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Enhancement of the antibody-dependent cellular cytotoxicity of human peripheral blood lymphocytes with interleukin-2 and interferon α

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Abstract. Antibody-dependent cellular cytotoxicity (ADCC) is regarded as an important mechanism by which monoclonal antibodies (mAb) can exert an antitumour effect in vivo. It may be possible, therefore, to enhance the therapeutic efficacy of mAb by cytokines that are able to enhance the ADCC of human $CD3^-$, $CD56^+$, $CD16^+$ natural killer (NK) cells. We investigated in vitro the effects of recombinant interferon α (rIFNα) and recombinant interleukin 2 (rIL-2), alone or in combination, on the ADCC of human peripheral blood NK cells. Both cytokines enhanced the ADCC of the human effector cells. rIFNα induced a maximally increased ADCC after an exposure of human effector cells to 20 IU/ml for 15–30 min, while rIL-2 induced optimal ADCC after incubation of the cells for 2 days in 20–50 U/ml. We now show that activation of the NK cells with a combination of rIL-2 *and* rIFNα induced significantly higher levels of ADCC than either cytokine alone. The highest ADCC was induced if the cells were first exposed to rIL-2 before rIFNα was added to the culture. Culture of NK cells in medium or rIL-2 decreased the expression of FcγRIII (CD16), indicating that intensity of CD16 expression and level of ADCC are not directly correlated, although blocking experiments with a mAb directed against CD16 showed that this FcγR was essential for ADCC of the human effector cells.

Key words: Interleukin-2 – Interferon α – ADCC – Human NK cells

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Introduction

In the past decade immunotherapy trials with monoclonal antibodies (mAb) have been performed in patients with malignant disease and, although some impressive responses were observed that indicate the validity of the approach, the overall effects were limited [14]. The therapeutic efficacy of mAb, not coupled to toxins or radioisotopes, depends on the recruitment of host effector systems including complement, antibody-dependent cellular cytotoxicity (ADCC) and/or phagocytosis/cytostasis of antibody-coated tumour cells by cells of the reticulo-endothelial system.

The lymphokine interleukin-2 (IL-2), produced by activated T-helper cells, is an important regulatory lymphokine that affects many different cells of the immune system, including T and B lymphocytes, monocytes and natural killer (NK) cells (for review see [31]). Clinical trials have been performed in patients with malignant disease with recombinant IL-2 (rIL-2 [30]) alone or in combination with autologous lymphokine-activated killer (LAK) cells or tumour-infiltrating lymphocytes (TIL) [29]. Although the overall clinical responses were again limited, some impressive tumour regressions were documented, which encourage further attempts to improve the efficacy of this form of therapy.

Interferons (IFN) are a family of related cytokines produced by a variety of haematopoietic and non-haematopoietic cells, which were originally described as soluble factors that inhibit viral infection and replication [3]. Recombinant $IFN\alpha_2$, the most common subtype of IFNα, has been tested in clinical trials in patients with malignant disease and favourable responses were documented. $IFN\alpha_2$ can enhance the cytolytic activity of human peripheral blood NK cells and modulate IL-2-dependent LAK cell generation [16, 17, 32, 35]. However, the anti-neoplastic effect in the clinical trials was most likely due to a direct anti-proliferative effect of $IFN\alpha_2$ on the tumour cells.

In addition to activation of the antibody-independent cytotoxic activity of lymphocytes, both rIL-2 and natural IFNα and IFNγ have been shown to enhance the ADCC of

these effector cells [2, 16, 18, 24]. However, systematic kinetics and dose/response experiments dealing with this effect of rINFα have so far not been published.

Immunotherapy experiments with anti-idiotypic mAb in combination with rIL-2 or rIFNα, performed in a syngeneic murine B lymphoma model, indicated that with these cytokines the therapeutic efficacy of the mAb could be significantly enhanced [4–6]. We have shown that a combination of mAb (anti-CD19) therapy with rIL-2 synergistically enhances the antitumour activity against a Burkitt lymphoma transplanted in nude mice [36, 37]. These data indicate that in these murine models ADCC indeed is an important mechanism by which mAb mediate antitumour effects.

Since it is known that the most important ADCC effector cells in man are lymphocytes with the features of NK cells [26], we investigated, as a prelude to clinical immunotherapy trials, the effects of the cytokines rIL-2 and rIFNα, alone or in combination, on the ADCC of human peripheral blood NK cells. Both cytokines enhanced the ADCC activity of the human effector cells, and activation with a combination of rIL-2 and rIFNα enhanced the ADCC to a significantly higher extent than each cytokine alone. Furthermore, we show that although the ADCC of the human effector cells was dependent on the function of the FcγRIII (CD16), the level of ADCC was not correlated with the number of FcγRIII molecules expressed on the effector cells.

Materials and methods

Cell lines and cell culture conditions. The human cell lines used were Jiyoye, M14 and K562 (American Type Culture Collection, Rockville, USA). The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, non-essential amino acids, 100 IU penicillin/ml, 100 μg kanamycin/ml and fetal calf serum (FCS, 10% v/v, PAA Laborgesellschaft, Austria).

Monoclonal antibodies. The mAb used in immunofluorescence experiments and in cytotoxicity assays were: 32.2 (CD64) [21], CIKM5 (CD32) [22], kindly provided by Dr. G. Pilkington, Melbourne, Australia; NKI-L15 (CD11a) [19], NKI-L16 (CD11a) [20], CLB LFA 1/1 (CD18) [23], CLB-FcRgran.1 (CD16) [38] and F(ab)$_2$ fragments of CLB-FcRgran.1, kindly provided by Dr. T. W. J. Huizinga, CLB, Amsterdam; and AT1 (CD38) (A. Hekman, unpublished), 4F2 [15], T3b (CD3) [33], CD14 (Beckton Dickinson, Mountain View, Calif., USA), Leu19 (CD56), Beckton Dickinson) and R24.3 (rat IgG2b anti-HLA-class-II) [36]. R24.3 mAb was produced and purified (≥95% pure as determined by sodium dodecyl sulphate/polyacrylamide gel electrophoresis analysis) by Euroclone Bv, Amsterdam, The Netherlands.

