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C. Mariat<sup>1</sup>, L. Absi<sup>2</sup>, F. Berthoux<sup>1</sup>

## Harmful Effect of Anti-Class II Antibodies in Kidney Transplant Patients who Experienced an Acute Rejection Episode

The presence of anti-lymphocytes antibodies is associated with the occurrence of acute rejection after kidney transplantation but few is known on their role after the rejection episode. We conducted a retrospective study in kidney transplant recipients who experienced a biopsy proven acute rejection episode to analyse the influence of anti-lymphocytes antibodies on clinical outcome.

Anti-lymphocytes antibodies were detected before and after transplantation and characterized for isotype, class I and class II targets and donor specificity. 76 kidney recipients were included and analysed for steroid resistance of acute rejection, serum creatinine and 1-year actual graft survival.

The presence of anti-lymphocytes antibodies was noticed in 80% of patients. Anti-lymphocytes antibodies were associated with more frequent steroid resistant rejection episodes, higher creatinine at discharge and throughout the first year post transplantation and with a worse graft survival, at the condition they were of the IgG isotype, donor-specific, and they recognized class II targets.

We conclude that donor-specific anti-class II IgG antibodies are deleterious in the subgroup of kidney transplant recipients who develop an acute rejection.

### Key words:

kidney transplantation, acute rejection, humoral response, anti-class II antibodies

### *Negative Folgen von Anti-Klasse-II-Antikörpern auf nierentransplantierte Patienten nach akuter Abstoßung*

*Das Vorliegen von Anti-Lymphozyten-Antikörpern steht in Zusammenhang mit dem Auftreten von akuten Abstoßungsepisoden nach Nierentransplantation, jedoch ist nur wenig darüber bekannt, welche Rolle sie nach der Abstoßungsepisode spielen. Wir führten eine retrospektive Studie an Nierentransplantatempfängern durch, die eine biopsisch gesicherte Abstoßung erlitten hatten, um den Einfluss von Anti-Lymphozyten-Antikörpern auf das klinische Ergebnis zu analysieren.*

*Anti-Lymphozyten-Antikörper wurden vor und nach Transplantation entdeckt und nach Isotyp, Klasse-I- und Klasse-II-Ziele sowie Spenderspezifität charakterisiert. 76 Nierenempfänger wurden in die Studie aufgenommen und auf Steroidresistenz der akuten Abstoßung, auf Serum-Kreatinin und 1-Jahresüberleben ihres Transplantates untersucht.*

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Bei 80% der Patienten waren Anti-Lymphozyten-Antikörper vorhanden. Die Anti-Lymphozyten-Antikörper wurden mit häufigeren steroidresistenten Abstoßungsepisoden, höherem Kreatinin bei Entlassung sowie während des ersten Jahres nach Transplantation und mit einem schlechteren Transplantatüberleben assoziiert, sofern sie den IgG-Isotyp aufwiesen, spenderspezifisch waren und Klasse-II-Ziele erkannten.

Wir schlussfolgern daraus, dass sich spenderspezifische Anti-Klasse-II-IgG-Antikörper in der Subgruppe von Nierentransplantatempfängern mit akuter Abstoßung negativ auswirken.

### Schlüsselwörter:

Nierentransplantation, akute Abstoßung, humorale Antwort, Anti-Klasse-II-Antikörper

## Abbreviations

CDC: complement dependant cytotoxicity

CFM: flow cytometry

DSA: donor-specific antibodies

NSA: non-specific antibodies

The role of anti-lymphocytes antibodies has been known for long in kidney transplantation. In 1966, these antibodies were described [1]. Following the discovery of the consequences of anti-HLA antibodies, cross-matches were subsequently implemented [2, 3]. It was firstly identified that preformed anti-class I antibodies were responsible of acute rejection episodes and worst graft outcomes [3]. If the cellular response became the core of immunological studies, the humoral response gained important popularity during the past years. The concept of an acute humoral rejection was even coined to clarify this mechanism and the Banff classification was changed [4]. Monitoring of anti-HLA antibodies after transplantation also appeared useful to predict patients at risk of acute rejection [5, 6]. Alloreactive antibodies are distinguished on different basis. The isotype is of importance: IgG antibodies are generally considered as harmful, in opposition to IgM antibodies. Donor-specific antibodies should be differentiated from non-specific antibodies [7, 8]. Lastly, the class-I or class-II targets may alter the clinical influence of antibodies, although the debate is still open [9].

Since the presence of anti-HLA antibodies seemed to be associated with the occurrence of acute rejection, we focused our interest on alloreactive antibodies in the subgroup of kidney transplant patients who experienced an acute rejection episode, trying to know in this selected population whether antibodies were harmful.

