Activation of NF-κB in human endothelial cells induced by monoclonal and allospecific HLA antibodies

John D. Smith, Charlotte Lawson, Magdi H. Yacoub and Marlene L. Rose

National Heart & Lung Institute, Imperial College School of Medicine, Harefield Hospital, Harefield, Middlesex UB9 6JH, UK

Keywords: chronic rejection, endothelial cells, HLA antibodies, NF-κB, transplantation

Abstract

Chronic graft rejection, characterized by a gradual occlusion of grafted vessels, is the most serious complication following heart and kidney transplantation. Although often associated with chronic production of anti-HLA and anti-endothelial antibodies, the precise role of antibodies in chronic rejection remains uncertain. Here we have investigated whether HLA-specific antibodies, either monoclonal or derived from patients, cause endothelial cell activation. Thus we investigated tyrosine phosphorylation, NF-κB activation and cell proliferation in human umbilical vein endothelial cells (HUVEC) or microvascular endothelial cells from adult human heart (CMEC).

Ligation of monomorphic determinants of MHC class I molecules (using the mAb W6/32) on the surface of HUVEC caused an increase in tyrosine phosphorylation of proteins of mol. wt ~75–80 kDa. Similarly, ligation of monomorphic determinants on both CMEC and HUVEC resulted in increased NF-κB binding compared to controls (by 74.4 and 52.5%, P = 0.001) and this was enhanced by addition of secondary antibody. Two HLA-specific mAb resulted in a 277 and 170% increase in NF-κB-binding activity compared to controls. Four patient samples containing HLA antibodies were used against HLA-specific HUVEC and four samples were incubated with HUVEC bearing irrelevant antigens. Patient sera alone enhanced NF-κB binding by 27–186%, but only when added to HUVEC bearing relevant antigens. W6/32 and allospecific antibodies from patients significantly enhanced HUVEC proliferation, measured by uptake of [3H]thymidine. In conclusion, activation of NF-κB by human anti-HLA antibodies demonstrates their potential role in pathogenesis of chronic vascular occlusive disease following transplantation.

Introduction

It is well established that the presence of HLA antibodies reactive with donor HLA antigens prior to renal or heart transplantation is associated with increased graft failure, often in the form of hyperacute rejection (1–3). Traditionally, antibodies are detected using a complement-dependent cytotoxicity assay. It is therefore assumed that these antibodies act by fixing complement components leading to lysis and death of the target cells in vivo. Human endothelial cells constitutively express MHC class I and class II molecules (4), and are likely to be the first donor cells to be affected by antibody responses. In recent years it has become apparent that antibodies may be acting in a more subtle way to damage endothelial cells. Chronic rejection of solid organ transplants, characterized by a progressive occlusive vasculopathy in donor grafts, is the most common long-term complication following heart or kidney transplantation (5,6). Both diseases have been associated with a chronic production of allo-antibodies (7–10), but the precise role of these antibodies has remained elusive. The demonstration that mAb against monomorphic MHC class I determinants induce endothelial cells to produce fibroblast growth factor receptors and proliferate in vitro (11) suggests anti-HLA antibodies may be activating endothelial cells in vivo. It is known that activated endothelial cells can produce a number of cytokines, chemokines and growth factors (12,13) which could conceivably promote the intimal proliferation seen in transplant-associated graft occlusive disease. That MHC molecules can act as signal transducers has been previously demonstrated in human T and B cells, where it was shown that ligation of MHC class I molecules resulted in tyrosine kinase activity (14,15).
The objective of this study, therefore, was to determine whether HLA antibodies, produced by patients, against specific HLA molecules induce signal transduction events within human umbilical vein endothelial cells (HUVEC) and human cardiac microvascular endothelial cells (CMEC). The end-point chosen was activation of the transcription factor, NF-$\kappa$B, since this is a key transcription factor involved in activation of a number of different ‘pro-inflammatory’ genes (16).

