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Evaluation of CR1 expression in neutrophils from chronic myeloid leukaemia: relationship between prognosis and cellular activity

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Summary. The expression of the complement receptor CR1 has been evaluated using an immunoalkaline phosphatase staining method on peripheral blood neutrophils and granulocyte precursors from 22 patients with chronic myeloid leukaemia (CML) and 15 healthy subjects. The immunocytochemical labelling pattern of CR1 was evaluated semiquantitatively on cell smears using three different anti-CR1 monoclonal antibodies. The scoring method showed that seven patients with CML had a marked reduction in CR1 expression which did not change with *in vitro* stimulation of neutrophils with phorbol-myristate-acetate (PMA) whereas control cells responded to PMA, increasing the receptor level two-fold. In addition, functional analysis of neutrophils with low CR1 expression from CML patients showed a very low cytolytic activity against K562 tumour target, suggesting a relationship between the cellular content of CR1 and neutrophil tumouricidal activity. The involvement of CR1 in neutrophil-mediated lysis is consistent with complete lack of tumour toxicity following receptor neutralization by anti-CR1 monoclonal antibodies.

Interferon therapy improved CR1 expression and the cytolytic response of neutrophils in three out of five CML patients with a moderately low CR1 score. CML patients non-responding to interferon therapy and those with a very low CR1 score, independent of the clinical stage, progressed more rapidly into the advanced clinical stage and blastic crisis.

Several neutrophil functions, including chemotaxis, phagocytosis and tumour cell cytotoxicity, are mediated by membrane receptors, whose biological role has not yet been fully established (Malech & Gallin, 1987; Hemler, 1988). The complement receptor, CR1, is a biologically active molecule present in a variety of cell types including neutrophils, monocytes, eosinophils, erythrocytes, B-lymphocytes, a subset of T-lymphocytes and other non-haematopoietic cells (Fearon, 1979; McMichael, 1987; Lambris, 1988). CR1 (CD35) recognizes specifically the C3b fragment of C3 and binds C3bi, C3c and C4 fragments with lower affinity. The biological effects of the interaction between CR1 and C3 fragments are numerous and depend on both the nature of ligands and the cell type involved.

Neutrophils in whole blood express relatively few CR1 antigens although the receptor level can be up-regulated. When neutrophils are activated with appropriate stimuli, such as formyl-methionyl-leucyl-phenylalanine (FMLP), C5a, phorbol-myristate-acetate (PMA), tumour necrosis factor (TNF), ionophore A23187, or interferon (IFN), the surface expression of CR1 receptor rapidly increases (O'Shea *et al.* 1985; Malbran *et al.* 1988; Castoldi & Lanza, 1989). This phenomenon might be due to the translocation of CR1 to the cell surface from the latent pool stored in the cytoplasmatic granules. In this study we examined, using monoclonal antibodies, the potential role of the CR1 receptor in triggering and/or modulating cell functions, such as chemotaxis, post-phagocytic superoxide production, granule enzyme release and cytotoxicity in neutrophils from healthy subjects and patients with CML. We present evidence which suggests a relationship between the cytolytic activity and CR1 expression.

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MATERIALS AND METHODS

Patients. 22 patients with chronic myeloid leukaemia (CML) were examined. The patients were diagnosed according to standard morphological, cytochemical and clinical criteria. Peripheral blood samples were analysed at diagnosis

and at monthly intervals after commencement of chemotherapy or alpha-interferon (α-IFN) treatment. The follow-up study lasted 18 months. 17 patients received cytostatic drugs (hydroxyurea or busulphan) and five received recombinant α-2a IFN (Roferon-Roche) (9–12 MU daily) for at least 18 months. All had splenomegaly, Ph^1 chromosome and a reduced leucocyte alkaline phosphatase score. In several patients the presence of the Bcr-Abl chimaeric gene was demonstrated at molecular level. Using the classification of Tura *et al* (1981) the patients were grouped as follows: 10 with low risk; seven intermediate risk, and five high risk.

Controls. 15 healthy subjects from the technical staff of the haematology laboratory were chosen as the control group.

