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Binding of mouse monoclonal antibodies to human leukaemic cells via the Fc receptor: a possible source of 'false positive' reactions in specificity screening

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SUMMARY

Mouse monoclonal antibodies (MoAbs) of different classes and subclasses directed against antigens not expected to be present on human cells have been screened by indirect immunofluorescence using flow cytometry for binding to human non-lymphocytic leukaemic cells and normal peripheral blood leucocytes. Antibodies of the IgG2a and IgG3 subclass, but not of the IgG1, IgG2b or IgM class bound to the blast cell and monocyte populations in a peripheral blood mononuclear cell preparation from a patient with acute myelomonocytic leukaemia. $F(ab')_2$ fragments of an anti-salmonella antibody of IgG2a subclass failed to bind, indicating that the results were not due to cross-reactivity with antigens of the cell membrane, thus implicating the Fc region in binding. Furthermore, binding was partly blocked by inclusion of 10% heat-inactivated normal rabbit serum in the assays. IgG2a bound to varying degrees to the leukaemic cell populations in seven of the nine non-lymphocytic leukaemic specimens tested, but no binding to normal peripheral blood mononuclear cells or granulocytes was detected. The results emphasize the importance of including appropriate controls when screening MoAbs for binding to various types of human cells.

Keywords monoclonal antibodies Fc binding human leukaemic cells fluorescence activated cell sorting

INTRODUCTION

Many cellular receptors for the constant region of homologous immunoglobulin molecules (Fc receptors) bind some xenogeneic immunoglobulins. For example, Fc receptors on certain human cell types avidly bind rabbit but not goat immunoglobulins (Alexander & Sanders, 1977). Such non-antigen specific binding has the potential to give rise to false positive reactions when xenoantisera are used for detection of cell surface antigens. With the development of the hybridoma technology for production of mouse monoclonal antibodies (MoAbs) and its application to the study of surface antigens on human cells, it is important to determine the extent and class/subclass specificity of Fc binding of mouse immunoglobulins to various human cell types.

In the course of screening MoAbs raised against human blood cells for reaction with a panel of cryopreserved peripheral blood mononuclear cells from patients with acute leukaemia, we observed binding to blast cell populations by a negative control mouse monoclonal antibody which is specific for a cytoplasmic protein antigen of salmonella. This finding led us to examine the immunoglobulin class and subclass specificity of the binding. We observed significant binding of several preparations, both monoclonal and conventional, of mouse IgG2a and three preparations of IgG3,

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but no appreciable binding of other IgG subclasses or IgM. Binding of IgG2a could be detected with a substantial proportion of the leukaemic populations tested, but not with normal peripheral blood leucocytes. Many workers screening MoAbs for binding to human cells use supernatant from the IgG1 secreting myeloma P3/X63-Ag8 (X63) as negative control, or indeed no control at all except for the anti-immunoglobulin indicator reagent alone. The results of this study demonstrate the inadequacy of such controls at least in studies using human leukaemic cells.

MATERIALS AND METHODS

MoAbs. Except where otherwise indicated, MoAbs used in this study were prepared and cloned in this laboratory essentially as described by Oi & Herzenberg (1980). Positive control antibodies were 7B6c and 2B2.F7, IgG2a and IgM, respectively, which detect HLA-A,B,C monomorphic determinants (L. Ashman, unpublished). MoAbs specific for antigens not expected to be present on human cells were as follows. Sal-2 (IgG1), Sal-3 (IgM), Sal-4 (IgG2b) and Sal-5 (IgG2a) react with cytoplasmic proteins from *Salmonella enteritidis* 11RX (O'Connor & Ashman, 1982). X63-IgG1 was derived from myeloma P3/X63-Ag8 (cf. Köhler & Milstein, 1975). A monoclonal IgM specific for the enzyme pyruvate carboxylase was a gift from Ms K. Oliver, Department of Biochemistry, University of Adelaide. FMC-19 (IgG3) and FMC-21 (IgG2a) (Johnson *et al.*, 1981) were kindly supplied by Dr A. Johnson, Flinders Medical Centre, Adelaide, South Australia. J606 (purified IgG3 myeloma protein) and an ascites fluid containing the FLOPC-21 IgG3 myeloma protein were obtained from Litton Bionetics, Kensington, Maryland, USA. The FLOPC-21 IgG3 was purified by Dr P.L. Ey of the Department of Microbiology and Immunology, The University of Adelaide, by affinity chromatography on protein A–Sepharose 4B by the method of Ey, Prowse & Jenkin, (1978) and contained 4% IgG2a, 1% IgG1 and no detectable IgG2b by solid phase radioimmunoassay.