Isolation and cytokine activation of the LL subpopulation of human PBL. Peripheral blood lymphocytes (PBL) of healthy volunteers were fractionated by means of centrifugal elutriation as described previously [12]. The large lymphocyte (LL) fraction was used in all our experiments. This LL fraction was enriched for CD3$^-$, CD16$^+$, CD56$^+$ NK cells (25%–40% CD3$^-$, 60%–75% CD3$^+$) and contained no B lymphocytes and up to 5% monocytes. The isolated LL were washed once with DMEM/FCS and suspended in DMEM/FCS at $(2-3) \times 10^6$ cells/ml. rIL-2 was a generous gift from Eurocetus BV, Amsterdam [3] and rIFNα (Roferon) was obtained from Hoffmann-La Roche BV, Mijdrecht, the Netherlands. The LL were activated by culturing at 37° C in humidified air containing 5% CO_2 in medium supplemented with the indicated amounts of one or both of these cytokines [rIL-2 concentrations are given in Cetus U (1 Cetus U = 6 IU) and rIFNα concentration in IU]. Activating the cells with a combination of both cytokines was performed without washing the cells to remove the cytokine to which the cells were exposed first. In order to remove the cytokine with which the LL were activated the cells were extensively washed with complete medium before being used in the cytotoxicity assay or for immunophenotyping.

Immunofluorescence. Cells were incubated in the appropriate dilution of mAb [$(0.5-1) \times 10^5$ cells in 25 μl] in V-bottom 96-well microtiter plates (Greiner). For the determination of background staining the cells were incubated with mAb K8, which reacts only with the idiotype of the tumour cells of a non-Hodgkin lymphoma patient [28]. Bound mouse Ig was detected with fluorescein-isothiocyanate (FITC)-conjugated F(ab)$_2$ fragments of goat anti-(mouse Ig) (GAM/FITC, Tago, Burlingame, Calif., USA). All incubations were performed for 30 min at 4° C and after each incubation the cells were washed once with 100 μl phosphate-buffered saline supplemented with 0.5% (w/v) bovine serum albumin and 0.02% (w/v) sodium azide. Fluorescent staining was analysed with a Facscan cytofluorometer (Becton Dickinson). Antigen density is expressed as the fluorescence index (FI) calculated on the basis of fluorescence intensity (mean channel number, n_{mc}) according to the following formula:

$$FI = \frac{n_{mc}\text{ positive population with test mAb} - n_{mc}\text{ negative control}}{n_{mc}\text{ negative control}}$$

ADCC assay. ADCC was determined with short-term ^{51}Cr-release experiments [39]. All ADCC experiments were performed with the R24.3 mAb, a rat IgG2b mAb, which detects a common epitope on HLA class II molecules, and the class II$^+$ Burkitt cell line Jiyoye was used as a target. Non-activated and cytokine-activated LL were washed with medium before they were mixed with 10^3 ^{51}Cr-labelled Jiyoye cells (labelling with ^{51}Cr: 6.4 MBq/10^6 cells incubated at 37° C for 60–120 min, sp. act. ^{51}Cr = 13–22 GBq/mg chromium; Amersham, Buckinghamshire, England) at effector-to-target cell (E/T) ratios varying from 100 : 1 to 0.3 : 1 in triplicate in the absence or presence of the indicated amounts of R24.3 mAb in 96-well round-bottom microtiter plates (Sterilin). The final volume was 200 μl. Subsequently the plates were centrifuged for 2 min at 1000 rpm and incubated for 4 h at 37° C in humidified air containing 5% CO_2. After this incubation period the plates were centrifuged again (2 min, 1000 rpm) and 100 μl supernatant was harvested and the ^{51}Cr content was determined in a gamma counter (Packard). The percentage specific label release was calculated according to the following formula:

$$\text{Specific } ^{51}\text{Cr release (\%)} = 100 \times (T-S)/(M-S)$$

where T = ^{51}Cr (cpm) in test sample, M = maximal releasable label in 2% (v/v) Triton X-100, 0.5% (w/v) sodium dodecyl sulphate, 1% (w/v) sodium deoxycholate and 10 mM EDTA, and S = spontaneously released label from target cells alone. The spontaneous label release never exceeded 15% of the maximal releasable radioactivity.

Calculation of cytotoxicity (lytic units) and statistical analysis. For calculating lytic units from ^{51}Cr-release curves, the curves were fitted to the von Krogh model [^{51}Cr release = $A(1+k/ET)^{-N}$] [27] after a variance-stabilizing 2/3 power transformation using non-linear least-squares regression. Subsequently we tested whether all the curves obtained from one donor could be described using identical A *and* N values; this is equivalent to testing whether the only difference between the different curves from one donor is a parallel horizontal shift in a ^{51}Cr-release versus log (ET) plot. If this assumption was not justified (P <0.05), we tested whether at least the A *or* the N values could be considered to be equal. If this also was not possible, for every different curve an individual N and A value was determined. In all statistical comparisons of different curves the F-test was used. In this, the denominator was always based on all data form the donor, not just the curves to be compared. By doing this it was often possible to increase the degrees of freedom of the denominator of the F-test from 6 to 18.