Immunoalkaline phosphatase staining technique. The expression of CR1 was evaluated using an APAAP immunoalkaline phosphatase method in peripheral blood neutrophils and myeloid precursors as first described by Cordell *et al* (1984). Briefly, the peripheral blood slides and cytocentrifuge preparation from resting and activated neutrophils were fixed in acetone, rinsed in tris buffer saline and then incubated with monoclonal antibodies (MoAb) followed by a two-step incubation with the rabbit anti-mouse immunoglobulins and the APAAP complexes (Dakopatts). Finally, slides were counterstained in Meyer's haemalum solution. The immunocytochemical staining patterns of CR1 were evaluated semiquantitatively in cell smears by testing three different anti-CR1 monoclonal antibodies: J3D3, CB04, J8B10. The specificity of these MoAbs for the CR1 receptor in neutrophils and other blood cells was established at the Third and the Fourth International Conference on Human Leukocyte Differentiation Antigens (McMichael, 1987; Malavasi *et al*, 1990). The MoAbs were shown to identify three different epitopes on the neutrophil surface membrane. The scoring method was as follows: absence of granules = 0; a few scattered granules in the cytoplasmic membrane or directly within the cell cytoplasm = 1; a moderate number of cytoplasmic or membrane granules = 2; strongly positive with numerous granules = 3; very strongly positive with a great number of coarse granules generally obscuring the nucleus = 4. The sum of 100 cells gives a score with a possible range from 0 to 400.

Monoclonal antibodies. J3D3 Moab, IgG1 subclass, and J8B10 MoAb, IgM class, were kindly provided by Dr Kazatchkine, Paris (1 mg/ml), and the CB04 MoAb, IgM class, by Dr Malavasi, Turin (1 mg/ml). The CR1 expression in the patients was tested using the optimal dilution found for each MoAb during preliminary tests in the control group (J3D3 1 in 10; CB04 1 in 5; J8B10 1 in 10).

The effect of the MoAbs was investigated by incubating 1×10^6 neutrophils in Krebs-Ringer phosphate containing glucose (0·1%) (KRPG) (pH 7·4) for 30 min at 20°C, in the presence of an antibody concentration from the optimal dilution found as above for each MoAb to a dilution of 1 : 100. Parallel experiments were run as negative controls and were as follows: (1) buffer alone; (2) R1.30, an anti-β_2 microglobulin, IgG2b class (Baricordi *et al*, 1985); (3) GF 26.7.3, IgG2b class (Traniello *et al*, 1988); (4) MF 25.1, IgG1 class (Traniello *et al*, 1988); (5) Leu11, an anti-FcIII receptor (Becton Dickinson).

Cells. Neutrophils were purified using the standard procedure of dextran (Pharmacia) sedimentation, followed by centrifugation in lymphocyte separation medium (Flow S.P.A.) and hypotonic lysis of erythrocytes (Lanza *et al*, 1987; Spisani *et al*, 1989a). The cells (95–98% pure neutrophils and >98% viable as determined by trypan blue exclusion) were washed and resuspended in KRPG at a concentration of 50×10^6 cells/ml.

Human leukaemic K562 cells were used as target for neutrophil cytotoxicity. Culture medium consisted of α-medium (Gibco) supplemented with 10% fetal calf serum (Flow S.P.A.) in 5% CO_2, 80% humidity. K562 cells were labelled with 1·85–3·70 MBq of $Na_2{}^{51}CRO_4$ (Sorin) for 1 h at 37°C. After incubation the target cells were washed three times with the culture medium and resuspended in KRPG.

Granule enzyme release. $2{\cdot}5 \times 10^6$ neutrophils (with or without treatment with MoAbs) in 0·5 ml of KRPG were activated by PMA (Sigma) (0·1 μg/ml) or by FMLP (Sigma) (10^{-6} M) for 15 min. The cells were incubated with 5 μg/ml cytochalasin B (Sigma) for 15 min prior to the activation by FMLP. The activity of released β-glucuronidase was determined after incubation for 3 h with phenolphthalein-glucuronate (Sigma) as substrate. The lysozyme release was quantitated nephelometrically by the rate of lysis of a cell wall suspension of *Micrococcus lysodeicticus* (Sigma) (Spisani *et al*, 1989b). Enzyme release was expressed as a net percentage of total enzyme content released by 0·1% Triton X-100.

