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Novel antibodies associated with unexplained loss of renal allografts

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Abstract. Using an endothelial/epithelial hybrid cell line, three different non-HLA antibody types have been identified by flow cytometry in patients who have rapidly rejected multiple renal allografts. These antibodies may be classified as anti-endothelial-monocyte, anti-activated endothelial cell, or anti-epithelial cell.

Key words: Antibodies, and loss of renal grafts – Non-HLA antibodies, loss of renal grafts – Rejection, multiple, kidneys, novel antibodies

The existence of antibodies to the endothelial cell and monocyte antigen system and their occurrence in renal transplant recipients have been well documented [1–3, 5, 7, 13, 14]. Anti-endothelial-monocyte antibodies may also be responsible for the loss of some non-HLA-identical grafts [1, 7], including those in patients who rapidly reject multiple grafts, despite negative cross-matches and no evidence of production of antibodies specific to the donor tissue. It has been suggested that these antibodies may be responsible for up to 80% of HLA-identical graft losses [4]. However, not all HLA-identical graft losses can be attributed to these antibodies [8, 9].

In the majority of studies, endothelial cell antibodies have been assayed by investigating antibody binding to biopsy material or by cytotoxicity testing using umbilical vein endothelial cells (UVEC) and/or monocytes as targets. These techniques demonstrate antibody activity directed against unstimulated endothelial cells (EC). Antibodies specifically cytotoxic to cytokine-activated EC have been shown in some patients with Kawasaki's syndrome [10, 11]. If such antibodies were a contributory factor in patients who have rejected grafts, they would not be detected by the techniques previously used.

We report the use of an endothelial/epithelial fusion cell line to screen for endothelial-monocyte antibodies and antibodies against tumour necrosis factor (TNF)-activated EC. This cell line is easily grown, provides large numbers of cells for screening and, as an added dimension, allows the detection of antibodies directed against the epithelial component of the cell. Antibodies against epithelial cells have been demonstrated in the sera of patients who have lost grafts to recurrent disease [15] but have not been previously implicated in graft rejection.

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Patients and methods

Sera from 28 patients who had lost renal transplants, 12 transplanted patients who had not experienced any rejection episodes and 12 non-transplanted patients were screened against an endothelial/epithelial cell line (EAhy 926) [6]. The patients who had lost their transplants were selected on the basis of biopsy evidence of antibody-mediated rejection in the absence of specific anti-donor HLA antibodies.

The EAhy 926 cells were cultured in RPMI 1640 (Flow Laboratories, Rickmansworth, UK) with 10% fetal calf serum (Globefarm, Esher, UK). Cells were harvested for screening by incubation with Puck's saline A (Gibco, Oxbridge, UK) containing 0.2% EDTA for 30 min at room temperature. Both unstimulated cells and cells that had been stimulated by the addition of recombinant TNF alpha (10 ng/ml) to the culture medium 18 h prior to harvesting were used in the screening runs. Cells were washed twice and resuspended in a solution of phosphate-buffered saline (PBS) azide at a concentration of 10^7–10^8/ml. Then 30 µl of cells were incubated with 20 µl of serum for 30 min at 22°C. After two washes, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-human IgG or anti-human IgM (Dakopats, High Wycombe, UK) for 20 min on ice. Cells were then washed twice and resuspended in 500 µl of PBS azide. Samples were analysed by flow cytometry (FACStar, Becton Dickenson).

The same normal AB serum was used to define negativity in each run. Sera that gave positive reactions with either TNF-activated EAhy or stimulated and unstimulated EAhy were then tested with three further cell types: UVEC, A549 cells (the epithelial parent cell line of EAhy 926) and the U937 monocyte cell line. All cell lines were cultured and harvested by the same methods as EAhy 926. Sera were tested with both unstimulated and TNF-stimulated UVEC and A549. Tests with each cell type were repeated two to four times.

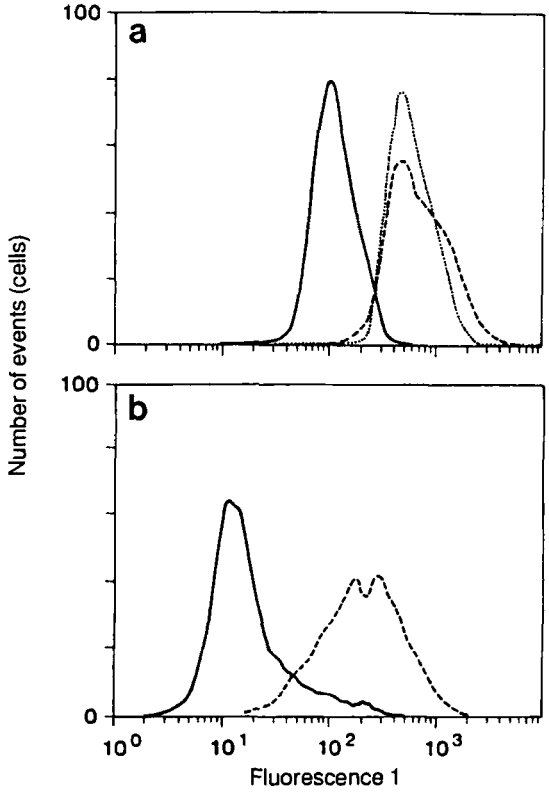

Fig. 1 a, b. Control (—) IgG binding to: **a** TNF-activated (----) and non-activated (.....) umbilical vein endothelial cells *(UVEC)*; **b** U937 (----)

