British Journal of Haematology, 2002, **117**, 821–827

Evidence that continued remission in patients treated for acute leukaemia is dependent upon autologous natural killer cells

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Received 19 July 2001; accepted for publication 17 December 2001

Summary. Although it has been known for more than 40 years that allogeneic immune responses cure leukaemias after bone marrow transplantation, autologous leukaemia-specific immunity remains controversial and its impact upon survival has not been established. Here we have tested 25 patients with *de novo* acute leukaemias, while in remission at completion of their anti-leukaemia therapy, for evidence of autologous cytolytic immunity to their leukaemic cells taken and cryopreserved at disease presentation. We have measured this degree of cell-mediated cytotoxicity *in vitro* and termed it 'leukaemia cytolytic activity' (LCA). Patients whose disease ultimately relapsed had significantly lower LCA than those who remained in remission beyond 2 years ($P < 0{\cdot}001$); the absence of LCA when in remission predicted subsequent relapse within 2 years with a sensitivity of 100% and specificity of 77%. LCA was mediated *in vitro* by $CD56^+/CD8\alpha^+/CD3^-$ natural killer cells. We propose that it is this immune response, rather than the chemotherapy *per se*, which is responsible for continued remission and that measurement of LCA in patients at completion of therapy may be used as an indicator of risk of subsequent relapse. Patients lacking this response will require further treatment, either with an allogeneic donor transplant or an alternative immunotherapeutic strategy.

Keywords: cell-mediated cytotoxicity, immunotherapy, CD56, CD8, CD69.

It was shown as early as 1956 that an allogeneic immune response to residual leukaemic cells, rather than the conditioning with radiotherapy, cures leukaemia in a murine model (Barnes *et al*, 1956). Immune-mediated cure of residual leukaemia in the clinical setting has been suggested by observations of an increased risk of leukaemia relapse after T cell-depleted allogeneic bone marrow transplantation (BMT) when compared with non-manipulated BMT and an association between graft-versus-host disease (GvHD) and disease-free survival (Horowitz *et al*, 1990). Furthermore, an increased incidence of leukaemia relapse has been reported after aggressive GvHD prophylaxis with cyclosporin A (Bacigalupo *et al*, 1991) or lymphocyte depletion (Horowitz *et al*, 1990). The most convincing direct evidence of allogeneic anti-leukaemia activity has been provided by the long-lasting remissions following donor leucocyte infusions (DLI) to treat relapse of chronic myeloid leukaemia (CML) after allogeneic stem cell transplantation (Kolb *et al*, 1990). Given that the donor immune response to leukaemia is paramount in the cure of patients after allogeneic stem cell transplant who have received extensive chemo- and radiotherapy, we were interested in the role of autologous immunity in the control or eradication of leukaemia, with or without autologous stem cell transplantation.

We, and others, have recently reported the detection of leukaemia-specific autologous cytolytic immunity to presentation leukaemic cells. We have termed this 'leukaemia cytolytic activity' (LCA) and report it as a percentage lysis of leukaemic blasts in an *in vitro* assay. In recipients of autologous bone marrow transplants for acute leukaemias we demonstrated LCA during remission which was lost in patients who subsequently relapsed. The second of these patients recovered high levels of LCA after further chemotherapy and interferon-alpha treatment. Both she and the patient who maintained LCA throughout follow-up remain in complete molecular remission more than 6 years later (Lowdell *et al*, 1997). In addition to these findings, two groups from the MD Anderson Cancer Center have

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described autologous cell-mediated immunity to chronic leukaemias. The first report was of autologous natural killer (NK) cell responses to presentation chronic lymphocytic leukaemia (CLL) (Robertson *et al*, 1996), while the most recent described the presence of $CD8^+$ T cells in chronic myeloid leukaemia (CML) patients after interferon-alpha treatment which were specific for a proteinase III epitope, a molecule hyper-expressed on CML blasts (Molldrem *et al*, 2000).