Cell locomotion. Chemotaxis assay was performed in a modified Boyden chamber, using Wilkinson's technique (Wilkinson, 1974) and evaluated by the method of leading front (Zigmond & Hirsch, 1973). The chemoattractants used were FMLP (10^{-8} M), casein 'Hammarsten' (Merck) (1 mg/ml) and a pool of fresh sera (15%) activated by 25 μg/ml lypopolysaccharide (LPS, *E. coli*, 0127, B8 D.I.D.).

Superoxide production was monitored continuously in a temperature controlled spectrophotometer by the reduction of ferricytochrome c (Sigma) inhibited by superoxide dismutase, as described previously (Traniello *et al*, 1988). Briefly, in this assay both sample cuvettes contained 1×10^6 neutrophils (with and without treatment with MoAbs), 100 nmol cytochrome c (Sigma) and KRPG buffer in a total volume of 1 ml. Superoxide production was calculated from the change in linear rate of absorbance using a millimolar extinction coefficient for cytochrome c of 15·5 at 550 nm. The results were expressed as net nmoles of O_2^- calculated from the difference between stimulated neutrophils and resting neutrophils alone. Aliquots of the cells were activated by PMA (0·1 μg/ml) for 10 min, by FMLP (10^{-6} M) for 5 min or by opsonized zymosan (Sigma) for 15 min. Neutrophils were incubated with 5 μg/ml cytochalasin B for 5 min prior to activation by FMLP or opsonized zymosan.

Cytotoxicity. Neutrophil cytotoxicity was studied by ^{51}Cr release assay using human erythroleukaemic K562 cells as the target (Gavioli *et al*, 1990; Lanza *et al*, 1988). Briefly, $0{\cdot}25 \times 10^6$ K562 cells prelabelled with 1·85–3·70 MBq of $Na_2{}^{51}CRO_4$ were mixed with $2{\cdot}5 \times 10^6$ neutrophils (with and without treatment with MoAbs). The cytolytic reaction was initiated by the addition of appropriate concentrations of PMA (2·5–10 ng/ml), followed by centrifugation in order to

Table I. CR1 expression and neutrophil function. Neutrophils from 15 controls, 10 CML patients with normal score and five CML patients with low score and seven CML patients with a very low score were studied. CR1 score was evaluated by treating 1×10^6 neutrophils for 30 min at 37°C with and without 10 ng/ml of PMA, and subsequently incubated with MoAb J8B10. Superoxide production was detected by activating 2×10^6 neutrophils with opsonized zymosan (OZ) for 15 min at 37°C. Results are expressed as net nmoles. Cytotoxicity was detected by activating $2{\cdot}5 \times 10^6$ neutrophils with 10 ng/ml of PMA for 2 h at 37°C in the presence of $2{\cdot}5 \times 10^5$ K562 target cells. Results are expressed as net percentage of ^{51}Cr release.

Subjects	CR1 score in resting cells	CR1 score in PMA-activated cells	Superoxide production by OZ	Cytotoxicity against K562 cells
Control ($n=15$, ±SD)	213±5	380±10	43±13	44±11
CML patients with normal CR1 score ($n=10$, ±SD)	202±12	384±13	45±9	40±11
CML patients with a moderately low ($P<0{\cdot}05$) CR1 score (range 170–130; $n=5$)				
A	166	189	45	32
B	140	ND	38	26
C	136	146	ND	23
D	152	167	ND	24
E	138	ND	48	ND
CML patients with a very low ($P<0{\cdot}001$) CR1 score (<130; $n=7$)				
F	126	130	39	4
G	128	ND	35	19
H	110	114	10	4
I	118	115	ND	13
L	116	120	49	26
M	110	122	44	17
N	76	83	41	6

facilitate effector–target cell contact. After 2 h at 37°C, the net percentage of ^{51}Cr release was measured in the cell free supernatant and calculated using the formula $(E-S)/(T-S) \times 100$, where E represents the cpm released in the presence of the effector cells, T the cpm released after lysing target cells with 0·2% Triton X-100 and S the cpm spontaneously released by target cells in the absence of effector cells (which did not exceed 10%). At the doses used no reagent induced spontaneous ^{51}Cr release. Assays were performed in duplicate.

Statistical analysis. Differences in the CR1 score in the CML patients were evaluated using the Wilcoxon test; Wilcoxon matched-pairs signed ranks test was used for analysing CR1 score changes during interferon-treatment.

