HLA-Antibody Testing: The Immune Phagocytosis Inhibition Test Is Superior to the PRA-STAT and NIH Lymphocytotoxic Test With Respect to Specificity

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We compared the specificity and sensitivity of four different methods for the detection of antibodies specific for HLA antigens. The NIH version of the complement-dependent cytotoxic test (CDC) was used as the gold standard to which we compared two Fcγ receptor (FcγR)-dependent immune phagocytosis inhibition tests (IPI) and one commercial enzyme-labelled immunosorbent assay (ELISA) with soluble HLA class I-antigen preparations bound to the plate (PRA-STAT). Both IPI tests are based on the fact that HLA-antibodies specifically bind to antigens on the monocyte surface via their Fab portion, and in so doing block a neighbouring FcγR with their Fc region. This blockade prevents phagocytosis of IgG-coated red blood cells (RBCs), which can be measured either microscopically (IPI_m) or photometrically (IPI_p). The four assays were used in blind tests on 20 human alloantisera or monoclonal antibodies

with known HLA-antigen reactivities. Additionally, two monoclonal antibodies and one human serum were titrated to elucidate the sensitivity of each test. After all tests were completed, the identities of the samples were disclosed. Both IPI methods detected and identified all clinically relevant HLA class I and class II specific antibodies. In contrast, the CDC was not able to detect noncytotoxic HLA-antibodies and HLA class II specific antibodies; however, it detected clinically insignificant IgM lymphocytotoxins. The PRA-STAT assay enabled identification of all cytotoxic and noncytotoxic IgG antibodies with specificity for HLA-class I antigens. With respect to sensitivity, the CDC and the IPI methods were superior to the PRA-STAT. These facts demonstrate the advantage of IPI methods in the detection of clinically relevant HLA-antibodies. *J. Clin. Lab. Anal.* 15:334–341, 2001. © 2001 Wiley-Liss, Inc.

Key words: HLA antibody; immune phagocytosis inhibition (IPI); complement-dependent cytotoxic test; PRA-STAT

INTRODUCTION

Since the introduction of the complement-dependent cytotoxic (CDC) crossmatch by Terasaki and McClelland (1) this assay has become the gold standard for detection of HLA-antibodies prior to transplantation. However, this test can not detect noncytotoxic antibodies. Because in its standard version the test is performed on mononuclear cells, containing a high percentage of T-lymphocytes, it is not possible to determine exact reactivities with HLA class II antigens, which are restricted to B-lymphocytes and monocytes. These antibodies may induce transplant rejection (2–4). On the other hand, the CDC gives positive results with non-HLA-specific IgM lymphocytotoxins, which proved to be of no consequence in transplant rejection (5). Furthermore, the clinical significance of HLA-specific IgM antibodies producing positive CDC results is still a matter of controversy (5–8). Thus, the test-specific disadvantages of the assay forced the development of alternative tests. The flow-cytometric crossmatch is indepen-

dent of complement fixation, and therefore detects cytotoxic as well as noncytotoxic IgG antibodies. A corresponding fluorochrome-conjugated secondary antibody is used for the detection. However, false-positive results, difficulties in the detection of HLA-DR antibodies (9,10), and the necessity of providing an expensive flow cytometer pushed the search

Abbreviations: CDC = complement-dependent cytotoxic test; FcγR = Fcγ receptor; IPI_m = immune phagocytosis inhibition test with microscopic evaluation; IPI_p = immune phagocytosis inhibition test with photometric evaluation; ELISA = enzyme-labelled immunosorbent assay; RBC = red blood cell; MNC = mononuclear cells; PRA = panel reactivity of antibodies.

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for alternative methods. The Fc γ receptor (Fc γ R)-dependent immune phagocytosis inhibition (IPI) assay (11) uses the characteristic of IgG HLA antibodies to form three molecule complexes in that the antibody binds with its F(ab') portion to the HLA antigen, and with its Fc portion to a neighbouring Fc γ R. Thus the assay is independent of complement fixation and exclusively detects HLA IgG antibodies. Evaluation of results can be either microscopic (IPI_m) (11) or photometric (IPI_p) (12,13). A high association has been demonstrated by the IPI_m between HLA antibody detection in sera from high-risk patients with preexisting antibodies and transplant rejection (14). The clinical significance of non-complement-dependent IgG alloantibodies to HLA has been further demonstrated by other studies (7,10,15–17). A commercially available ELISA (PRA-STAT) assay with immobilized preparations of soluble HLA antigens was introduced some years ago. In the first versions this assay used HLA class I antigen preparations and was reported to detect HLA class I specific antibodies of the IgG type independent of complement fixation. A recently offered version also contains HLA class II antigens. A comparison of the specificity and sensitivity of HLA antibody detection was performed between the standard lymphocytotoxic test, IPI_m, IPI_p, and the PRA-STAT assay in a blind trial using 20 human alloantisera and human monoclonal antibodies.