## Methods

Our policy for immunological monitoring is to detect anti-lymphocytes antibodies 1/ every 3 months prior transplantation in the patients on the waiting list and in case of blood transfusion; 2/ at the time of the transplantation with a comprehensive cross-match; 3/ systematically after transplantation at month 3, 6 and 12, then on an annual basis; 4/ in case of suspicion of an acute rejection episode with a cross-match using the frozen lymphocytes from the donor.

Detection of anti-lymphocytes antibodies was a complement dependant lymphocytotoxicity technique (CDC) using 30 HLA-typed lymphocytes. A systematic search of anti-class II antibodies was done with a panel of 20 B lymphocytes after cell separation and adsorption on platelets. Depolymerization by dithiothreitol allows telling IgG from IgM antibodies. Since 2002 we have introduced detection with the ELISA and the Luminex® methods.

Cross matches were done against T and B lymphocytes after a magnetic separation with anti-CD3 and anti-CD19 monoclonal antibodies, at 4 and 22°C, with

and without DTT, with historical and present sera. The CDC technique was in use since the beginning, but we introduced the flow cytometry (CFM) cross match in 1998. A combined incubation of donor's lymphocytes and recipient's serum with a PE-labelled anti-CD3, a PC5-labelled anti-CD19 and a FITC-labelled secondary antibody allowed a one-step technique before analysis on a flow cytometer.

We conducted a retrospective study in all patients who experienced a biopsy-proven acute rejection episode within the first six months after transplantation. We considered all the patients transplanted between 1996 and 2004. We chose the year 1996 as modern immunosuppressants became available. Rejections were classified using the 1997 Banff classification and a grade  $\geq$  I was required to include the patients. The observation period lasted one year. Patients were compared using the standard characteristics. The clinical influence of the immunization against lymphocytes was evaluated on the following parameters: sensitivity to steroid treatment of acute rejection episodes, serum creatinine at discharge, at 3, 6 and 12 months; and graft loss. Steroid resistance was defined as the need for a complementary treatment in case of an inadequate recovery of renal function after administration of 30 mg/kg of methylprednisolone.

### Statistical analysis

Patient's characteristics were compared between groups for continuous variables using t-test and for categorical variables by the Chi-square test or by Fischer's exact test. An alpha value of  $< 0.05$  was used to determine statistical difference.

## Results

Among the 372 kidney grafts performed during this period, 76 patients encountered at least one acute rejection episode (20%). They were 48 men and 28 women, aged  $49 \pm 13$  at the time of grafting (17-73 years). The mean dialysis period was  $46 \pm 76$  months (median 22 months). Mean HLA mismatches were  $1.1 \pm 0.6$  for HLA-A,  $1.2 \pm 0.6$  for HLA-B and  $1.0 \pm 0.5$  for HLA-DR.

The induction treatment was basiliximab in 9 patients, daclizumab in 17 patients, anti-thymocyte globulins in 14 patients and none in 36 patients. Tacrolimus was given to 41 patients and ciclosporine to 35. Azathioprine was given to 55 patients, mycophenolate mofetil to 15 and rapamycin to 5. All patients received prednisolone.

First acute rejection episodes were graded Ia in 29 (38%), Ib in 3 (4%), IIa in 23 (30%), IIb in 7 (9%) and III in 14 (18%). Mean onset of first rejection was  $36 \pm 38$  days (median 22). Steroid resistance was noticed in 34 patients (37%). We noticed a second rejection episode in 28 patients (37%).

The actual graft survival at one year was 75%. Grafts were lost because of rejection (14 patients), surgical complications (2 patients), de novo or recurrent nephritis (2 patients) or death (1 patient).

The nadir of serum creatinine before rejection was  $218 \pm 148$   $\mu\text{mol/L}$ . Creatinine was  $236 \pm 194$   $\mu\text{mol/L}$  at discharge,  $207 \pm 128$   $\mu\text{mol/L}$  at 3 months and  $187 \pm 99$   $\mu\text{mol/L}$  at 1 year.

#### Immunisation before transplantation

The peak PRA was  $<5\%$  in 45 patients (59%), between 5 and 80% in 18 patients (24%) and  $\geq 80\%$  in 11 patients (14%). The analysis of isotypes and specificities showed anti-class I IgG in 14 patients (18%), anti-class I IgM in 10 patients (13%), anti-class II IgG in 9 patients (12%), anti-class II IgM in 18 patients (24%).