In this study, we have monitored patients in remission after either chemotherapy or autologous BMT (aBMT). We initially determined a potential cut-off for a protective level of leukaemia cytolytic activity (LCA) from the maximum detected LCA level of the first three patients in the study who relapsed after chemotherapy. We then prospectively measured LCA of recipients of chemotherapy with and without autologous BMT to determine the predictive value of this test for relapse, in order to assess the role of the autologous immune response in the cure of leukaemia. Our results demonstrate that chemotherapy alone or in conjunction with radiotherapy and autologous BMT is unlikely to eradicate the acute leukaemia but may de-bulk the disease sufficiently for a protective immune response to arise. This observation may have a major impact on our understanding of the treatment of leukaemia and on the design of therapeutic strategies in the future.

MATERIALS AND METHODS

Twenty-five patients were studied, the clinical details of whom are presented in Table I. Seventeen consecutive adult patients, who presented with *de novo* acute leukaemias (14 AML; three ALL) and from whom presentation leukaemia from bone marrow or peripheral blood was cryopreserved, were studied. In addition, eight children with common acute lymphoblastic leukaemia (cALL) were studied retrospectively from previously cryopreserved presentation and follow-up bone marrow and peripheral blood samples. Peripheral blood and bone marrow samples were obtained with informed consent at different time points during treatment. These samples included leukaemic blasts at the time of initial disease presentation and remission bone marrow and peripheral blood. Mononuclear cells were isolated by density gradient separation (Lymphoprep,

Table I. Patient characteristics.

Patient ID	Age at treatment	Diagnosis	Treatment	Current status (months in CR)	Lowest LCA	Highest LCA
RFH-01	18	AML M2	Cy/TBI aBMT	CR (98)	18·5	27·7
RFH-02	50	AML M4	Bu/Cy aBMT	CR (72)	13·8	30·1
RFH-03	35	AML M4	Bu/Cy aBMT	CR (58)	15·2	15·2
RFH-04	29	AML M2	Baltimore × 2; FLAG/Ida × 2	CR (58)	16·6	22·6
RFH-05	40	AML M5a	Baltimore × 2	CR (50)	17·6	18·3
CGH-01	69	AML M4	Ara C & Mitozantrone × 2	CR (51)	14·5	18·5
CGH-02	66	AML	Ara C & Mitozantrone × 3	CR (50)	11·3	15·3
CGH-03	36	AML M4	MRC AML XII × 4	CR (40)	21·0	24·0
SBH-01	12	c-ALL	UKALL XI	CR (76)	–	9·0
SBH-02	13	c-ALL	UKALL XI	CR (57)	–	74·4
SBH-03	11	c-ALL	MRC ALL 97	CR (48)	–	15·6
SBH-04	8	c-ALL	MRC ALL 97	CR (34)	0·0	0·0
SBH-05	7	c-ALL	MRC ALL 97	CR (56)	–	37·0
SBH-06	9	c-ALL	MRC ALL 97	CR (26)	–	5·3
SBH-07	7	c-ALL	MRC ALL 97	CR (28)	–	7·4
SBH-08	13	c-ALL	MRC ALL HR1	CR (27)	–	18·4
RFH-06	53	APML-M3	ADE + ATRA	CR (71)	22·4	30·9
RFH-07	27	T-ALL	Cy/TBI aBMT	Relapsed (5)/Deceased	–	6·4
RFH-08	47	AML M1	Bu/Cy aBMT	Relapsed (8)/Deceased	0·0	0·0
RFH-09	48	AML M6	Bu/Cy aBMT	Relapsed (24)/Deceased	0·0	0·0
RFH-10	72	AML M4 Ph +	Baltimore × 2	Relapsed (21)/Deceased	0·0	10·9
RFH-11	8	Pre B-ALL	UKALL; FLAG/Ida × 2	Relapsed (14)/Deceased	–	3·7
RFH-12	57	AML M0	MAE × 2; MACE × 1	Relapsed (2)/Deceased	0·0	0·0
CGH-04	56	B-ALL	MEGA III × 3	Relapsed (1)/Deceased	0·0	0·0
CGH-05	72	AML M4	Ara C & Mitozantrone × 3	Relapsed (2)/Deceased	0·0	8·2

aBMT, autologous bone marrow transplant; ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; APL, acute promyelocytic leukaemia; ADE, cytarabine (Ara-C), daunorubicin, etoposide; ATRA, all *trans* retinoic acid; Baltimore, modified timed sequential therapy (Vaughan *et al*, 1980); Bu/Cy, busulphan/cyclophosphamide; FLAG/Ida, fludarabine, Ara-C, granulocyte colony stimulating factor, Idarubicin (Deane *et al*, 1998); MAE, mitozantrone, Ara-C, etoposide; MACE, amsacrine, Ara-C, etoposide; MEGA III, cyclophosphamide, vincristine, bleomycin, methotrexate, prednisolone;

MRC ALL97, UKALL XI and MRC ALL HR1, Medical Research Council (MRC) acute lymphoblastic leukaemia trials protocols; TBI, total body irradiation; RFH, Royal Free Hospital, London; CGH, Colchester General Hospital; SBH, St Bartholomew's Hospital.