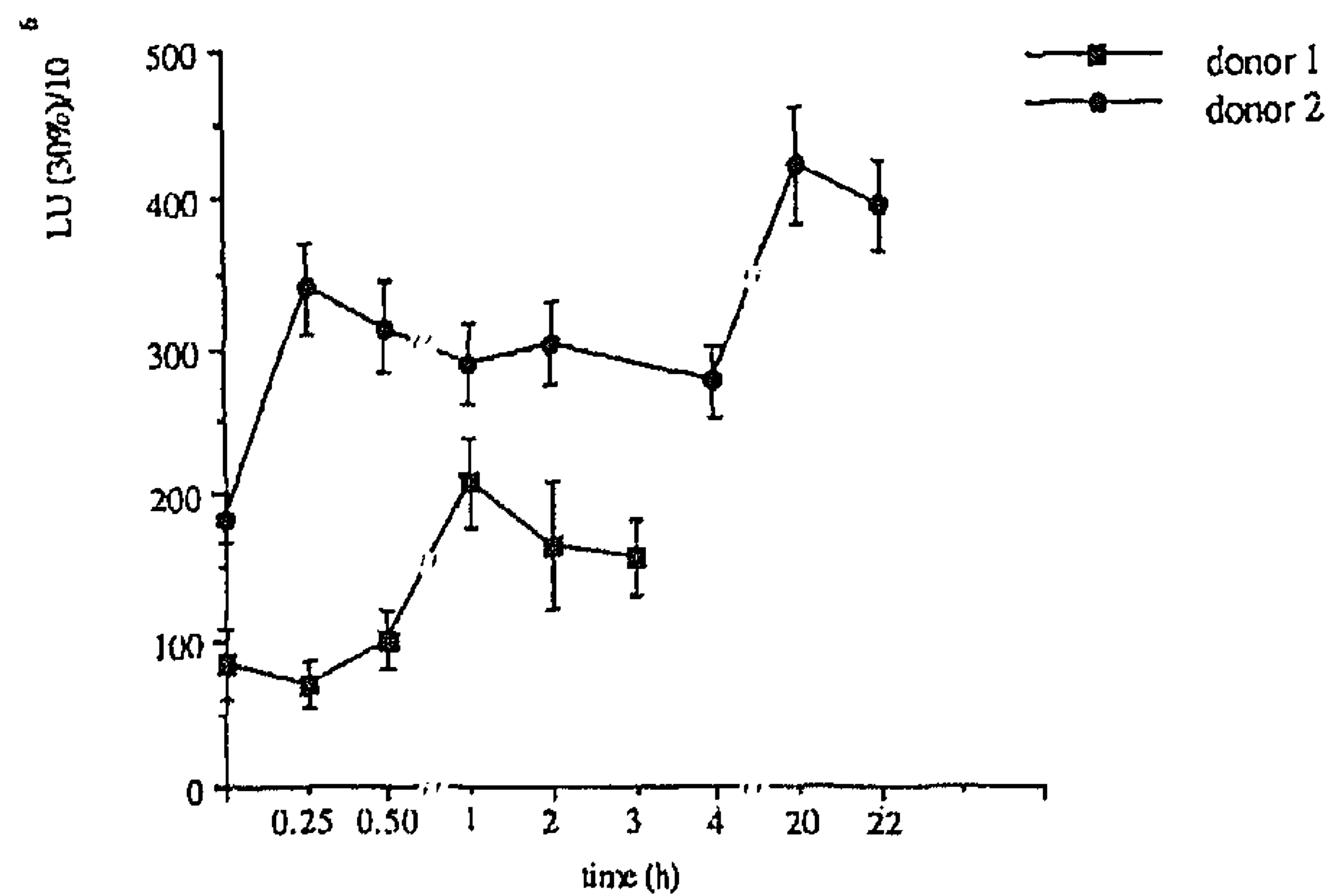

Fig. 1. Large lymphocytes (*LL*) were cultured for the indicated time in 50 IU recombinant interferon α (*rIFNα*)/ml. The results of two independent experiments are shown. Cytotoxicity is expressed as LU ± SE. Cytotoxicity in the absence of mAb was not more than 25% of the cytotoxicity in the presence of R24.3 mAb. The LL fractions from donors 1 and 2 contained 20% and 27% CD16⁺ cells respectively

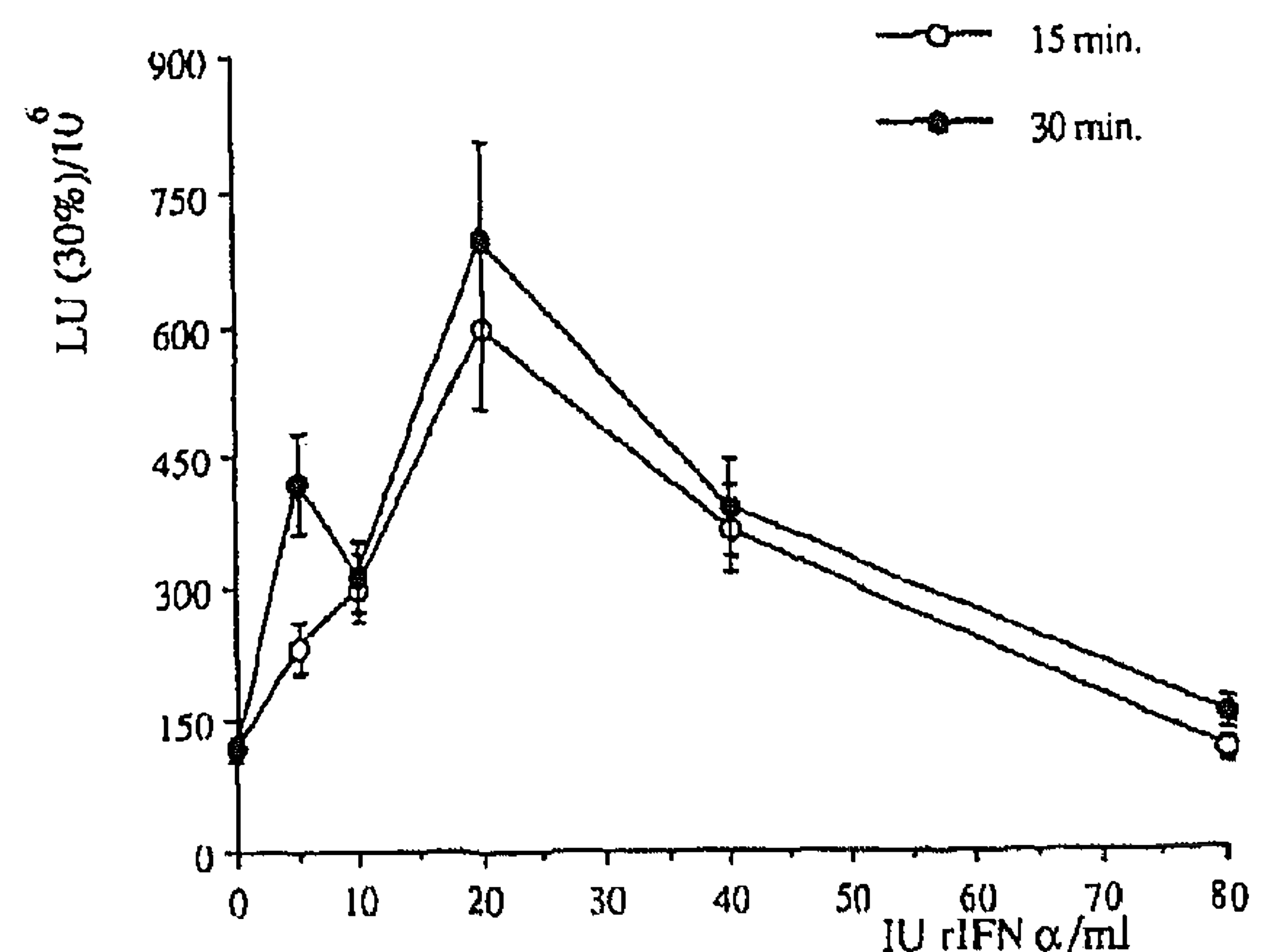

Fig. 2. The LL were activated for 15 min or 30 min with the indicated concentration of rIFNα. Cytotoxicity is expressed as LU ± SE. The LL fraction from this donor contained 28% CD16⁺ cells

