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Identification of repertoires of surface antigens on leukemias using an antibody microarray

We have previously described a microarray of cluster of differentiation (CD) antibodies that enables concurrent determination of more than 60 CD antigens on leukocytes. This procedure does not require protein purification or labeling, or a secondary detection system. Whole cells are captured by a microarray of 10 nL antibody dots immobilized on a nitrocellulose film on a microscope slide. Distinct patterns of cell binding are observed for different leukemias or lymphomas. These haematological malignancies arise from precursor cells of T- or B-lymphocytic, or myeloid lineages of hematopoiesis. The dot patterns obtained from patients are distinct from those of peripheral blood leukocytes from normal subjects. This microarray technology has recently undergone a number of refinements. The microarray now contains more CD antibodies, and a scanner for imaging dot patterns and software for data analysis provide an extensive immunophenotype sufficient for diagnosis of common leukemias. The technology is being evaluated for diagnosis of leukemias with parallel use of conventional diagnostic criteria.

Keywords: Antibody / Cluster of differentiation antigens / Leukemia / Lymphoma / Microarray
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1 Introduction

A limited immunophenotype (15–20 CD antigens) is used with clinical, hematological, morphological, cytochemical and genetic criteria to diagnose and classify tumors derived from hematopoietic and lymphoid tissues [1–3]. Different leukemias and lymphomas develop from mutations in precursor cells in lineages of differentiation from pluripotent stem cells to mature leukocytes of lymphoid (T, B or NK cells) or myeloid (neutrophils, basophils, eosinophils, macrophages, platelets or erythrocytes) origin. The blood, bone marrow and lymphoid tissues of normal subjects contain mixed populations of cells at various stages of differentiation. Each type of mature leukocyte or precursor cell has a distinct immunophenotype or subset of surface antigens called CD (cluster of differentiation) antigens. In patients with leukemia, a clonal malignant cell may predominate, changing the leukocyte population in peripheral blood. Usually, the immuno-

phenotype of the leukemia resembles the precursor cell from which it originated. Leukemia cells sometimes express unusual combinations of CD antigens. For example, chronic lymphocytic leukemia (CLL) often arises from the B cell lineage but expresses CD5 normally found on T but not B cells.

The Venn diagram shown in Fig. 1 provides a rapid guide to the expression of CD antigens and other surface molecules on leukocytes of different lineages: T, B, myeloid

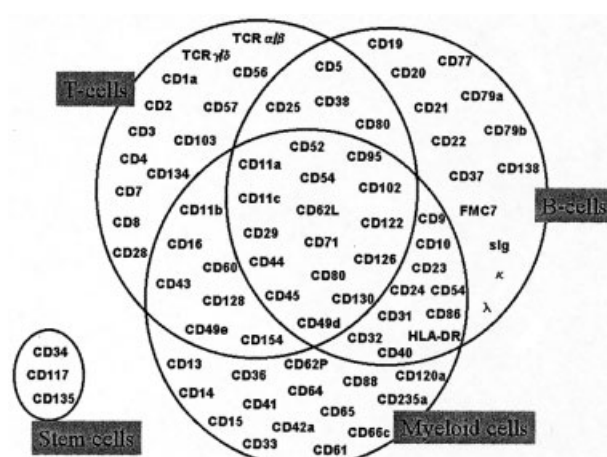


Figure 1. Venn diagram showing the distribution of CD antigens and other antigens on the surface of leukocytes, namely T cells, B cells, myeloid cells and stem cells.

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Abbreviations: CD, cluster of differentiation; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; PBL, peripheral blood leukocytes; T-LPD, T-cell lymphoproliferative disorder

and stem cells [4–6]. Some antigens, such as CD4 and CD37, are shown with the cell type showing major expression (T cells and B cells, respectively), although low level expression is found on other cells.

Flow cytometry is currently used for immunophenotyping leukemia cells. However, this technique is time-consuming, expensive both in use of fluorescently tagged CD antibodies and instrumentation, and requires a high level of expertise. A more extensive membrane proteome of leukemias may be sufficient to provide a diagnosis of leukemias and could indicate suitable targets for immunotherapy. Therapeutic antibodies are already used to treat patients with relapsed leukemia or lymphoma, *e.g.*, Rituximab (anti-CD20), Mylotarg (anti-CD33), Campath-1H (anti-CD52) [7], but use of these antibodies is limited by toxicity towards normal cells expressing these antigens. Drugs are required which target only the abnormal cells of a patient with minimal side-effects; hence the need to discover novel surface antigens or combinations of antigens on the cancer cells of patients.

