Histol Histopathol (2006) 21: 1115-1124

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Review

Comparison of the established standard complement-dependent cytotoxicity and flow cytometric crossmatch assays with a novel ELISA-based HLA crossmatch procedure

W.W. Altermann[1], B. Seliger[1], S. Sel[2], D. Wendt[1] and G. Schlaf[1]

[1]Institute of Medical Immunology, Tissue Typing Laboratory and

[2]Clinic and Dispensary of Ophtalmology, Martin-Luther University Halle/Saale, Germany

Summary. The detection of donor-specific anti-HLA antibodies by standard procedures such as complement-dependent cytotoxicity assay (CDC) or flow cytometric (FACS) analysis is limited by its low sensitivity and the quality of the donor cells. Therefore, an ELISA-based technique was employed using solid phase-immobilized monoclonal antibodies to capture HLA class I or class II molecules of the donor, respectively. In this HLA class I and class II antibody monitoring system (AMS) the donor-specific anti-HLA antibodies from the sera of recipients bind to the HLA molecules of the donor which have been immobilized by monoclonal antibodies (mAb) recognizing non-polymorphic epitopes. Upon binding of donor-specific anti-HLA antibodies they are recognized by secondary enzyme-conjugated anti-human immunoglobulin (Ig) antibodies. A newly established modification of the standard protocol allows the differentiation between bound antibodies of the IgG and IgM isotype. Furthermore, this assay was adapted for investigating small amounts of solid tissue of donors from whom no other cells (e.g. from blood) were available. We here provide an overview of the classical crossmatch methods with their advantages and limits. In addition, the design of the novel AMS-ELISA is described in terms of quality and sensitivity of the approach using exemplary cases of different application. The selected cases show that the AMS-ELISA represents a valuable tool for the post-transplantation monitoring of donor-specific anti-HLA antibodies during reaction crisis, after transfusion reactions and in particular cases of tissue transplantations lacking single cells.

Offprint requests to: W.W. Altermann/G. Schlaf, Institute of Medical Immunology, Tissue Typing Laboratory (GHATT), Magdeburger Straße 16, D-06097 Halle/Saale, Germany. e-mail: gerald.schlaf@medizin.uni-halle.de

Key words: Complement-dependet cytotoxicity (CDC), Crossmatch (CM), Enzyme-linked immunosorbent assay (ELISA), Human leukocyte antigen (HLA), Post-transplantation monitoring

Introduction

Patel and Terasaki (1969) described for the first time that antibodies which are directed against antigens of donor lymphocytes are associated with hyperacute rejections in recipients of renal allografts. Subsequent studies provided evidence that antibodies directed against HLA antigens of the donor are a prominent cause for hyperacute rejection (Ahern et al., 1982; Chapman et al., 1986). Indeed, a negative crossmatch (CM) between the recipient's serum and lymphocytes of the donor is hitherto regarded as the best predictor for short-term survival of renal allografts. The standard method for the detection of donor-specific antibodies directed against HLA class I and/or class II molecules is the complement-dependent lymphocytotoxicity (CDC) assay which was developed more than thirty years ago. However, this crossmatch technique sometimes failed to identify antibodies as non-complement-fixing alloantibodies or low antibody concentrations were not detected. These data suggested that more sensitive assays were urgently required in order to detect low to marginal antibody concentrations which may be relevant for the clinical outcome. As a result the CDC-CM was modified using secondary anti-human immunoglobulin (Ig) antibodies in addition to the primary donor-specific antibodies. This variant termed anti-human-globulin (AHG-) enhanced CDC-CM resulted in a considerably higher sensitivity due to an increase in the antibody-mediated complement activation (Gebel and Bray, 2000; Karpinski et al., 2001). Furthermore, the flow cytometric

crossmatch (FACS-CM) was developed (Garovoy et al., 1983). This method detects low antibody titers (Bittencourt et al., 1998), but may result in false positive crossmatch analyses due to antibodies which are not directed against HLA antigens (Christiaans et al., 1996; Kerman et al., 1999). Therefore, a positive FACS-CM does not necessarily correlate with a poor transplantation outcome concerning graft rejections (Kerman et al., 1999; Lobashevsky et al., 2000). Non-complement-activating alloantibodies can be detected by the more sensitive FACS-CM and have been reported in other studies to be associated with an increased allograft rejection despite the absence of a positive CDC-crossmatch (Scornik et al., 1994; Scornik, 1995). In order to avoid these problems a solid phase immunoassay based on a flow cytometric procedure named FlowPRA™ was designed which utilizes purified HLA molecules immobilized on the surfaces of microparticles (Pei et al., 1998; Rebibou et al., 2000, Gebel et al., 2001, 2002; Khan et al., 2003). The advantage of this method is its high sensitivity and particularly its independence from the cell quality.

In general the crossmatch procedure performed prior to transplantations only demonstrates the degree of presensitization, but does not identify all of the recipients undergoing transplant rejection. So far, the dominating method for the diagnosis of an acute post-transplantation rejection is an invasive biopsy (Böhmig and Regele, 2003; Nickeleit and Mihatsch, 2003; Böhmig et al., 2005). As less invasive procedures, many approaches, including flow cytometric crossmatch analyses were employed for monitoring donor-specific immune responses and consequently detecting possible rejections in tissue transplant recipients (Piazza et al., 1998). Recently a novel ELISA-based crossmatch technology (Antibody-Monitoring System-AMS; GTI Diagnostics, WI, USA) exhibiting high sensitivity and independence of the quality of donor cells was developed. This method was optimized for post-transplant monitoring and successfully implemented for the determination of allograft rejections or transfusion reactions which had not been detectable by classical CDC assays using donor lymphocytes and selected panel cells from peripheral blood lymphocytes (PBL) or from chronic lymphatic leukaemia (CLL).

