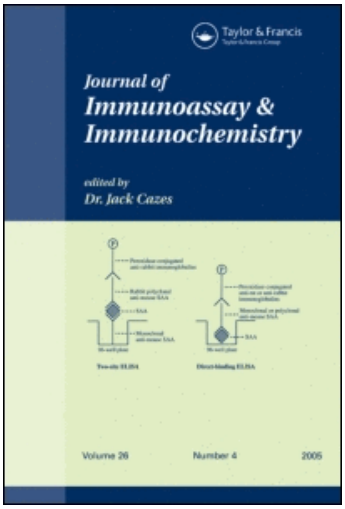

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The Rose Bengal Assay for Monoclonal Antibodies to Cell Surface Antigens: Comparisons with Common Hybridoma Screening Methods

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THE ROSE BENGAL ASSAY FOR MONOCLONAL ANTIBODIES TO CELL SURFACE ANTIGENS: COMPARISONS WITH COMMON HYBRIDOMA SCREENING METHODS

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ABSTRACT

An automated colorimetric procedure for detection of antibodies specific for cell surface antigens (1) has been compared for specificity and sensitivity to other methods of hybridoma supernatant screening. The Rose Bengal colorimetric assay (RBA) compared favourably in these respects with whole cell radioimmunoassay and indirect immunofluorescence with manual or flow cytometric analysis (FACS). A major advantage of the method is that it allows a large number of samples to be screened in a comparatively short time. Unlike other semi-automated colorimetic assays, such as ELISA, the procedure does not require cell fixation, which can destroy some antigenic determinants. The original assay of O'Neill and Parish (1) has been modified to give increased sensitivity and also to enable the detection of erythrocyte specific antibodies by elimination of the dye staining step and direct measurement of haemoglobin by spectrophotometry. The RBA allows detection of monoclonal antibodies (MoAb's) binding to only a proportion of the cells in a sample, which is an important feature when hybridoma supernatants are screened for reactivity to a minor cell population, for example against leukaemic cell samples with low percentages of blast cells.

(KEY WORDS: Hybridoma screening; cell surface antigens)

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INTRODUCTION

The development of the monoclonal antibody technology (2) has enabled immunologists and cell biologists to greatly expand their knowledge of cell surface antigens. However, the screening of hybridoma supernatants for their specificity represents a very large and time consuming proportion of the process. It is desirable to determine specificity of hybridomas as early as possible in the process to eliminate the need for maintaining cultures that are not of interest, in view of the time and expense involved. Screening methods which have been employed include whole cell radioimmunoassay (3), manual indirect immunofluorescence (4) and microrosette assays (5) all of which are quite labour intensive. Enzyme linked immunosorbent assays (ELISA), while being less labour intensive, require cell fixation and endogenous enzyme blocking, which may result in loss of antigenic determinants (6) (our unpublished results). Recently, a colorimetric assay has been described (1), which allows rapid and sensitive assay of antibodies specific for cell surface antigens. Briefly, this technique uses 96 well microtitre trays coated with a purified antibody to mouse immunoglobulin (Ig). These are incubated with test antibody, which, if specific, binds target cells to the tray. Bound cells are quantitated by uptake of Rose Bengal dye, and measurement of absorbance with an automated ELISA reader enables rapid determination of antibody specificity and titre. This

paper deals with our experience with the assay in screening hybridoma supernatants. It includes a comparative study using a human cell line and MoAb's of known specificity, in which the RBA was compared with other assays in common use: the whole cell indirect radioimmunassay (RIA), and indirect immunofluorescence with manual and flow cytometric scoring. In addition, a modification is described for the detection of erythrocyte specific MoAbs.