DNA microarrays have been used to identify distinct gene expression patterns for a number of leukemias and lymphomas [8, 9]. However, this procedure is complex, empirical and unsuitable for routine diagnosis of hematological disorders. There is an uncertain relationship in cells between mRNA and protein levels and their subsequent post-translational modification. Protein profiling, including high-throughput analytical protein microarrays [10–12], more accurately reflects cell type and function. There have been some recent developments in the use of antibody microarrays [13–15] and new antibodies or fragments can be added to microarrays for large-scale screening for antigens. Potential problems of immobilizing protein molecules on solid surfaces include maintenance of tertiary conformation and binding activity [16–18].

In 2001, we described a novel CD antibody microarray (LD array*) which enabled semi-quantitative identification of the expression of CD antigens on leukemia and lymphoma cells from the peripheral blood of patients [19]. While many of the antibody chips described to date have been designed to detect, quantify or analyze proteins such as cytokines in solution [20–22], the LD array captures cells from suspension, the number of cells captured by each CD antibody being roughly proportional to the density of cells expressing the relevant antigen. The original antibody microarray consisted of 60 dots of CD antibodies against surface antigens on human leukocytes. When a cell suspension was incubated for 30 min with the microarray, cells bound only to those antibody dots

for which they expressed the corresponding CD antigen. A mixed population of cell types, such as normal peripheral blood leukocytes, showed a characteristic pattern of binding reflecting the subpopulations (T, B and myeloid cells) of leukocytes. The Histopaque fraction of leukocytes from leukemia patients showed different patterns of binding, reflecting the immunophenotype of the clonal leukemia. In contrast to most protein and DNA microarrays, this method uses cell capture and does not require fluorescent tags or other detection systems.

This CD antibody microarray has been expanded and modified for automated imaging and analysis. Accumulation of immunophenotypes for a wide range of patients has enabled recognition of consensus patterns of CD antigen expression for common hematological malignancies. Software has been developed for distinguishing normal and abnormal leukocyte populations, and determining the lineage and stage of maturation of the leukemia cells.

2 Materials and methods

2.1 Construction of CD antibody microarrays

The procedure is based on that described previously [19, 23]. A PixSys 3200 Aspirate and Dispense System (Cartesian Technologies, Irvine, CA, USA) was used to construct duplicate microarrays over an area of approximately 15×19 mm. Each microarray consisted of 90 different 10 nL antibody dots (including isotype controls) air-dried on a film of nitrocellulose (18×27 mm) on a glass microscope slide (FAST Slides; Grace Biolabs, Bend, OR, USA; supplied by Schleicher and Schuell, Keene, NH, USA). Antibodies were purchased from the following companies: Coulter and Immunotech from Beckman Coulter (Gladesville, NSW, Australia), Pharmingen from BD Biosciences (North Ryde, NSW, Australia), Biosource International from Monarch Medical (Stafford City, Qld, Australia), Serotec from Australian Laboratory Services (Sydney Markets, NSW, Australia), Sigma-Aldrich (Castle Hill, NSW, Australia). Antibody solutions were reconstituted as recommended, frozen in aliquots at -20°C with 0.1% w/v BSA (Sigma-Aldrich), and used at concentrations ranging from 50–1000 $\mu\text{g/mL}$, as supplied for flow cytometry. After application of the antibody dots (10 nL) in a rectangular array, the nitrocellulose was blocked with 5% w/v skim milk (Diploma; Bonlac Foods, Melbourne, Vic., Australia) in PBS (90 min at room temperature or overnight at 4°C), washed in two changes of water, dried, and stored at 4°C with desiccant. Each batch of slides was tested with cell lines and/or frozen peripheral blood leukocytes or leukemia cells of known immunophenotype to check antibody binding activities.