The MoAbs X63 IgG1, Sal-2 IgG1, Sal-4 IgG2b and Sal-5 IgG2a were purified from ascites fluid as described by Ey *et al.* (1978) and quantitated by A_{280nm} using $E^{1\,cm}_{280\,nm}$ of 1·4 for a 1 mg/ml solution (Hudson & Hay, 1980). All other MoAbs were used as dilutions of culture supernatant and were quantitated by competitive solid phase radioimmunoassay using purified immunoglobulin of the same class/subclass as standard. The IgM standard was a gift from Dr P.L. Ey.

Immunoglobulin heavy chain isotypes were determined by immunodiffusion in agarose using culture supernatants concentrated 10 times by centrifugation with Centriflo-25 membrane cones (Amicon, Danvers, Massachusetts, USA). The isotype specific reagents used were obtained from Meloy (Springfield, Virginia, USA) and were raised in goats. In several cases (see above) results were confirmed by the pH of elution from protein A–Sepharose during purification.

$F(ab')_2$ fragments of Sal-5 IgG2a were prepared by digestion with Pepsin (twice crystallized, lot 60F-8056, Sigma, St Louis, Missouri, USA) at a ratio of immunoglobulin: pepsin of 50:1 in acetate buffer pH 4·5 for 21 h at 37°C. Undegraded immunoglobulin was removed by binding to protein A-Sepharose 4B.

Polyclonal immunoglobulins. IgG2a from serum of normal or sheep erythrocyte immunized mice was purified as described by Ey *et al.* (1978) and quantitated by $A_{280nm.}$

Human cells. The mononuclear cell fractions from the peripheral blood of patients with acute leukaemia were prepared and cryopreserved as previously described (O'Keefe & Ashman, 1982). Mononuclear cells and granulocytes were prepared from heparinized blood samples from normal donors using Ficoll/Angiograffin/Urovisin, density 1·114 g/ml, essentially as described by Ferrante & Thong (1980).

Cell binding assay. Human cells, 10^6 in 50 μl of Ca^{2+}-Mg^{2+}-free phosphate-buffered saline containing 0·1% sodium azide and 0·1% bovine serum albumin were incubated with 50 μl of mouse immunoglobulin, diluted as indicated in the same buffer, for 1 h on ice. Cells were washed three times with 1 ml of the same buffer, then incubated for 1 h on ice with 50 μl of a 1/10 dilution of fluorescein labelled affinity purified goat antibody to mouse IgG + IgM (H + L chains) (lot CL08, Kirkegaard and Perry, Gaithersburg, Maryland, USA). This dilution of fluorescent antibody was shown to be sufficient to give maximal fluorescence when used to detect MoAb binding to a

monomorphic determinant on HLA-A,B,C molecules which are major cell surface glycoproteins. Labelled cells were washed twice with 1 ml of buffer, resuspended in 1 ml of 1% paraformaldehyde in phophate-buffered saline and stored in the dark at 4°C for 1–3 days (cf. Lanier & Warner, 1982) before analysis by flow cytometry using a FACS IV. Fifty thousand cells per sample were analysed.