MATERIALS AND METHODS

Monoclonal Antibodies and Sera

All MoAb's used were prepared and cloned in this laboratory, except for FMC 14 (7), a kind gift of Dr H. Zola, essentially using the method of Oi and Herzenberg (8). MoAb's used as negative controls [Sal-2(IgG1), sal-5 (IgG2A)] were raised against cytoplasmic antigens of Salmonella enteritidis (9). Positive control polyclonal antiserum was raised against normal human tonsillar lymphocytes in mice. Goat antibody to mouse Ig(G+M) was raised using pooled, purified monoclonal antibodies of various classes. Antibodies were precipitated from the serum with 40% ammonium sulphate and purified by affinity chromatography on columns of purified polyclonal mouse IgG and IgM coupled to Sepharose 4B (Pharmacia, Uppsala

Sweden). The purified antibodies to murine IgG and IgM were pooled and antibody cross reactive with human Ig was removed using a human Ig-Sepharose 4B column.

Purified goat anti-mouse Ig(G+M) was labelled with fluorescein isothiocyanate (FITC) by the method of Rinderknecht (10), and with I^{125} by the method of Salacinski et al (11).

Cell Lines

DAUDI, a human Burkitt Lymphoma derived cell line was obtained from Dr H. Zola, Flinders Medical Centre, South Australia. The line was maintained in RPMI 1640 (GIBCO, G.I. USA) with 15 mM HEPES and 10% FCS (Flow, Aust.) without bicarbonate/carbon dioxide buffering. Human AML cells used in immunization and screening were prepared and cryopreserved as previously described (12). Other human cell lines (RC2A, U937, HL60, K562, BALM-1 and MOLT-4) were obtained and maintained as previously described (13).

The Rose Bengal Assay

Linbro flexible 96 round-bottomed well microtitre trays (Flow, VA, cat. No. 76-364-05) were coated with goat anti-mouse Ig(G+M) antibody by incubation with 100 µl per well of affinity purified antibody at 5 µg/ml at 4°C over night. Trays were washed 3 x with isotonic Phosphate Buffered Saline (PBS) pH7.4 by flooding and flicking. Undiluted or serial 1/2 dilutions of

hybridoma supernatants in PBS pH7.4 + 0.1% Bovine Serum Albumin (BSA) prepared in separate trays, were added in 100 ul aliquots to the goat anti-mouse Ig(G+M) coated trays. The trays were incubated at 4°C for 60 min, then washed 3 x as previously. Target cells, 2-5 x 10^5 in 100 µl of RMl 1640 (Gibco, Grand Island, USA) + 10% Foetal Calf Serum (FCS) + 0.1% sodium azide (to prevent active adherence or capping) were added to the wells and incubated at 4°C for 120 min. In later experiments, trays were first incubated for 30 min at 37°C, followed by 90 min at 4°C. After incubation, excess cells were flicked out and 100 ul of Rose Bengal dye (0.25% w/v in PBS) (Faulding, Australia) was added to each well, followed by incubation at 4°C for 15 min. Different batches of Rose Bengal were found to vary greatly in their suitability for use in the assay. The trays were washed 3 x as above, then 100 µl of Sodium Dodecyl Sulphate (SDS) solution (1% w/v in water) was added to each well to lyse bound cells and release the dye. The OD_{540}, corresponding to the absorbance maximum of Rose Bengal, was read on a Titertek Multiskan ELISA reader (Flow). Wells having an OD_{540} more than twice that of negative control wells were considered to be positive.

Modification for Anti-erythrocyte Antibodies

Trays coated with goat anti-mouse Ig(G+M) were washed and incubated with MoAb as described above. Human erythrocytes were

added to each well as 100 µl of a 0.5% suspension in PBS pH7.4 + 0.1% BSA, incubated at 37° for 30', followed by 90' at 4°C. Trays were washed 3 x with PBS pH7.4 to remove unbound cells. Instead of staining with Rose Bengal, cells were lysed with SDS solution, and haemoglobin was measured directly at OD_{410}, which corresponds to its absorbance maximum in the presence of 1% SDS.