**Methods**

**Cell culture**

HUVEC were isolated as described previously (17), CMEC, from adult human heart, were isolated and cultured as described by McDouall et al. (18). All cells were maintained in M199 culture medium with 2 mM L-glutamine (Gibco, Paisley, UK), 150 U/ml penicillin/streptomycin (Gibco), 20% FCS (Sigma, Poole, UK) and 10 ng/ml heparinized endothelial cell growth factor (Boehringer Mannheim, Lewes, UK) on gelatin-coated tissue culture flasks. Confluent monolayers of single isolates between passages 4 and 6 were used for all experiments.

**HLA typing of endothelial cells**

HUVEC and CMEC were HLA typed by one of two methods. (i) Using lymphocytes isolated from cord blood or peripheral blood in a standard microcytotoxicity assay using well-characterized antisera. (ii) By PCR-SSP techniques for HLA-A and HLA-B (19) alleles using DNA extracted from umbilical cord blood or peripheral blood.

**Serum samples and antibodies**

Serum samples were collected from a number of patients who had previously undergone human aortic valve replacement surgery and who had been shown to have produced HLA-specific antibodies (20). Their sera was investigated for lymphocytoxic activity using a panel of HLA-A, -B and -DR typed lymphocytes from 40 healthy volunteers and 20 patients with chronic lymphocytic leukemia (CLL) using a microcytotoxicity technique previously described (21). Sera were selected which demonstrated activity against HLA class I antigens, these sera did not have activity for MHC class II antigens as determined by the cytotoxicity assay or subsequent screening by ELISA for class II antigens (Lambda Antigen Tray ELISA from One Lambda, VH Bio, Newcastle, UK). Non-HLA antibodies were defined as sera showing IgM cytotoxic activity for all peripheral blood lymphocytes but negative for reactivity with CLL and negative in the ELISA for class I and class II antigens (Lambda antigen Tray ELISA as above). IgG antibodies were purified from patient serum samples using HiTrap protein G columns (Pharmacia) as previously described (22). A small panel of mAb was also used for this study. These were W6/32 (anti-HLA class I), CR11-351 (anti-HLA-A2,28) and 205-116 (anti-HLA-B13,27, provided by Dr K Gelsthorpe, Sheffield UK).

**Treatment of endothelial cells**

HUVEC or CMEC were plated onto 9 cm gelatin-coated dishes and allowed to become confluent. Once confluent, the cells were washed in serum-free M199 and then incubated with 5% FCS for 18 h in order to minimize activation by serum. Immediately prior to use the medium was removed and the cells washed once in serum-free M199 before being incubated in 2 ml M199 with 5% FCS. mAb were diluted to ~50 µg/ml in 199 culture medium. Patients’ IgG samples were diluted to 200 µg/ml. Confluent monolayers of HUVEC or CMEC were treated with media, mAb or patient samples for 30 min, washed and treated with media, rabbit anti-mouse Ig mAb (RAM; Z0259 from Dako, Cambridge, UK) or rabbit anti-human Ig mAb (RAH; A0107 from Dako) for a further 30 min. Positive controls were incubated with 100 U/ml of recombinant tumor necrosis factor (TNF)-α (Genzyme, West Malling, UK) for 30 min.

**Tyrosine phosphorylation and IκB determination**

Treated endothelial cells were harvested and lysed into 1% SDS buffer for global tyrosine phosphorylation determination by SDS–PAGE and Western blotting. Then 25 µg of total cell lysates was loaded onto 12% acrylamide gels and resolved by SDS–PAGE at 60 mA for 1.5 h. The proteins were then transferred onto nitrocellulose membranes by Western blotting. Blots were probed with an anti-phosphotyrosine antibody (Upstate Biotechnology, New York, NY). This was then incubated with a horseradish peroxidase-conjugated RAM mAb (PO260 from Dako, Cambridge, UK). The blots were then treated with ECL reagents (Amersham, Little Chalfont, UK) and visualized by exposure to autoradiography film (Amersham). For IκB-α, 25 µg of cytosolic extracts was loaded onto gels and blotted onto nitrocellulose membranes. Blots were probed with a rabbit anti-IκB-α antibody (Santa Cruz; from Insight Biotechnology, London, UK), followed by a goat anti-rabbit Ig antibody (Jackson, from Stratex, Luton, UK) and visualized as described above.