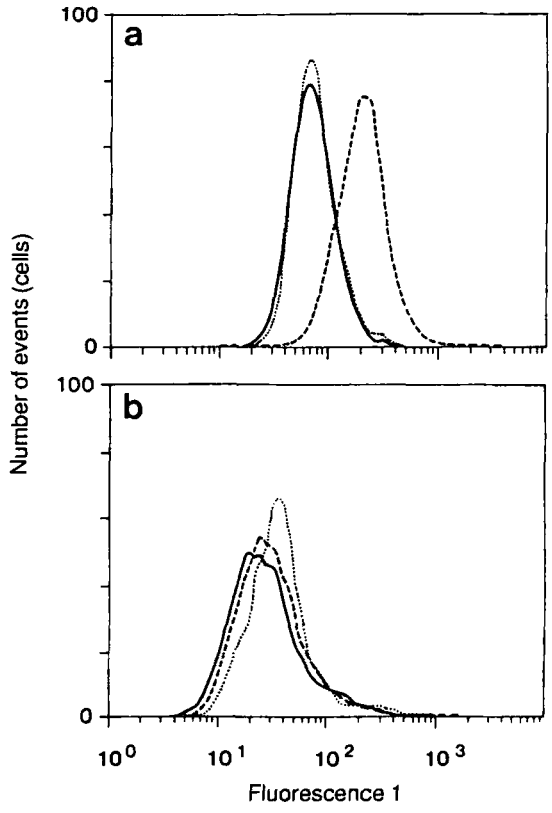

Fig. 2 a, b. Control (—) IgG binding to: **a** TNF-activated (----) and non-activated (.....) EAhy 926; **b** TNF-activated (----) and non-activated (.....) A549

Results

Sera from 52 patients were screened against EAhy 926. Seven positive reactions were obtained from patients who had lost renal transplants; no positive reactions were obtained from patients who had never experienced graft rejection. Five of the patients who were not transplanted gave positive reactions that were all of the same type. These results were reproducible on repeated testing. Three different types of reactions were observed.

The serum of three patients contained IgG that bound to both TNF-stimulated and unstimulated EAhy 926. They also showed IgG binding to TNF-stimulated and unstimulated UVEC and to U937 (Fig. 1 a, b), but they did not bind to A549 epithelial cells. These antibodies were therefore classified as anti-endothelial-monocyte. Of the three patients in this group, one lost two HLA-A, B, C, DR-identical grafts in less than 1 week, one lost two non-identical grafts in less than 1 month, and the third lost four non-identical grafts, although the anti-endothelial-monocyte antibody was only implicated in one of these losses. Conventional lymphocytotoxic cross-matches were performed and were negative in every case. Additional flow cytometry cross-matches were performed for two of these transplants and were also negative.

Two patients had IgG that bound strongly to TNF-activated EAhy 926 or UVEC but weakly or not at all to unstimulated cells. Figure 2 shows the results of one of these patients. This patient received a living related graft from his mother that had a 1 haplotype match. He then received a second graft from a sibling and a third from a cadaver donor, both of which were HLA class I and class II-identical and were negative on conventional cross-matching. All three grafts were lost in less than 1 month. Figure 2 a shows the presence of antibody directed specifically against TNF-activated cells in a serum sample 1 month prior to transplantation with the HLA-identical sibling. There was no binding to A549 cells (Fig. 2 b) or to U937. This antibody was classed as being anti-activated-endothelial cell.

Two patients had an IgM antibody that reacted with both activated and non-activated EAhy 926. One of these patients lost four grafts, including living related transplants from her father (who had a single B locus mismatch) and from an HLA-A, B, C, DR-identical sibling. Standard antibody screening did not reveal any anti-HLA antibodies prior to the loss of her third graft. Figure 3 shows the results of a serum sample taken before the patient received her third graft. The IgM binds to A549 (Fig. 3 a) as well as to EAhy 926, but there is no binding to UVEC or to U937 (Fig. 3 b, c). This antibody therefore appears to be directed at epithelial cells. Stimulation of EAhy 926 and A549 did not alter the intensity of the antibody binding in these sera.

The IgM anti-epithelial antibody was also found in five non-transplanted children. All of these children had characteristic epithelial skin lesions, and one went on to develop severe renal impairment.