Nyegaard, Oslo, Sweden) and cryopreserved in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 50% fetal calf serum (FCS) and 10% dimethyl sulphoxide (DMSO) (Sigma, Poole, UK) in the vapour phase of liquid nitrogen (all tissue culture reagents were purchased from Gibco, Paisley, UK). After chemotherapy or aBMT heparinized blood samples were collected at regular intervals and tested immediately or cryopreserved as above for future analysis.

Cell-mediated cytotoxicity and LCA. All samples of patient peripheral blood mononuclear cells were tested for NK-cell function against the NK-sensitive erythroblastoid leukaemia cell line, K562. All samples showed normal or enhanced levels of lysis (data not shown) and were then tested for NK-cell function against autologous target cells. Cell-mediated cytotoxicity was determined by flow cytometry as previously described (Lowdell *et al*, 1997), using peripheral blood mononuclear cell samples taken at or beyond 3 months post aBMT or induction of complete remission (CR) by chemotherapy as effector cells. Autologous leukaemic and normal bone marrow mononuclear cells were labelled with the red fluorescent dye PKH-26 (Sigma) and were used as target cells. PKH-26 labelled cells showed high levels of fluorescence and could be resolved from non-labelled effector cells. The membrane dye was stable and there was no transfer to non-labelled effector cells during the assay period (data not shown).

Patient-derived peripheral blood mononuclear cells were monocyte-depleted and incubated in triplicate with labelled targets at a ratio of 10:1 for 4 h. After the incubation period, the lysis of leukaemic blasts was determined by uptake of the vital dye propidium iodide as measured by flow cytometry [FACSCAN or FACS VANTAGE, Becton Dickinson (BD), Oxford, UK]. Background target cell death was determined from cells incubated in the absence of effector cells. LCA was calculated as follows:

(Mean percentage lysis of autologous blasts
– % background lysis) – (Mean percentage lysis of autologous normal mononuclear cells
– % background lysis)

Selection of NK cells and NK cell subsets. We have previously shown that the cells activating in response to autologous leukaemic blasts in this 4 h assay are $CD56^+/CD3^-/CD8\alpha^+$ natural killer cells (Lowdell *et al*, 1997). In these studies we have been able to select specific NK-cell subsets for analysis of anti-leukaemia activity. The $CD56^+$ NK cells were positively selected by magnetic sorting (VS column, MACS, Miltenyi Biotec, Cologne, Germany) and further sorted into $CD8\alpha^+/CD3^-$ and $CD8^-/CD3^-$ fractions by flow cytometry (FACS VANTAGE, Becton Dickinson). Both fractions were > 95% pure as determined by flow cytometry. Each fraction was tested for lytic activity against leukaemic blasts using the cytotoxicity assay described above.

Measurement of cell activation. Expression of the C-type lectin CD69 on the surface of effector cells following co-incubation with leukaemic blasts was determined by flow cytometry as previously described (Craston *et al*, 1997) and its cell surface localization visualized by immunofluorescent microscopy using a Hoechst 33258 nuclear counterstain (Bainbridge & Macey, 1983). CD69 is an early activation antigen expressed on NK and T lymphocytes.

Cell activation was also assessed by flow cytometric measurement of release of intracellular calcium. Effector cells were loaded with Fluo-3 for 30 min at 37°C (Molecular Probes) and then the background fluorescence at 570 nm was measured. Having established the baseline level of free calcium, anti-CD8α chain antibody (OKT8, from the American Tyoe Culture Collection, ATCC) was added in excess to the sample and the fluorescence monitored for a further 10 min. The effect of cross-linking the CD8 molecule was measured by addition of polyclonal goat anti-murine IgG before or after the addition of OKT8. Anti-CD16 was added to parallel cultures to provide a positive control.