RESULTS

(a) *Immunocytochemical analysis of neutrophil CR1 receptor*

Table I shows the expression of CR1 receptor using the reference antibody J8B10 in neutrophils from patients with CML and from control subjects.

In the control adult group, the CR1 expression measured by immunocytochemical labelling was very consistent, mean score (range 198–220) 213±5 SD. The large majority of the cells tested (at least 100 per subject) had a score of 2, only occasionally 1 or 3; no cells had strong reactivity (4) or absence of expression (0). In neutrophils the staining had a diffuse and fine granular distribution in both the cytoplasm

Table II. Clinical profile and CR1 score exhibited by CML patients with a very low CR1 score. Seven CML patients were analysed at diagnosis and after 18 months. Clinical stages were evaluated using the classification of Tura. CR1 score was evaluated in the resting neutrophils as described in Table I.

	At diagnosis		After 18 months of observation	
Patient	Clinical stage	CR1 score in resting cells	Clinical status*	CR1 score in resting cells
F	II	126	BC	64
G	III	128	BC	92
H	I	110	PR	112
I	II	118	BC	48
L	II	116	PR	122
M	III	110	BC	44
N	III	76	BC	55

* BC = Blastic crisis; PR = Partial remission.

Table III. Effect of α-interferon therapy on CR1 expression and cytotoxicity of CML neutrophils. Five CML patients were analysed before starting α-IFN therapy and again after 8 months of treatment. Clinical stages were evaluated using the classification of Tura. CR1 score was evaluated in the resting neutrophils as described in Table I. Cytotoxicity was measured and the values are expressed as described in Table I. The criteria for evaluating the response to IFN were those described by Talpaz (Talpaz *et al.* 1986).

		Before treatment		After 8 months of alpha-IFN treatment		
Patient	Clinical stage	CR1 score in resting cells	Cytotoxicity	CR1 score in resting cells	Cytotoxicity	Response to IFN*
B	II	140	26	235	40	CR
C	II	136	23	240	35	PR
D	I	152	24	248	38	PR
H	I	110	4	135	10	SD
I	II	118	13	126	15	SD

* SD = Stable disease; PR = Partial remission; CR = Complete remission.

and plasma membrane (Fig 1A). Analyses of the bone marrow aspirate smears showed that CR1 expression was detectable from the promyelocyte stage with the strongest staining intensity (mean score of 3) in the myelocytic stage. In seven of 22 with CML the CR1 was extremely low (76–130) and in five moderately low (130–170). In the remaining 10 patients the score was in the normal range. There was a wide range of CR1 expression in the individual neutrophils in 18 cases (score 0–4) and there were qualitative abnormalities in CR1 staining including complete disappearance of antibodies in a variable number of neutrophils in each patient (5–32%) Fig 1B, C. Similarly, myeloid precursors showed severe quantitative and qualitative alterations in the CR1 immunocytochemical staining.

Neutrophils from the controls and the 10 CML patients with normal CR1 score responded well to PMA stimulation, increasing the level of surface receptor up to two-fold. In contrast, neutrophils from the 12 patients with a low CR1 score (< 170) did not respond to PMA treatment at the concentrations tested (10–100 ng/ml) (Table I).

(b) *Clinical characteristics*

A relationship was observed between the decrease in CR1 score and the stages of increasing severity laid down by Sokal *et al* (1984) and Tura *et al* (1981). Follow-up studies clearly demonstrated that patients with the lowest CR1 score went into blastic crisis with the highest frequency (Table II). Conversely, all the patients (five) who had developed blastic crisis had a very low CR1 immunocytochemical score (below 100). In five patients (three with a score below 170 and two below 130) undergoing α-IFN treatment a progressive increase in the neutrophil CR1 score and a complete recovery of normal CR1 expression was observed within 8 months in three out of the five patients (Table III). This increase in CR1 score was found to be associated with an improvement in the disease (fall in WBC count below 10×10^9/l; reduction of splenomegaly in one patient and disappearance in two; recovery to near normal bone marrow haematopoiesis; decrease in the number of Ph^1 positive marrow cells). Two of five patients who had received α-IFN did not increase CR1 expression and were considered clinically non-responsive to α-IFN. Cytostatic treatment became necessary to control the disease. The criteria of response were those established by Talpaz *et al* (1986).