**Electrophoretic mobility shift assay for determination of NF-κB**

Between 1 and 5 µg of nuclear extract was incubated with 0.175 pmol [α-32P]dCTP-labelled oligodeoxynucleotides complementary to the consensus sequence of the NF-κB DNA binding sites (Promega, Southampton UK), in a reaction mix which also contained 10 mM Tris–HCl, pH 7.5, 20 mM NaCl, 1 mM EDTA, 5% glycerol, 1.5 pmol irrelevant oligodeoxynucleotide and 2.5 ng poly(dI–dC) in a final reaction volume of 20 µl. Following incubation at room temperature for 1 h, samples were loaded onto prerun 6% native polyacrylamide gels. Gels were fixed for 5 min and exposed to phosphoimage screens (Kodak, Rochester, NY) for at least 3 h. The screens were then visualized using a Storm 860 phosphoimage analyser (Molecular Dynamics, Sunnyvale, CA) and intensity of the bands measured using Image Quant software (Molecular Dynamics) as arbitrary units. NF-κB activation was measured as the amount of binding to the 32P-labelled complementary oligodeoxynucleotide corresponding to the consensus sequence of the NF-κB binding site in experiments and controls. The intensity of bands of experiments were compared to band intensity of controls (cells treated with RAM or RAH alone) and results expressed as mean percentage increase binding in experiments compared to controls. TNF-α was used as positive control since it has been shown to upregulate NF-κB (23).
Proliferation assays

HUVEC were plated out into 96-well tissue culture plates at a concentration of 1x10^4 cells/well in M199 and 5% FCS, but did not contain endothelial cell growth factor, and incubated overnight at 37°C. The next day, mAb controls or allospecific serum samples were added to the cultures with at least 5 replicates for each treatment. [³H]Thymidine (Amersham) was added at the same time (10 μl of medium containing 1 μCi/well) and incorporation measured after 24 h by scintillation counting in a Wallac MicroBeta 1450 counter. Results are expressed as percentage increase in c.p.m. of experiments above cells treated with normal human serum (NHS; individual serum samples taken from a panel of five healthy volunteers).

Statistics

Comparison of means were calculated using Student’s t-test. P < 0.05 was considered to be statistically significant.

Results

Tyrosine phosphorylation

Incubation of HUVEC for 30 min with W6/32 (which binds to monomorphic determinants of MHC class I) had no effect on its own but additional cross-linking with RAM-induced increased tyrosine phosphorylation of protein or proteins of ~75-80 and 200 kDa (Fig. 1, n = 4). Increased tyrosine phosphorylation of proteins of similar size was also seen after cross-linking PA2.6 mAb (also directed against HLA class I) (data not shown).

NF-κB transcription factor activity

Effect of mAb directed against monomorphic class I determinants. In these experiments, both CMEC and HUVEC were used as target cells; they behaved the same as far as response to antibodies was concerned. Incubation of CMEC for 30 min with W6/32 alone resulted in a 74.1% increase binding of NF-κB compared to controls (CMEC treated with RAM alone, P = 0.001, n = 7). However, addition of the cross-linking antibody (RAM) resulted in a further increase in NF-κB binding of 94% above controls in the same seven experiments P = 0.003 (Fig. 2 A and B). Similarly, W6/32 alone caused a 52.5% increase in NF-κB binding (P = 0.001) in HUVEC and cross-linking W6/32 on the surface of HUVEC caused a 74.1% increase in NF-κB activity above control (RAM)-treated cells (P = 0.001, n = 7) (Fig. 3). In a single experiment the mAb PA2.6, which is also directed against monomorphic determinants of HLA class I molecules, when cross-linked with RAM also resulted in increased NF-κB binding activity of 41% when compared to RAM-treated CMEC alone.

Effect of HLA allele-specific mAb and allospecific HLA antibodies. W6/32 binds to monomorphic determinants of MHC class I (24). In order to discover whether antibodies directed against polymorphic epitopes can induce signalling, the HLA-specific mAb CR11-351 (anti-HLA-A2) and 205-116 (anti-B13,27) were used on CMEC bearing the specific antigens (n = 2). The HLA-specific mAb CR11-351 and 205-116 resulted in 58 and 34% increase in NF-κB binding to the NF-κB consensus sequence compared to media controls. However, when these antibodies were cross-linked on the surface of CMEC the increase in NF-κB binding was 277% for CR11-351 and 170% for 205-116 (data not shown).