Indirect Immunofluorescence

Binding assays to investigate specificity of MoAb's using indirect immunofluorescence were performed as previously described (14). Manual scoring was performed using an Olympus microscope (Model BH2/BH-RFL-W) with epi-illumination. A minimum of 200 cells per sample were scored. Flow cytometry was carried out at Flinders Medical Centre, South Australia, using a FACS IV (Becton Dickinson). Before analysis, labelled cells were fixed in 1% paraformaldehyde (15) and stored for up to 2 days at 4°C in the dark. Fifty thousand cells were analysed per sample.

Whole Cell Radioimmunoassay

Whole cell RIA was performed in the same way as indirect immunofluorescence, except that ^{125}I-labelled goat anti-mouse Ig(G+M) was added in the second stage instead of FITC-labelled goat anti-mouse Ig(G+M). After washing away excess radiolabel,

tubes were counted in a Packard auto-gamma spectrometer. Hybridoma supernatants which brought about binding of more than twice as much radiolabel to the cells as the negative control antibodies were considered "positive". Marginal cases were designated (+/-).

Haemagglutination Assay

Hybridomas producing antibodies to human erythrocyte antigens were prepared by Ms S. Niutta in this laboratory. Human erythrocytes (50 µl of a 0.5% solution in PBS pH7.4 + 0.1% BSA) were incubated with 50 µl aliquots of serial 1/2 dilutions of the hybridoma supernatants for 45 minutes at room temperature in Linbro flexible round bottomed 96 well Microtitre trays. Haemagglutination titre was determined by visual inspection.

RESULTS

Optimisation

It was noted that some human Acute Myeloid Leukaemia (AML) samples gave very low binding in the RBA, even though adequate cell numbers were used. To overcome this, an incubation at 37°C for 30 min at the cell binding stage was included in the protocol prior to a 90 min incubation at 4°C. This modification, when used with cell lines, markedly improved

the sensitivity of the assay. This sensitivity increase was most pronounced when low cell numbers were used. With 10^5 DAUDI cells per well, the sensitivity was eight fold greater than with the original protocol using the same MoAb. Figure 1 shows the titration of the MoAb SB1 (IgG1) on DAUDI cells. The concentration of undiluted SB1 was 10 μg/ml, thus the end point of 1/64, obtained with the 37° incubation, was equivalent to 16 ng of MoAb per well. When the entire cell binding incubation was carried out at 4°C, the end point occurred at a dilution of 1/8, corresponding to 125 ng of MoAb per well.

The increase in cell binding gained by the inclusion of an incubation at 37°C is thought to be due to increased fluidity of the cell membrane allowing more antigen to come into contact with specific antibody.

Comparison with Whole Cell RIA

For this comparison, 20 MoAb's of various subclasses were assayed for binding to DAUDI cell surface antigens. Of these 20 MoAb's, six were found to be negative and twelve were positive in both assays. There were two MoAb's which gave discordant results. One MoAb, PA5, was positive as determined by the RBA, and unable to be confidently assigned by RIA, i.e. (+/-). Another MoAb, QA4, was negative by RBA and positive by RIA. Subsequent investigation revealed that QA4 had an affinity for the plastic tubes used in the RIA, causing a false positive