#### Transplant cross matches

The CDC transplant cross-match was negative in 61 patients (80%) and positive in 15 patients (20%). The positive cross-match was due to an historical anti-B antibody in 6 cases (1 IgG, 5 IgM), an historical anti-T antibody in 3 cases (3 IgM) and a present anti-B antibody in 6 cases (3 IgG, 3 IgM). Since 1998, a CFM cross match was also performed either prospectively or retrospectively. Concordance between CDC and CFM cross matches is given in table 1. Discrepancies with a negative CDC cross match and a positive CFM cross match were always due to the presence of an anti-B IgG. The mirror case with a positive CDC cross-match and a negative

CFM cross-match was seen in presence of IgM antibodies.

#### Rejection cross matches

At the time of acute rejection episode, 28 patients had a positive cross-match (37%). We found an anti-T IgG in 2 patients (7%), an anti-T IgM in 5 patients (18%), an anti-B IgG in 15 patients (53%) and an anti-B IgM in 11 patients (32%).

#### Systematic detection

The routine control of anti-lymphocytes antibodies detected 3 other patients who developed an immunisation.

As a whole, 61 patients (80%) developed some kind of immunisation. Antibodies were specific of the donor in 36 patients (47%) and non-specific in 25 patients (33%). Specificities and isotypes of these antibodies are given in table 2. Donor specific antibodies were found more frequently than non specific antibodies, but of interest, anti-class II antibodies were predominant.

#### Clinical outcomes

The presence of an immunization prior transplantation mildly influenced the outcome. Incidence of steroid resistant rejection was more frequent, 32%, 59%

and 67% in non-immunized, immunized and highly immunized patients, respectively ( $P=0.01$ ). Graft losses (14%, 28% and 33% respectively; NS), and 1-year creatinine were not statistically different. Nor the presence of a positive transplant cross match had an influence on these same evaluation criteria.

We analysed the influence of the presence of anti-lymphocytes antibodies, whenever they were detected. The presence of donor specific antibodies had a strong influence when compared to non-specific antibodies or to the absence of any immunization (Table 3). They were associated with an increase in the frequency of steroid resistance and of graft losses, as well as an increase in serum creatinine at discharge. We subsequently analysed the isotype of these donor-specific antibodies. When of the IgM isotype, they had no influence on the clinical outcomes. Only IgG donor-specific antibodies were influential (data not shown). Lastly, we compared the clinical outcome regarding which HLA class these donor-specific antibodies recognized (table 4). It appeared that anti-class II antibodies strongly affected all the evaluation criteria: Steroid resistant rejection episodes were more frequent and graft loss occurred more frequently.

Tab. 1: Concordance between CDC and CFM transplant cross-matches

Transplant cross match	Number	Antibodies
CDC- / CFM-	43	
CDC+ / CFM+	5	
CDC- / CFM+	7	All anti-B IgG
CDC+ / CFM-	6	All IgM

Tab. 2: Specificities and isotypes of non specific and donor-specific antibodies

Antibodies	DSA	NSA
anti-class I IgM	5	4
anti-class I IgG	3	4
anti-class II IgM	16	12
anti-class II IgG	12	6

Tab. 3: Influence of anti-lymphocyte immunization on clinical outcome

Antibodies	None	Non specific	Donor specific
Steroid resistance #	4/15 (27%)	14/25 (66%)	24/36 (66%)
Creatinine at discharge #	159+53	226+180	279+232
1-year Creatinine	166+80	192+117	195+93
Graft losses #	1/15 (7%)	3/25 (12%)	15/36 (41%)

# P&lt;0.05

Tab. 4: Influence of donor specific antibodies on clinical outcome

Antibodies	None	Anti-Class I	Anti-Class II	Anti-Class I & II
Steroid resistance #	18/41 (44%)	2/4 (50%)	16/19 (84%)	6/12 (50%)
Creatinine at discharge	201+146	195+125	289+232	299+174
1-year Creatinine	181+102	161+57	210+94	193+116
Graft losses #	4/41 (10%)	0/4 (0%)	10/19 (53%)	5/12 (42%)

# P&lt;0.05

## Discussion

Complement-dependant cytotoxicity remains the standard method to characterize anti-lymphocytes antibodies. Unfortunately, CDC lacks sensitivity, reproducible panels of cells and inter laboratories reproducibility. More recently, ELISA and Luminex® techniques have been proposed [10]. They have a better sensitivity for both anti-class I and anti-class II antibodies, but do not identify IgM antibodies [11]. These techniques are presently used for screening and identification of anti-lymphocyte antibodies; nevertheless, CDC is still mandatory in our country. We introduced modern techniques on a routine basis in 1998 for antibodies detection and in 2002 for CFM cross-matches, but all the patients of our study were retrospectively analysed. Of importance, following studies by Cardella [12], we allowed grafting through a positive transplant cross-match, with the only exception of current anti-T IgG antibodies.