* This array was named after Mrs Lee Dixon who died of acute myeloid leukemia

2.2 Binding of leukocytes to the LD array

The methods for collection of peripheral blood from patients and normal subjects, leukocyte purification on Histopaque-1077 and incubation with the CD antibody microarray were as described previously [19], except more cells were used for the larger arrays ($4\text{--}6 \times 10^6$ cells in $300\text{ }\mu\text{L}$ to cover a surface area of 486 mm^2). Unbound cells were then gently washed off with PBS, arrays were fixed for at least 20 min in PBS containing 10% v/v formaldehyde and washed in PBS. Most cell suspensions were tested in PBS containing 1 mM EDTA, but cells from patients with acute myeloid leukemia were generally tested in PBS containing heat-inactivated human AB serum (2% v/v Sigma-Aldrich) to minimize nonspecific Fc receptor binding.

2.3 Data recording and analysis

Bound leukocytes were initially imaged using non-conventional dark-field microscopy and a digital camera [19], and subsequently using a scanner and data analysis software (Medsaic, Eveleigh, Australia). This software quantifies the density of cell binding on each antibody dot and uses a pattern recognition algorithm to identify the leukemia. Bar charts generated by the software for a defined region of each dot in the microarray show the average brightness of captured cells above background scaled from 0–255.

2.4 Flow cytometry

FITC- or phycoerythrin-conjugated antibody ($10\text{--}20\text{ }\mu\text{L}$; BD Biosciences) was incubated at room temperature for 15 min with volumes of blood containing approximately

10^6 leukocytes/mL. After addition of FACS lysing solution (BD Biosciences) and 30 min incubation at room temperature to lyse red blood cells, samples were analyzed on a FACSCalibur (Becton Dickinson, San Jose, CA, USA) using Worklist Manager, MultiSET, or CellQUEST software.

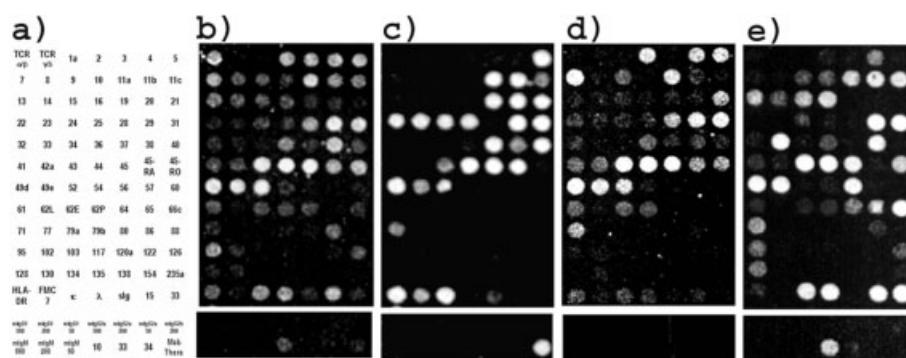
3 Results and discussion

3.1 Immunophenotypes of leukemias

Images for cells captured on CD antibody microarrays are shown in Fig. 2 for normal peripheral blood leukocytes (PBL), chronic lymphocytic leukemia (CLL), T cell lymphoproliferative disease (T-LPD) and a transformed chronic myeloid leukaemia (CML), the addresses of the CD antibodies are shown in Fig. 2(a). Only one of duplicate microarrays is shown for leukocytes fractionated from each blood sample. A CD antigen found on all leukocytes is used as a positive control, and murine isotype control antibodies against the various classes of human immunoglobulin allow detection of any non-specific binding of cells to antibody dots by Fc receptors.

Each antibody dot is approximately $500\text{ }\mu\text{m}$ in diameter and at saturation holds $1500\text{--}2000$ cells, determined by counting cells captured on dots (not shown). This correlates well with the theoretical number of cells incubated over each antibody dot (N) when 4×10^6 cells are spread over the area of nitrocellulose ($18 \times 27\text{ mm}$) on which the array is constructed.

$$N = \frac{\pi \times 0.25^2 \times 4 \times 10^6}{18 \times 27} = 1616 \text{ cells}$$



The intensities of dots in an array reflect approximately the proportion of cells expressing each CD antigen. However, the number of cells captured by an antibody dot also depends on the number of antigen molecules on the cell surface and the affinity of the antibody-antigen interaction. The average number of cells bound *per* dot (determined microscopically) correlates well with average binding density values from the image analysis software, and generally correlates with results from flow cytometry (Table 1). The few discrepancies between flow cytometry and microarray results could be attributed to the flow cytometry being done with a total lymphocyte population, while the microarray results were obtained with mononuclear cells, platelets and some granulocytes from Histopaque fractionation. A fluorescently-tagged soluble antibody may have better access to some surface antigens on cells than an antibody immobilized on a surface. Occasionally, cells may fail to bind to antibody dots if the number of antigens *per* cell is below the threshold required for cell capture. The discrepancy for CD22 (low

by flow cytometry; high by microarray) may result from the use of different clones of antibodies (leu-14 for flow cytometry; SJ.101H11 for microarray).