Table 1. Antibody-dependent cellular cytotoxicity (ADCC) of large lymphocytes (LL) activated with a combination of recombinant interleukin-2 (rIL-2) and recombinant interferon α (rIFNα)[a]

Donor	$CD16^+$ (%)	Cytotoxicity (LU 30%/10^6 effector cells) (SE, $n = 6$) after activation with			
		Medium	rIL-2*1	rIFNα*2	rIL-2+rIFNα*3
1	38	212 (20)	314 (23)	286 (23)	417 (30)
2	26	12 (2)	25 (5)	12 (3)*4	85 (14)
3	21	12 (3)	70 (6)	15 (4)*4	192 (30)
4	32	446 (74)	2236 (510)	817 (153)	5734 (1609)
5	17	70 (8)	440 (67)	323 (47)	1666 (322)
6	20	63 (5)	239 (13)	94 (6)	521 (30)
7	35	79 (13)	438 (76)	143 (23)	1816 (565)
8	30	11 (1)	47 (7)	26 (4)	103 (15)
9	21	9 (2)	71 (7)	28 (3)	102 (10)
10	19	30 (3)	154 (15)	74 (7)	394 (40)

[a] The *F*-test was used in all comparisons. LL were used immediately after isolation except for cells from donors 1, 2 and 4, which had been stored in liquid nitrogen before use. The LL were cultured overnight in medium or in medium supplemented with rIL-2 (50 U/ml; donor 4: 25 U/ml). rIFNα (25 IU/ml) was added to the appropriate cultures for 30 min. The cells were washed once and mixed with 10^3 ^{51}Cr-labelled Jiyoye target cells at six E/T ratios in the presence of R24.3 mAb (1 μg/ml). Cytotoxicity is expressed as lytic units (30%)/10^6 effector cells ($LU_{30\%}$) and the SE is shown in parentheses. One lytic unit is defined as the number of effector cells needed to kill 30% of 10^3 target cells and was calculated as described in Materials and methods. Cytotoxicity (LU) in the absence of mAb was not more than 15% of the value in the presence of mAb

*1 $P \leq 0.02$ versus medium

*2 $P \leq 0.03$ versus medium, except *4 not significantly different versus medium

*3 $P \leq 0.02$ versus rIL-2 alone

Results

rIFNα or rIL-2 activation of the ADCC of LL

The LL obtained from 22 random donors were tested for cytotoxic activity after activation with rIFNα. In these experiments we noted variability in the reaction of the LL to rIFNα stimulation. The LL of 4 out of these 22 donors did not respond with increased ADCC, while more than 80% (18/22) did show increased ADCC after rIFNα activation compared to ADCC of LL cultured in medium. The kinetics of rIFNα activation of the ADCC of L fractions obtained from two random donors, tested in two separate experiments, is shown in Fig. 1. The antibody-independent activity of the LL from these donors was below the detection limit (<1 LU) and is therefore not indicated. Using the LL from donor 2 the largest gain in ADCC was seen after incubation for 15 min with 50 IU/ml, with some additional increase after 20 h of activation. Maximal ADCC of the LL from donor 1 was reached after 60 min exposure to this dose of rIFNα. Although, the LL from donor 1 (Fig. 1) needed a 60-min exposure to reach maximal ADCC, the results of other experiments showed that the minimal time needed to reach maximal ADCC with 50 IU rIFNα/ml was 15 min for LL obtained from most donors (see donor 2, Fig. 1).

In subsequent experiments the dose/response relation of rIFNα activation of LL was determined. The results of a representative experiment are shown in Fig. 2. Exposure of the LL from this donor to 20 IU rIFNα/ml for 15 or 30 min induced maximal ADCC. Other dose/response experiments gave similar results, although the LL obtained from some donors needed a minimum of 50 IU rIFNα/ml for the induction of maximal ADCC.

In Table 1 the effect of IFNα and/or IL-2 activation on the ADCC of the LL of 10 healthy donors is shown. These results show that there is a considerable variation in the baseline level of ADCC and that LL from different donors react differently to activation with these cytokines. Despite this variability, however, the ADCC of LL obtained from all these donors significantly increased after exposure to rIL-2. The results of other experiments with rIL-2 alone (data not shown) showed that culture of LL from most donors for 2–3 days with 10–50 U rIL-2/ml increased their ADCC to the maximal level, while for the induction of maximal antibody-independent cytotoxic activity more