RESULTS

Binding of mouse immunoglobulins to human acute myelomonocytic leukaemic cells

Fig. 1 shows fluorescence and scatter histograms for specimen 81/13 incubated with fluorescein labelled goat anti-mouse IgG + IgM alone, and with Sal-5 IgG2a which is specific for a salmonella protein antigen or 7B6c IgG2a which binds to an HLA-A, B,C monomorphic determinant (positive control) followed by the fluoresceinated reagent. The goat anti-mouse Ig was chosen rather than a rabbit anti-mouse reagent since goat immunoglobulins are known to bind less to human Fc receptors (Alexander & Sanders, 1977). A shoulder on the fluorescence histogram for the goat anti-mouse reagent alone (Fig. 1a) indicated very slight binding of this reagent to a proportion of the cells, a finding which was confirmed in other experiments. When the cells were incubated with Sal-5 IgG2a two peaks were observed, with 60% of the cells in the positive peak. This specimen contains 25% blast like cells and 55% monocytes, presumably mostly leukaemic. The IgG2a may be binding to the monocytes as well as to some of the blast cells. The peak channel on the fluorescence histogram was channel 70 on gain setting 4. This compared with a peak channel of 91 on gain setting 2 (equivalent to channel 182 on gain 4) for the anti-HLA-A,B,C positive control at the same concentration. Thus the binding of the IgG2a control was appreciable compared with that of the monoclonal antibody against a major cell surface antigen.

The dose–response for binding of Sal-5 and 7B6c to 81/13 cells is shown in Fig. 2a. Maximum binding occurred using 0·25 μg of immunoglobulin per 10^6 cells in 0·1 ml in both cases. Also shown in Fig. 2a are dose response data for the IgM anti-HLA-A,B,C positive control and Sal-3 IgM. Only trace binding of IgM was observed. Dose–response data obtained in a separate experiment for Sal-2 (IgG1), Sal-4 (IgG2b), FMC-21 (IgG2a) and FLOPC-21 (IgG3) relative to the IgG2a positive control 7B6c are shown in Fig. 2b. FMC-21 and FLOPC-21 bound to the cells whereas no binding was observed with the IgG1 or IgG2b antibodies. Similarly X63 IgG1 and the anti-pyruvate

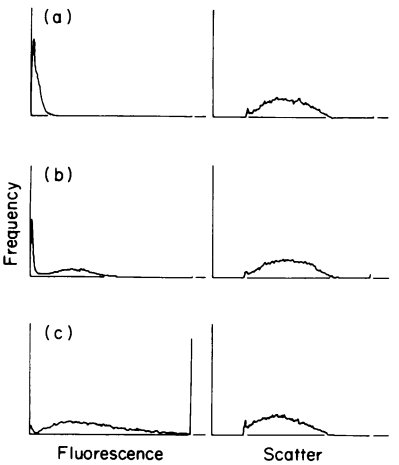

Fig. 1. Flow cytometric analysis of the binding of Sal-5 IgG2a and an anti-HLA-A,B,C MoAb to acute myelomonocytic leukaemic cells (81/13). Cells were incubated with mouse MoAb as indicated, and bound antibody detected using fluorescein labelled goat anti-mouse IgG + IgM antibody. Fluorescence histograms indicate the frequency of cells of a given fluorescence intensity. Scatter histograms give an indication of the size distribution of the population. (a) No mouse antibody; fluorescence gain 4, peak channel 4 (shoulder 13). (b) Cells incubated with Sal-5 IgG2a (0·5 μg/10^6 cells) fluorescence gain 4, peak channel 70, 60% of cells positive. (c) Cells incubated with anti-HLA-A,B,C (7B6c): fluorescence gain 2, peak channel 91; 98% of cells positive.