Immunophenotypes of leukemias and lymphomas obtained with the LD array are being compiled in an extensive database to determine consensus patterns. A relational database has been developed for diagnosis of hematological malignancies using immunophenotyping by flow cytometry [24]. Although only 42 CD antibodies were used with a small number of patient samples for each disease category, the results suggested that differential diagnoses could be made solely from extensive immunophenotypes. The LD array currently uses 90 CD antibodies, and could be extended further.

Image analysis software has been developed which automatically detects positive dots (captured cells) in the microarray, integrates dot volumes, constructs bar charts for average binding density for each dot, and uses pattern

Table 1. CD antigen expression of leukocytes determined by flow cytometry and CD antibody microarray

Antibody	CLL-11			T-LPD-32		
	Flow cytometry		Dot array	Flow cytometry		Dot array
	% positive ^{a)}	Cells/dot		% positive ^{a)}	Cells/dot	
			Binding density ^{b)}			Binding density ^{b)}
CD2	8	145	13	92	>1000	108
CD3	7	120	6	7	27	1
CD4	4	112	6	84	>1000	97
CD5	99	>1000	160	82	>1000	91
CD7	2	146	14	91	>1000	127
CD8	2	115	6	3	25	1
10	2	74	0	ND ^{c)}	6	2
11c	14	480	72	ND	71	4
CD19	93	>1000	167	7	106	5
CD20	94	>1000	177	ND	74	1
CD22	14	>1000	188	ND	110	8
CD23	90	>1000	185	ND	67	1
CD25	ND	>1000	174	4	91	2
38	12	500	89	ND	143	17
CD45	100	>1000	180	100	>1000	157
FMC7	56	750	144	ND	60	1
HLA-DR	93	>1000	172	9	132	17
κ	63	>1000	190	ND	128	18
λ	0	48	1	ND	102	13