The discovery of a decreased graft survival due to preformed anti-HLA antibodies was done in the early 70's [2, 3], and was very largely confirmed since that period. The role of antibodies appearing after transplantation was a more recent finding [13-15] and their negative impact was demonstrated [6, 16,

17]. It appeared that post-transplant antibodies were of a great influence when donor-specific [18]. A negative impact of both anti-class I and anti-class II donor-specific post transplant antibodies has been demonstrated, although production of anti-class II antibodies was more indicative of chronic rejection [19]. A strong association between donor-specific antibodies and acute humoral rejection was recently documented [20].

Anti-B lymphocytes antibodies have very contradictory consequences [21, 22]. They comprise both anti-class I and anti-class II antibodies, both IgG and IgM isotypes and such a heterogeneity might explain why. Confusion between various antibodies recognizing B lymphocytes jeopardized the interpretation of immunological studies. The recent progresses in the detection and the identification of antibodies have clarified these confusing data and it is now possible to identify anti-class II antibodies without any doubt [13]. Anti-class II antibodies may recognize HLA-DR, -DP and -DQ molecules [23].

Taking for granted that a majority of anti-lymphocytes antibodies have a harmful role on graft outcome, we focused our study in patients who did develop an acute rejection episode. Inclusion period was restricted to the modern era of transplantation, not to be confused

with less powerful strategies. Antibodies were detected at various times prior or after transplantation, either on clinical guidance or by systematic detection. The main end-point, actual graft survival, was strongly associated with the presence of anti-lymphocytes antibodies. The pooled analysis showed a 6-fold increase in graft loss due to donor-specific antibodies when compared to the absence of antibodies and a 3-fold increase when compared to the presence of non specific antibodies. The class-II specificity conferred to the donor-specific antibodies a strong influence.

In these selected patients, we confirmed the harmful effect of preformed antibodies but failed to identify any worse outcome after grafting through a positive cross-match. Donor specific IgG antibodies, whatever the time of their isolation, badly influenced graft survival. We identified a strong frequency of anti-class II antibodies, which were associated with more frequent steroid resistance and graft failure.

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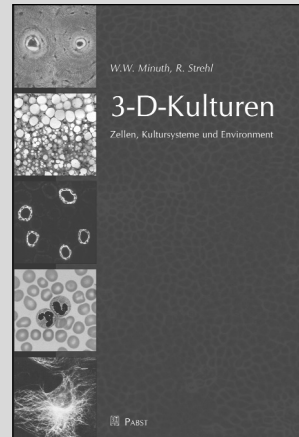
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## 3-D-Kulturen Zellen, Kultursysteme und Environment



Es gibt viele Bücher, in denen beschrieben wird, wie Zellen kultiviert werden. Das Buch jedoch zeigt, wie aus zweidimensionalen Zellkulturen dreidimensionale (3-D) Gewebestrukturen entstehen können. Es bietet eine Einführung in die Welt von innovativen 3-D-Kulturen, die in der Tumorbiologie, der pharmazeutischen Forschung, in den verschiedenen Feldern der experimentellen Biomedizin, im Bereich der zukünftigen Stammzelltherapie und beim Tissue engineering Verwendung finden. Das Buch ist leicht verständlich geschrieben und somit besonders geeignet für die im Labor arbeitenden technischen Mitarbeiter, für Studierende und junge Wissenschaftler/innen der Medizin, Biologie, Pharmazie, Biomaterialforschung und Biotechnologie.

Anschaulich wird zuerst der Übergang von der klassischen Zellkultur zur 3-D-Kultur beschrieben. Informiert wird über die unterschiedlichen Arten der Zell- und Gewebekulturen, über die Auswahl der Medien und über die verschiedenen Arbeitstechniken. In Verbindung mit vielen Abbildungen werden möglichst anschaulich die technischen Voraussetzungen, aktuelle Entwicklungen und die biomedizinischen Perspektiven mit 3-D-Kulturen behandelt.

Besondere Bedeutung hat die kritische Bewertung der entstehenden 3-D-Kulturen. Ziel der Experimente ist, dass histiotypische Eigenschaften in den 3-D-Kulturen entstehen und die Ausbildung von atypischen Eigenschaften vermieden wird. Deshalb wird intensiv über die Bewertung der Differenzierung in den entstehenden Geweben informiert. Besondere Bedeutung hat diese Frage beim Arbeiten mit Stammzellen. Es reicht nicht aus, die Stammzellen zu isolieren und zu vermehren, vielmehr sollen daraus funktionelle Gewebe entstehen, die sicher und damit risikolos angewendet werden können.

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