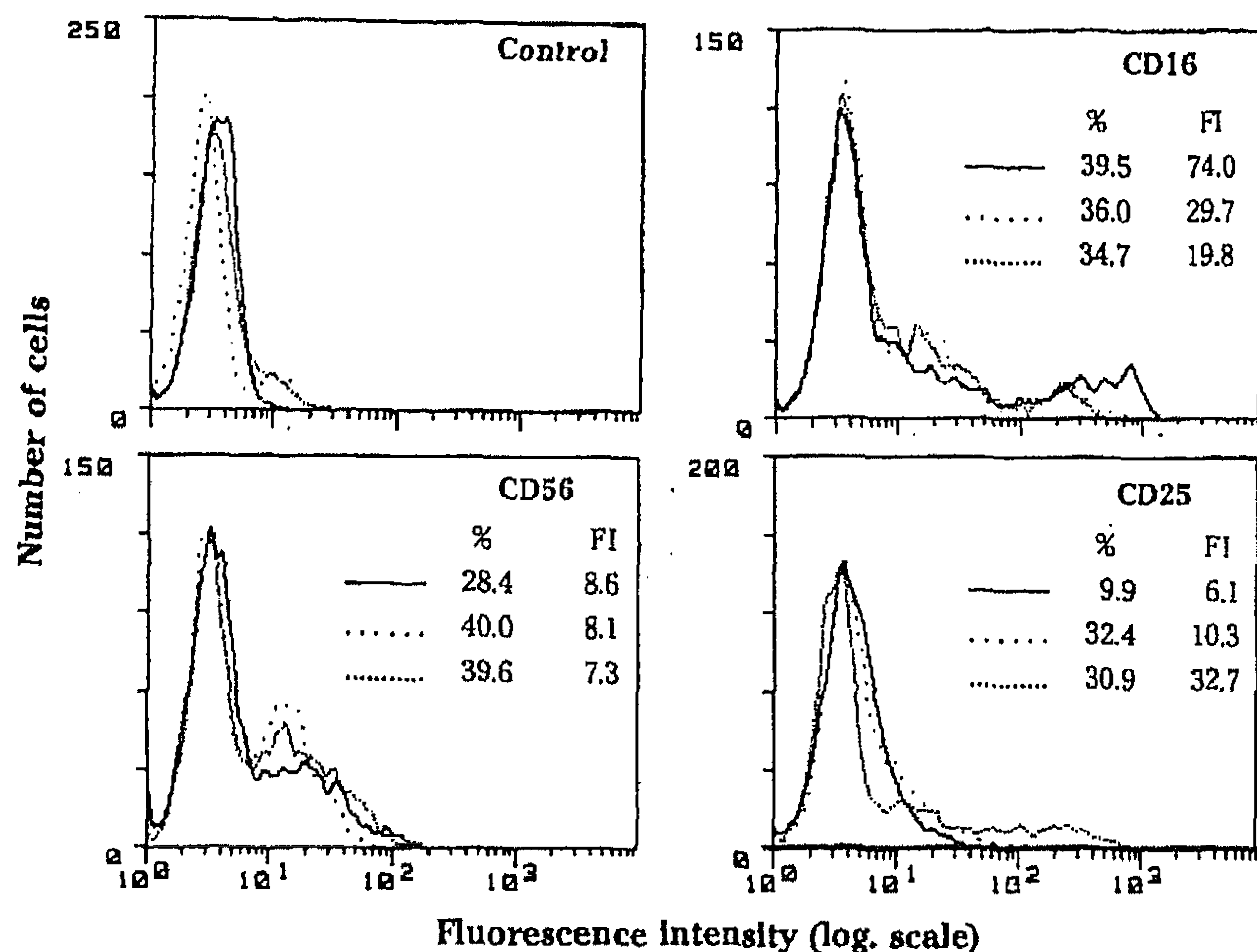

Fig. 3. Single-colour histograms of a LL fraction. —, Fresh; ..., day 2, medium; ......, day 2, 15 U rIL-2/ml. Cells were stained with an irrelevant anti-idiotype mAb K8 (*Control*), CLB-FcRgran.1 (*CD16*), Leu19 (*CD56*) or TB30 (*CD25*). Antigen density is indicated with the fluorescence index (*FI*)

rIL-2 and/or a longer exposure time were needed. These results confirm data published by other investigators [18, 24].

Activation of LL with a combination of rIL-2 and rIFNα

Since it is known that the induction of LAK cell activity with IL-2 can be modulated by a number of other cytokines, including IFNα [17, 32, 35], we investigated the effect on ADCC of simultaneous activation of LL with rIL-2 and rIFNα. In initial experiments a number of variables were tested including time of rIL-2 activation and time of rIFNα addition. In some of these experiments the LL were activated for 3 days with rIL-2, and rIFNα was present either during the entire activation time or only for the last few hours. The results of these experiments (data not shown) indicated that maximal effects were achieved by pre-activating the cells with rIL-2 prior to the addition of IFNα. Moreover, pre-activation of the cells with rIL-2 followed by an exposure to rIFNα induced a significantly higher ADCC than either cytokine alone at the same dose levels and exposure times. Subsequently, the LL from ten different donors were tested using the same activation schedule (Table 1). As already stated above, the ADCC of LL cultured in medium alone was very variable. It is noteworthy, however, that LL cultures from all 10 donors showed significantly higher ADCC after activation with rIL-2 and that 8/10 showed significantly higher ADCC after activation with rIFNα, although the effect of rIL-2 was much more pronounced compared to the effect of rIFNα. Activation of the cells from all these donors with rIL-2 followed by a 30-min exposure to rIFNα significantly increased the ADCC compared to the increase with rIL-2 alone (Table 1, $P \leqslant 0.02$). It is important to note that this was also the case with cells from the donors 2 and 3, who did not respond to rIFNα alone.

Phenotype of freshly isolated and cultured LL

In immunofluorescence experiments the phenotype of freshly isolated LL and LL cultured in medium, rIL-2 and/or rIFNα was determined. It should be noted, however, that the cytotoxic activity was only determined for cells cultured in medium with or without rIL-2 and/or rIFNα. Culture of the LL in medium alone or in medium with rIL-2 resulted in a decrease of the average FcγRIII (CD16) expression per cell and also in very small decrease of the percentage of FcγRIII$^+$ cells. One experiment is shown in Fig. 3. In the fresh LL, 39.5% of the cells were CD16$^+$ with a fluorescence index (FI) of 74, while these parameters changed to 36.0% CD16$^+$, FI = 29.7 for the cells cultured in medium and 34.7% CD16$^+$, FI = 19.8 for the cells cultured in rIL-2. Additional immunofluorescence experiments showed that the decrease of CD16 expression was not reproducibly different between LL cultured in medium and LL cultured in rIL-2. Since culture of the LL with rIL-2 consistently induced an increase of the ADCC activity and a decrease of the average CD16 expression, it can be concluded that the level of ADCC is not correlated with the intensity of CD16 expression.

In the immunofluorescence experiments a number of other cell-surface markers were also tested. No significant changes were observed in the expression of HLA class II, CD3, CD32, CD64, CD18, and CD11a. The NK cell marker CD56 (Fig. 4), the cell-adhesion molecule ICAM-1 and the activation markers CD38 and 4F2 increased after culture of the cells in rIL-2 (data not shown). The results of Figs. 3 and 4 also show that rIL-2 activation increased the expression of the 55-kDa chain of the IL-2 receptor (CD25) and of NKI-L16, an activation epitope on the leucocyte-function-associated antigen 1 (LFA-1) [13, 20].

LL activated with rIFNα were tested for the same markers as described above and only the expression of CD38, CD56, 4F2 and ICAM-1 increased. Incubation of