Eight patient IgG samples were used in experiments to determine whether allospecific HLA antibodies were capable of NF-κB activation. Four of these samples were used against endothelial cells bearing the specific HLA determinants (specificity confirmed by both HLA typing and flow cytometry analysis of IgG binding) and the remaining four were used with endothelial cells bearing irrelevant HLA determinants. The patients samples tested with specific endothelial cells were BR (anti-HLA-A2), KD (anti-HLA-A2,28), VS (anti-HLA-A1, 2) and CM (anti-HLA-B44), and those with HLA non-specific cells were WA (anti-HLA-A3), PS (anti-HLA-B44), MM (anti-HLA-A10, B8) and SH (anti-HLA-A2) (see Table 1). In addition NHS (diluted to 5%) was also used in three separate experiments. The samples (BR, KD, CM and VS) incubated with HLA-specific endothelial cells induced increased binding of NF-κB ranging from 27 to 186% compared to controls and 47% to 198% when these antibodies were cross-linked (Table 1). In contrast, when HLA-specific anti-sera (WA, PS, MM and SH) was incubated with HUVEC bearing irrelevant antigens there was no increase in NF-κB binding (range –25 to –9%). Similarly, NHS did not result in an increase in binding compared to controls (range –36 to 13%).

The results of four separate experiments are shown in Figs 4 and 5. Enhanced NF-κB binding resulted from incubating HUVEC bearing A2 antigens with IgG containing anti-A2 antibodies (Fig. 4a) or CMEC bearing A1 antigens with IgG containing anti-A1 antibodies (Fig. 4b). However, IgG containing anti-A2 antibodies did not enhance NF-κB binding by HUVEC expressing A1 antigens (Fig. 4d) and normal serum did not enhance NF-κB binding compared to RAH alone (Fig. 4c).

Effect of HLA antibodies on IkB-α levels in endothelial cells. NF-κB is normally found, bound to one of its inhibitors, IkB, in the cytosol. Upon receipt of an appropriate signal, IkB becomes phosphorylated and releases NF-κB which translocates into the nucleus and binds to its specific site in the promoter region of numerous genes. The phosphorylated IkB rapidly becomes ubiquinated and degraded. Therefore, following NF-κB activation a decrease in the level of IkB should also be observed. Incubation of HUVEC with W6/32, W6/32 plus RAM and TNF-α were able to cause a decrease in the level of IkB-α, at 30 min, as determined by SDS–PAGE and Western blotting (Fig. 6, lanes 3,4 and 9). However, patient samples (from BR and KD) that caused an increase in NF-κB binding activity failed to reduce the level of IkB-α at the same time in the same experiments (Fig. 6, lanes 5–8).

HUVEC proliferation

In five experiments W6/32 binding to HUVEC induced proliferation of endothelial cells as measured by [³H]thymidine uptake at 24 h, ranging from 22 to 49% increased above control levels (P = 0.015, data not shown). In five experiments, sera from eight patients containing allospecific antibodies was added to HLA-specific endothelial cells and cell proliferation measured at 24 h (Table 2, sera 1–15). This treatment signific
Endothelial cell activation by HLA antibodies

Fig. 1. Induction of tyrosine phosphorylation by cross-linking W6/32 on HUVEC. HUVEC were incubated for 30 min with medium alone (lanes 1 and 2), RAM alone (lanes 3 and 4), and W6/32 (lanes 5 and 6) and W6/32 followed by RAM (lanes 7 and 8) before being lysed and subjected SDS–PAGE (A). The peptides separated by electrophoresis were transferred to nitrocellulose and probed with mouse anti-phosphotyrosine antibody. Binding of antibody was detected using peroxidase-conjugated rabbit anti-mouse Ig and visualized with ECL reagents. It can be seen from the scanning densitometry (B) data that proteins representing phosphorylated tyrosine residues have been induced by W6/32 and cross-linking antibody (lanes 7 and 8) of ~75–80 and 200 kDa. In contrast, there was no increase in density of proteins of mol. wt 40 kDa, showing equal loading of lysates in all lanes. Representative experiment from four.

