Variable detection of myeloid antigens in childhood acute lymphoblastic leukaemia

M R Howard, L Thomas, M M Reid

Abstract

Aims—To determine whether the use of different sources of anti-CD13 and anti-CD33 monoclonal antibodies leads to discrepant results in childhood acute lymphoblastic leukaemia (ALL), which might contribute to the wide variation in the reported incidence of myeloid antigen expressing ALL in childhood.

Methods—Stored leukaemic cells from 10 children with previously defined myeloid positive ALL were examined. A range of commercially available anti-CD13 and anti-CD33 monoclonal antibodies, directly conjugated with phycoerythrin or fluorescein isothiocyanate, or both, was used. Positively reacting cells were detected by flow cytometry.

Results—There was a noticeable discordance between the different commercial sources of antibody and between the two fluorochromes in their ability to detect myeloid antigens, as well as variation in the intensity of staining. For CD13, one antibody reacted with eight cases and another with only four. Similarly, CD33 was detected in all 10 cases by one antibody and in only three by another.

Conclusions—The lack of any consistent pattern of results suggests that various commercial antibodies against the same CD antigen might recognise different epitopes and that the number of molecules per cell might vary from case to case. These observations partly explain the variation in reported incidence and the failure to establish the clinical importance of myeloid positivity, and they highlight the importance of standardisation in multicentre studies in which immunophenotypic data are collected.

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The co-expression of myeloid and lymphoid associated antigens in acute lymphoblastic leukaemia (ALL) is well recognised. A review of early studies of childhood ALL, comprising over 2000 cases, concluded that 7% were labelled by monoclonal antibodies to myeloid associated antigens.¹ However, the incidence of myeloid antigen expressing ALL (myeloid positive ALL) in different paediatric series ranges from less than 5% to almost 30%.²⁻¹⁰ This discrepancy can be partly explained by use of different panels of monoclonal antibodies. Thus, in addition to the well established

myeloid antigens CD13 and CD33, different workers have variably included antibodies to other "myeloid" antigens including CD11, CDw12, CD14, CD15, CD36, and CDw65. Significant differences occur even where incidences are derived from the use of monoclonal antibodies to the same CD antigens. Thus in two recent studies the incidence of childhood ALL expressing CD13 or CD33, or both, was 4% and 16%, respectively.^{5,10}

Standardisation of the definition of myeloid positive childhood ALL is important because several groups have drawn entirely different conclusions concerning its clinical importance.^{3-7,9} It is important to clarify the factors leading to the wide range of reported incidence. In this study we compared the use of different commercial sources of anti-CD13 and anti-CD33 monoclonal antibodies in a group of children with previously diagnosed myeloid positive ALL. Our objective was to establish whether the use of different monoclonal antibodies affected the diagnosis of myeloid positive ALL.

Methods

Between December 1991 and November 1993, leukaemic cells from all 47 new cases of ALL in children aged less than 15 years presenting within the Northern Health Region of England were immunophenotyped in the Department of Haematology at the Royal Victoria Infirmary. The diagnosis of ALL was based on the morphological and cytochemical criteria of the French American British (FAB) cooperative group.¹¹ There were 42 cases of B cell precursor and five cases of T-ALL. Twenty (43%) cases (all B cell precursor ALL) expressed myeloid antigens, defined as > 20% of mononuclear cells expressing CD13, or CD33, or both, as demonstrated by immunofluorescence using a FACscan flow cytometer. Of these 20 myeloid positive ALL cases, 10 had sufficient cryopreserved leukaemic cells to permit repeat immunophenotyping and were included in this study. Four further children with ALL (two B cell precursor and two T-ALL cases) not expressing myeloid antigens before (that is, CD13 and CD33 negative) and one adult case of acute myeloid leukaemia FAB M5a, previously strongly expressing CD33, were included for comparison.

MONOCLONAL ANTIBODIES

The original definition of myeloid positive ALL was based on results of staining with

Department of
Haematology, Royal
Victoria Infirmary,
Newcastle upon Tyne,
NE1 4LP

M R Howard
L Thomas
M M Reid

Correspondence to:
Dr M M Reid

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Percentage of mononuclear cells expressing CD13/CD33 antigens with the specified monoclonal antibody and fluorochrome in 15 cases of acute leukaemia

Case No	Diagnosis	Monoclonal antibody-fluorochrome								
		BD13-PE	BD33-PE	SC13-PE	SC33-PE	SC13-FITC	CC13-PE	CC33-PE	CC33-FITC	HLA-DR
1	BP-ALL*	71	23	53	22	78	67	63	3	90
2	BP-ALL	65	77	38	79	12	53	79	27	92
3	BP-ALL	7	19	<1	51	2	2	40	<1	99
4	BP-ALL	53	<1	3	56	21	30	23	10	99
5	BP-ALL	5	10	<1	9	10	4	63	11	82
6	BP-ALL	47	37	9	39	34	15	87	6	97
7	BP-ALL	50	23	1	10	30	24	62	40	99
8	BP-ALL	93	16	73	72	74	88	53	1	98
9	BP-ALL	44	74	3	69	38	15	95	61	99
10	BP-ALL	47	9	26	67	65	29	44	3	90
11	BP-ALL	<1	15	1	16	29	<1	19	<1	94
12	BP-ALL	23	<1	2	62	50	4	27	1	96
13	T-ALL	4	1	1	4	1	3	13	3	4
14	T-ALL	5	2	3	4	18	<1	13	2	7
15	AML	27	99	13	99	55	8	44	97	97

ALL = acute lymphoblastic lymphoma; AML = acute myeloid leukaemia; BD = Becton Dickinson; S = Serotec; C = Coulter Electronics; PE = phycoerythrin; FITC = fluorescein isothiocyanate.