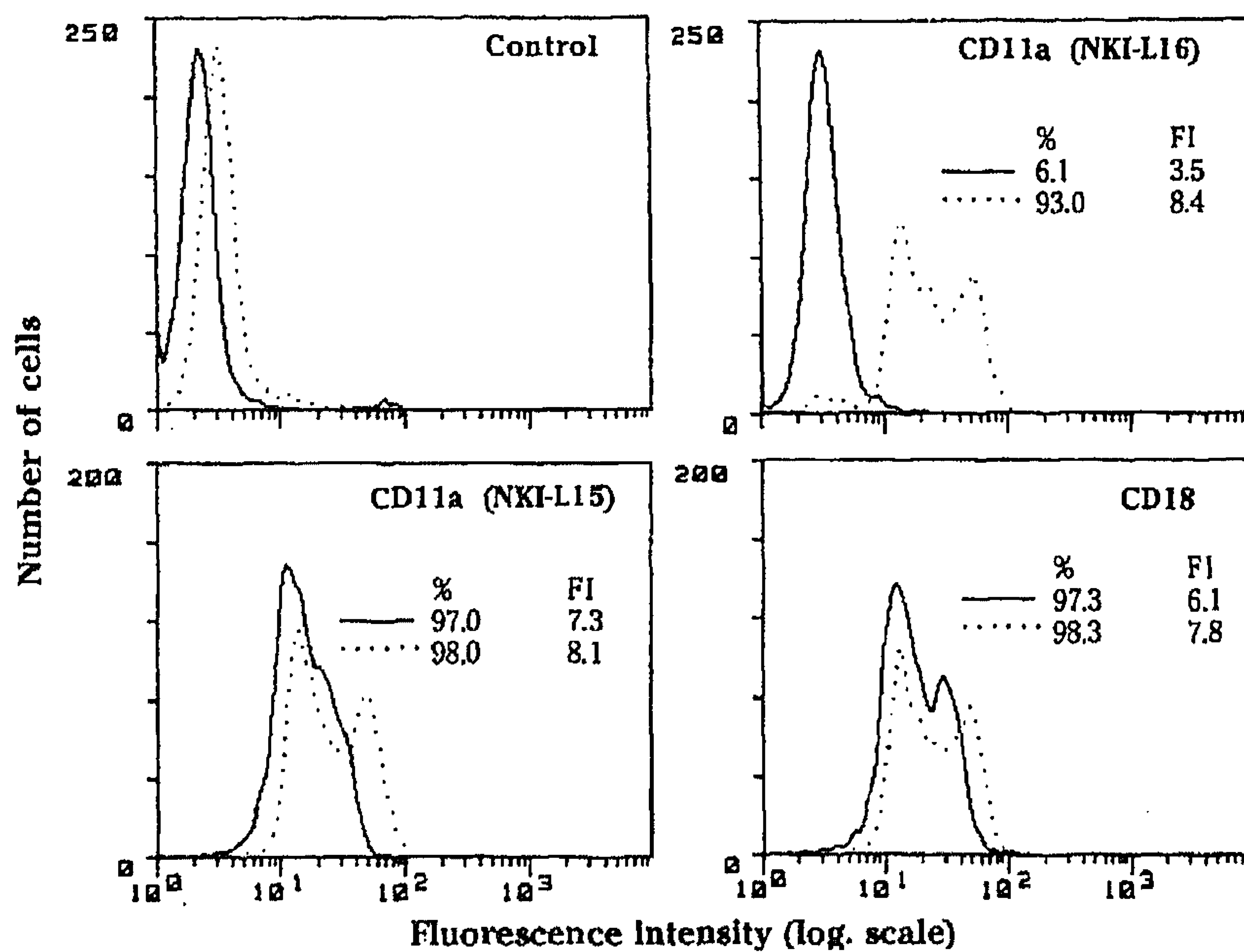

Fig. 4. Single-colour histograms of a LL fraction cultured for 2 days in (—) medium or (...) 25 U rIL-2/ml. Cells were stained with an irrelevant anti-idiotype mAb K8 (*Control*), NKI-L16 (*CD11a*), NKI-L15 (*CD11a*) or CLB-LFA 1/1 (*CD18*). Antigen density is indicated with the fluorescence index (*FI*)