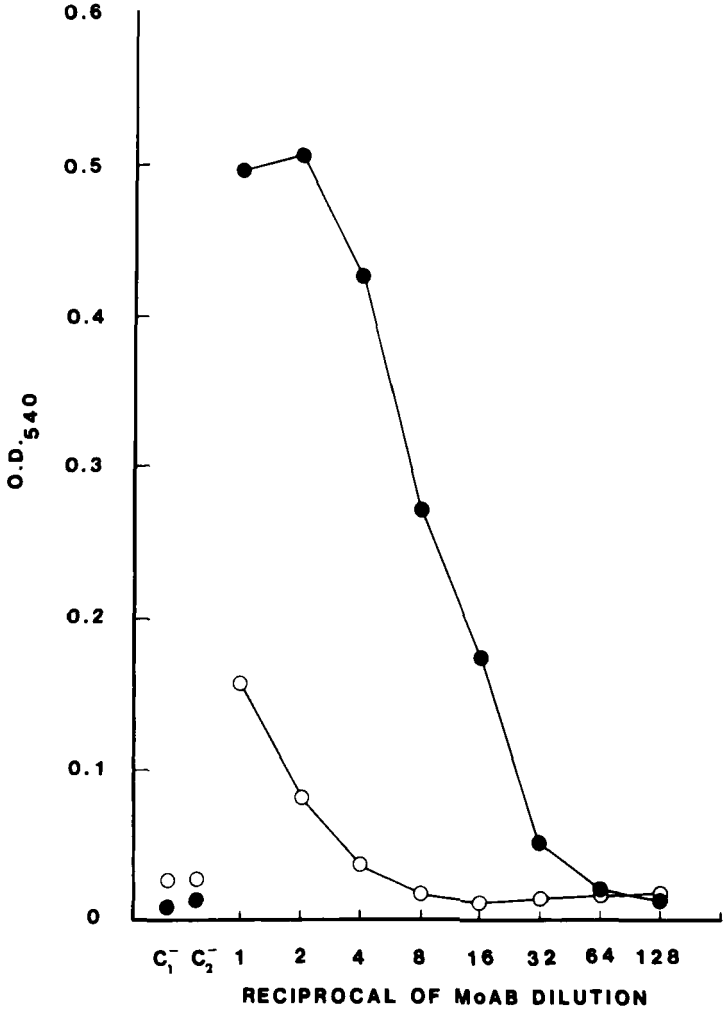

Figure 1

Titration of MoAb SB1 (IgG1) on DAUDI cells 10^5 per well by RBA. (●): Cell binding step at 4°C for 120 min. (○): Cell binding step initially at 37°C for 30 min, followed by 4°C for 90 min. C_1^- and C_2^- are, respectively, Sal-2 (IgG1) and Sal-5 (IgG2A). Points are the average of duplicate assays.

result. Resuspension of the QA4 labelled cells and transfer to a fresh tube before counting reduced the radioactivity by 60% and resulted in this antibody being classified as negative. In contrast, transfer of cells labelled with other positive MoAbs to fresh tubes prior to counting resulted in a reduction in counts of 10-15%. Comparative data for the RBA and RIA are shown in Figure 2. Statistical analysis of the data by the Spearman Rank Correlation test (16) gave a correlation coefficient, r_s, of 0.694, which has a $p < 0.001$.

Comparison with Manual Indirect Immunofluorescence

The same MoAbs used in the RIA comparison were tested for binding to DAUDI cells by manual indirect immunofluorescence. The results of the assay in terms of positivity and negativity were in total agreement with the RBA (data not shown). Antibody QA4 was negative, and PA5 was weakly reactive, with about 30% of DAUDI cells scored as positive.

Comparison with Indirect Immunofluorescence Scored by Flow Cytometry

This comparison, using 19 of the 20 MoAbs used previously, shows FACS analysis, like manual indirect immunofluorescence, to be in agreement with the RBA in assigning positivity or negativity. MoAb PA5 showed weak binding to DAUDI cells with a peak channel fluorescence intensity of 38 on a