The final measure of cell activation by anti-CD8α antibody was a 'reverse' cytotoxicity assay against the NK-resistant murine plasmacytoma cell line, P815. These cells bear the murine Fcγ-R. Freshly isolated human NK cells were incubated with murine anti-human CD8α antibodies OKT8 (ATCC), RPA-T8 (Pharmingen, BD) or Leu2a (BD), washed to remove non-specifically bound antibody, and then incubated for 20 h with ^{51}Cr-labelled P815 cells.

RESULTS

Analysis of cytotoxicity in vitro

In an initial study of the first six patients in this prospective study, three relapsed early after completion of chemotherapy (median duration of CR 2 months). The highest individual level of LCA detected in these patients was 8·2%. The mean of the highest detected LCA for these three patients was 4·0% with a standard deviation of 4·1. We hypothesized that the LCA in CR may be predictive of relapse, so we established a theoretical predictive level for relapse of mean + 2 × standard deviation of the three early relapse patients, i.e. 12·2%.

As shown in Table I, eight of the 25 patients in this study relapsed during the study with a median duration of CR of 7 months (range 1–24). All the remaining 17 patients have maintained CR beyond 24 months (median 50 months; range 25–98). LCA was measured on multiple occasions in patients during CR after completion of treatment. The maximum LCA (LCA-max), the minimum LCA (LCA-min) and the mean LCA (LCA-mean) of the group of patients who remain in CR beyond 24 months all exceeded the respective values from the group who relapsed (Fig 1). All differences were highly significant ($P < 0·001$), which implies that this cytotoxicity index may be a predictor of outcome in these patients. Using the threshold value of 12·2% determined from the first three relapsing patients, we assessed the sensitivity and specificity of this assay by stratifying all the patients with respect to the highest recorded LCA for each

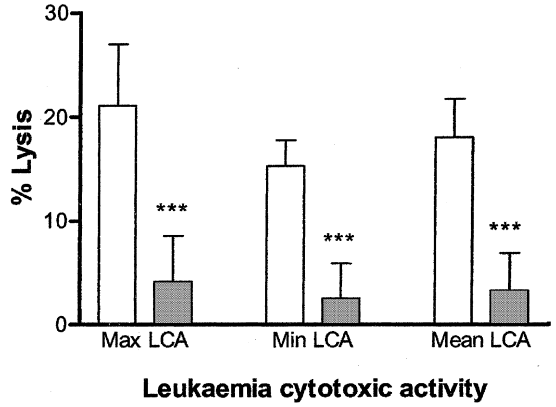

Fig 1. LCA levels of all patients with follow-up beyond 2 years during CR. CR > 2 years, open bars; relapsed, shaded bars; ***$P < 0{\cdot}01$.

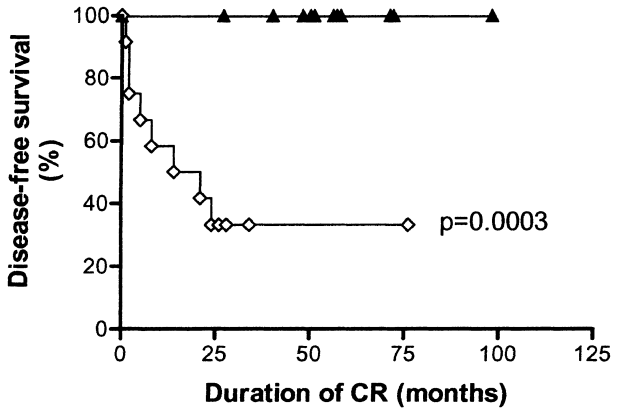

Fig 2. Prediction of disease-free survival by virtue of LCA-max activity. < 12·2% LCA, ◇; > 12·2% LCA, ▲.

patient. Of the eight relapsing patients, none had LCA > 12·2% at any time during CR. In contrast, all of the adult patients remaining in remission beyond 2 years and 50% of the paediatric cALL patients had LCA above this threshold. This gives the assay a sensitivity of prediction of relapse at 2 years of 100% and a specificity of 77%.

Figure 2 shows the respective leukaemia-free survival curves (Kaplan & Meier, 1958) of the 25 patients divided into the two groups with respect to their maximum LCA measured during CR. Patients falling above the threshold have a significantly greater probability of leukaemia-free survival ($P = 0{\cdot}0003$).