*BP-ALL = B cell precursor ALL. Percentages in bold type are positive results (> 20%).

Becton Dickinson monoclonal antibodies. The following antibodies were used in the study: (a) anti-CD13 antibodies: Becton Dickinson, CD13-phycoerythrin (PE) (anti-Leu M7), clone L138, IgG1 κ ; Coulter Electronics, San Jose, USA, CD13-PE (My7), clone 336, IgG1 κ ; Serotec, Hialeah, USA, CD13-PE, and CD13-fluorescein isothiocyanate (FITC), IgG clone B-F10; (b) anti-CD33 antibodies: Becton Dickinson, Oxford, UK, CD33-PE (anti-Leu M9), clone P67-6, IgG1 κ ; Coulter Electronics, CD33-PE and CD33-FITC (My9), clone 906, IgG2b κ ; Serotec, CD33-PE, IgG clone WM-54. Leukaemic cells from all 15 cases included in the study were immunophenotyped with all of the above antibodies. HLA-DR expression was also measured to assess the accuracy of electronic gating in those with B cell precursor ALL and the case with AML.

IMMUNOPHENOTYPING

Patients' cells were originally separated on a Ficoll-Hypaque gradient and preserved in liquid nitrogen. For this study, cells from all cases were thawed, washed, and stained on the same day in one batch. Cells were adjusted to a concentration of 2×10^6 /l in 20% normal rabbit serum in buffered saline (pH 7.2). A total of 2×10^5 cells was added to each tube along with the volume of fluorochrome labelled monoclonal antibody specified in the manufacturer's instructions. A single batch of antibody from each supplier was used. The volume was made up to 50 μ l with normal rabbit serum in buffered saline. Tubes were incubated at 4°C for 30 minutes. Cells were then washed in 3 ml buffered saline and fixed in 0.5% paraformaldehyde in buffered saline.

FLOW CYTOMETRY

A Becton Dickinson FACscan was used for flow cytometric analysis. Three thousand events were collected using the Consort 30 acquisition program with four parameter data collection: FSC = forward light scatter; SSC = 90° angle light scatter; FL1 = fluorochrome channel 1 (FITC); FL2 = fluorochrome channel 2 (PE). Analysis was performed using Becton Dickinson LYSIS software.

Leukaemic cells were gated electronically using the FSC/SSC dot plot. The percentage of positive cells was derived from the appropriate fluorescence histogram. An isotype matched negative control was used to set acceptable negative or positive discrimination, permitting up to 2% "positivity" in the negative control samples. Results were deemed positive if >20% of leukaemic cells expressed a myeloid surface antigen.

Results

The table shows the percentage of mononuclear cells expressing CD13/CD33 antigens with the specified monoclonal antibody and fluorochrome for the 10 B cell precursor ALL cases and the five others. In all but the two T-ALL cases (cases 13 and 14) mononuclear cells were strongly HLA-DR positive. The 10 myeloid positive ALL cases (cases 1–10) were originally defined using the same Becton Dickinson antibodies used in this study. On repeat testing, cases 3 and 5 no longer fulfilled the criteria for myeloid positivity using these two Becton Dickinson antibodies. Conversely, of the four ALL cases previously defined as myeloid negative (cases 11–14), case 12 was positive for CD13 on retesting with the Becton Dickinson antibody.

The results show a noticeable discordance between different commercial sources of the monoclonal antibodies anti-CD13 and anti-CD33 and between the two fluorochromes. Thus for CD13-PE, the Becton Dickinson reagent identified nine ALL cases as being myeloid positive compared with six and four cases using Coulter and Serotec reagents, respectively. When Serotec CD13-FITC and CD13-PE were compared, the FITC conjugated reagent identified nine ALL cases as being myeloid positive compared with only four using the PE conjugated antibody. Similar discrepancies were seen with CD33-PE. The Coulter reagent identified 11 cases as myeloid positive, the Serotec reagent nine and the Becton Dickinson five only.

Both cases of T-ALL reacted negatively with all antibodies in the study. The single case of AML demonstrated a positive reaction with all anti-CD33 reagents and with two of

Figure 1 Histograms showing positivity for CD13 compared with the appropriate negative control (blocked in population) in case 7. The y axis shows number of cells; x axis shows intensity of fluorescence. A Becton Dickinson CD13-PE; B Serotec CD13-FITC; C Serotec CD13-PE; D Coulter CD13-PE.

the anti-CD13 antibodies (Becton Dickinson CD13-PE and Serotec CD13-FITC).