a) Percentage of positive cells in a gated lymphocyte population

b) Average dot brightness above background in units scaled from 0–255

c) ND, not determined

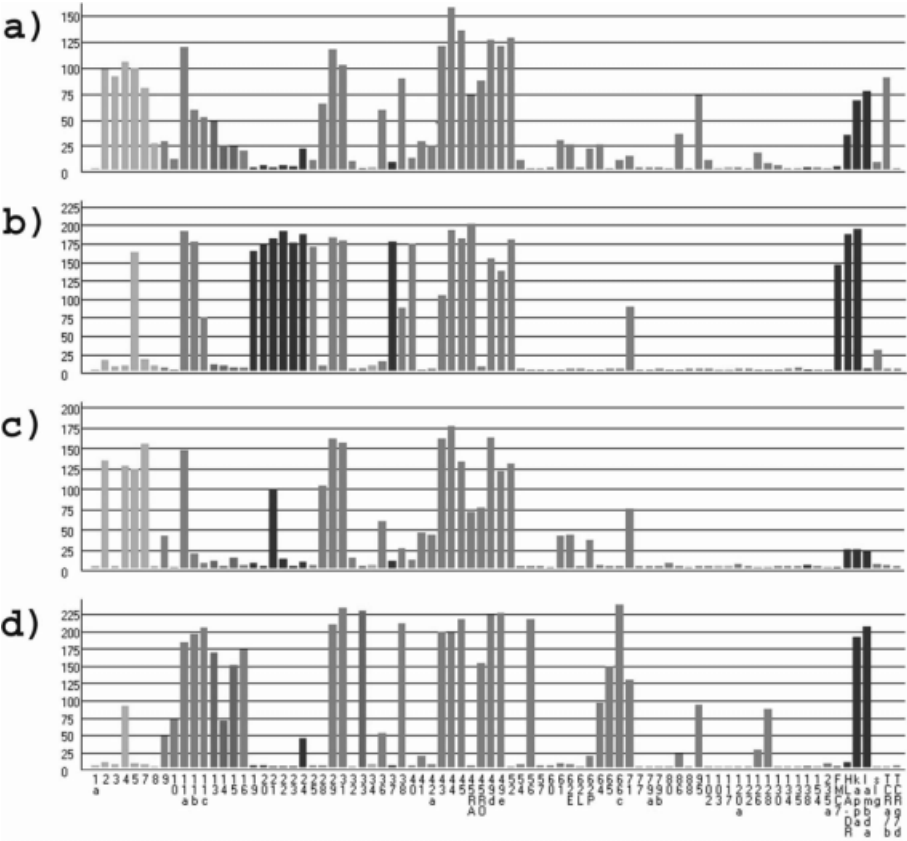


Figure 3. Bar charts generated in colour by image analysis software from the scanned microarray images of Fig. 2(b–e). (a) Normal subject PBL-46; (b) patient CLL-11; (c) T-LPD-32; (d) patient transformed CML-71. Markers routinely used for flow cytometry are shown as pale grey for T cells, black for B cells and dark grey for myeloid cells. All other antibodies are shown in medium gray. Numbers on the x axis refer to antibodies against the corresponding CD antigens. Values on the y axis are average image brightness above background for each antibody dot scaled from 0–255. Samples from patients and normal subjects are identified as for Fig. 2.

recognition to match the dot pattern for a patient to a stored library of consensus patterns for leukemias to provide a diagnosis. Bar charts corresponding to the dot images in Fig. 2 are shown in Fig. 3. Different patterns are obtained for normal PBLs (Fig. 3(a)), CLL (Fig. 3(b)), T-LPD (Fig. 3(c)) and transformed CML (Fig. 3(d)). Because the microarray concurrently tests for expression of many CD antigens, unusual antigens on malignant cells may be identified as potential targets for immunotherapy.

3.2 Reproducibility and background binding

When cells are tested on duplicate arrays, results are reproducible, as shown for CLL-85 in Fig. 4(b) and (c). Both arrays showed high density binding on CD5, CD9, CD11a, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD29, CD31, CD37, CD40, CD43, CD44, CD45, CD45RA, CD49d, CD52, CD54, CD71, CD95, HLA-DR, lambda light chain and Mabthera (Roche CD20).

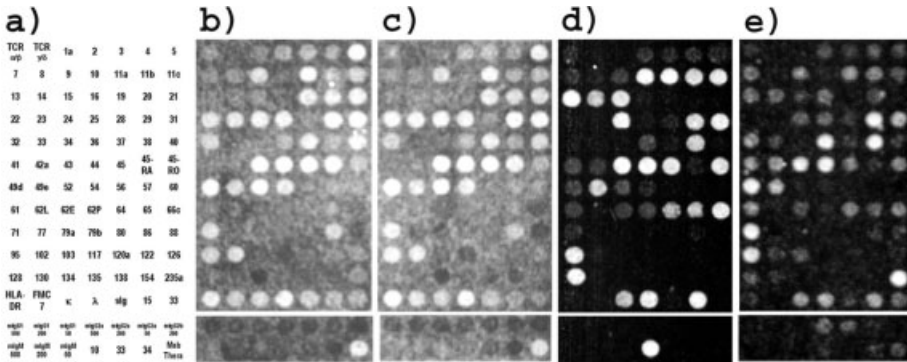


Figure 4. Binding patterns of human leukocytes. (a) key; (b, c) duplicate analyses of patient CLL-85; (d) normal subject PBL-111 with granulocytes not removed; (e) normal subject PBL-37 contaminated with >50% RBC. Numbers in the key, and samples from patients and normal subjects are identified as for Fig. 2.

CLL-85 also showed some nonspecific binding to the surface of the blocked nitrocellulose which did not interfere with the analysis.