MATERIALS AND METHODS

Sera and Monoclonal Antibodies

Fourteen different human alloantisera with known reactivities for HLA antigens, including negative controls and the human monoclonal antibodies H6D3 (kindly provided by J. Harpprecht, Kiel, Germany (18)), BRO11F6 and SN607D8 (both kindly provided by Arend Mulder, Leiden, The Netherlands (19)), and two different dilutions of BRO11F6 and SN607D8, were aliquoted. BRO11F6 was reactive to HLA-A11 with cross-reactivity to HLA-A3, and SN607D8 had reactivity to HLA-A2 and cross-reactivity to HLA-A28. Encryption of the test samples was carried out by W.-M. Becker and A. Petersen (Department of Allergology, Forschungszentrum Borstel, Germany). The human alloantisera were obtained from HLA-immunized patients and pregnant women, and were provided by U. Cassens (Münster, Germany) and J. Neppert (Kiel, Germany). Heat-inactivated human serum from blood group AB donors (iAB) was used as the negative control, and the HLA class I specific monoclonal antibody w6.32 (20) was used as the positive control.

One set of sera and monoclonal antibodies was sent to U. Cassens (Münster) for performance of the IPI_p. The other sets remained in Kiel, Germany, where the CDC, IPI_m, and PRA-STAT were performed. The test cell panels were designed so that each known HLA-antibody specificity could be detected with a corresponding antigen. The cells were isolated from the

blood of HLA-typed blood donors from the Institute of Transfusion Medicine, Kiel. HLA-class I typing was performed by classical serology testing (lymphocytotoxic test). The test cell panel in Münster derived from local donors. The test cell panels in Kiel and Münster were not identical.

Preparation of Test Cells

Mononuclear cells (MNCs) were isolated from heparinized human blood from HLA-typed blood donors by density gradient centrifugation on ficoll ($d = 1.078$) (Lymphoflot, Biotest, Dreieich, Germany). After centrifugation for 20 min at 800 g the MNCs were harvested from the interphase and were washed twice in PBS with Ca²⁺ and Mg²⁺ at pH 7.4 (subsequently referred to as washing buffer). The MNCs were suspended in washing buffer at 2×10^6 /mL for the CDC and the IPI_m, and for the IPI_p at 1×10^6 /mL in culture medium (RPMI 1640 (Gibco, Eggenstein, Germany) with 10% heat inactivated fetal calf serum (FCS) (Gibco) and 1% gentamycin (Gibco)). Indicator RBCs were isolated from heparinized blood from group 0, Rh-D positive individuals. RBCs were washed and adjusted to a 5% suspension in washing buffer for the IPI_m, and to 1×10^8 /mL in isotonic NaCl solution for the IPI_p.

Lymphocytotoxic Test

The CDC was performed in its standard version (according to Terasaki and McClelland (1)) on a cell panel of 50 HLA-typed donors. Briefly, 1 μ L of MNC suspension was incubated together with 1 μ L of heat-inactivated serum for 30 min at room temperature under oil within the well of a Terasaki plate. Next, 5 μ L of rabbit complement was added and incubated for 60 min at room temperature. Cells were stained with 3 μ L of eosine solution per well and fixed with 6 μ L of formaldehyde (10%). After staining, the percentage of dead cells was evaluated microscopically under phase contrast. Cytotoxicity scores were classified according to the 1984 Histocompatibility Workshop (21). Scores ≥ 4 were positive.

IPI_m Assay

The IPI_m was performed as described previously (11), on the same cell panel as the CDC. Briefly, 30 μ L of MNC suspension was allowed to adhere to the reaction fields of adhesion test slides (12 reaction fields per slide; Dunn, Asbach, Germany) for 60 min at 37°C. After washing, 30 μ L of heat-inactivated test serum (1:10 in washing buffer) was added and incubated for another hour at 37°C. A second incubation period (1 hr at 37°C) followed after washing, during which 30 μ L of anti-D coated RBC suspension was added. After rinsing with 0.9% sodium citrate, the adhesion slides were air dried and stained with Wright's eosin-methylene blue solution (Merck, Darmstadt, Germany). The percentage of monocytes with ingested RBCs was evaluated, and the relative phagocytosis rate of the sample ($prel_s$) was determined

as $p_s \times 100 : p_{i.ab} (\%)$ (p_s = phagocytosis rate of the sample; $p_{i.ab}$ = phagocytosis rate of i.AB serum). The cutoff was determined from the mean $- 3 \times$ standard deviation of the negative controls from earlier experiments (11) at 30.4%; all results below that value were termed positive. For the sensitivity studies, the results of the IPIIm were expressed as the relative inhibition of the immune phagocytosis ($irel_s$) to enable comparisons to be made with the CDC and the PRA-STAT. The $irel_s$ was calculated as $100 - prel_s (\%)$. Thus, a $prel_s$ cutoff of 30.4% corresponded to an $irel_s$ cutoff of 69.6%. The initial sample dilution required for the test (1:10) was not included in the calculation of the sample dilutions for the sensitivity studies.