In addition to discordance between proportions of blasts which reacted with individual antibodies, the intensity of fluorescence also varied. Examples of such variation in cases 7 and 10 are presented in figs 1 and 2.

Discussion

This study was prompted by the apparently high incidence (43%) of myeloid positive results in new cases of childhood ALL presenting to this centre since December 1991. Cases before this period were excluded from analysis because detection of CD13 and CD33 was accomplished using a variety of reagents and techniques, and the high proportion of myeloid positive cases had not become apparent.

While the incidence of myeloid positive ALL in our cases seems higher than in other reported series of childhood ALL, any comparison with other series is fraught with difficulty. Firstly, panels of monoclonal antibodies

used to define "myeloid positivity" vary as do the required number of positive cells. We restricted our "myeloid" panel in this study to CD13 and CD33 as only these two reagents are universally regarded as myeloid specific. Data for particular antibodies are often difficult to extract from other reports. Where data for CD13/33 positivity are accessible, results remain inconsistent although the level of overall positivity is generally lower than in our cases.^{4,10} Other possible reasons for discrepancies include different patient groups, differences in immunophenotyping methodology (such as choice of electronic gates, thresholds for discriminating between positive and negative cells, and the use of indirect immunofluorescence or direct conjugates) and the use of different monoclonal antibody sources. There is no evidence from results of our participation in the National External Quality Assurance Scheme for flow cytometric immunophenotyping (unpublished results) that subtle differences in cell preparation, gating, or interpretation are responsible for a consistent bias in our laboratory.

Figure 2 Histograms showing positivity for CD33 compared with the appropriate negative control (blocked in population) in case 10. The y axis shows number of cells; x axis shows intensity of fluorescence. A Becton Dickinson CD33-PE; B Coulter CD33-PE; C Serotec CD33-PE; D Coulter CD33-FITC.

In contrast to such potential methodological variation, it is relatively easy to compare different monoclonal antibody sources. As many centres using flow cytometry now use directly conjugated antibodies, we decided to compare a variety of commonly available, directly conjugated, commercial monoclonal antibodies to determine if this is an important variable.

To minimise possible variation in results arising from the inevitable changes in laboratory personnel, recalibration of equipment and use of different batches of reagents which would accompany a prospective study carried out over several years, all tests were performed on the same day by a single operator on stored samples. The use of stored, frozen samples might have affected the expression of myeloid antigens or particular epitopes by the leukaemic cells. However, it seems unlikely that this in itself would lead to the important differences observed between antibodies.

The results show extreme variability between these various anti-CD13 and anti-CD33 monoclonal antibodies in the definition of myeloid positive ALL. The degree of discordance, both in proportions of labelled cells and intensity of fluorescence, suggests that antibodies to the same CD antigen were directed against different epitopes on the cell surface and that there was variation in the number of molecules of particular epitopes from case to case. Discrepancy was not limited to different commercial sources of antibody. A difference in fluorochrome could fundamentally alter the results. The only area of consistency emerging from the study of patients with ALL was that both cases of T-ALL were identified as myeloid negative by all reagents. This observation is supported by other unpublished local experience; no case of T-ALL, whether in adults or children, investigated at presentation to our centre has been myeloid positive.

Use of different commercial sources of monoclonal antibodies against putative myeloid antigens probably at least partly accounts for the enormous range of reported "myeloid" positivity in ALL. Studies of myeloid positive ALL do not routinely state the source of reagents. Our observations suggest that this is a serious omission. Clinicians should be cautious in their interpretation of studies suggesting a prognostic role (or otherwise) for myeloid positivity in childhood ALL. Their own laboratories may be using an apparently identical panel of monoclonal antibodies, but differences in the source of antibody or fluorochrome may lead to the definition of different subgroups of patients with different clinical outcomes.

The high rate of myeloid positive ALL defined by some reagents may also possibly lead to diagnostic confusion. For example, there is potential for ambiguity in the diagnosis of null ALL. Should the blasts of some such patients react with anti-CD13 or anti-CD33 antibodies, these cases could be confused with

the FAB defined diagnosis AML-MO.¹² To avoid further confounding the results of clinical studies by inaccurate diagnosis, it is important to understand the range of reactivity of the specific monoclonal antibodies used.

Are myeloid antigens worth detecting in childhood ALL? If we continue using antibodies indiscriminately, myeloid positive results will remain uninterpretable. A more consistent picture of the incidence and clinical importance of what we term myeloid positive ALL will emerge only when there is increased standardisation of monoclonal antibody panels with explicit description of the identity of the antibodies. In the shorter term it is important for coordinators of prospective studies of childhood ALL which gather immunophenotypic data to ensure that participating centres specify, and ideally standardise, the source and fluorochrome of their monoclonal antibodies.

In conclusion, we have shown that the use of discrete, commercially available monoclonal antibodies in childhood ALL leads to important differences in the identification of "myeloid" positivity. This observation partly explains the wide range of reported incidence of myeloid positive ALL and the failure to establish its clinical significance.

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