Whole blood cannot be used directly on microarrays, as the predominant red blood cells (RBC) interfere with binding of leukocytes to antibody dots. RBC and granulocytes must be removed by centrifugation through Histopaque (density 1.077 g/mL). If granulocytes or RBC constitute >50% of the total population, the binding of these contaminating cells may result in an incorrect diagnosis due to their dominating contribution to the binding pattern. In the normal sample PBL-111 (Fig. 4(d)), granulocytes were not removed and constituted 74% of the total leukocyte population. The bright dot pattern differed markedly from that of PBL-46 shown in Fig. 2(b), and was characteristic for granulocytes (CD10, CD11a, CD11b, CD11c, CD13, CD15, CD24, CD29, CD31, CD43, CD44, CD45, CD45RO, CD49e, CD66c, CD71, CD95, κ , λ). The presence of contaminating RBC (>50%) in PBL-37 (Fig. 4 (e)) resulted in the appearance of a bright CD235a (glycophorin A) dot and poor T cell binding (Fig. 4(e)).

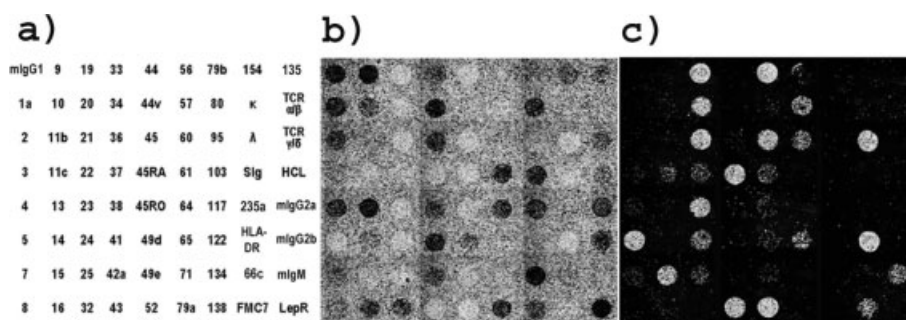
Contaminating RBC can be removed by osmotic lysis in 0.15 M ammonium chloride, 10 mM potassium hydrogen carbonate, without significantly altering the immunophenotype of the mononuclear cell population (not shown). Granulocytes are generally removed by centrifugation through Histopaque-1077 (400 $\times$ g, 30 min, no brake). However, granulocytes may be more difficult to remove from the blood of patients with a variety of inflammatory disorders, where an increase in granulocyte numbers is common and a change in granulocyte density may also occur. Conditions such as infection, burns, allergy and other inflammatory disorders, bone marrow infiltration with nonhematological disseminated malignancy, and the postsplenectomy state, involve changes in leukocyte populations [25, 26] and need to be differentiated from hematological malignancies.

Nonspecific background binding of cells to the nitrocellulose is minimized by washing them to remove exogenous nutrients, and the addition of EDTA to the suspension me-

dium. Occasionally, leukemias and lymphomas show unacceptable background binding, even with EDTA, which obscures cell capture by antibody dots. Such leukocyte samples can be retested in the presence of 0.1% w/v sodium azide, which inhibits the metabolic activity and hence phagocytosis by cells (Fig. 5). However, cells killed by formaldehyde fixation or prolonged exposure to sodium azide show little or no binding to the antibody dots, suggesting that some metabolic activity is required for specific cell capture, perhaps for processes such as antigen capping [27]. Interestingly, the heavy background binding of the CLL in Fig. 5(b) was inhibited by several murine monoclonal antibodies on the array (black dots). The CLL cells may have a differential affinity for the proteins used to block the microarray.

The binding patterns for PBLs and CLLs on the LD array have been described [19]. Data are being collected from many patients with other types of leukemias and lymphomas to define consensus patterns and the software for analysis of microarray images is undergoing further development. For low level leukemias, the software must recognize an abnormal binding pattern in the presence of a high background of PBLs. Changes in leukocyte subpopulations occurring during infection, inflammation, allergy and other disorders, must be distinguished from changes due to hematological malignancies. Extensive data collection and analysis will enable such issues to be addressed. Previous data suggest that the LD array can detect clonal CLL populations as low as $2 \times 10^9/L$ in peripheral blood. The minimal level of detection for a leukemia or lymphoma will vary depending upon whether it is of T or B cell or myeloid origin, given that the levels of normal leukocytes of these categories are different in peripheral blood.