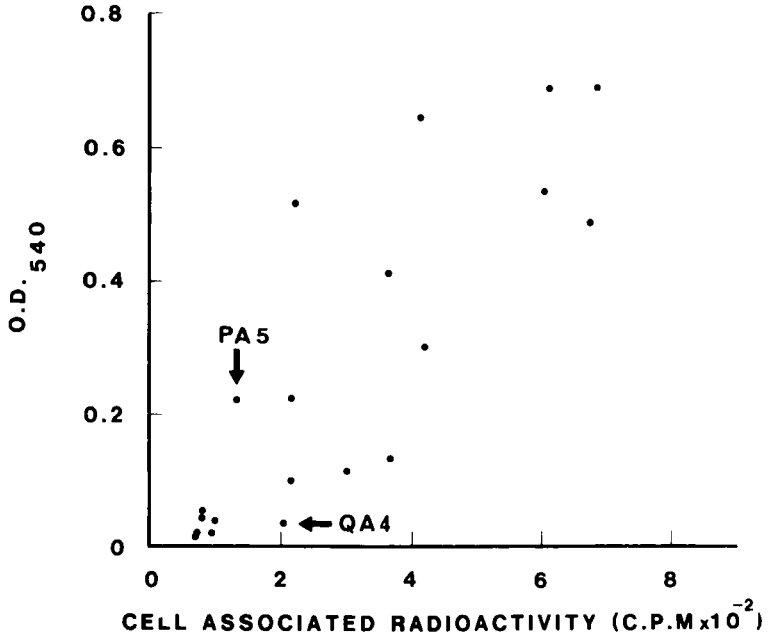

Figure 2

Comparison of RBA and RIA of Mo Ab on DAUDI cells. Cell numbers used were 3 x 10^5/well in the RBA and 5 x 10^5/tube in the RIA. Points are the average of duplicate assays. Arrowed points are discussed in the text (r_s = 0.694, $p < 0.001$).

logarithmic scale compared to a control negative value of 30. This result, along with that of manual indirect immunofluorescence and RIA, highlights the deficiency of the RIA in resolving positivity of MoAb to weakly binding antigens, or antigens present on a small proportion of cells. Figure 3 shows a comparison of data from the RBA and FACS analysis. Spearman Rank correlation coefficient, r_s, for this set of data was

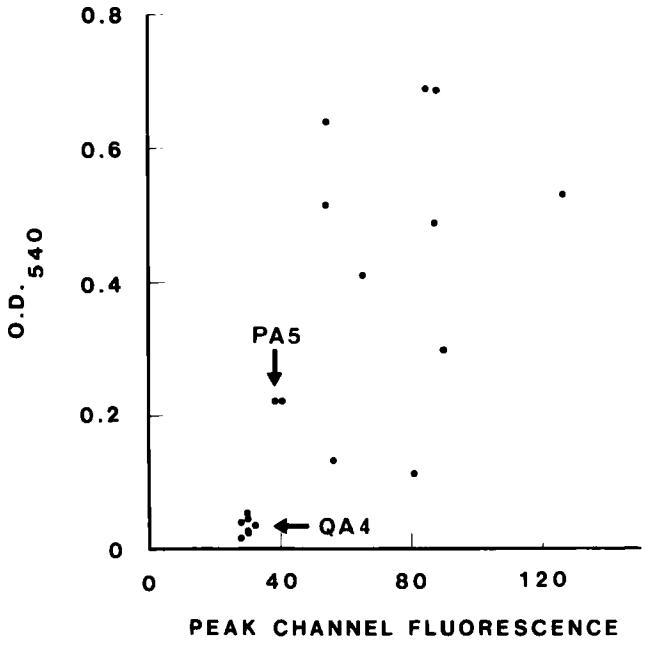

Figure 3

Comparison of RBA and indirect immunofluorescence (with FACS analysis log gain). Cell numbers used were 3 x 10^5/well in the RBA and 6 x 10^5/tube in the FACS analysis. Points are the average of duplicate assays. Arrowed points are discussed in the text (r_s = 0.578, $p < 0.01$).

0.578 corresponding to $p < 0.01$. The spearman Rank correlation coefficient for the comparison of the RIA and FACS data was 0.796, corresponding to $p < 0.001$.

Results with Other Human Cell Lines and Cell Types

A similar comparison has been performed using the human myelomonocytic cell line RC2A (17) (data not shown) with similar

success. Other human cell lines which have been used successfully in the RBA include U937, HL60, K562, Molt 4 and BALM-1. In addition, human cell types such as mononuclear and granulocyte fractions of normal peripheral blood, normal tonsillar lymphocytes, concanavalin A stimulated T cell blasts, normal and leukaemic bone marrow cells, and numerous myeloid leukaemia samples have been used successfully in the assay. Some leukaemic samples gave extremely low binding in the assay, which was overcome by inclusion of the 37°C incubation step, without a subsequent increase in negative control binding. Other leukaemic samples gave high background binding which obscured weakly positive MoAbs. At the suggestion of Dr C.R. Parish, this was countered by the inclusion, after the MoAb binding step, of a separate incubation in which wells were treated for 30' at 4°C with RPMI 1640 + 10% heated FCS before addition of cells. FCS for this purpose was incubated for 30' at 80°C.