All patients investigated in the present study were

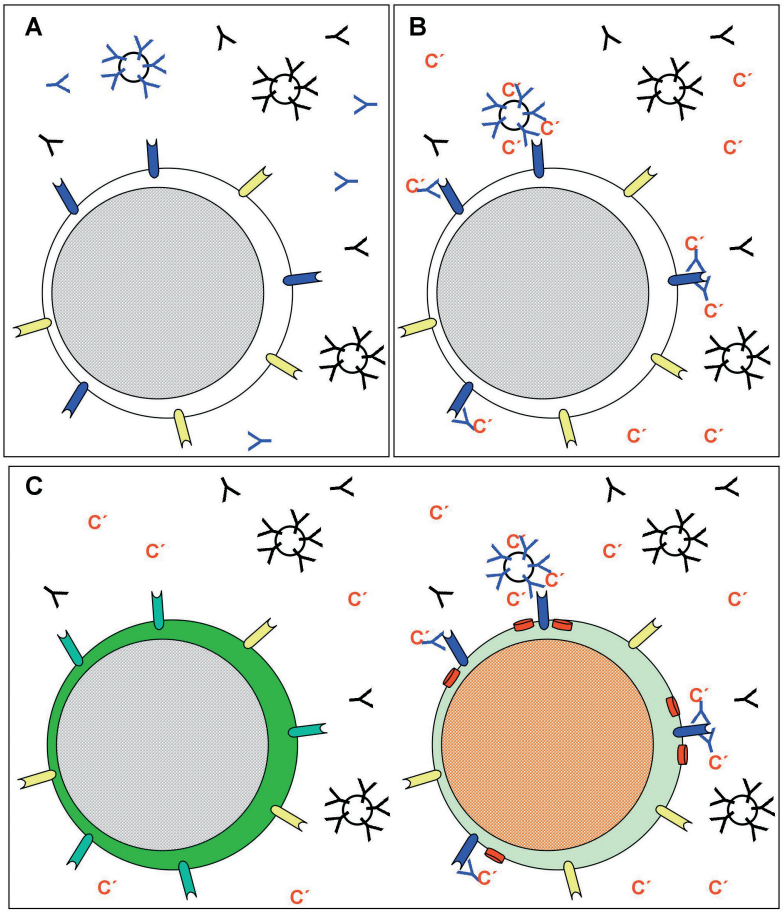

Fig. 1. Scheme of the classical CDC-crossmatch as standard procedure. **A.** Antibodies (monomeric IgG and pentameric IgM) of the recipient's serum bind to the HLA-molecules (blue and yellow) of lymphocytes isolated from the donor's blood. **B.** Activation of the complement cascade of added rabbit complement (C´) by the antibodies (blue) bound to the corresponding HLA-molecules. **C.** Positive reaction by ethidium bromide staining (red colour) of the nuclei of lethal cells which have been lysed by the complement system (right) in contrast to negative reaction by acridine orange staining (green colour) of vital cells to which no antibodies had bound and, which consequently have not been lysed by the complement added. The red cylinders symbolise Membrane Attack Complexes (MAC) as the final products of all pathways of complement activation.

attended by the University Hospital of the Martin Luther University (MLU) Halle-Wittenberg. The outer rims of rejected cornea allografts and sera from the cornea recipients were kindly provided by the Clinics of Ophthalmology of the MLU/Halle-Wittenberg. Sera from patients who suffered from a transfusion reaction due to a transfusion of thrombocyte concentrates and blood samples of the respective donors as well as serum from a patient suffering from sepsis as cause for a transfusion were provided by the Department of Transfusion Medicine of the University Hospital.

Complement-derived cytotoxicity (CDC)- crossmatch standard assay - its technical description, advantages and disadvantages

The conventional CDC-CM assay dictated by the Eurotransplant Foundation is at present the standard method to detect antibodies against HLA-antigens of donor cells in a potential recipient. These donor-specific antibodies may result in acute or hyperacute rejections of allografts. As schematically represented in Figure 1, after isolation by Ficoll density gradient centrifugation, donor lymphocytes were incubated with the serum of the recipient (Fig. 1A). Subsequently, complement components of rabbit serum were added which are activated via the classical pathway of complement activation only by antibodies that have been bound in the first incubation step (Fig. 1B). The result is positive by the existence of bound complement-activating cytotoxic antibodies of the IgG/IgM isotype against cellular antigens of the donor. The readout of this assay is performed by fluorescence microscopy and the reaction is defined on the basis of a score system with values of 0, 2, 4, 6 and 8 (Table 1) according to standard protocols of the National Institute of Health (USA). The appearance of only a few dead lymphocytes visible by a red staining pattern due to the DNA-intercalator ethidium bromide must be interpreted as a positive result, whereas vital lymphocytes exhibit a green staining pattern through the active uptake of acridin orange (Fig. 1C). Methodological modifications enhancing the sensitivity of the assay are recommended which use separate T- or B-lymphocytes freshly isolated with antibody-coated magnetic beads (System Dynal, Oslo, Norway). However, due to relatively high costs and the complicated and time consuming handling this procedure is not performed in all laboratories. Although this isolation method is essential in particular when anti-HLA class II antibodies have to be identified [the HLA-class II bearing cells are only about 15 % of the peripheral blood leukocytes (PBL)] it is not required according to the Eurotransplant guidelines for crossmatch procedures. In contrast to the serological determination of the HLA phenotype of a given donor using the micro-lymphocytotoxicity test (MLCT) the background value has to be <10 % to obtain interpretable results. This low background value can sometimes not be reached due to long term (≥ 2 days) or inadequately stored blood samples, high loss of blood of the donor or an irritation of the donors' lymphocytes due to the pharmaceutical treatment of donors.

The classical CDC-CM was further modified. The binding of the primary donor-specific antibodies was followed by the incubation with secondary anti-human IgG antibodies (AHG-enhanced CDC-CM) (Fig. 2). This secondary "layer of antibodies" resulted in an enhancement of the complement activation and, thus, increased the sensitivity of the CDC-CM. However, the drawback of this procedure may be an increased number of damaged lymphocytes due to the additional incubation step as "stress factor" which may result in a higher background and possibly in uninterpretable results.

Flow cytometry crossmatch (FACS-CM) – its "pros" and "cons"

In comparison to the conventional CDC-CM the

Table 1. Score system for the analysis of the standard Complement Dependent Cytotoxicity-Crossmatch Assay (CDC-CM) as percent of dead (red coloured) cells which are the result of the complement-mediated lysis (positive cells).