There was no significant correlation between the level of LCA-max and duration of remission in the patients who relapsed; implying that a threshold level of activity is required and that the effect is 'all or nothing' (Fig 3).

Identification of LCA effector cells in vitro

We have shown previously that the cells activating in response to autologous leukaemic blasts were $CD56^+/CD8wk^+/CD3^-$ natural killer cells and that these cells express CD69 upon incubation with the leukaemic blasts (Lowdell *et al*, 1997, 1999). We confirmed these findings in three further patients (data not shown), and in two patients we were able to isolate highly purified (> 95%) subsets of NK cells from their peripheral blood for functional testing against the presentation leukaemic blasts and remission bone marrow mononuclear cells (BMMC). In both cases the LCA activity was mediated by the $CD56^+/CD8\alpha^+$ cells and not the $CD56^+/CD8^-$ cell fraction (an example is shown in Fig 4), thus confirming the observations from the cell activation experiments described previously.

In cross-linking studies using a CD8-alpha chain-specific monoclonal antibody, we found that the molecule could induce NK-cell activation as measured by release of intracellular calcium (Fig 5A–C) and by upregulation of CD69 on the surface (data not shown), but could not activate the lytic machinery as detected by a reverse cytotoxicity assay against P815 cells (Fig 5D). In addition, cells sorted on the basis of CD8 expression were able to mediate LCA despite

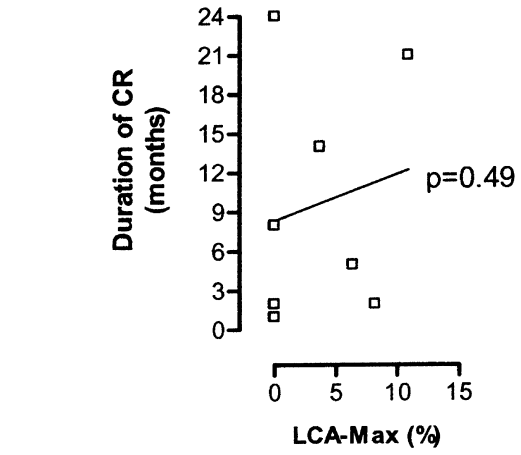

Fig 3. Correlation between LCA-max and duration of CR in patients who relapsed.

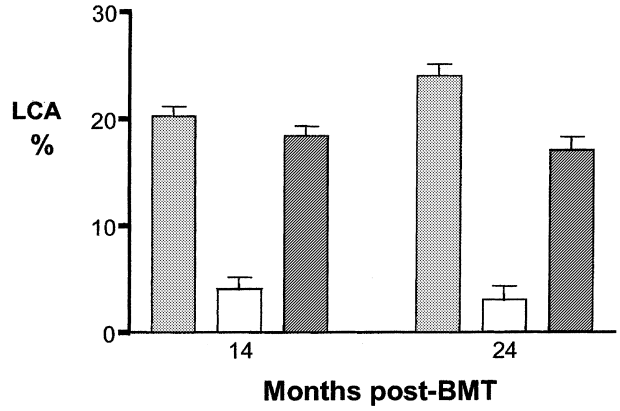

Fig 4. Leukaemia-specific cytotoxicity mediated by NK-cell subsets: non-selected, lightly shaded bars; $CD56^+/8^-$, open bars; $CD56^+/8\alpha^+$, heavily shaded bars.