IPIp Assay

The IPIp was performed as described previously (12), with minor modifications, on a panel of 10 donors. Briefly, 100 μ L of MNC suspension was incubated in each well of a 96 F-well microtiter plate (Greiner, Nürtingen, Germany) for 60 min at 37°C. Nonadherent cells were removed by washing the plate with PBS, pH 5.5 (IPIp buffer), and 200 μ L of culture medium was added to the monocyte layer in each well. Next, 20 μ L of test sample or control (positive control: w6.32; negative control: culture medium) in a 1:10 dilution was added and incubated for another 60 min. After washing with IPIp buffer, 200 μ L of culture medium and 50 μ L of anti-D coated RBC suspension was added and incubated for 60 min at 37%. After washing twice, the noningested RBCs were removed by hypotonic shock (bidistilled water : PBS = 2:7), followed by a further wash. Then 100 μ L of 0.1% SDS in IPIp buffer was added to lyse the monocyte membranes. Free hemoglobin was determined by the peroxidase reaction. For this, 100 μ L of TMB solution (2 mg of tetramethylbenzidine (Sigma, Deisenhofen, Germany) in 1 mL of ethanol, 19 mL of IPIp buffer, and 5 μ L of H_2O_2 (30%, Sigma) per mL of substrate) was added, and the optical density (OD) was read by an ELISA reader (Behring, Marburg, Germany) at 650 nm (reference 450 nm). Each determination was performed in triplicate and the arithmetic mean was calculated. The phagocytosis values of the test samples were reported as percentage of the negative control, calculated from the OD values. Values lower than 30% were considered positive. For the sensitivity studies the relative phagocytosis inhibition was calculated; accordingly, the cutoff was at 70% phagocytosis inhibition.

PRA-STAT

The PRA-STAT assay, HLA-class I antigen antibody detecting version (22), was purchased from Nextran/Baxter (Munich, Germany). The assay was performed in accordance with the manufacturer's instructions, and the plates were read by an automatic ELISA reader (Behring, Marburg, Germany) at 492 nm. The test prerequisite 1:101 dilution of the sample was not included in the calculation of the dilution steps used for the study. The manufacturer's recommendations were followed with respect to criteria for validity of measurement

and cutoff values. The ratio factor of each single well was set at 0.35 as recommended by the manufacturer, so that the cutoff value was calculated as $(OD_{sample} - OD_{unspec. background}) \times 0.35$. The PRA-value of each serum sample was calculated according to the formula: PRA-value (%) = number of wells with positive reactions $\times 100/46$. Samples with a PRA-value > 6.52 % were declared to be antibody positive. The HLA-specificities were calculated by computer software (Soft STAT) provided by the manufacturer.

To determine the specificity of each assay, the number of actually positive reactivities was related to the number of expected positive results according to the HLA type of the cells. Additional reactivities were compared for the different assays to find out if they were false-positive or correct additional results. The sensitivity of the different assays was determined by the titrations of one human polyclonal antiserum with polyspecific cytotoxic HLA-antibodies, and two human monoclonal antibodies. These antibody titrations were tested in each assay with the corresponding cells.

RESULTS

Test Specificity

After completion of all tests the test sample encryption was decyphered. The samples were grouped according to known specificities or characteristics. In group I, human monoclonal antibodies are listed. Three different dilutions of BRO11F6 and SN607D8, and neat culture supernatant of H6D3 were compared for their reactivity in each assay (Table 1). CDC, IPIIm, and PRA-STAT found the HLA-A11 specificity of BRO11F6 as well as its cross-reactivity with HLA-A3 at the highest concentration, while the IPIp did not detect any cross-reactivity. Amongst the samples at the lowest concentration only the IPIIm had one positive result. For each assay the observed number or percentage of positive reactions was less than expected. The IPIIm produced the results closest to those expected. The IPIp gave two false-positive results of unknown specificity. With respect to the mab SN607D8, the test results were comparable to those of the mab BRO11F6. Both the HLA-A2 reactivity and the HLA-A28 cross-reactivity of SN607D8 were recognized in the CDC, IPIIm, and PRA-STAT, while the IPIp missed the cross-reactivity. The IPIIm and CDC were the only assays able to detect both reactivities with the three different dilutions of the antibody. False positivity was observed with the CDC technique in two cases with unknown specificity. Again, the IPIIm produced the results which stack closest to those expected.

