Quantitative analysis of human leucocyte antigen expression during culture of Epstein-Barr virus-transformed cell lines using the Dako QIFIKIT

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ABSTRACT

Pre-existing donor-specific human leucocyte antigen (HLA) antibodies in renal allograft recipients result in hyperacute and accelerated graft failure. These antibodies can be detected in flow cytometric assay systems using HLA-characterised Epstein-Barr virus (EBV)-transformed B-lymphocyte cell lines. Confident assay performance is predicated by the expression of HLAs on the EBV-transformed B-cell line surface. Surface HLA expression of three EBV-transformed B-cell lines that had previously been used as part of a potential organ recipient serum screening panel at St James' University Hospital, Leeds, are assessed for changes in the level of HLA expression over the nominal culture duration of eight days using the QIFIKIT (Dako, Denmark), a quantitative flow cytometry kit for assessing cell surface antigens. A comparison of the mean fluorescence intensity (MFI) of the known antigen levels of the beads via a calibration graph permits determination of the antibody binding capacity of the cell lines. Results showed that HLA expression is not consistent throughout the cell culture, with optimal expression occurring during day 2 of culture. Inconsistent HLA expression demonstrated during the cell culture means that no assumption of the level of HLA expression can be made, and that cell lines used as part of a screening panel should have their HLA expression in cell culture determined.

KEY WORDS: B lymphocytes.
Epstein-Barr virus.
Surface antigen.

Introduction

Pre-existing donor-relevant human leucocyte antigen (HLA) antibodies in an organ transplant recipient result in hyperacute and accelerated graft failure.[1] To avoid this consequence, laboratories supporting transplant programmes engage in longitudinal serum screening for the detection of HLA antibodies and crossmatch the donor and recipient immediately prior to transplant.

Serum screening can be performed by various means. Contemporary techniques utilise flow cytometric platforms that are extremely sensitive and permit simultaneous screening for HLA class I and II antibodies. The Leeds' screening programme includes the use of Epstein-Barr virus (EBV)-transformed B lymphocytes to detect anti-HLA by a flow cytometric method.[2]

Anti-HLA in the test serum binds to the Class I and II antigens on the EBV-transformed B-cell surface, which is then detected by binding to a fluorochrome-conjugated antibody specific to anti-human IgG (or IgM) using a flow cytometer. It was reasoned that should levels of HLA expression by the cells vary over the duration of culture, then this would influence assay sensitivity. To assess this possibility the commercially available QIFIKIT (Dako, Denmark) was used to determine the target antigen expression of cell lines.

Materials and methods

QIFIKIT permits the quantitative determination of cell surface antigen using an indirect immunofluorescence technique, with antigen quantity expressed in antibody binding capacity (ABC) units. The kit comprises two sets of 10-µm diameter beads, each coated with antigen. One set contains two bead populations, one with a low-level antigen coating and the other with a high-level coating, which serve as a guide for a window for analysis. The second set of beads contains six populations of beads, each covered with an increasing level of primary mouse anti-human CD5 monoclonal antibody (Mab) molecules (clone ST1, isotype IgG_{2A}).

When stained with a fluorescein isothiocyanate (FITC)-conjugated antibody (if the FITC:antibody ratio is fixed) then mean fluorescence intensity (MFI) correlates with the ABC of the bead; thus, the greater the amount of mouse Mab, the higher the ABC and the MFI. Measurement of the MFI of each bead group then can be used to plot a calibration graph of MFI against ABC of each bead (Fig. 1), permitting the determination of an unknown sample by extrapolation from the graph. For this to be successful, however, the antibody used must be a mouse antibody present in the assay at saturation level, and the FITC-conjugated antibody must also be an anti-mouse secondary antibody at saturation level.

Three EBV-transformed cell lines (29036, 27827 and 29962), originally gifted from the Welsh Blood Service and used routinely as part of a serum screening panel, were selected and recovered from liquid nitrogen storage. The thawed cell

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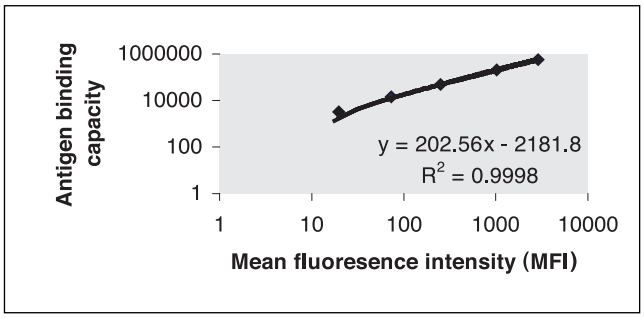

Fig. 1. Example of a calibration graph showing the linear regression equation and correlation coefficient.