SCORE	DEAD CELLS (%)	INTENSITY OF THE REACTION
1	≤ 10	negative
2 = +	10-20	doubtful positive
4 = ++	20-40	weakly positive
6 = +++	40-80	positive
8 = ++++	80-100	strongly positive

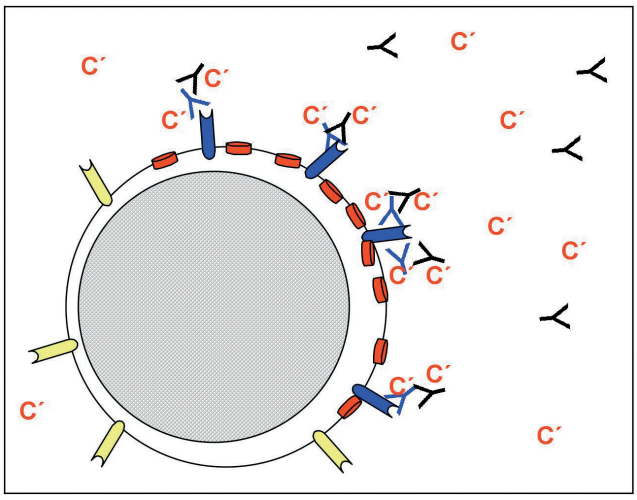

Fig. 2. Design of the anti-human globulin (AHG)-enhanced CDC-crossmatch. Modification of the classical CDC-crossmatch through the use of secondary anti-human IgG antibodies to enhance the activation of complement.

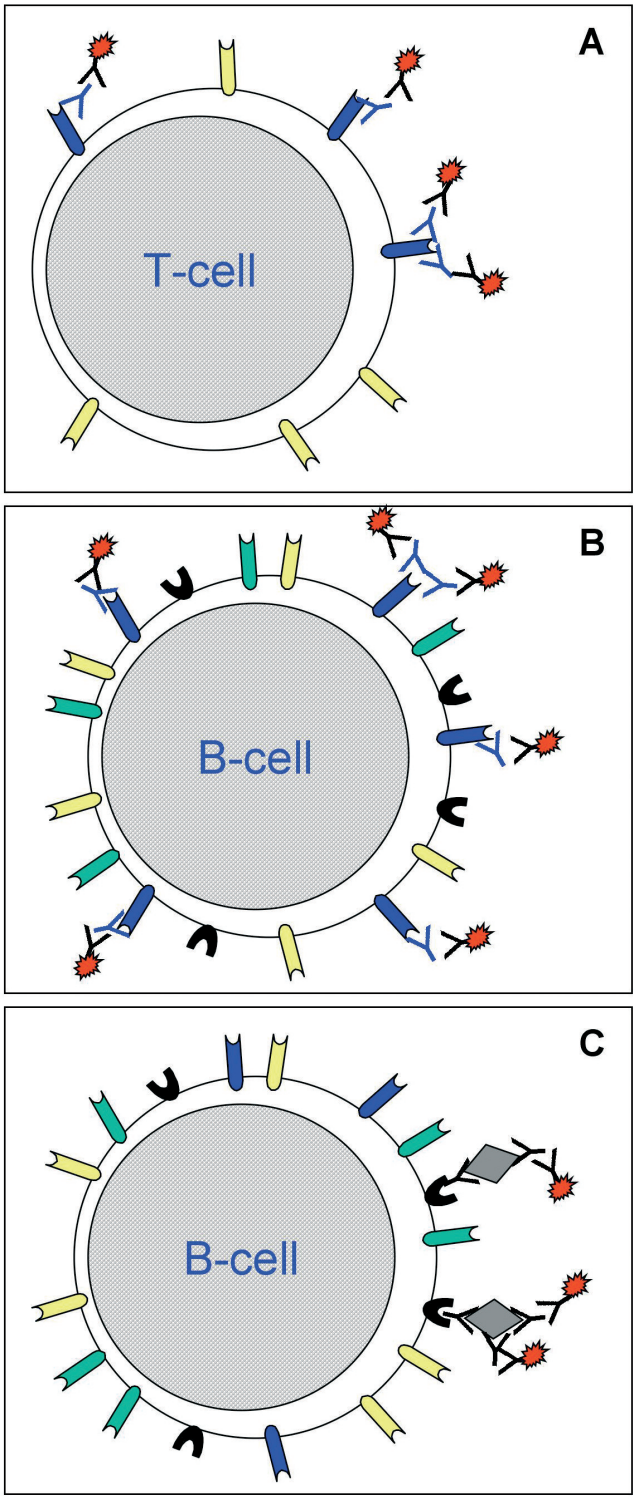

Fig. 3. Schematic diagram of the Flow Cytometry crossmatch (FACS-CM). In contrast to the CDC-CM with a complement mediated vital- or lethal staining of lymphocytes, the FACS-CM is based upon an indirect immunostaining using fluorescence dye-labelled secondary antibodies. In contrast to the FACS-CM of only HLA-class I bearing T-cells **(A)** the outcome of the B-cell crossmatch **(B)** may be falsified by irrelevant immune complexes **(C)** which through their Fc-fragments directly bind to Fc-receptors (black horseshoes) of B-cells.

FACS-CM procedure has a higher sensitivity which is in the range of that of the AHG-enhanced CM (Scornik et al., 1997). This procedure is not based on vital or lethal staining as demonstrated for the CDC-CM but on an indirect immune staining procedure using secondary fluorescence-labelled antibodies (Fig. 3). Therefore, it allows the detection of both low complement-activating, but also of complement-independent anti-donor antibodies. The outcome of this assay may be influenced by irrelevant antigen-antibody complexes in the recipient's serum through binding of Fc-fragments to the Fc-receptors. These are expressed at high quantities on the surfaces of antigen-presenting cells, in particular B-cells, which are separated and cytometrically analyzed to detect anti-HLA class II antibodies of the recipient. In Figure 4 two representative histograms of a FACS-CM are shown which demonstrate that this kind of assay may be interpreted wrongly. In contrast to the anti-class I (T-cell) CM (right) in which no anti-HLA class I antibodies were bound, the same procedure using B-cells led to a histogram of increased background intensity in comparison to the serum of the negative control. Neither the anti-HLA class II ELISA with solid phase-coated HLA class II antigens (Quikscreen-ELISA, GTI diagnostics, Waukesha, USA) (Worthington et al., 2001) nor the CDC-CM using isolated B-cells, both of which had been performed prior to the FACS-CM, had shown any positive signal thus most probably indicating a false positive FACS-signal. In contrast to T-lymphocytes, B-lymphocytes express HLA class II molecules constitutively and HLA class I molecules at a higher density than T-cells.