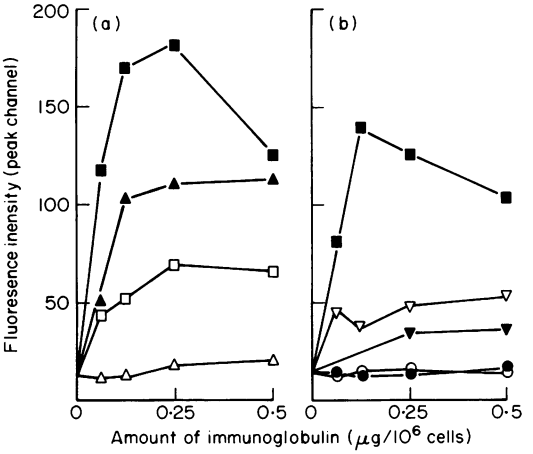

Fig. 2. Concentration dependence of the binding of different mouse immunoglobulin classes and subclasses to human acute myelomonocytic leukaemic cells. The figure shows fluorescence intensity (peak channel) for the fluorescent cell population as a function of immunoglobulin concentration. (a) Results for Sal-5 IgG2a (□——□); anti-HLA-A,B,C IgG2a (■——■); Sal-3 IgM (△——△); anti-HLA-A,B,C IgM (▲——▲). (b) Results for Sal-2 IgG1 (○——○); Sal-4, IgG2b (●——●); FMC-21 IgG2a (▽——▽); and FLOPC-21 IgG3 (▼——▼); with anti-HLA-A,B,C IgG2a (■——■) as positive control.

carboxylase IgM failed to bind whereas polyclonal normal mouse IgG2a and anti-sheep erythrocyte IgG2a gave binding comparable with that of Sal-5 and FMC-21. FMC-19 (IgG3) bound to the cells to a similar extent to FLOPC-21 whereas J606 (IgG3) gave lower but still significant binding (data not shown). Whereas the anti-HLA-A,B,C antibodies bound to almost all cells in the sample, the other IgG2a and IgG3 antibodies bound to only approximately 60% of the cells in all cases.

Fc dependence of binding of Sal-5 to 81/13 cells

Removal of the Fc fragment of Sal-5 IgG2a by digestion with pepsin and passage through protein A–Sepharose caused the loss of binding to 81/13 cells. Data obtained using $(F(ab')_2$ fragments

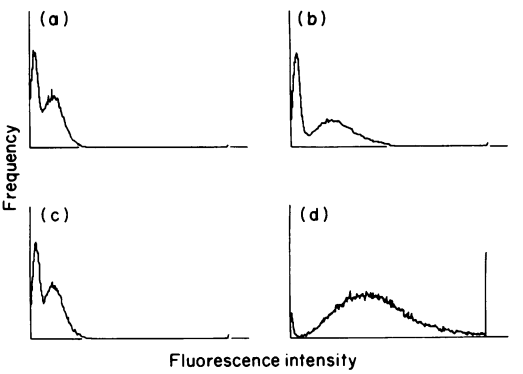

Fig. 3. Dependence on the Fc region of binding of Sal-5 IgG2a to acute myelomonocytic leukaemic cells 81/13. Cells were labelled with intact or $F(ab')_2$ fragments of Sal-5 IgG2a as described in Methods except that fluorescein labelled $F(ab')_2$ rabbit anti-mouse $F(ab')_2$ (1·25 $\mu g/10^6$ cells) was used as detecting reagent. Fluorescence histograms shown are: (a) negative control: 81/13 with detecting reagent alone; (b) 81/13 with intact Sal-5 IgG2a (0·5 $\mu g/10^6$ cells); (c) 81/13 with $F(ab')_2$ fragments of Sal-5 IgG2a (0·5 $\mu g/10^6$ cells) and (d) positive control: 81/13 with IgG2a anti-HLA-A,B,C. Peak fluorescence channels were 30, 50, 30, 200 for a–d respectively. Sixty per cent of cells were positive in all cases except (d) where 100% of cells were positive. Scatter histograms were identical in all cases and are not shown.

prepared in this way are shown in Fig. 3. In this experiment the detecting reagent was fluorescein labelled rabbit $F(ab')_2$ anti-mouse $F(ab')_2$. This ensured that any loss of binding was not due to the inability of the second antibody to react with the $F(ab')_2$ fragments of the mouse immunoglobulin. Slight binding was observed with the detecting reagent alone, presumably due to the presence of a trace of intact immunoglobulin.

Blocking of Sal-5 binding by normal rabbit serum

Inclusion of 10% heat-inactivated normal rabbit serum reduced binding of Sal-5 (0·5 $\mu g/10^6$ cells) as evidenced by a reduction of peak fluorescence intensity from channel 37 to channel 25, but had no effect on the binding of the anti-HLA-A,B,C MoAbs.

