leukemic blasts express monocyte-associated markers such as 63D3 and M522, with 63D3 staining lower percentages of the leukemia cells. The nonleukemic cells in our samples were reduced to minimal levels by the expanded leukemia cells. Normal T cells, identified by MoAb OKT3, were less than 2% in all but one patient. HNK-1 cells were 0.5% or less in five out of six samples, and VEP13 cells were not detectable. Thus, for five patients, negligible percentages of normal lymphocytes were found, suggesting that normal monocytes found in these preparations are also negligible.

Cytotoxicity of the AMoL cells was measured using the Wehi 164 mouse fibrosarcoma as a target cell. We had previously shown that this target cell, when pretreated for three hours with actinomycin D, becomes selectively susceptible for killing by human monocytes.[16] Actinomycin D also is able to increase susceptibility to lysis with other target cells, such as fresh human leukemia cells, but it is not effective in cell lines such as K562 or MOLT 4.[23]

Killer cell activity against the Wehi 164 target could be readily demonstrated in four of six leukemic samples, while little or no lysis of the NK cell targets K562 and MOLT 4 was observed. The killing of Wehi 164 appeared to be mediated by the AMoL cells in three out of four cases, as a contribution of normal cells is unlikely with the negligible percentages of normal cells, with the absence of NK cell activity against K562 and with the high counts of leukemic blasts.

With respect to donor Hu, taking together the marker analysis (7% OKT3-positive T cells) and the cytotoxic data (9% lysis of K562), we conclude that normal cells in this sample are present in amounts such that a contribution of normal monocytes to lysis of actinomycin D-treated Wehi 164 is very likely. Conversely, for the three patients Pi, Ec, and Wa, we conclude that normal cells do not contribute to any relevant extent to the monocyte-mediated lysis and that specific release from Wehi 164 is exerted by the clonal leukemic cells.

The inability to demonstrate high killing activity for K562 in these three patients and in two further cases of AMoL (experiment 2) is in contrast to a report by Hokland et al,[4] who described two cases of AMoL with killing activity for K562. Those cases might be transformants of different types of clones of the heterogeneous normal monocyte population, which is reported to also contain cells that lyse K562.[24,25] We did, however, detect some lysis of the MOLT 4 NK cell target (Table 2) in cases with no detectable HNK-1 cells (Table 1). This low level of lysis, in fact, might be mediated by the clonal AMoL cells. Confirming our conclusion with regard to a significant contamination with normal cells in patient Hu, a medium level of cytotoxicity with a specific release of 30% at 100:1 E:T was observed with the MOLT 4 target.

The cytotoxicity against Wehi 164 appeared to correlate with the expression of the 63D3 cell surface antigen. Similar to our findings, Dayton et al[9] demonstrated induction of several MoAb-defined cell surface antigens after conditioned medium induction in promyelocytic cell lines to correlate with the appearance of ADCC. Separation experiments, however, to elucidate whether or not the cells carrying any of these markers are the effector cells were not performed.

Hence, we attempted to demonstrate directly the linkage of 63D3 and cytotoxicity in the 63D3-positive samples by performing cell sorting. Our data demonstrate that within a leukemic population of monoblasts, the 63D3-positive cells contain the killer cell activity. Cooper and Kubagawa outlined a model for B cell leukemias that includes the possibility of a limited in

vivo differentiation of the malignant cells.[26] With respect to AMoL, one might speculate that the 63D3-positive fraction of clonal leukemic cells found in four of six patients results from differentiation of the leukemic clone from 63D3-negative precursors to 63D3-positive mature monocytes with cytotoxic potential. This concept is supported by the cytochemical findings, which indicate that the 63D3-positive AMoL cells contain a higher percentage of cells positive for NAS. Whether these 63D3-positive cells are able to kill their own progenitor cells is a hypothesis testable by cell sorting.

The 63D3 MoAb[10] selectively binding to human monocytes was reported to define a cell surface molecule of 200 kd molecular weight present on almost all adherent peripheral blood mononuclear cells. Functional analysis of 63D3-positive cells, thus far reported, demonstrated that these cells contain a population that has accessory cell function,[27] but the 63D3-defined molecule is not present on dendritic cells.[28] We presently do not know whether or not the 200 kd molecule defined by 63D3 is directly involved in the lytic interaction or whether it is only associated with the stage of maturation of monocytes in which they are capable of killing the Wehi 164 target. In order to answer these questions, studies of modulation and blocking with the 63D3 MoAb would be required.

The capability of AMoL to lyse the monocyte-specific target Wehi 164, as demonstrated in the present report, offers the possibility to study such questions at the clonal level.

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