The novel antibody monitoring system (AMS) ELISA procedure-description of the method

For the novel AMS-ELISA, lymphocytes from peripheral blood or spleen were prepared according to standard procedures by Ficoll density gradient centrifugation. The interphase was removed and after washing with phosphate-buffered saline (PBS) lymphocytes were subjected to lysis for 30 min on ice using a prediluted lysis buffer (TRIS-buffered non-ionic detergents) provided by the supplier. The outer corneascleral rim was cut into pieces by a scalpel and subsequently homogenized in PBS using mortar, pestle and glass powder. The homogenized cornea material was transferred into a 1.5 ml tube, immediately centrifuged (5000xg, 5 min) and then lysed for 1 h on ice in the respective lysis buffer. The donors' cell lysates were either used immediately or frozen at -80°C. Depending on the HLA class of recipients' antibodies to be identified the lysates were diluted 1:8 (class I) or 1:4 (class II), respectively, with lysate dilution buffer delivered with the AMS assay (GTI diagnostics, Waukesha, USA). Lysate was then pipetted into the wells of the ELISA-strips which had been precoated with monoclonal capture antibodies directed against monomorphic structures of the HLA molecules followed

by an incubation for 45 min at 37°C (Fig. 5A). The solution of the donor material was then discarded and the strips were washed with TRIS-buffered saline (TBS) containing 0.2% TWEEN 20 and 0.1% NaN_3 to remove unbound proteins. After four-fold dilution of recipients' sera with sample dilution buffer [PBS containing bovine serum albumin (0.5 % w/v), murine serum (5% v/v) and 0.1% NaN_3 (w/v)] they were incubated for 45 min at 37°C (Fig. 5B). Upon consecutive washing steps to remove unbound antibodies and other serum proteins, secondary alkaline phosphatase-conjugated goat anti-human IgG antibodies or, alternatively, according to our modified protocol, goat anti-human IgM/A/G antibodies (GTI diagnostics, Waukesha, USA) were applied to the ELISA at a dilution of 1:100 using PBS containing 0.1% NaN_3 (Fig. 5C). This modified and optimized protocol allows the differentiation between the recipient's bound anti-HLA antibodies of the IgM or IgG isotype. It enables the determination whether the immune response was due to a first contact or to a booster immunization induced by the donor's HLA molecules. Following a third incubation period for 45 min the colour reaction using p-nitrophenylphosphate (PNPP) was performed according to the supplier's instructions (Fig. 5C). The colour reaction was stopped with 3 M NaOH and readout was performed. A serum sample was positive when its value was two-fold the value of the negative control. In addition, for the validation of data three controls were included:

(i) The positive control serving as control of reagents

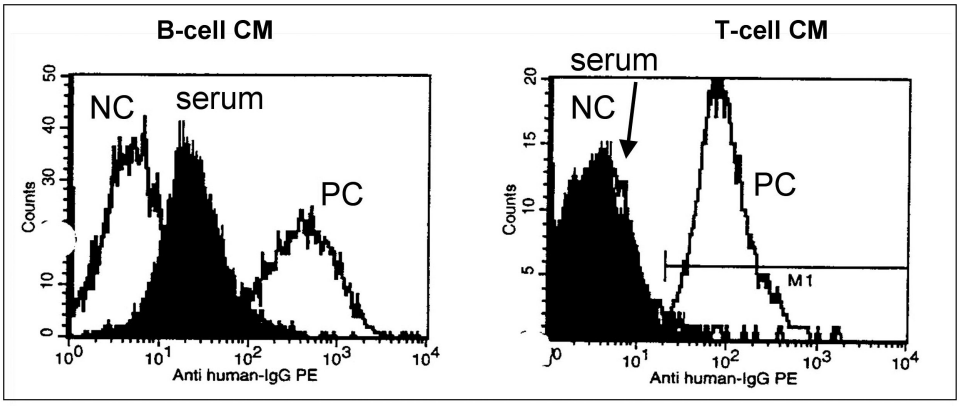

Fig. 4. FACS-CM of B-cells and T-cells as shown by FACS-histograms. In contrast to the FACS-CM of T-cells the result of which is unequivocally negative, the B-cells provide a histogram of weakly increased intensity which due to this faint intensity does not lead to an interpretable result.

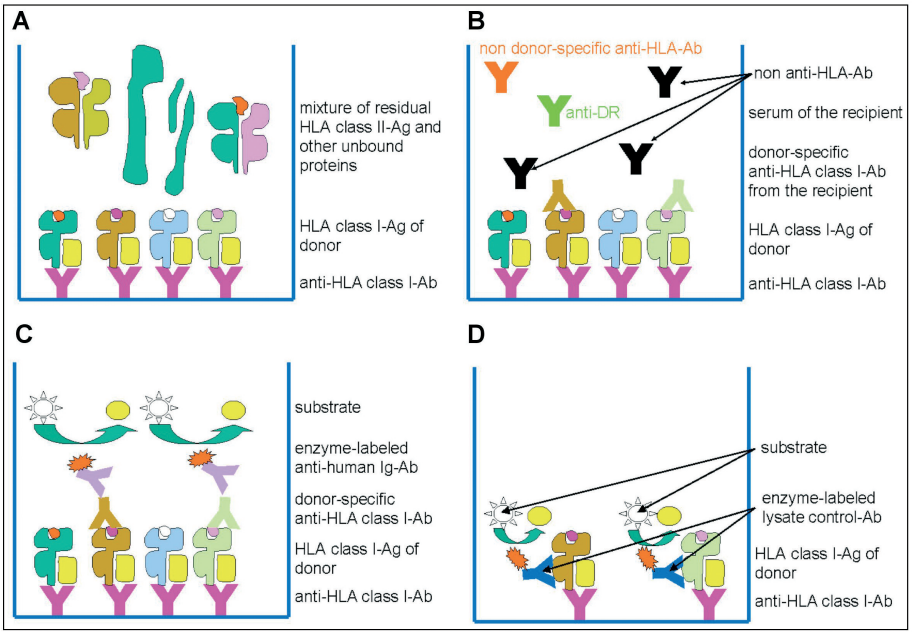

Fig. 5. Flow diagram of the AMS-ELISA for the detection of HLA-class I molecules. **A.** Binding of the solubilized HLA class I molecules of the donor by anti-class I monoclonal capture antibodies recognizing a monomorphic epitope on these molecules. **B.** Binding of the donor-specific anti-HLA antibodies out of the recipient's serum which detect the immobilized HLA-molecules of the donor. **C.** Recognition of the recipient's bound donor-specific anti-HLA class I antibodies by an alkaline-phosphatase-conjugated secondary antibody (anti-human IgG or IgM/G/A) and subsequent colour reaction. **D.** Lysate control consisting of alkaline phosphatase-conjugated monoclonal antibodies for detection (against different monomorphic structures of HLA class I molecules) to confirm the immobilization of solubilized HLA molecules by the solid-phase bound capture mAb. The AMS-ELISA variant for the detection of HLA-class II molecules is designed correspondingly.

consists of freeze-dried control lymphocytes and a HLA class I- and class II- positive serum sample. After the rehydration of the dried lymphocytes the cell lysate was employed to detect the HLA molecules by the positive human serum (included in the reagents of the AMS assay) thus demonstrating the functionality of the lymphocyte lysis buffer and of the other reagents provided by the supplier. The resulting OD value should be ≥ 1.000.