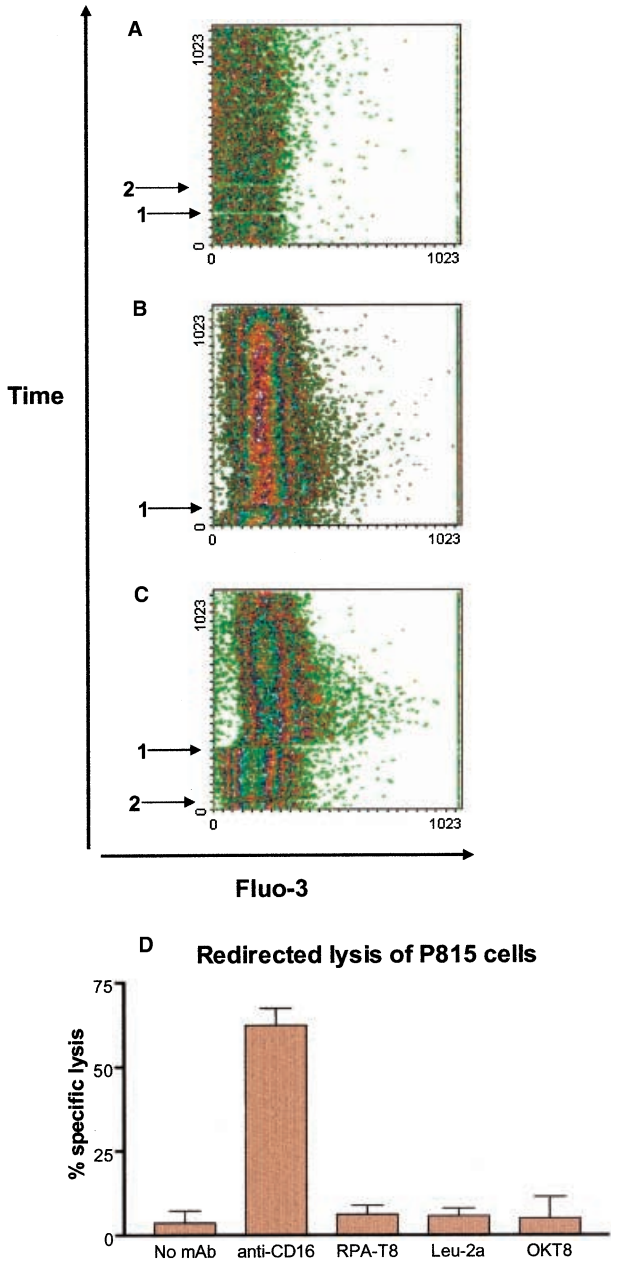

Fig 5. Intracellular calcium flux after stimulating isolated NK cells with (A) 1 – Leu-2a followed by, 2 – goat anti-murine IgG; (B) 1- OKT8; (C) 2 – goat anti-murine IgG followed by, 1 – OKT8. The increasing fluorescence of Fluo-3 after the addition of OKT8 antibody is indicative of the release of free cytosolic $Ca2^{+}$. (D) Graph showing the effect of pre-incubation with anti-CD8α monoclonal antibodies or anti-CD16 (positive control) on the reverse cytotoxicity of P815 cells.

continued binding of the anti-CD8 monoclonal antibody. Effector:target cell conjugation led to the induction of CD69 expression on the effector cells and, intriguingly, capping of the CD69 molecule at the point of effector:target cell conjugation (Fig 6). This was not due to binding of the anti-CD69 antibody to the FcγR on the AML blasts, as

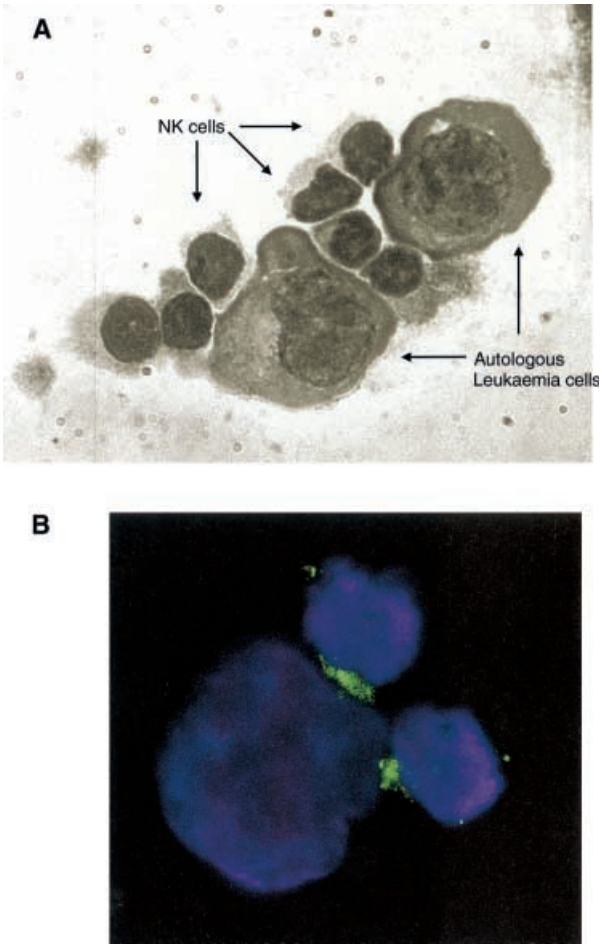

Fig 6. (A) Photomicrograph of NK/AML conjugates after 60 min co-incubation. (B) Photomicrograph of CD69 expression (green) on NK cells conjugated to autologous AML blasts counterstained with the blue nuclear dye H33258.