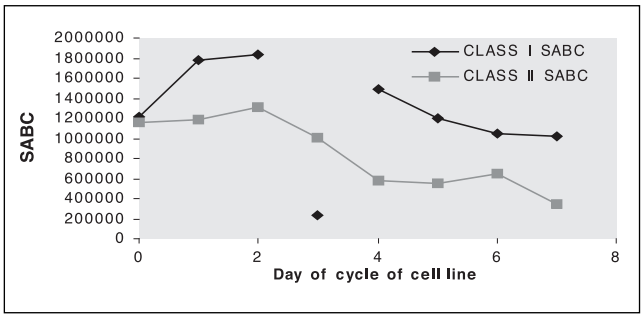

Fig. 2. Specific antibody binding of cell line 27827.

lines were resuspended in 10 mL RPMI 1640 medium containing 10% fetal calf serum (FCS), 5 mL L-glutamine (L-Glu) and 5 mL penicillin/streptomycin (pen/strep). Cultures were incubated at 37°C in a 5% CO_2 incubator.

After 24 h the culture medium was aspirated carefully, taking care to avoid disturbing the cells, and 10 mL fresh medium was added. The three cell lines were then expanded by removal of spent medium and replacement with fresh as required until a concentration of 5 x 10^5 cells/mL in 300 mL fresh medium was reached.

Each cell line was then inoculated into 24 culture flasks (50 mL) containing 10 mL RPMI 1640 medium containing FCS, pen/strep and L-Glut), to achieve a cell seeding of 5 x 10^3 mL. The 24 flasks were labelled in replicates of three, day 0 to day 7. The cell lines were incubated at 37°C in a CO_2 incubator until their day of analysis. Day 0 analysis was performed immediately using QIFIKIT (Dako) and a FACScan flow cytometer (Becton-Dickinson).

To 14 test tubes (labelled 1–14) were added 100 µL each cell line and each primary mouse monoclonal antibody (Table 1). These were incubated for 30 mins at 4°C and then 3 mL phosphate-buffered saline/bovine serum albumin/azide (PBS/BSA/azide) was added to each tube and gently vortex-mixed. The tubes were then centrifuged at 1100 rpm for 5 min and then the PBS/BSA/azide wash and centrifugation steps were repeated.

Table 1. Tube identity and corresponding cell line and antibody strain.

Tube	Cell line	Antibody
1	Set beads vial 1	–
2	Calibration beads vial 2	–
3	29036	CI isotype control
4	29036	CI antibody
5	29036	CII isotype control
6	29036	CII antibody
7	27827	CI isotype control
8	27827	CI antibody
9	27827	CII isotype control
10	27827	CII antibody
11	29962	CI isotype control
12	29962	CI antibody
13	29962	CII isotype control
14	29962	CII antibody

FITC-conjugated goat anti-mouse (10 µL) with a known antibody:fluorochrome ratio was added and the tubes were incubated in the dark at 4°C for 45 min. The tubes were then centrifuged at 1100 rpm for 5 min, the supernatant was aspirated, and then washing with PBS/BSA/azide (x2) was undertaken, as previously.

The set-up and calibration beads supplied with the kit were processed along with the cell lines, using the above method from the FITC-conjugate incubation stage. The cell lines and beads were then analysed immediately (or within 2 h if stored at 4°C) by flow cytometry.

A reference curve was produced from which to determine the specific antigen binding of the cell lines for a particular antibody, in accordance with the manufacturer's instructions. Antibody binding was quantified by plotting the MFI of the QIFIKIT calibration beads (Tube 2) against the ABC of each bead group, as stated in the manufacturer's protocol. A separate calibration graph was drawn for each day of the cell culture, ensuring accurate determination of antibody binding.

Using the calibration graphs, ABC was determined for Class I and II antibodies for each cell line by linear regression analysis. The Microsoft Excel program was used to calculate the linear regression equation into which the MFI of the cell line during a particular staining could be entered and thus antibody binding calculated. As a measure of accuracy, the correlation coefficients (R^2) were considered. The closer the R^2 value was to one, the greater the accuracy of the extrapolation.

Once the ABC had been calculated for each antibody and antibody isotype negative control, the specific antibody binding capacity (SABC) of each cell line to each antibody could be determined. The SABC was determined by subtracting the ABC of the isotype control from the ABC of the antibody used to stain Class I and Class II HLAs.