(c) *Functional analysis of neutrophils*

Chemotaxis, superoxide anion production, granular enzyme release and tumoricidal activity were examined in order to evaluate a functional difference in neutrophils with low CR1 expression. In addition, neutrophils were triggered with opsonized zymosan in order to study the involvement of this antigen in opsonin-dependent activation of phagocytosis and post-phagocytic superoxide production.

The results showed that cell motility induced by FMLP and LPS-activated serum and degranulation induced by soluble stimuli, did not differ from the control (not shown). Similarly, a normal amount of superoxide anion was generated from neutrophils stimulated with PMA and opsonized zymosan (Table I), confirming that opsonins other than C3b were involved in the phagocytic process (Malavasi *et al.* 1990). In contrast, when neutrophils with low score of CR1 were activated with PMA and then exposed to K562 erythroleukaemic cell line, the cytotoxicity was dramatically depressed (Table I), suggesting a correlation between low surface expression of CR1 and defective cytolysis. It is worth noting that in these neutrophils there was a complete inability to increase surface CR1 receptor by PMA treatment, because of the failure of granular storage of the antigen, as demonstrated by immunocytochemical assays.

In order to find an explanation for the reduced cytolysis, neutrophils from 15 controls, from 10 CML patients with normal CR1 score and seven CML with very low content of antigen (< 130) were labelled with the specific anti-CR1 J8B10 MoAb before testing the cell function. The data demonstrated that CR1 neutralization did not interfere with the function of chemotaxis, superoxide generation and degranulation (not shown), but greatly affected the lytic activity towards K562 target cells. In control and CML