(ii) The lysate controls (Fig. 5D) consist of two alkaline phosphatase-conjugated anti-HLA class I or II mAbs for direct detection by recognizing monomorphic structures of these molecules, respectively. Both lysate controls must exhibit a significant clear signal (OD ≥ 0.900) which confirms the binding of the HLA class I or class II antigens, respectively, to the immobilized capture antibodies.

(iii) The steps of the negative control are exactly those of the positive control with the difference that for detection of the donor's HLA molecules an irrelevant human serum is used which is negative for HLA antigens bound by the monoclonal capture antibodies. The value of the recipient's serum sample under investigation must exceed two-fold the value of the negative control (≤ 0.300) to be classified as positive.

Exemplary studies performed with the AMS-ELISA

Detection of a transfusion reaction induced by anti-HLA antibodies

Sera from a patient suffering from a transfusion reaction after receiving a concentrate of thrombocytes was obtained from a blood sample directly before and nine days post-transfusion and analyzed by both AMS-ELISA and the CDC crossmatch procedure with a cell panel consisting of 30 PBL-derived cells. As shown in Table 2A anti-HLA class I antibodies were not detectable in the patient's serum taken before the transfusion by using the CDC screening method. In contrast, the serum taken 9 days after the transfusion clearly contained anti-HLA antibodies since it recognized 26 out of 30 (i.e. 87%) panel cells. The recognized antigen of this serum sample was afterwards identified as Bw6, detectable up to a dilution of 1:128 using the CDC procedure. Using the AMS-ELISA, donor-specific anti-HLA class I antibodies were already found in the pre-transfusion serum (Table 2B). The serum obtained nine days after the transfusion and exhibiting a positive reaction in CDC-CM also showed reactivity in the AMS-ELISA up to a dilution of 1:256 with the IgG-specific secondary antibody as well as with the IgM/G/A-specific conjugate (Table 2B). As expected, antibodies against HLA class II molecules were not detectable since thrombocytes express HLA class I, but not HLA class II surface molecules (Cosgrove et al., 1988; Pocsik et al., 1990).

Detection of donor-specific anti-HLA antibodies using the cornea allografts after their transplantation

In the case of cornea transplantation only the central part of the cornea is transplanted, whereas the outer rim is stored in sterile buffer for the detection of a possible bacterial contamination. In general, a crossmatch is not performed since the cornea is regarded as an immune privileged tissue. However, it is postulated that local immunoreactions may lead to the clouding of the

Table 2. Comparison of the sensitivity of CDC-CM and AMS-ELISA as shown by the analyses of sera collected from a patient before and after a transfusion reaction.

(A) CDC screening

Serum	negative control	serum taken prior to transfusion	serum taken 9 days after transfusion	positive control
positive cells/ 30 PBL panel cells	0	0	26	30

(B) AMS-ELISA

	IgG-conjugate			IgM/G/A-conjugate		
	negative control	serum taken prior to transfusion	serum taken 9 days after transfusion (1:256)	negative control	serum taken prior to transfusion	serum taken 9 days after transfusion (1:256)
anti-HLA class I	0.145	n.t.	**0.543**	0.135	**0.628**	**0.285**
anti-HLA class II	0.080	n.t.	0.045	0.093	n.t.	0.037

Sera collected from a patient before and after a transfusion reaction were analysed by both assays. Positive OD values (bold numbers) were at least double the values of the corresponding negative controls. All sera were four-fold prediluted using lysate dilution buffer. This predilution factor is not implicated in the dilutions (1:256) at which the serum taken after the transfusion was used. n. t.: not tested due to the lack of serum.

allogenic graft due to marginal expression of HLA antigens. Although cell mediated immunity has been described to be the main mechanism of corneal allograft rejection (Niederkorn, 2001), complement activating alloantibodies were also shown to have an influence on corneal allograft rejection in mice (Hedge et al., 2002). Furthermore, the studies of Roy et al. (1992) and of Boisjoly and coworkers (1993) strongly suggest that the development of posttransplant antibodies against donor HLA antigens in contrast to so-called panel-reactive pretransplant antibodies which only show antibodies against HLA antigens of selected panel cells in general, but not donor-specific antibodies, represents a high risk of corneal allograft rejection. In addition Des Marchais and coworkers (1998) demonstrated that a donor-recipient CM is a useful procedure for the selection of recipients for corneal transplantation in patients who are presensitized by an anterior graft or a previous corneal rejection. In particular the AMS-ELISA fulfils the donor-specificity required by the studies of Roy et al. (1992) and Boisjoly et al. (1993) as this assay exclusively detects donor-specific alloantibodies.

In order to determine whether the cornea allograft rejection presented in the following case was due to donor-specific anti-HLA class I antibodies of the recipient the AMS-ELISA was performed using lysate of the outer cornea rim for the extraction of the donor's HLA class I molecules. As demonstrated in Table 3 both the monospecific anti-IgG and the polyspecific anti-IgG/M/A antibody detected anti-HLA class I antibodies in the recipient's serum using this method. In contrast, the CDC crossmatch which, although usually not done for cornea grafts, had been performed in this case prior to the cornea transplantation, had not detected allogenic antibodies.

Identification of a false positive CDC crossmatch caused by the serum of a sepsis patient

Serum from a 12 year old sepsis patient treated by granulocyte concentrate transfusion was employed for three CDC crossmatches to identify anti-HLA antibodies. A positive reaction was always obtained with scores between 2 and 4 (Table 1). In contrast, the AMS-ELISA used as a reference method did not identify anti-HLA class I antibodies in the same serum sample (Table 4). This discrepancy of data strongly suggests that the positive CDC crossmatch was not caused by anti-HLA antibodies. It rather reflects the existence of immune complexes or uncontrolled complement activation which are present in patient's serum undergoing severe sepsis and, thus, may lead to a false positive outcome of a CDC crossmatch.