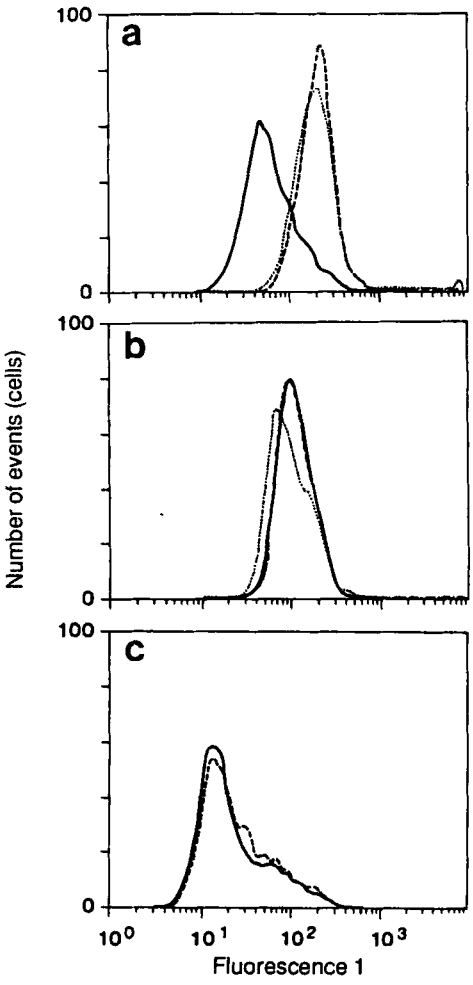

Fig. 3a–c. Control (—) IgM binding to: **a** TNF-activated (----) and non-activated (.....) A549; **b** TNF-activated (----) and non-activated (.....) *UVEC;* **c** U937 (----)

Discussion

Using an endothelial/epithelial hybrid cell line, we have identified three antibody types in seven patients who have lost a total of 20 grafts, 5 of which were HLA-identical. Antibodies to HLA class I or class II cannot explain the results given by these antibody types for several reasons. First, one case of each antibody type was associated with HLA-identical graft loss. Second, monoclonal class I antibodies show no inhibition of binding of the sera described, and class II is not expressed on normal endothelial or epithelial cells or TNF-activated endothelial cells, as shown by monoclonal antibody binding. Third, antibody was shown in a patient where conventional screening showed a complete absence of lymphocytotoxic antibody. Fourth, monoclonal and allo-antibodies to HLA class I react with both activated and non-activated endothelial cells. Lastly, monoclonal and allo-antibodies to HLA class II do not react with EAhy or A549 cell lines.

Type 1 is an anti-endothelial-monocyte antibody that has been implicated in graft rejection by several studies [1–9]. This antibody was present at the time of graft rejection in three of our patients, including one who lost two HLA-identical grafts. However, one patient in whom this antibody was identified (after he had lost two grafts) subsequently received an HLA-identical third cadaver kidney, which is still functioning at 3 months. Therefore, the role of this antibody in the graft rejections remains unclear, although it still appears to be a strong candidate in cases where there is evidence of antibody-mediated rejection despite the lack of anti-HLA antibodies. It is possible that close monitoring of our patient with immediate response to any suspected rejection episode has resulted in the successful course of this transplant to date, despite the presence of anti-endothelial-monocyte antibodies at the time of transplantation.

Antibody type 2 was directed against TNF-activated EC. Two patients had this antibody. The graft history of one is not well documented, as this patient received a kidney in the very early years of transplantation, before full typing records were kept. The second patient has been extensively studied at our centre, as he lost two identical grafts. This patient did have a number of anti-HLA antibodies but these could not be responsible for rejection of identical grafts, and flow cytometry cross-match was negative in this patient. The development of an antibody to activated endothelial cells prior to the patient's receiving an identical kidney may therefore explain this loss. It has been shown that transplantation causes upregulation of class I and II MHC antigens [12]. It is possible that endothelial cell activation markers may also be upregulated following transplantation. This would provide a target for anti-activated endothelial cell antibodies and lead to antibody-mediated graft rejection.

Two patients were found to have a third type of antibody. This was an IgM anti-epithelial cell antibody. One of these patients has lost four renal allografts. The antibody was shown to be present in this patient before she developed any anti-HLA antibodies, and in this period three grafts, including one identical kidney, were lost. The second patient lost three grafts and produced only low levels of anti-HLA antibodies until the time of loss of the third graft. Both patients experienced very severe vascular rejection episodes leading to the loss of their grafts. The presence of the anti-epithelial antibody at the time of rejection suggests that it may be responsible for the loss of these grafts.

The anti-epithelial antibody was also found in five children who had not received transplants, one of whom went on to develop severe renal impairment necessitating dialysis. This suggests that the anti-epithelial antibody is an auto-antibody that may be responsible for the development of renal disease.

By employing the EAhy 926 cell line we have screened sera against TNF-stimulated and unstimulated cells and have been able to identify three non-HLA antibody types. Only those patients giving positive reactions need be further tested against other cell types to classify their antibodies. Two of the antibodies we have demonstrated have not previously been described in cases of graft rejection. These antibodies may explain a number of antibody-

mediated graft losses that cannot be attributed to anti-HLA antibodies.

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