parallel studies with anti-CD56 did not show the same capping phenomenon (data not shown).

NK-mediated leukaemia cytolytic activity is non-major histocompatibility complex (MHC) restricted

PBMC samples from five patients with known autologous LCA were tested for their ability to lyse AML blasts from donor patients with different French–American–British (FAB) types. These data are presented in Fig 7 and show that the cytolytic activity was not restricted to autologous blasts but could be measured against allogeneic blasts of the same or different FAB type (Bennett *et al*, 1976). Interestingly, AML M4 cells from one patient were resistant to lysis by all the donor NK cells. These blasts were also resistant to lysis by autologous NK cells.

DISCUSSION

The graft-versus-leukaemia response to residual disease after related or non-related donor stem cell transplantation

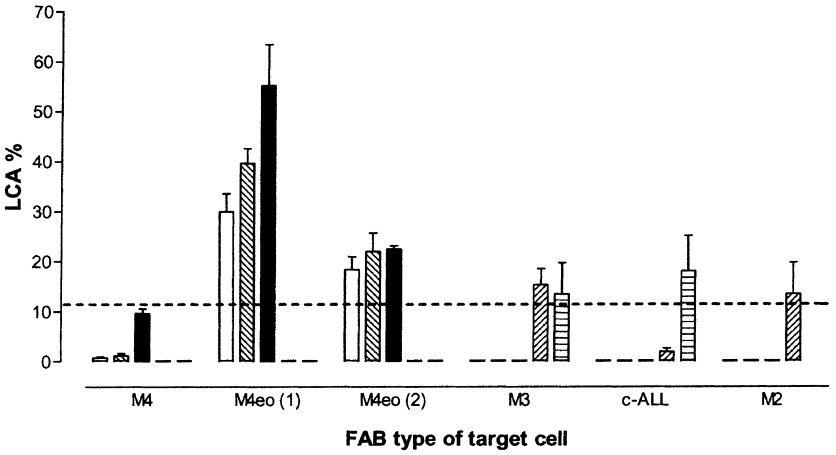

Fig 7. Non-MHC restriction of LCA activity. NK effector cells derived from five patients with documented autologous LCA whose leukaemias had been of the following FAB-types: AML M4eo CGH-03, black bars; APML M3 RFH-06, right cross-hatched bars; AML M2 RFH-01, left cross-hatched bars; not tested, __; AML M4eo RFH-02, open bars; AML M2 RFH-04, horizontally hatched bars; were tested for lytic activity against a variety of acute leukaemias from allogeneic donors. The FAB type of the allogeneic target leukaemic cells is shown on the *x*-axis. The dashed line indicates the LCA cut-off associated with protection from relapse as described in Fig 2.

is well established and the success of therapeutic donor leucocyte infusions in the treatment of relapse has been cited as 'proof-of-principle' (Horowitz *et al*, 1990; Kolb *et al*, 1990; Slavin *et al*, 1995). In contrast, the concept of spontaneous autologous immune responsiveness to leukaemic blasts remains speculative. We have previously reported an association between the detection of LCA and leukaemia-free survival in patients after autologous BMT (Lowdell *et al*, 1997), but were not able at that time to determine whether the development of LCA activity was the cause of the prolonged survival or an effect of the process of autologous transplantation. However, in an extensive study of patients with a variety of acute leukaemias treated by chemotherapy alone, it was shown that the levels of NK-cell activity against the NK-sensitive cell line K562 increased when the patients were in remission and, in all 15 patients studied longitudinally, a reduction in NK activity to pre-remission levels was associated with relapse within 10 weeks (Tratkiewicz & Szer, 1990). Those data suggested that functional NK responses may be important in the control of residual leukaemic cells but there was no direct evidence that NK cells could lyse autologous leukaemic cells. In contrast, a group using a similar dye-exclusion assay for cell-mediated killing, as used here, demonstrated specific lysis of autologous CLL cells in three out of five patients after chemotherapy alone (Robertson *et al*, 1996). No data were presented on the relationship between this activity and survival.