Screening of an Anti-AML Fusion: Comparison with RIA

Primary screening of supernatants of hybridomas, which had been prepared from spleen cells of mice immunized with peripheral blood mononuclear cells (containing 86% blasts) from a patient with AML, was carried out simultaneously by RIA and RBA. Of the 84 supernatants assayed, 21/84 could be confidently assigned positive according to the RBA results. Of the 21

supernatants only 11 could be confidently assigned as positive by the RIA, the rest were negative. A further 3 supernatants were unable to be confidently assigned i.e. (+/-) by RIA, but were negative by the RBA. Another supernatant (arrowed in Fig. 4) gave a strong positive result by RIA, but was negative by the RBA. It is suspected that the reason for this result was similar to that given by the MoAb QA4 (see above), that is, non-specific binding to the tube. However, further investigation of this antibody was not carried out. The MoAbs of interest from this fusion were further characterized by manual indirect immunofluorescence. Some clones were apparently unstable and stopped producing antibody, but all those which remained positive by RBA were also positive by indirect immunofluorescence.

Figure 4 shows a regression plot of the results. The Spearman Rank correlation coefficient, r_s, was 0.912, corresponding to a $p < 0.001$.

Detection of MoAbs Specific for Minor Cell Populations

Mixtures of the human myelomonocytic cell line RC2A, and the human promyelocytic cell line HL-60 were used to examine the ability of the RBA to detect a MoAb binding to a population of cells making up only a proportion of the cell sample tested. The anti-Ia MoAb FMC14 (7) binds to RC2A cells but not to HL60 cells. A total of 2.5×10^5 cells per well were added to goat

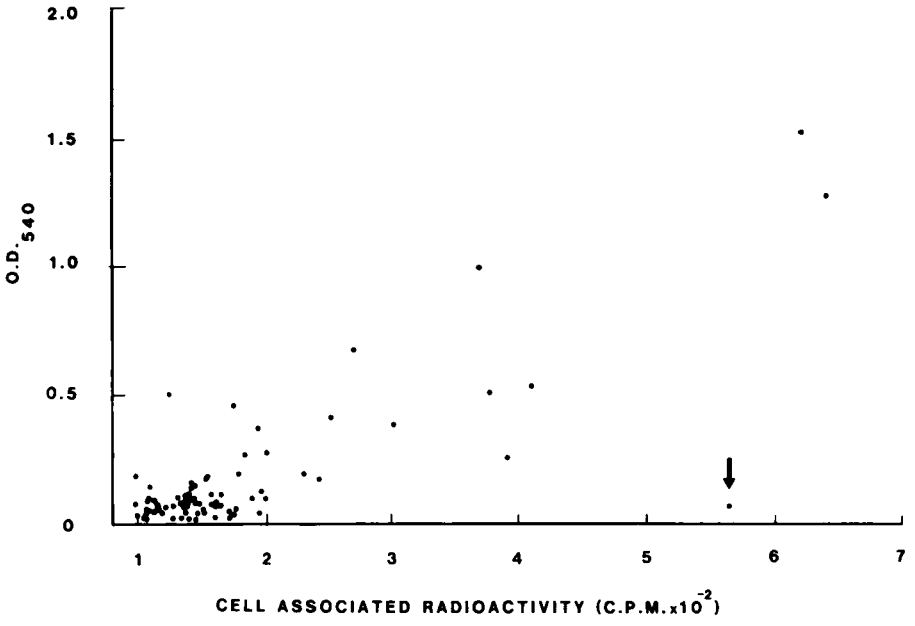

Figure 4

Primary screening of hybridomas against AML cells by RBA and RIA. The figure shows a regression plot of results obtained by the two assay methods. Points are the average of duplicate assays. Arrowed point is discussed in the text (r_S = 0.912, $p < 0.001$).

anti-mouse Ig(G+M)-coated microtitre trays to which FMC-14 had been bound. The proportion of RC2A cells in the mixture ranged from 5% to 100%. Table 1 shows that the RBA is able to detect MoAbs specific for a minor population of cells comprising 5-10% of the total population.