Each B cell expresses only one kind of antibody light chain (κ or λ), enabling distinction of malignant monoclonal B cell populations from normal mixed B cell populations (c.f. Fig. 2(b), (c)). However, some myeloid cells may have nonspecific binding to κ and λ antibody dots even though they do not express immunoglobulins on their surfaces, e.g., the transformed CML in Fig. 2(e). This may be due



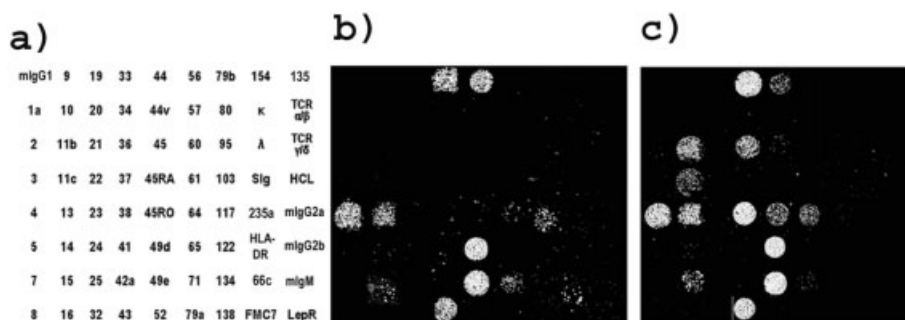


Figure 6. Changes in the immunophenotype of HL-60 cells induced by all-*trans* retinoic acid (ATRA). (a) key; (b) untreated HL-60 cells, (c) HL60 cells treated with 200 nm ATRA for 7 d.

to the presence of soluble immunoglobulins bound passively to Fc receptors on the surfaces of such cells [28, 29]. Since neutrophils also tend to bind to κ and λ antibodies (Fig. 4(d)), the presence of contaminating neutrophils in mononuclear cell preparations may mask binding of low levels of malignant B cells to these antibodies.

3.3 Drug-induced changes in immunophenotype

Acute myeloid leukemia arises from mutations in precursor cells which then proliferate rather than differentiate to mature leukocytes. It has been suggested that molecules capable of inducing differentiation in acute promyelocytic leukemia may be potentially useful as therapeutic agents [30, 31]. The CD antibody microarray may be useful for monitoring changes in the immunophenotype of leukemia cells during treatment with such agents. This is demonstrated in Fig. 6, which shows immunophenotypic changes induced in the human promyelocytic leukemia cell line, HL-60, treated for 7 d with 200 nm all-*trans* retinoic acid which induces differentiation of myeloid precursors into neutrophils [32]. Several such drugs are under investigation in our laboratory with leukemia cell lines.

3.4 Nitrocellulose slides

Many surfaces bind CD antibodies and after blocking, the resultant array can be used to capture cells, but we are using FAST nitrocellulose slides [33]. The porous nitrocellulose film on FAST slides allows complete adsorption of antibodies, preventing the smearing of antibody drops that sometimes occurs on shiny surfaces during blocking and washing. Leukemia cells do not normally bind non-specifically to blocked FAST slides. FAST slides become translucent when moist, allowing scanning using transmitted light. In general, antibodies are more stable when dry on nitrocellulose in a microarray at 4°C, than stored in aqueous solution at 4°C. Most CD antibodies consistently capture cells when used at concentrations of greater than 200 $\mu\text{g}/\text{mL}$.

4 Concluding remarks

The LD array enables rapid, concurrent screening of leukocyte suspensions for expression of many CD antigens. Cells captured on the CD antibody microarray can be imaged directly with an optical scanner without staining or labeling, and image analysis software gives immediate results. Extensive immunophenotypes of leukemias and lymphomas obtained with the LD array can be used for diagnosis of patients, and for basic research in cell biology.

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5 References

- [1] Bain, B. J., *Leukaemia Diagnosis*, 2nd edition, Blackwell Science, Oxford 1999, pp. 1–200.
- [2] Harris, N. L., Jaffe, E. S., Diebold, J., Flandrin, G. *et al.*, *J. Clin. Oncol.* 1999, 17, 3835–3849.
- [3] Jaffe, E. S., Harris, N. L., Stein, H., Vardiman, J. W. (Eds.), *World Health Organization Classification of Tumours; Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues*, IARC Press, Lyon 2001, pp. 1–302.
- [4] Pharmingen, *Immunology Today Immune Receptor Supplement* 1997, poster insert.
- [5] Kishimoto, T., Kikutani, H., von dem Borne, A. E. G. K., Goyert, S. M. *et al.* (Eds.), *Leucocyte Typing VI; White Cell Differentiation Antigens*, in: *Proc. Sixth Int. Workshop and Conference*, Kobe, Japan, November 1996, Garland Publishing, New York & London 1998, pp. 1109–1220.
- [6] <http://www.serotec.com/>
- [7] Carter, P., *Nat. Rev. Cancer* 2001, 1, 118–129.
- [8] Golub, T. R., *Curr. Opin. Hematol.* 2001, 8, 252–261.