Identification of anti-HLA class I antibodies not detected by classical CDC crossmatch as a cause for an acute kidney/liver rejection

A 53 year old woman with an HLA typing of A2,24; B62,35; Cw4,-; Bw6,-; DR1,13; DR52; DQ5,6 who had received a combined liver/kidney graft rejected the liver after 4 days and the kidney graft after an additional seven days. Serum from the patient taken prior to the transplantation had contained several anti-HLA class I antibodies (anti-A1, -A10, -A11, -A19) as demonstrated by a panel reactive activity of 53% for PBL and 94% for

Table 3. Detection of anti-HLA class I, but not of anti-HLA class II antibodies of a cornea transplant recipient by the AMS-ELISA.

	IgG conjugate		IgM/G/A conjugate	
	negative control	cornea lysate	negative control	cornea lysate
anti-HLA class I	0.226	**0.647**	0.174	**0.459**
anti-HLA class II	0.080	0.089	0.056	0.063

Positive OD values (bold numbers) were at least double the values of the corresponding negative controls. The pre-transplantation serum which did not show an effect in the CDC crossmatch was used at a four-fold dilution. The AMS-ELISA was performed with the recipient's serum taken prior to cornea transplantation.

Table 4. AMS-ELISA -detection of a false positive CDC-CM with a serum sample from a patient who suffered from severe sepsis.

	IgG conjugate		IgM/G/A conjugate	
	negative control	PBL lysate of the donor	negative control	PBL lysate of the donor
anti-HLA class I	0.126	*0.210*	0.141	*0.212*

positive controls

	IgG conjugate		IgM/G/A conjugate	
	control of reagents	lysate control	control of reagents	lysate control
anti-HLA class I	2.458	2.165	not done	not done

The negative OD values (italics) clearly demonstrate that there were no donor-specific anti-HLA (class I and class II) antibodies detectable. Both positive controls (control of reagents and lysate control) which due to the lack of serum were only performed for antibodies of the IgG-isotype showed the faultless work of the assay.

CLL. Despite this high panel reactivity the CDC crossmatch performed twice with the pre-transplant serum in different laboratories did not display any cytotoxic activity against the HLA phenotype of the donor (A2,24; B7,39; Cw2,7; Bw 6,-; DR7,12; DR52,53; DQ2,3). In contrast, the AMS-ELISA detected a positive reaction of the patient's serum with HLA class I molecules of the donor (Table 5) which most probably was the cause of the graft rejection. Again the direct comparison of both assays, the CDC crossmatch procedure and the AMS-ELISA, underscores the high sensitivity of the novel method. Donor-specific anti-HLA antibodies of the recipient's serum were identified by the AMS-ELISA, but not by the classical CDC crossmatch procedure.

Falsification of the outcome of the AMS-ELISA by serum of a patient suffering from rheumatoid arthritis

A female patient suffering from rheumatoid arthritis showed positive CDC auto-crossmatch outcomes with scores always ranging between 2 and 4 (Table 1). After requests concerning her clinical state it became apparent that the person suffered from rheumatoid arthritis. Therefore, both the patient's sera of three consecutive blood collections and her PBL were employed in the AMS-ELISA and, in parallel, in the CDC procedure. As demonstrated in Table 6, the first and the second collection of sera obtained after an interval of only two days showed positive outcomes in both assays, whereas the third collection obtained three weeks later resulted in ELISA outcome values of 50-70% which decreased when compared to the previous collections. Interestingly the corresponding result of the CDC crossmatch procedure was negative again indicating the minor sensitivity of this assay. The serum of the last collection (two months later), however, showed a negative outcome in both assays. It is noteworthy that together these data suggest that a rheumatoid attack with a decreasing pathology and no anti-HLA antibodies was monitored, which was overcome at the last sample collection.

Advantages and limits of the AMS-ELISA in comparison with the established crossmatch-procedures

Concerning the transfusion reaction (Table 2) and the acute liver/kidney allograft rejection (Table 5) the comparison of both assays demonstrates a significantly increased sensitivity of the AMS-ELISA. In the pre-transfusion serum of the recipient this assay detected anti-HLA class I antibodies (Table 5), whereas the antibody concentration was too low to exhibit an effect in the CDC crossmatch. The transfusion (Table 2) led to a booster immunization with an increased anti-HLA class I (anti-Bw6) antibody titer and finally resulted in a positive CDC crossmatch. The higher sensitivity of the ELISA in comparison with the CDC method becomes strikingly apparent when the four-fold predilution of the serum used for the AMS-ELISA is additionally calculated for the values of Table 2B. For the pre-transfusion serum a four-fold dilution was sensitive enough to detect anti-HLA class I antibodies using the AMS-ELISA, whereas in the CDC crossmatch even

Table 5. Detection of anti-HLA class I antibodies by AMS-ELISA prior to a combined kidney/liver transplantation.

	IgG conjugate		IgM/G/A conjugate	
	negative control	lysate of donor spleen cells	negative control	lysate of donor spleen cells
anti-HLA class I	0.110	**0.642**	0.157	**0.518**
anti-HLA class II	0.098	0.129	0.064	0.114

Results of the AMS-ELISA (OD values) performed with a serum sample taken prior to the transplantation of a patient who afterwards lost the graft most probably due to anti-HLA class I antibodies. The positive OD values (bold numbers) demonstrated the existence of donor-specific anti-HLA class I antibodies in the patient's serum taken prior to the transplantation whereas anti-HLA class II antibodies were not demonstrable.

Table 6. Influence on the outcome of the AMS-ELISA and the CDC auto-crossmatches by serum samples from an acute rheumatoid arthritis patient.