Detection of MoAbs to Erythrocyte Surface Antigens

The modified protocol for detecting MoAbs specific for human erythrocyte antigens was equally as accurate as the

TABLE 1

RBA using Mixtures of 'Positive' and 'Negative' Cells.

% RC2A	% HL-60	OD_{540}
100	0	1.14
75	25	0.9
50	50	0.73
25	75	0.30
10	90	0.12
5	95	0.05
0	100	0.02

The RBA was carried out on mixtures of RC2A cells (FMC-14 positive) and HL-60 (FMC-14 negative) using the MoAb FMC-14 (IgG1). Cell numbers were 2.5 x 10^5/well in total. The negative control MoAb Sal-2 (IgG1) gave an OD_{540} of 0.02 to 0.025 on both cell lines.

standard haemagglutination assay. In many cases, the assay gave an increase in sensitivity of 8-fold or better over the haemagglutination assay (data not shown).

DISCUSSION

The preceding results show that the RBA is able to accurately identify MoAbs specific for cell surface antigens. Statistical analysis of the data from experiments in which the RBA was compared with RIA and immunofluorescence revealed a high correlation between the results of the different assays. However, the regression plots appeared to be non-linear, which

may indicate that the results obtained by RBA were not simply a function of antigen density.

Apart from its accuracy, the RBA offers a significant advantage over RIA and immunofluorescence due to its rapidity and non labour intensive nature. The RBA overcomes three additional major disadvantages encountered with the RIA: the non-specific binding of antibodies to the RIA tubes, the resolution of weakly reacting MoAbs from negative MoAbs, and, as with immunofluorescence, damage to cells due to repeated centrifugation and resuspension.

The RBA has a very low control negative background OD_{540} which allows easier identification of 'weakly positive' MoAbs which cannot always be confidently assigned as positive or negative by RIA. Typically, such antibodies give results 30 to 60% above background radioactive binding, whereas in the RBA they give an OD_{540} of 0.1 - 0.2 compared with a control negative OD_{540} of 0.01 - 0.05. This ability is enhanced greatly by the use of the modified protocol, in which the cell binding step is initially carried out at 37°C, as the negative control binding is not increased. The necessity for repeated centrifugation and resuspension of the target cells can raise the 'background' in RIA and immunofluorescence due to cell damage. Cell lines, because of their fragility, are especially vulnerable. The RBA avoids this problem as the cells are added

at a late stage of the assay and subsequent manipulations are gentle.

Whole cell ELISA assays, while being rapid to perform, have the disadvantage of requiring fixation of the target cells, which can result in loss of antigenic determinants (6) (our unpublished results). The RBA uses cells in physiologic, buffered medium during the binding process, ensuring retention of antigenic determinants.

The RBA has the ability to detect of the order of 20 ng per well of specific antibody to a cell surface antigen. While this is not as sensitive as an optimised ELISA assay, which can detect of the order of 1-10 ng of specific antibody per test well, for the purpose of screening hybridoma supernatants, this level of sensitivity is entirely adequate, as supernatants of antibody producing hybrids usually contain more than 1 μg of MoAb per ml. The RBA enables detection of MoAb's specific for minor populations in cell samples, which is advantageous when screening hybridoma supernatants against heterogeneous cell samples. A modified protocol allows detection of erythrocyte specific MoAb's, in many cases with greater sensitivity than a haemagglutination assay.

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TO WHOM REQUESTS FOR REPRINTS SHOULD BE MADE

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