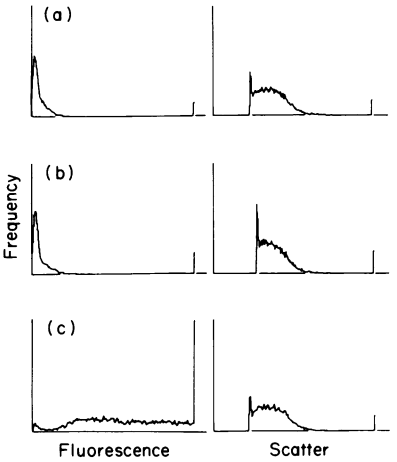

Fig. 4. Flow cytometric analysis of the binding of Sal-5 IgG2a and an anti-HLA-A,B,C MoAb to normal peripheral blood mononuclear cells. Details were as described for Fig. 1. (a) No mouse antibody: fluorescence gain 8, peak channel 7. (b) Cells incubated with Sal-5 IgG2a (0·5 $\mu g/10^6$ cells): gain 8, peak channel 7. (c) Cells incubated with IgG2a anti-HLA-A,B,C (0·5 $\mu g/10^6$ cells): gain 4, dual peaks channels 100, 256; 100% positive.

Fc binding of mouse MoAbs to other cell types

Sal-5 (IgG2a), Sal-2 (IgG1), Sal-4 (IgG2b), Sal-3 (IgM) and J606 (IgG3) were examined for binding to peripheral blood mononuclear cells and granulocytes from a normal donor. No binding was observed in any case, although strong binding of the positive control antibodies 2B2-F7 and 7B6c was observed. The results obtained with Sal-5 and 7B6c on mononuclear cells are shown in Fig. 4.

Leukaemic cell populations from eight other patients with non-lymphocytic leukaemia were examined for binding of Sal-5 IgG2a. These specimens were chosen for their varying proportions of blast cells and leukaemic monocytes in an effort to determine which cell types were involved in Fc binding. The results are summarized in Table 1, and shown in detail for specimen 81/53 (acute monocytic leukaemia) as an example in Fig. 5. It appears that leukaemic cells with monocytic differentiation as well as some but not all blast cells have Fc receptors for mouse IgG2a. However specimen 81/23 was an exception to this pattern and a larger series is necessary to accurately define the relationship between cell differentiation and expression of Fc receptors which can bind mouse immunoglobulins. The important point is that the possession of such Fc receptors appears to be quite common in human non-lymphocytic leukaemic cell populations.

DISCUSSION

Fc receptors are widely distributed on normal and leukaemic leucocytes, and may bind xenogeneic as well as homologous immunoglobulins (Maheu *et al.*, 1981). This raises the possibility that Fc

Table 1. Binding of IgG2a to non-lymphocytic leukaemia cells from different patients. The table shows the results obtained using Sal-5 IgG2a (0·5 $\mu g/10^6$ cells) in the cell binding assay with the peripheral blood mononuclear cell fraction from patients with active leukaemia

Patient	Diagnosis*	Differential count		% cells binding	Fluorescence intensity (peak channel)	
		% blasts	% monocytes	IgG2a	Control†	IgG2a
81/13	AMML(M4)	25	55	60	13	70
80/36	AMML(M4)	81	7	50	4	20
81/53	A MoL(M5)	85	6	96	16	48
81/17	A MoL(M5)	80	0	92	26	42
81/51	AML(Ml)	79	0	0	4	4
81/34	AML(Ml)	96	0	27	7	55
81/23	CMML	20	35	0	7	8
80/24	CMML	0	80	96	26	45
80/14	CMML	1	90	93	21	95

* Abbreviations: AMML = acute myelomonocytic leukaemia; AMoL = acute monocytic leukaemia; AML = acute myeloid leukaemia; CMML = chronic myelomonocytic leukaemia. FAB classifications (where applicable) are given in brackets.

† The control consisted of cells incubated with the fluorescein labelled goat anti-mouse reagent alone.