stantly increased cell proliferation ranging from 23 to 106% above levels seen with NHS. This effect was not simply due to the presence of growth/survival promoting factors in patients’ sera; thus patients’ sera containing irrelevant allo-specific IgG HLA antibodies or sera containing IgM non-HLA antibodies incubated with endothelial cells (Table 2, sera
Endothelial cell activation by HLA antibodies

Fig. 2. NF-κB binding activity in CMEC. CMEC were incubated with medium alone (lane 1), RAM alone (lane 2), W6/32 alone (lane 3), W6/32 plus RAM (lane 4) or TNF-α (100 U/ml, lane 5) for 30 min and nuclear extracts prepared. Binding of nuclear extracts to 0.175 pmol [α-32P]dCTP-labelled oligodeoxynucleotides complementary to the consensus sequence of the NF-κB DNA binding sites is shown in the gel (A), retention of the labelled oligonucleotide being found in lanes 3, 4 and 5. Quantitation of the amount of retention of labelled oligodeoxynucleotide was done by exposure to phosphoimage screens and the percentage increase in binding to labelled probe produced by different procedures measured by densitometry (y-axis) in (B). It can be seen that W6/32 alone, W6/32 plus RAM and TNF-α caused significant increases in NF-κB binding compared to anti-mouse Ig treatment alone. Results expressed as mean percent increase (±SD) derived from seven experiments.

Discussion

This study has confirmed that antibodies against HLA determinants can activate endothelial cells, providing a mechanism whereby chronic production of antibodies could play a part in the pathogenesis of chronic rejection or transplant-associated coronary artery vasculopathy. Previous studies have shown that mAb against HLA class I monomorphic determinants (W6/32) are capable of causing activation of signal transduction events within endothelial and smooth muscle cells (11, 25). The end-points used were expression of fibroblast growth factor receptor and endothelial cell proliferation for endothelial cells (11), and tyrosine phosphorylation, cell proliferation and expression of fibroblast growth factor receptor in smooth muscle cells (25). Here we have extended these observations using anti-HLA antibodies derived from patients, specific for HLA antigens, mAb against specific HLA determinants and NF-κB activation as marker of endothelial activation.

Chronic rejection is the most serious complication following solid organ transplantation; it affects 40% of patients 5 years after cardiac transplantation (6). It is an occlusive vascular disease, the occlusion consisting of proliferating and migrating smooth muscle cells and myofibroblasts with deposition of extracellular matrix proteins (26). Unlike conventional atheroma, the disease is characterized by diffuse and concentric lesions which can affect the entire arterial tree and includes venous lesions, suggesting a pan-vascular disease (27). The endothelial cells are usually intact, unlike antibody-mediated hyperacute rejection which is characterized by microvascular destruction (28, 29). These features would be consistent with the hypothesis that chronic production of antibodies against donor endothelial cells results in activation but not destruction of such cells. Chronic production of anti-HLA antibodies (9, 10) and anti-endothelial antibodies (30–32) has been reported in patients with transplant-associated coronary artery disease.

NF-κB is a family of nuclear transcription factors found in nearly all cell types that were first described as a dimer that bound to the enhancer of the Ig κ light chain (33). It normally exists in the cytoplasm in a dormant form bound to an
Table 1. Effect of allospecific HLA antibodies on NF-κB binding activity in both HLA specific and irrelevant endothelial cells

<table>
<thead>
<tr>
<th>Antibody specificity</th>
<th>Endothelial cell HLA type</th>
<th>Antibody IL-1/TNF (% change)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Alone (% change)</td>
</tr>
<tr>
<td>BR A2</td>
<td>A2 A29</td>
<td>160</td>
</tr>
<tr>
<td>KD A2</td>
<td>A2 A29</td>
<td>186</td>
</tr>
<tr>
<td>CM B44</td>
<td>A3 A24 B44 B62</td>
<td>34</td>
</tr>
<tr>
<td>VS A1, A2</td>
<td>A1 A31 B35 Cw4</td>
<td>27</td>
</tr>
<tr>
<td>NHS NEG</td>
<td>A1 A24 B44 B63</td>
<td>−40</td>
</tr>
<tr>
<td>NHS NEG</td>
<td>A1 B8 B44</td>
<td>13</td>
</tr>
<tr>
<td>WA A3</td>
<td>A1 A24 B44 B63</td>
<td>−9</td>
</tr>
<tr>
<td>PS B44</td>
<td>A3 A23 B41 B52</td>
<td>−20</td>
</tr>
<tr>
<td>MM B8</td>
<td>A3 A23 B41 B52</td>
<td>−25</td>
</tr>
<tr>
<td>SH A2</td>
<td>A1 B8 B44</td>
<td>−12</td>
</tr>
</tbody>
</table>