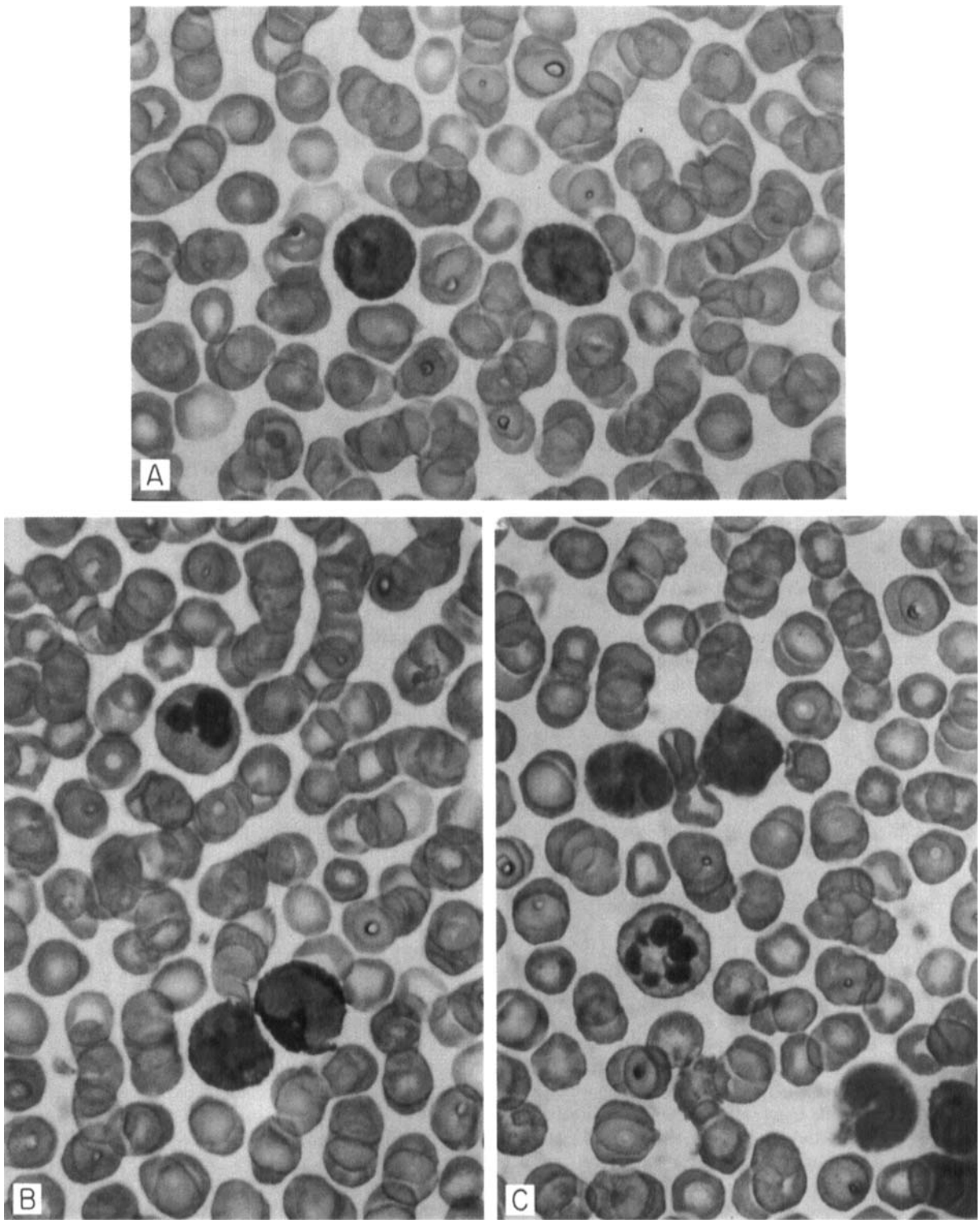

Fig 1. Neutrophil CR1 expression from a normal subject (A), a patient with CML, showing a lack of reactivity in a hyposegmented cell (B), or in a hypersegmented neutrophil (C). Peripheral blood smear. APAAP technique. J8B10 monoclonal antibody. Magnification × 1000.

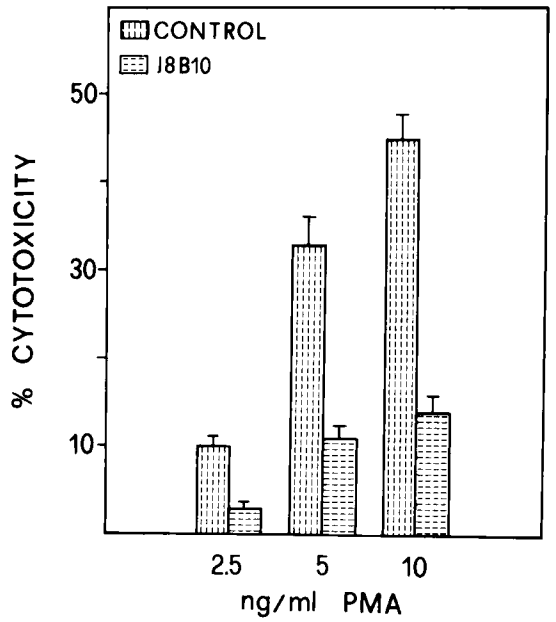

Fig 2. Inhibition of neutrophil-mediated cytotoxicity by MoAb J8B10 anti-CR1. Neutrophils from 15 control subjects were incubated with MoAb J8B10 and with buffer alone. Neutrophils were then assayed for cytotoxicity in an effector-to-target ratio of 10:1. The lytic reaction was then initiated by the addition of varying concentrations of PMA (2·5–10 ng/ml). After 2 h at 37°C the net percentage ±SD of ^{51}Cr release was evaluated. The basal level of target lysis was less than 10%; all experiments were done in duplicate.