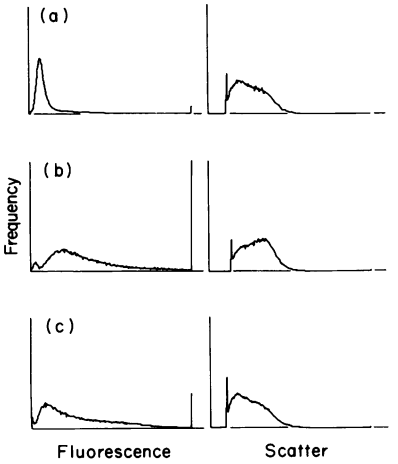

Fig. 5. Flow cytometric analysis of the binding of Sal-5 IgG2a to acute monocytic leukaemic cells 81/53. Details were as described for Fig. 1. (a) No mouse antibody: fluorescence gain 4, peak channel 16. (b) Cells incubated with Sal-5 IgG2a (0·5 $\mu g/10^6$ cells) fluorescence gain 4, peak channel 48, 96% of cells positive. (c) Cells incubated with IgG2a anti-HLA-ABC positive control (0·5 $\mu g/10^6$ cells): fluorescence gain 1, peak channel 24, 100% positive.

binding of mouse MoAbs to human cells could occur and be mistakenly interpreted as indicating the presence of the particular antigen on the cell. Furthermore, Fc receptors for homologous immunoglobulins are known to be class and subclass specific (Unkeless, Fleit & Mellman, 1981), to differ on various cell types (e.g. Fleit, Wright & Unkeless, 1982) and to vary with cell activation (Ezekowitz, Bampton & Gordon, 1983). Therefore each Ig subclass must be considered separately with respect to the possibility of Fc binding to each cell type.

We first observed strong binding of the IgG2a control MoAb, Sal-5, with a peripheral blood mononuclear cell population from a patient (code 81/13) with acute myelomonocytic leukaemia, M4 according to the FAB classification (Bennett *et al.*, 1976). That this was due to Fc binding and not an unexpected cross-reaction was clearly shown by the following findings: (i) one other unrelated monoclonal and two polyclonal IgG2a preparations gave similar results; (ii) $F(ab')_2$ fragments of Sal-5 were not bound and (iii) the binding was partly blocked by inclusion of 10% heat-inactivated normal rabbit serum in the incubations. Three different monoclonal IgG3 preparations also bound to this cell population, but little or no binding of IgM or other IgG subclasses was detected. The subclass specificity parallels that found for binding of mouse antibodies to homologous macrophages where IgG2a and IgG3 have separate Fc receptors from IgG1 and IgG2b which share the same receptor (Unkeless *et al.*, 1981; Diamond & Yelton, 1981).

A preliminary survey of non-lymphocytic leukaemic cell populations from nine different patients indicated that the Fc binding of mouse IgG2a antibodies to 81/13 cells was not an isolated case. Indeed cells in seven of the nine specimens examined displayed significant binding of Sal-5. Binding appeared to occur with some blast cell and leukaemic monocyte populations, however a larger series is necessary to define the relationship of Fc receptor expression for mouse IgG2a to cell differentiation. We did not observe Fc binding of any mouse MoAb to normal peripheral blood granulocytes or mononuclear cells, however monocyte enriched populations were not examined and so the possibility of Fc binding to normal monocytes cannot be entirely dismissed.

The results of the study demonstrate the importance of using appropriate control immunoglobulins in specificity studies of mouse MoAbs reacting with human cells. The differences in subclass specificity of the Fc receptors means that X63 IgG1, which is widely used as a negative control, is an inappropriate control for antibodies of IgG2a or IgG3 subclass. Several widely used commercial MoAbs, e.g. OKT3 and OKT8 (Ortho, Raritan, New Jersey, USA) are of IgG2a subclass, and it is therefore particularly important that studies using these antibodies with cells from patients with various disease states should include an IgG2a control antibody. In our own experiments (data not shown) we observed that OKT3 bound strongly to 78% of the cells in specimen 81/13 indicating, presumably, Fc binding to the monocyte/blast population as well as specific binding to normal T cells. We were able to reduce substantially although not entirely eliminate Fc binding of IgG2a to specimen 81/13 by including 10% heat-inactivated normal rabbit serum, since rabbit immunoglobulin binds effectively to human Fc receptors. Thus, inclusion of such Fc blocking reagents during incubation can minimise complications due to Fc binding, although care must be taken to ensure that these immunoglobulins are not recognized by the second antibody where indirect assays are used.

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