Results

Figure 2 demonstrates the effect that cell culture age has on the antibody binding of both Class I and Class II antibodies. Specific antibody binding of Class I antibodies was higher than that of Class II antibodies over the duration of culture.

The SABC of the Class I antibody increased rapidly from day 0. Specific antibody binding of cell line 29036 rose from 1,209,156 at day 0 to an SABC of 1,780,101 on day 1. This level was maintained to day 2, after which the SABC decreased by 4000–5000 sites per day. Similar results for Class I antibodies were obtained for cell line 29962.

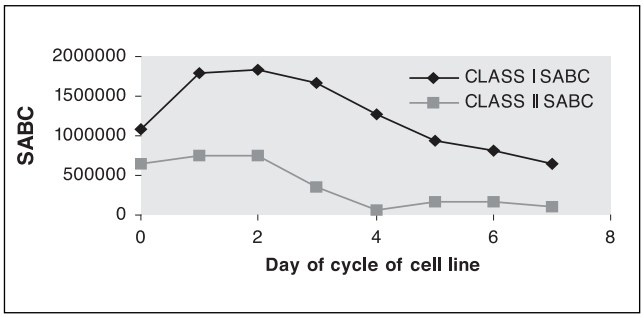

Fig. 3. Specific antibody binding of cell line 29036.

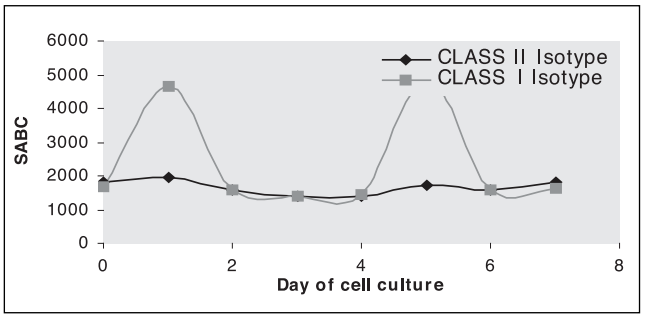

Fig. 4. Specific antibody binding capacity of isotype controls by cell line 29036.

The greatest SABC of Class I antibody for all three cell lines was seen over the two-day period after cell line culture was initiated. After a rapid increase in SABC on day 1, cell line 27827 (Fig. 2) and cell line 29036 (Fig. 3) SABC continued to rise but increased only by approximately 50,000 binding sites. This trend was mirrored for Class II binding levels. Results over the duration of the cell culture demonstrate that maximal specific antibody binding capacity is greatest during the first days of cell culture.

Analysis of isotype control binding capacity to cell lines was carried out to establish if binding of Class I and Class II isotype controls increased during cell culture. This would indicate that as the specific antibody binding capacity of Class I and II antibodies falls with the increased duration of culture, a rise in background binding is seen. However, this was not the case. Figure 4 clearly shows that background binding remained the same throughout the period of culture for cell line 29036. Similar results were obtained for the other cell lines (results not presented)

Discussion

Quantitative determination of cell line ABC showed conclusively that cell line HLA expression increases during the initial period of culture. Maximal ABC for cell lines 29036 and 27827 was at day 2, while cell line 29962 showed maximal ABC at day 1. Thereafter, levels decreased daily for all cell lines, and by day 7 each cell line ABC had decreased to below that of day 0. These findings are important to the cell lines used as targets in serum screening, as it might be expected that assay sensitivity and antigen levels are related. To ensure maximal HLA antigen expression the cell lines need to be harvested during the first two days.

The present work highlights the need to assess target antigen expression levels in cultured cells used in serum screening tests, as variation may result in changed assay sensitivity over time and confound efforts to monitor patients effectively. It is therefore advisable that cell lines used for screening purposes should first have their optimum stage of cell culture determined for SABC, and it should not be assumed that cell lines share the same characteristics. The Dako QIFIKIT provides a simple and robust technique for such determinations and is well suited to the routine laboratory.

Additional work may also determine the fate of the surface antigens that are lost during cell culture. Cells have been shown to excrete surface antigen in a soluble form.[3] If this is true then monitoring of HLA in the cell culture medium could detect the secreted HLA, and it would be expected that the secreted HLA antigens in the medium would increase as the SABC of the cell line decreased. Theoretically, measurement of the medium's HLA antigen volume could then provide an indirect method of assessing the SABC of the cell lines. □

References

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