Fig. 4. Effect of patients’ IgG on NF-κB activation. HUVEC bearing specific HLA-A2 antigens were incubated with patients’ IgG anti-A2 antibodies (patient BR, a), HUVEC or CMEC bearing HLA-A1 antigen was incubated with patients’ sera containing anti-A1 antibodies (patient VS, b), normal serum (c) or patients’ IgG containing anti-A2 antibodies (patient SH, d) for 30 min. Cells were also incubated with media alone (not shown), media plus RAH, human anti-HLA IgG plus RAH and TNF-α. The amount of binding of nuclear extracts to [α-32P]dCTP-labelled oligodeoxynucleotides complementary to the consensus sequence of the NF-κB DNA binding sites is shown on the y-axis as density measurements in arbitrary units. It can be seen that in two instances human IgG anti-HLA significantly enhanced binding of NF-κB compared to RAH controls (a and b) which was not increased by addition of cross-linking antibody. Normal serum (c) or anti-A2 added to irrelevant cells (d) had no effect on NF-κB binding to HUVEC. These are the results of individual experiments.

inhibitory protein, IκB, of which several forms exist (IκB-α, IκB-β, IκB-γ, IκB-ε) (34). IκB is activated by specific IκB kinases, and on phosphorylation it undergoes rapid ubiquitination and proteolysis by non-specific proteases (35). This unveils nuclear localization signals on the Rel proteins, which results in the rapid translocation of NF-κB to the nucleus. In the nucleus NF-κB binds to the consensus sites in the promoter sequence of the inducible genes
Endothelial cell activation by HLA antibodies

Fig. 5. Gel showing effect of patients’ IgG on NF-κB activation. A2-bearing HUVEC were incubated with media alone (lane 1), media plus RAH (lane 2), IgG anti-HLA-A2 from patient BR (lane 3), IgG anti-HLA-A2 from patient BR plus RAH (lane 4), IgG anti-HLA-A2 from patient KD (lane 5), IgG anti-HLA-A2 from patient KD plus RAH (lane 6) and TNF-α (lane 7). Binding of nuclear extracts to [α-32P]dCTP-labelled oligodeoxynucleotides complementary to the consensus sequence of the NF-κB DNA binding sites is shown. Retention of labelled probe in lanes 3–7 can be seen.

Fig. 6. Effect of anti-HLA antibodies on IκB. A2-bearing HUVEC were incubated with medium alone (lane 1), media plus RAM (lane 2), W6/32 alone (lane 3), W6/32 plus RAM (lane 4), IgG anti-HLA-A2 from patient BR (lane 5), IgG anti-HLA-A2 from patient BR plus RAH (lane 6), IgG anti-HLA-A2 from patient KD (lane 7), IgG anti-HLA-A2 from patient KD plus RAH (lane 8) and TNF-α (lane 9). Binding of IκB to nitrocellulose strips is shown.

Fig. 7. Effect of patient allospecific antisera on cell proliferation. Sera containing allospecific IgG antibodies from four patients (MD, BG, JD and SW) or normal serum was incubated with HUVEC bearing relevant antigens (A). Allospecific antisera from two patients (PC and MM) and sera from two patients (RF and JH) containing IgM reactivity in Methods. Disappearance of cytosolic IκB-α was observed 30 min after treatment with W6/32, W6/32 plus cross-linking antibody and TNF-α (lanes 3, 4 and 9), but IκB-α was retained in the cytosol following treatment with patients’ anti-HLA antibodies (lanes 5–8). Representative of two experiments.