DATE OF COLLECTION	Nov. 29th	Dec. 1st	Dec. 20th	Feb. 16th	neg. control (average)
anti-HLA class I (IgG)	**0.677**	**0.737**	**0.239**	0.092	0.118
anti-HLA class I (IgM/G/A)	**0.483**	**0.549**	**0.322**	0.118	0.136
anti-HLA class II (IgG)	0.157	0.138	0.110	0.137	0.121
anti-HLA class II (IgM/G/A)	0.108	0.088	0.099	0.126	0.092
corresponding CDC auto-crossmatch	**positive (4)**	**positive (2)**	negative	negative	negative

The positive OD values (bold numbers) of the AMS-ELISA and the corresponding CDC crossmatches (outcome scores in brackets) together with the patient's clinical state clearly indicate the existence of rheumatoid factors which falsify the data of both assays.

undiluted serum did not show a cytotoxic effect. In addititon, for the serum taken nine days after the transplantation a dilution of 1:1024 was sensitive enough for detection by the ELISA, but a cytotoxic effect was only detectable up to a dilution of 1:128 employing the CDC crossmatch. In the case of the rejected liver/kidney allograft (Table 5) the recipient's serum sample was also four-fold pre-diluted for the AMS-ELISA, but not diluted for the CDC crossmatch. Despite the higher pre-dilution of the serum, donor-specific antibodies were clearly identified using the AMS-ELISA, whereas using the CDC crossmatch with undiluted serum such antibodies were not detectable.

In the case of cornea transplants the advantage of the AMS-ELISA due to its increased sensitivity in comparison to the classical CDC crossmatch is also shown (Table 3). In addition, not only lysates of lymphocytes but also of tissues such as cornea which generally are poor in cells can be used for HLA antigen isolation of a given donor. However, it is noteworthy that anti-HLA class II antibodies were not detectable in the recipient's serum presented in this study (Table 3) and five other sera (data not shown) which may be due to the lack of HLA class II expressing cells in the cornea tissue. The lysate controls using alkaline phosphatase-conjugated mAb for detection of anti-HLA class I or anti-HLA class II molecules, respectively, which are included in the AMS-ELISA confirmed this hypothesis. In contrast to the HLA class I-specific lysate control resulting in a significant signal, the HLA class II-specific lysate control using mAb which recognize monomorphic structures of the HLA class II molecules exhibited no reactivity (data not shown).

Furthermore, it is evident that the outcome of the AMS-ELISA may be influenced by acute rheumatoid attacks which is in accordance with many ELISA assays (Schlaf et al., 1998). Indeed, immune complexes such as rheumatoid factors or other Ig-aggregates often result in false positive outcomes of ELISA-based assays. Since the CDC-crossmatch showed the same phenomenon, the observed effect was not unexpected. Once more it is clearly demonstrated that outcomes derived from a single assay should not be used for final diagnosis without further analyses or without considering the clinical state of a patient.

In contrast to the formerly established Quikscreen-ELISA (GTI diagnostics, Waukesha, USA) using solid phase-coated HLA class I or class II glycoproteins (Worthington et al., 2001) the novel AMS-ELISA was designed using the sandwich-technology with a monoclonal capture antibody directed against monomorphic epitopes of HLA class I or class II molecules, respectively. This allows the detection of the recipient's individual antibodies which are directed against the captured HLA molecules of the donor. In contrast to the AMS-ELISA the antigen source of the Quikscreen-ELISA (to detect anti-class I antibodies) consists of a pool of more than 100 donations of platelets. The HLA class II antigens, HLA-DR and HLA-DQ, are purified from selected Epstein Barr virus-transformed cell lines and are manufactured in the same format as the HLA class I antigens (Fuller et al., 2000). The HLA molecules are purified by column chromatography and directly immobilized on the surface of microtiter plates. Theoretically, the AMS system has the advantage that an epitope loss of the HLA molecules due to their solid phase-coating may be avoided. Therefore, it would be interesting to determine the concordance between both assays. Other difficulties may arise from this direct coating of HLA antigens which have been described recently by Worthington and coworkers (2001) for the Quikscreen-ELISA class II antigen DQ. Due to its properties, the HLA-DQ antigen most probably does not avidly stick to the surface of the microtiter plate and is therefore washed off more easily. This results in false-negative discrepant outcomes concerning this antigen using the Quikscreen class II-ELISA (QSB), a problem which can be overcome by the sandwich technology of the AMS-ELISA.

Taken together this is the first study describing the use and optimisation of the AMS-crossmatch ELISA for the identification of donor-specific antibodies. This assay is a sensitive and reliable tool with obvious advantages over the classical CDC crossmatch. As shown by five exemplary cases in this comparative analysis the diagnostic outcome would have been improved for four of the patients by employing the AMS-ELISA as tool for monitoring antibodies in parallel to or instead of the classical CDC procedure. However, due to the limited number of cases (n=27) investigated so far in our lab and because of its time-consuming procedure (about five to six hours including the lysis of the cells) we do not suggest the general substitution of the CDC crossmatch by the AMS-ELISA since its use in the routine task of a tissue typing lab is limited by this aspect. In emergency duties in which the standard CDC crossmatch is performed in about 2.5 hours it cannot be substituted by an assay which requires at least twice this time. The AMS-ELISA may, therefore, rather be an additional assay applied for special cases of crossmatch analyses with no limitations of time (e.g. kidney grafts of living donors). In conclusion, our data nevertheless strengthen the urgent requirement for a novel monitoring procedure in addition to the conventional CDC crossmatch method currently employed by all laboratories.

References

Ahern A.T., Artruc S.B., Della Pelle P., Cosimi A.B., Russell P.S., Colvin R.B. and Fuller T.C. (1982). Hyperacute rejection of HLA-AB-identical renal allografts associated with B lymphocyte and endothelial reactive antibodies. Transplantation 33, 103-106.

Bittencourt M.C., Rebibou J.M., Saint-Hillier Y., Chabod J., Dupont I., Chalopin J.M., Herve P. and Tiberghien P. (1998). Impaired renal graft survival after a positive B-cell flow-cytometry crossmatch. Nephrol. Dial. Transplant. 13, 2059-2064.

Böhmig G. and Regele H. (2003). Diagnosis and treatment of antibody-

mediated kidney allograft rejection. Transpl. Int. 16, 773-784.

Böhmig G., Regele H. and Hörl W. (2005). Protocol biopsies after kidney transplantation. Transplant. Int. 18, 131-139.

Boisjoly H.M., Tourigny R., Bazin R., Laughrea P.A., Dube I., Chamberland G., Bernier J. and Roy R. (1993). Risk factors of corneal graft failure. Ophtalmology 100, 1728-1735.

Chapman J.R., Taylor C., Ting A. and Morris P.J. (1986). Hyperacute rejection of a renal allograft in the presence of anti-HLA-Cw5 antibody. Transplantation 42, 91-93.