The highly significant difference in disease-free survival between the patient groups in this study, divided by virtue of their LCA, suggests that these cells mediate an important anti-leukaemic effect. Furthermore, the lack of a relationship between duration of CR and level of LCA-mean in the relapsing patients implies that a minimum threshold of LCA is required over a period of time to eradicate or control residual leukaemic progenitors. If these leukaemia-reactive NK cells simply controlled the minimal disease then one might imagine that patients with intermediate levels of activity might relapse later than those with little or no detectable LCA, but this is not the case.

Our previous study reported restriction of the NK cell-mediated LCA to the $CD56^+/CD8\alpha^+/CD3^-$ subset (Lowdell *et al*, 1997) and we have now confirmed this with sorted NK-cell subpopulations. The role of the CD8α chain in the conjugation/cytotoxic process is intriguing as, while able to activate the NK cells, the signal is clearly insufficient to initiate the lytic process. The fact that cells sorted on the basis of CD8 expression and thus carrying anti-CD8 antibodies were capable of LCA suggests that either the molecule is not itself involved in the cellular response or that an alternative epitope to that which is bound by the OKT8 antibody is involved. Studies using antibodies reactive with different epitopes of the CD8α chain have been uninformative.

The recognition of tumour cells by NK cells is an area of active research for a number of groups and, although it has been shown that the induction of CD69 expression on NK cells parallels the acquisition of lytic activity (Lanier *et al*, 1988), this is the first evidence of co-localization of this molecule at the point of contact between effector and target cells in an autologous system. The ligand for CD69 is unknown but cross-linking of CD69 on NK cells can lead to activation and re-directed lysis in *in vitro* studies (Cebrian *et al*, 1988). CD69 is a type II integral membrane protein belonging to the family of C-type lectin receptors and as such is closely related to the members of the NKR-P1 family of NK-cell receptors. This group of NK receptors was first described on rat NK cells where they are involved in activation of cytotoxicity (Chambers *et al*, 1989). Intriguingly, the human homologue, NKR-P1A, is expressed only on a minor subset of NK cells and some T cells, and is an inhibitory receptor (Lanier *et al*, 1994).

The leukaemic blasts studied here consistently showed normal levels of MHC class I expression and, we assumed, provided appropriate MHC-restricted inhibition signals to autologous NK cells. This would suggest that an activation signal is being received by the NK cells and we hypothesize that this is through CD69, although the ligand on the leukaemic blast remains unknown.

Given the fact that LCA was mediated by NK cells in all cases, it was unsurprising that the response was shown to be non-MHC restricted. One of the leukaemias appeared resistant to NK-mediated lysis irrespective of the donor cells

tested and this patient relapsed rapidly after induction chemotherapy. The mechanism of evasion of LCA could not be investigated in these cells because of the lack of primary tissue, but it is possible that the leukaemic blasts failed to bind perforins secreted by the NK cells as reported recently (Lehman *et al*, 2000), raising the possibility that absence of LCA may not solely be a function of the patient's immune system but of that of the leukaemic blasts.

We believe that the results presented here establish a central role for innate immunity in the clearance or control of residual leukaemia after chemotherapy. These data may have important implications for the selection of patients who would benefit from allogeneic stem cell transplantation in first clinical remission and in the design of immunotherapeutic treatment strategies.

ACKNOWLEDGMENTS

We would like to thank Dr Atul Mehta and other members of the Clinical Haematology Team at the Royal Free Hospital for provision of clinical material. We are grateful to Professor Martin Glennie for provision of the P815 cell line. This work was supported by the Leukaemia Research Fund (R.C., M.W.L.), the Association for International Cancer Research (E.O.) and by the Foundation for Children with Leukaemia (M.W.L.). It was undertaken by the Royal Free Hampstead NHS Trust which received a proportion of its funding from the NHS Executive; the views expressed in this publication are those of the authors and not necessarily those of the NHS Executive.

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