- [9] Alizadeh, A. A., Ross, D. T., Perou, C. M., van de Rijn, M., *J. Pathol.* 2001, **195**, 41–52.
- [10] MacBeath, G., *Nat. Genet.* 2002, **32**, 526–532.
- [11] Ng, J. H., Ilag, L. L., *J. Cell. Mol. Med.* 2002, **6**, 329–340.
- [12] Constans, A., *The Scientist* 2002, **16**, 28–30.
- [13] Sreekumar, A., Nyati, M. K., Varambally, S., Barrette, T. R. *et al.*, *Cancer Res.* 2001, **61**, 7585–7593.
- [14] Delehanty, J. B., Ligler, F. S., *Anal. Chem.* 2002, **74**, 5681–5687.
- [15] Borrebaeck, C. A., Ekstrom, S., Hager, A. C., Nilsson, J. *et al.*, *BioTechniques* 2001, **30**, 1126–1130, 1132.
- [16] Service, R. F., *Science* 2001, **294**, 2080–2082.
- [17] Frederickson, R. M., *Chem. Biol.* 2002, **9**, 763–765.
- [18] Constans, A., *The Scientist* 2003, **17**, 35–36.
- [19] Belov, L., de la Vega, O., dos Remedios, C. G., Mulligan, S. P. *et al.*, *Cancer Res.* 2001, **61**, 4483–4489.
- [20] Haab, B. B., Dunham, M. J., Brown, P. O., *Genome Biol.* 2001, **2**, Research 0004–0004.13.
- [21] Lin, Y., Huang, R., Santanam, N., Liu, Y. G. *et al.*, *Cancer Lett.* 2002, **187**, 17–24.
- [22] Pawlak, M., Schick, E., Bopp, M. A., Schneider, M. J. *et al.*, *Proteomics* 2002, **2**, 383–393.
- [23] Christopherson, R. I., dos Remedios, C. G. 1998. An Assay. International Patent Application filed December 23, 1999, PCT/AU099/01156. ARIPO patent, Eurasian patent, European patent, OAPI patent, and 77 other national patents.
- [24] Nguyen, A. N., Milam, J. D., Johnson, K. A., Banez, E. I., *Am. J. Clin. Pathol.* 2000, **113**, 95–106.
- [25] Lewis, S. M., Bain, B. J., Bates, I. (Eds.), *Dacey and Lewis Practical Haematology*, 9th ed., Churchill Livingstone, London 2001, 65–100.
- [26] Calvano, S. E., Greenlee, P. G., Reid, A. M., deRiesthal, H. F. *et al.*, *J. Trauma-Inj. Infect. Crit. Care* 1988, **28**, 353–361.
- [27] Rao, P. E., Talle, M. A., Kung, P. C., Goldstein, G., *Cell. Immunol.* 1983, **80**, 310–319.
- [28] Gupta, S., Garrett, T., Straus, D., Good, R. A., *Int. Arch. Allergy Appl. Immunol.* 1980, **62**, 334–340.
- [29] Fernandes, G., Garrett, T., Nair, M., Straus, D. *et al.*, *Blood* 1979, **54**, 573–580.
- [30] Taimi, M., Chen, Z. X., Breitman, T. R., *Oncol. Res.* 1998, **10**, 75–84.
- [31] Chen, Z., Breitman, T. R., *Chin. Med. J.* 1997, **110**, 783–788.
- [32] Breitman, T. R., Selonick, S. E., Collins, S. J., *Proc. Natl. Acad. Sci. USA* 1980, **77**, 2936–2940.
- [33] Stillman, B. A., Tonkinson, J. L., *Biotechniques* 2000, **29**, 630–635.