Christiaans M.H., Overhof R., ten Haaft A., Niemann F., van Hoof J.P. and van den Berg-Loonen E.M. (1996). No advantage of flow cytometry crossmatch over complement-dependent cytotoxicity in immunologically well-documented renal allograft recipients. Transplantation 62, 1341-1347.

Cosgrove L.J., Vaughan H.A., Tjandra J.J., Thurlow P.J. and McKenzie I.F. (1988). HLA (class I) antigens on platelets are involved in platelet function. Immunol. Cell. Biol. 66, 69-77.

Des Marchais B., Bazin R., Boisjoly H.M., Laughrea P.A., Dube I., Lille S. and Roy R. (1998). Role of presensitization and donor-recipient crossmatching in corneal graft outcome. Cornea 17, 141-145.

Fuller K.E., Worthington J.E. and Martin S. (2000). ELISA screening for HLA-DR and -DQ specific antibodies-The Quikscreen B-Screen (Abstract). Hum. Immunol. 61, (Suppl. 1) 98.

Garovoy M.R., Rheinschmidt M.A., Bigos M., Perkins H., Colombe B., Feduska N. and Salvatierra N. (1983). Flow cytometry analysis: a high technology cross-match technique facilitating transplantation. Transplant. Proc. 15, 1939-1940.

Gebel H.M. and Bray R.A. (2000). Sensitization and sensitivity: defining the unsensitized patient. Transplantation 69, 1370-1374.

Gebel H.M., Bray R.A., Ruth J.A., Zibari G.B., McDonald J.C., Kahan B.D. and Kerman R.H. (2001). Flow PRA to detect clinically relevant HLA antibodies. Transplant. Proc. 33, 477.

Gebel H.M., Harris S.B., Zibari G. and Bray R.A. (2002). Conundrums with FlowPRA beads. Clin. Transplant. 16 (Suppl. 7), 24-29.

Hedge S., Mellon J.K., Hargrave S.L. and Niederkorn J.Y. (2002). Effect of alloantibodies on corneal allograft survival. Invest. Ophtalmol. Vis. Sci. 43, 1012-1018.

Karpinski M., Rush D., Jeffery J., Exner M., Regele H., Dancea S., Pochinco D., Birk P. and Nickerson P. (2001). Flow cytometric crossmatching in primary renal transplant recipients with a negative anti-human globulin enhanced cytotoxicity crossmatch. J. Am. Soc. Nephrol. 12, 2807-2814.

Kerman R.H., Süsskind B., Buyse I., Pryzbylowski P., Ruth J., Warnell S., Gruber S.A., Katz S., van Buren C.T. and Kahan B.D. (1999). Flow cytometry-detected IgG is not a contraindication to renal transplantation: IgM may be beneficial to outcome. Transplantation 68, 1855-1858.

Khan N., Robson A.J., Worthington J.E. and Martin S. (2003). The detection and definition of IgM alloantibodies in the presence of IgM autoantibodies using FlowPRA beads. Hum. Immunol. 64, 593-599.

Lobashevsky A.L., Senkbeil R.W., Shoaf J., Mink C., Rowe C., Lobashevsky E.S., Burke R., Hudson S., Deierhoi M.H. and Thomas J.M. (2000). Specificity of preformed alloantibodies causing B cell positive flow crossmatch in renal transplantation. Clin. Transplant. 14, 533-542 (erratum in Clin. Transplant. 15, 218).

Nickeleit V. and Mihatsch M.J. (2003). Kidney transplants, antibodies and rejection: is C4d a magic marker? Nephrol. Dial. Transplant. 18, 2232-2239.

Niederkorn J.Y. (2001). Mechanisms of corneal graft rejection. Cornea 20, 675-679.

Patel R. and Terasaki P.I. (1969). Significance of the positive crossmatch test in kidney transplantation. N. Engl. J. Med. 280, 735-739.

Pei R., Wang G., Tarsitani C., Rojo S., Chen T., Takemura S., Liu A. and Lee J. (1998). Simultaneous HLA class I and class II antibodies screening with flow cytometry. Hum. Immunol. 59, 313-322.

Piazza A., Adorno D., Poggi E., Borelli L., Buonomo O., Pisani F., Valeri M., Torlone N., Complone C., Monaco P.I., Fraboni D. and Casciani C.U. (1998). Flow cytometry crossmatch: a sensitive technique for assessment of acute rejection in renal transplantation. Transplant. Proc. 30, 1769-1771.

Pocsik E., Mihalik R., Gyodi E., Reti M., Paloczi K., Petranyi G.G., and Benczur M. (1990). Activation of lymphocytes after platelet allotransfusion possessing only class I MHC product. Clin. Exp. Immunol. 82, 102-107.

Rebibou J.M., Chabod J., Bittencourt M.C., Thevenin C., Chalopin J.M., Herve P. and Tiberghien P. (2000). Flow-PRA evaluation for antibody screening in patients awaiting kidney transplantation. Transpl. Immunol. 8, 125-128.

Roy R., Boisjoly H.M., Wagner E., Langlois A., Bernard P.M., Bazin R., Laughrea P.A. and Dube I. (1992). Pretransplant and posttransplant antibodies in human corneal transplantation. Transplantation 54, 463-467.

Schlaf G., Salje C., Wetter A., Stuertz K., Felgenhauer K. and Mäder M. (1998). Determination of synapsin I and synaptophysin in body fluids by two-site enzyme-linked immunosorbent assays. J. Immunol. Meth. 213, 191-199.

Scornik J.C. (1995). Detection of alloantibodies by flow cytometry: relevance to clinical transplantation. Cytometry 22, 259-263.

Scornik J.C., Brunson M.E., Schaub B., Howard R.J. and Pfaff W.W. (1994). The crossmatch in renal transplantation. Evaluation of flow cytometry as a replacement for standard cytotoxicity. Transplantation 57, 621-625.

Scornik J.C., Bray J.A., Pollak M.S., Cook D.J., Marrari M., Duquesnoy R. and Langley J.W. (1997). Multicenter evaluation of the flow cytometry T-cell crossmatch: results from the American Society of Histocompatibility and Immunogenetics–College of American Pathologists proficiency testing program. Transplantation 63, 1440-1445.

Worthington J.E., Robson A.J., Sheldon S., Langton A. and Martin S. (2001). A comparison of enzyme-linked immunosorbent assays and flow cytometry techniques for the detection of HLA-specific antibodies. Hum. Immunol. 62, 1178-1184.

Accepted April 17, 2006