Table 2. Effect of allospecific HLA antibodies on endothelial cell proliferation in both HLA-specific and irrelevant endothelial cells

<table>
<thead>
<tr>
<th>No.</th>
<th>Patient</th>
<th>Antibody specificity</th>
<th>Endothelial cell HLA type</th>
<th>Antibody alone (% change)</th>
<th>P valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MD</td>
<td>A2</td>
<td>A2 B7</td>
<td>80</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2</td>
<td>BG</td>
<td>A2</td>
<td>A2 B7</td>
<td>85</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>3</td>
<td>SW</td>
<td>B7</td>
<td>A2 B7</td>
<td>88</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>4</td>
<td>JD</td>
<td>A2</td>
<td>A2 B7</td>
<td>106</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>5</td>
<td>BG</td>
<td>A2</td>
<td>A2 A24</td>
<td>39</td>
<td>0.005</td>
</tr>
<tr>
<td>6</td>
<td>MD</td>
<td>A2</td>
<td>A2 A24</td>
<td>23</td>
<td>0.014</td>
</tr>
<tr>
<td>7</td>
<td>JD</td>
<td>A2</td>
<td>A2 A24</td>
<td>44</td>
<td>0.002</td>
</tr>
<tr>
<td>8</td>
<td>DG</td>
<td>A2 B7 B57</td>
<td>A2 A24</td>
<td>44</td>
<td>0.002</td>
</tr>
<tr>
<td>9</td>
<td>KW</td>
<td>A25 A26 B8</td>
<td>A3 A26 B7 B51</td>
<td>34</td>
<td>0.038</td>
</tr>
<tr>
<td>10</td>
<td>WA</td>
<td>A1 A3</td>
<td>A3 A26 B7 B51</td>
<td>21</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>11</td>
<td>KR</td>
<td>A3 B35</td>
<td>A3 A26 B7 B51</td>
<td>43</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>12</td>
<td>SW</td>
<td>B7</td>
<td>A3 A26 B7 B51</td>
<td>44</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>13</td>
<td>DG</td>
<td>A2 B7 B57</td>
<td>A2 A3</td>
<td>54</td>
<td>0.005</td>
</tr>
<tr>
<td>14</td>
<td>MD</td>
<td>A2</td>
<td>A2 A3</td>
<td>22</td>
<td>0.06</td>
</tr>
<tr>
<td>15</td>
<td>JD</td>
<td>A2</td>
<td>A2 A3</td>
<td>57</td>
<td>0.003</td>
</tr>
<tr>
<td>16</td>
<td>PC</td>
<td>A11</td>
<td>A3 A26 B7 B51</td>
<td>2</td>
<td>0.802</td>
</tr>
<tr>
<td>17</td>
<td>MM</td>
<td>B8</td>
<td>A3 A26 B7 B51</td>
<td>11</td>
<td>0.159</td>
</tr>
<tr>
<td>18</td>
<td>RF</td>
<td>IgM non-HLA</td>
<td>A3 A26 B7 B51</td>
<td>−7</td>
<td>0.453</td>
</tr>
<tr>
<td>19</td>
<td>JH</td>
<td>IgM non-HLA</td>
<td>A3 A26 B7 B51</td>
<td>5</td>
<td>0.523</td>
</tr>
</tbody>
</table>

aCompared to effect of NHS.

(GGGACTTTCC), which results in rapid increase in transcription and increased protein synthesis. Many stimuli activate NF-κB including pro-inflammatory cytokines (e.g. IL-1 and TNF-α), oxidants, viruses and activators of protein kinase C (36). NF-κB may induce many ‘inflammatory’ genes that encode for pro-inflammatory cytokines, chemokines that facil-
Endothelial cell activation by HLA antibodies

iterate transendothelial migration, inflammatory enzymes such as inducible NO synthase, cyclooxygenase (Cox-2), adhesion molecules and cytokine receptors (such as IL-2 receptor). It is thus not possible to say precisely what are the biological consequences of antibody activation of endothelial cells; many of the above effects would be relevant for accelerating or promoting growth of intimal lesions. We do know that serum from these patients (with aortic valve allografts) also contains IL-1 which can appear in the IgG fraction following separation through Centricron filters (21) and causes up-regulation of adhesion molecules when the sera is incubated with human endothelial cells (21). These particular sera did not contain either IL-1 or TNF-α (measured by ELISA, data not shown) and did not cause up-regulation of adhesion molecules. We are currently investigating effects down-stream of NF-κB activation to understand further what are the biological consequences of antibody-mediated activation of endothelial cells.