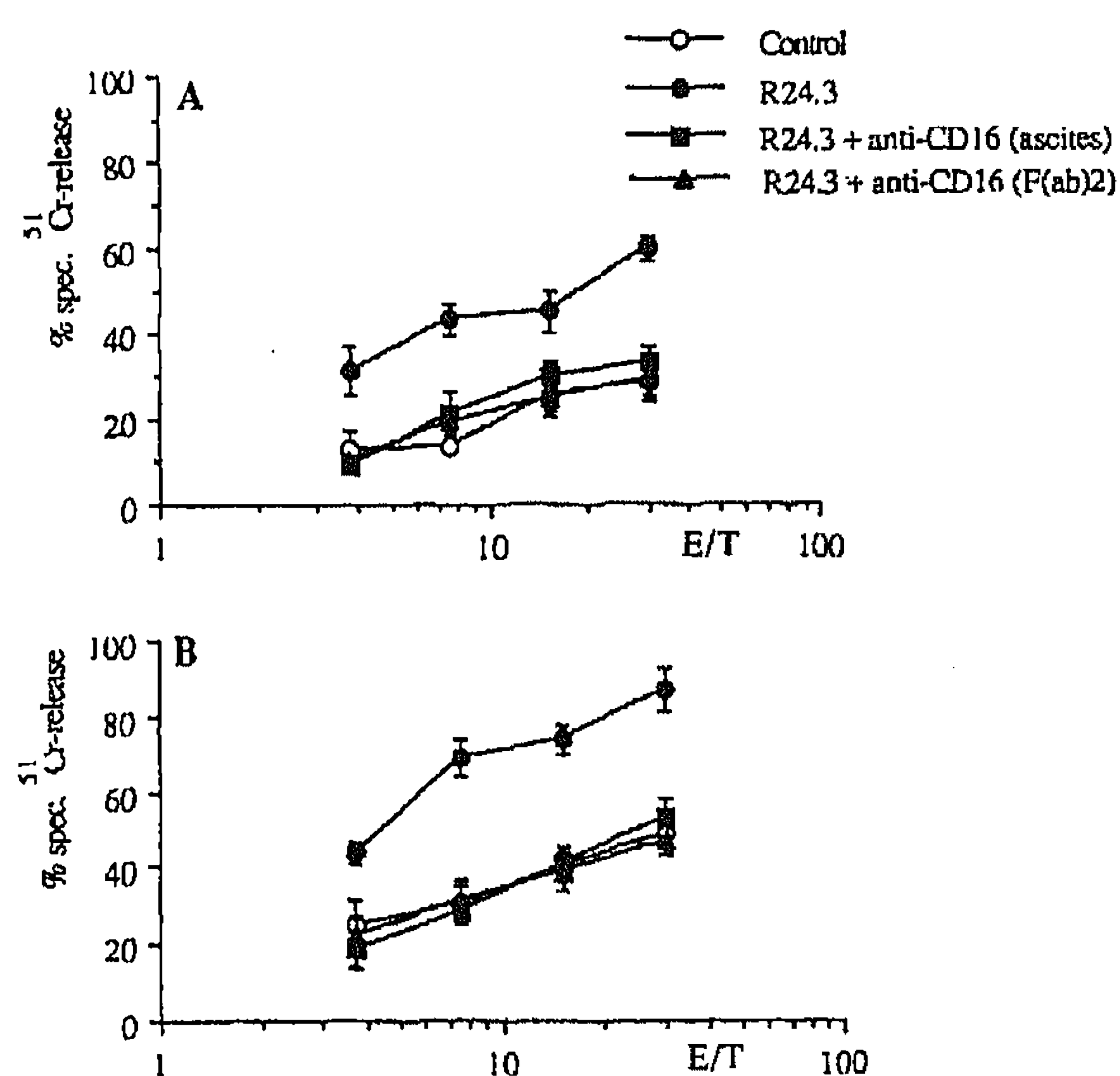

Fig. 5. Involvement of FcγRIII in the antibody-dependent cellular cytotoxicity of LL cultured for 2 days in medium (A) or medium supplemented with 25 U recombinant interleukin-2/ml (B). After activation the LL were washed with medium and mixed with ^{51}Cr-labelled Jiyoye target cells at four E/T ratios in triplicate in the absence (control) or presence of R24.3 mAb (1 μg/ml). CLB-FcRgran.1 (anti-CD16) mAb was added as ascites (1/500 final dilution) and F(ab)$_2$ fragments (10 μg/ml). Cytotoxicity is expressed as percentage specific ^{51}Cr release ± SD ($n = 3$)

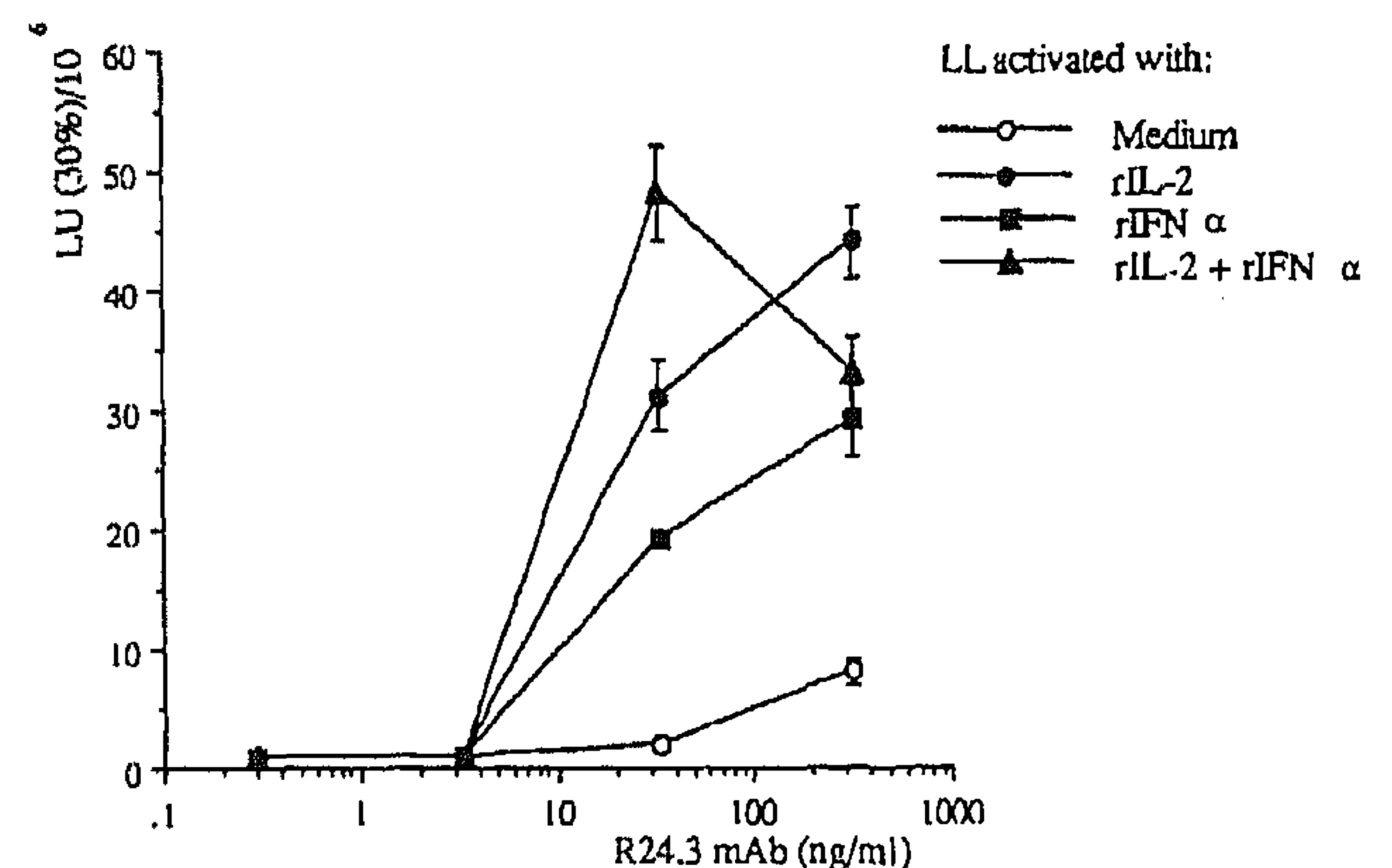

Fig. 6. LL were activated for 16 h with 50 U rIL-2/ml and for the last 30 min rIFNα was added to the appropriate cultures. Non-activated cells were cultured in medium alone. LL were mixed in vitro with ^{51}Cr-labelled Jiyoye target cells in the presence of the indicated amount of R24.3 mAb. Cytotoxicity is expressed as LU ± SE

LL with a combination of rIL-2 and rIFNα, according to the same schedule as used in the experiments shown in Table 1, did not show a qualitatively different change in phenotype from that induced by either cytokine alone, although the increase in expression of CD56 and CD54 was more pronounced (data not shown).

Involvement of FcγRIII (CD16) in the ADCC of LL

To test whether the FcγRIII (CD16) on the human effector cells was involved in the ADCC activity of LL, blocking experiments were performed with the anti-FcγRIII mAb CLB-FcRgran.1. This mAb is known to inhibit FcγRIII-mediated functions of granulocytes and monocytes [38]. In Fig. 5 a representative experiment is shown in which the anti-FcγRIII mAb was added to the cytotoxicity assay. The ADCC activity of the LL was completely blocked by CLB-FcRgran.1 mAb to the level of cytotoxicity observed in the absence of R24.3 mAb. This inhibition was observed with intact CD16 mAb (ascites) as well as with F(ab)$_2$ fragments. It is significant that the enhanced ADCC of LL activated with rIL-2 was also completely blocked with CLB-FcRgran.1 (Fig. 5 B) suggesting that it is unlikely that

other yet undefined FcγR species are involved in the effect of rIL-2 on the ADCC of LL.