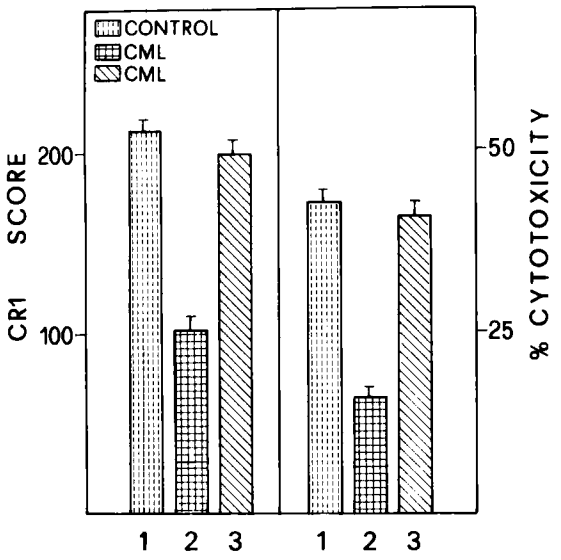

Fig 3. Relationship between neutrophil cytotoxicity and CR1 expression. Panel A: CR1 expression of resting neutrophils derived from 15 controls (1), seven CML patients with very low CR1 level (2) and and 10 CML patients with normal CR1 level (3). Panel B: Cytolytic activity of neutrophils derived from 15 controls (1), seven CML patients with very low CR1 level (2) and 10 CML patients with normal CR1 level (3) was detected as described in Table I. Data are means ±SD of 7–15 experiments performed in duplicate.

neutrophils expressing a normal amount of receptor, treatment with the specific MoAb almost completely abolished the cytotoxicity. The effect was evident at concentrations of the cell activator, PMA, from 2·5 to 10 ng/ml (Fig 2). Similar results were obtained using anti-CR1 MoAbs from the three different laboratories. Neutrophils with low CR1 score from CML patients who had received α-IFN were assayed for cytotoxicity within 8 months of therapy. As shown in Table III, in the three patients clinically responsive to α-IFN, the increase in CR1 expression was accompanied by an increase in neutrophil lytic activity to normal levels. On the other hand, in the two unresponsive patients neither the CR1 level nor the cytotoxicity were increased. This study has shown that neutrophils with low surface expression were also defective in cellular content of this CR1 antigen and it appears that there is a relationship between lytic activity and the CR1 score (Fig 3).

DISCUSSION

In this report, using an immunocytochemical method and the MoAbs against CR1, we show that patients with CML have both qualitative and quantitative alterations in the receptor expression. Most of them, including those with a CR1 score within the normal range, have a very varied expression of CR1 in the different myeloid elements. This supports the theory that, in CML patients, neutrophils are derived from a pathologic clone originating from a stem cell. The association between a low CR1 expression and an advanced clinical stage, together with the evidence that patients with decreased antibody activity have a higher risk of developing a blastic crisis, suggest an unfavourable prognosis when there is loss of this receptor in CML neutrophils. This is further confirmed by the finding that all the patients who had developed blastic crisis had a very low CR1 score. However, we cannot exclude *a priori* that the loss of CR1 expression in the myeloid population of CML patients may be a secondary event to the development of acute leukaemia.

The increase in the expression of the membrane structure upon activation is a general phenomenon (O'Shea *et al*, 1985; Malbran *et al*, 1988; Castoldi & Lanza, 1989). PMA markedly enhanced the extent of surface CR1 receptor in neutrophils from both controls and CML patients with normal CR1 score. CML neutrophils deficient in this antigen, in contrast, lack the ability to respond to PMA stimulation (Gavioli *et al*, 1989) and this may indicate a low CR1 content not only on the cell surface, but also in the granular pool.

CR1 antigen is known to mediate the binding of C3b coated particles to neutrophils and to enhance phagocytosis together with a respiratory burst. Nevertheless, the superoxide production induced by opsonized zymosan is not affected by the low content of CR1 receptor. This clearly demonstrates that immunoglobulin(s) and other fragments of C3 are also involved in the opsonization of zymosan particles. Neutrophil motility and degranulation are similarly unaffected by the low surface expression of CR1, also confirmed by the MoAb neutralization of this antigen in neutrophils with a normal score. Our data, on the contrary, demonstrate that CR1

antigen has a specific role in lytic activity towards tumour cells from PMA-activated neutrophils. CML patients with low expression and content of CR1 exhibit a very low neutrophil-mediated tumouricidal activity. This is consistent with the drop in lytic activity towards K562 target cells when neutrophils from control and CML patients with normal CR1 score are neutralized by specific anti-CR1 MoAb.

Support for the involvement of CR1 in neutrophil cytotoxicity is shown by the fact that three of five CML patie. ts respond to α-IFN therapy. The neutrophils of these patients when treated with α-IFN showed a progressive increase in the CR1 score accompanied by an increase in cytotoxicity, demonstrating the striking effect *in vivo* of this lymphokine on CR1 receptor. The other two patients with very low CR1 expression who did not respond to α-IFN therapy are an additional evidence of the severity of the disease in these subjects.

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