HUVEC are commonly used as model endothelial cells in studies of vascular biology. Here we combined the use of HUVEC with adult microvascular endothelial cells derived from human heart (18). CMEC were used, because they are more physiological and also because we have previously reported that they are considerably more sensitive to activation by IFN-γ than are HUVEC (37). However, as yet, we have not found any difference in sensitivity to HLA antibodies, using the measurements described in this paper.

The current results strongly suggest the antibody effect is mediated through antigen-specific binding and is not the results of binding to Fc receptors, which may be present on endothelial cells (38). Thus no effect was obtained using secondary antibodies alone and the HLA-specific antisera only produced an effect on endothelial cells bearing the specific HLA antigens. The mAb had some effect on their own, but this was usually enhanced by addition of secondary antibodies. This suggests that aggregation of HLA molecules (which would be achieved using mAb alone) is necessary for signal transduction. Interestingly, the IgG separated from human sera was not enhanced by addition of anti-human IgG antibody (apart from one sample, VS). It is likely that IgG complexes are already present in human sera and use of whole sera more truly reflects the situation in vivo. Patients’ sera was used to induce HUVEC proliferation because we found use of purified IgG fractions did not support HUVEC survival over the 24 h period. Whereas it is known that sera contains ‘survival/growth factors’, we believe the induced proliferation was caused by HLA-specific antibodies since it was only seen on HUVEC bearing relevant HLA antigens and the effect was always greater than that produced by normal serum. These results confirm the recent report by Bian and colleagues (39) that human anti-HLA alloantibodies have a proliferative effect on endothelium and smooth muscle cells. The same authors have also shown that anti-HLA antibodies enhance expression of fibroblast growth factor receptor on endothelial and smooth muscle cells, which is likely to be related to the effect on cell proliferation. Although it has been shown that NF-κB is required for the transcription of IL-2 in human T cells (40) leading to T cell proliferation, it is not known whether proliferation of endothelial cells is NF-κB dependent. It may be that activation of NF-κB and cell proliferation are independent pathways initiated by signal transduction through MHC class I molecules.

When using W6/32 in our studies we were able to show that binding and cross-linking of W6/32 to endothelial cells led not only to NF-κB activation but also a reduction in iNOS activity. However, when using allospecific antibodies from patient samples we were unable to show any decrease in iNOS-α. Possible explanations for this persistence of iNOS-α may be that inhibitors of NF-κB other than iNOS-α, such as IkB-β, IkB-ε, IkB-γ or Bcl-3 may be involved in the inhibition of NF-κB activation induced by human alloantibodies. Alternatively, there is evidence that the phosphorylation of a tyrosine residue of iNOS molecules, rather than serine residues as normally occurs, can lead to activation of NF-κB, but does not result in the degradation of the iNOS molecules (41).

In conclusion, allorreactive HLA antibodies have the ability to induce activation of endothelial cells indicated by increased signal transduction events and resulting in proliferation of endothelial cells. Further studies to elucidate the molecules produced following the activation of signalling pathways may help to understand the mechanisms involved in the rejection process following solid organ allografts.

Acknowledgements

This study was supported by the British Heart Foundation.

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLL</td>
<td>chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>CMEC</td>
<td>cardiac microvascular endothelial cells</td>
</tr>
<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>NHS</td>
<td>normal human serum</td>
</tr>
<tr>
<td>RAH</td>
<td>rabbit anti-human Ig</td>
</tr>
<tr>
<td>RAM</td>
<td>rabbit anti-mouse Ig</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
</tbody>
</table>

References

8. Costa, A. N., Scolari, M. P., Ianneli, S., Buscaroli, G. L.,


37 McDouall, R. M., Batten, P., Mc Cormack, A., Yacoub, M. H. and Rose, M. L. 1997. MHC class II expression on human heart microvascular endothelial cells: distinctive sensitivity to interferon-γ and natural killer cells. Transplantation 64:1175