Monoclonal antibody dose/response relationship

mAb titration experiments were performed to test whether the activation of LL with rIL-2 and/or rIFNα influenced the concentration of R24.3 mAb needed to induce ADCC. In Fig. 6 the results of one of these experiments are shown. Cytokine activation of LL did not decrease the minimal concentration of mAb (50 ng/ml) nor did it affect the optimum concentration of mAb (1000 ng/ml) that was needed for the induction of ADCC. The consistent effect of rIL-2 and/or rIFNα activation was that the cytotoxic activity at a given mAb concentration increased.

Discussion

In this paper we confirm other reports that show that the cytokines IL-2 and IFNα can enhance the ADCC of human peripheral blood lymphocytes [2, 18, 24, 16]. We show here that activation of PBL with a combination of rIL-2 and rIFNα enhanced the ADCC activity of these effector cells to a significantly higher extent than either cytokine alone.

Experiments performed to determine optimal conditions for the enhancement of the ADCC of LL with rIFNα showed that maximal ADCC was reached with doses of rIFNα between 20 and 50 IU/ml and that the LL had to be exposed for 15–30 min to rIFNα (Figs. 1 and 2). These conditions also induced optimal antibody-independent activity although the level of this activity induced by rIFNα was rather moderate and for some LL populations even below the detection limit of the ^{51}Cr-release assay (<1 LU). In contrast to the results with rIFNα, rIL-2 induced maximal ADCC at lower doses of rIL-2 and shorter exposure times in comparison with the conditions needed to induce maximal antibody-independent activity (data not shown [18, 24]).

The cytotoxicity data with rIL-2 and/or rIFNα-activated LL from random healthy donors showed that baseline antibody-dependent and antibody-independent cellular cytotoxicity was variable (illustrated in Table 1 with the LL from ten random donors). This variable baseline activity, however, did not preclude an enhancement of ADCC after rIL-2 and/or rIFNα activation. The LL of all donors shown in Table 1 responded to rIL-2 with significantly enhanced ADCC and eight of the donors did show enhanced ADCC after activation with rIFNα. Prior activation with rIL-2 resulted in a further increase in ADCC after exposure to rIFNα. It is notable also that the LL that did not react to rIFNα alone did respond after prior activation with rIL-2. This suggests that the activation state of the LL may determine whether or not they respond to rIFNα. On cells from IFNα-non-responsive donors, rIL-2 may induce receptors for IFNα [8].

A negative effect of rIFNα on LAK cell induction by IL-2 has been found by several investigators [32, 35]. However, one of these studies [35] also shows that exposure to rIFNα for 1 h enhanced the LAK activity of PBL pre-activated with rIL-2 for 4 days. In our experiments we also noticed that the time of addition of rIFNα was critical.

Our mAb dose/response relationship experiments (Fig. 6) confirm data published by Munn et al. [24] and show that rIL-2 and/or rIFNα activation increases the level of killing at a given mAb dose but does not reduce the minimal dose of mAb needed for ADCC.

Inhibition experiments performed with the mAb CLB-FcRgran.1 (Fig. 5) showed that the ADCC activity of LL cultured in medium or rIL-2 was mediated by the FcγRIII (CD16) recognized by this mAb. Surprisingly, culture of LL decreased the CD16 expression per cell (Fig. 4). These results indicate that, in contrast to the enhancement of the ADCC activity of murine lymphocytes by rIL-2, which has been suggested to be associated with an increase in the number of FcγRII+ cells [10], the mechanism of IL-2 enhancement of the ADCC of human lymphocytes is not an increase in the expression of FCγRIII or the induction of yet undefined FcγR species.

Nagler et al. [25] published data indicating that the CD16 expression on human peripheral blood NK cells is heterogeneous. They were able to discriminate three distinct CD3-negative NK cell subsets based on CD16 expression: CD16$^{neg.}$, CD16^{dim} and CD16^{bright} cells. They showed that the CD16^{dim} subset was as efficient in lysing anti-CD16-producing hybridomas as the CD16^{bright} subset, suggesting that the CD16^{dim} subset expresses sufficient CD16 molecules per cell to mediate ADCC. Furthermore, they showed that the CD16^{neg} and CD16^{dim} subsets proliferate more efficiently than the CD16^{bright} subset in response to low doses of rIL-2. This suggests that the reduction of CD16 expression we observed as a result of culture may be caused by the enhanced proliferation of the CD16^{neg} and/or CD16^{dim} subsets, thereby increasing their relative contribution to the cell population resulting in a decreased average CD16 expression.

A prominent effect of rIL-2 activation of the LL was the induction of the NKI-L16 determinant on he CD11a molecule. The anti-LFA-1 mAb NKI-L16 was shown previously to enhance LFA-1-mediated cell-cell interactions and the epitope recognized by this mAb is suggested to be expressed on activated forms of the LFA-1 molecule [20, 13]. This effect of rIL-2 suggests that activated LL may adhere more efficiently to target cells expressing ICAM-1/ICAM-2 thereby increasing their efficiency in killing these targets. The results of preliminary cluster experiments indeed show that activation with rIL-2 increased the adhesion of LL to Jiyoye target cells (data not shown). However, whether this enhanced adherence of effector cells to target cells is involved in the increased ADCC remains to be elucidated.

Our in vitro experiments suggest that the therapeutic efficacy of mAb may be enhanced by combination treatment of mAb with rIL-2 and rIFNα. Eisenthal et al. [11] showed that treatment of the murine B16 melanoma with rIL-2, rIFNα and mAb resulted in an antitumour effect on macrometastases that were resistant to treatment with rIL-2 and mAb alone.

In the experiments described in this paper we used a mAb that recognizes HLA class II antigens and this mAb

probably cannot be used in humans. However, a number of mAb of more relevant specificity have been described that indeed are able to mediate ADCC with human effector cells like the LYM-1 [7] and the CAMPATH-1 mAb [9].

The results of a clinical trial with long-term rIFNα treatment show that the treatment regimen employed caused a marked inhibition of B cell, T cell and NK cell function [34], again suggesting that rIFNα has differential effects, which are determined by the exposure times and the doses used.

The results presented in this paper indicate that clinical trials should be considered with mAb in combination with the cytokines rIL-2 and rIFNα. If in the treatment protocols the results of our in vitro experiments and those of other investigators in vivo are carefully considered, treatment with combinations of mAb, rIL-2 and rIFNα may be more effective than treatment with mAb and either cytokine alone.

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