The IgM mab H6D3 of the isotype IgM was known to react polyspecifically with lymphocytes in the CDC, and this observation was supported by the results obtained from our cell panel. The other tests were negative with this mab.

In order to have a better basis for comparison, we grouped the test sera. Within group II we gathered those alloantisera with known specificities (Table 2). Altogether, the number of false-negative reactions was very low in that group, but in

TABLE 1. Reactivities of the monoclonal antibodies (group 1) in CDC, IPI, IPIp, and PRA-STAT

Sample	Antibody clone/ characteristics/ known specificities		Detected known specificities				No. or percentage of positive reactions/ no. or percentage of expected positive reactions				No. or percentage of false negative reactions			
	no.	Dilution	CDC	IPIIm	IPIp	PRA-STAT	CDC	IPIIm	IPIp	PRA-STAT	CDC	IPIIm	IPIp	PRA-STAT
4	BRO 11 F6/	1:20	A11, 3	A11, 3	A11	A11, 3	5/16	12/16	2/5 ^a	8.7%/19.5%	11	4	4	10.8%
20	Cytotox. IgG1/	1:80	A11, 3	A11, 3	–	–	5/16 ^a	3/16	1/5 ^a	0%/19.5%	12	13	5	19.5%
25	HLA-A11xA3	1:160	–	A3	–	–	0/16	1/16	0/5	0%/19.5%	16	15	5	19.5%
5	SN 607 D8/	1:10	A2, 28	A2, 28	A2	A2, 28	13/36	35/36	4/4	30.4%/37%	23	1	4	6.6%
26	Cytotox. IgG1/	1:20	A2, 28	A2, 28	–	A2, 28	6/36	35/36	0/4	28.3%/37%	30	1	4	8.7%
11	HLA-A2x28	1:40	–	A2, 28	–	–	5/36 ^b	28/36	0/4	0%/37%	33	8	4	37%
9	H6D3/ Cytotox. IgM/ Lymphocytes	1:1	Broad reactivity	–	–	–	50/#	0/0	0/0	0%/0%	#	0	0	0

^aOne false positive reaction each with unknown specificity.^bTwo false positive reactions with unknown specificity.

—, no specificity detected; #, not possible to specify.

some cases additional specificities beyond those known were observed. The HLA-A31 specificity of serum no. 8 was not detected in the IPIp, as HLA-A31 positive cells were not included in the test panel. The other assays, along with HLA-A31, were also able to detect the specificities for HLA-A19, -A30, -A32, and -A33. The noncytotoxic alloantiserum with specificity for HLA-B44 and possible cross-reactivity to HLA-A1 was tested twice (nos. 10 and 23). The main specificity was detected by the IPI, IPIp, and PRA-STAT without false negativity, but not by the CDC. Additional reactivity was observed in each assay, and an additional 10 nonspecific results were obtained using the CDC.

Serum no. 19, with HLA-B7 specificity and possible cross-reactivity to HLA-A3, was demonstrated by the CDC, IPI, and

and IPIp. Although some reactivities were missed, additional specificities were found in the IPI and CDC.

The noncytotoxic serum no. 2, which was reactive to HLA-DR (except HLA-DR 7), was positive in the IPI, IPIp, and PRA-STAT, but negative in the CDC. Because the donor cells were not typed for HLA-DR it was not possible to determine the number of false-positive or false-negative reactions within the IPI, IPIp, and PRA-STAT.

The group III alloantisera were defined by general characteristics of test reactivity, e.g., cytotoxic, polyspecific, CDC positive, etc. Therefore, it was impossible to determine the number of false-positive or false-negative reactions. The results obtained by each assay for sera 3, 12, 13, and 15 were consistent with their original description. Of special interest were the results

TABLE 2. Reactivities of the group II sera with known specificities in CDC, IPI, IPIp, and PRA-STAT

Sample no.	Characteristics/ known specificities	Detected known specificities				No. or percentage of positive reactions/no. or percentage of expected positive reactions (main specificity)				No. or percentage of positive reactions and/or additional specificities			
		CDC	IPI	IPIp	PRA-STAT	CDC	IPI	IPIp	PRA-STAT	CDC	IPI	IPIp	PRA-STAT
1	iAB	—	—	—	—	0/0	0/0	0/0	0%/0%	0	0	0	0
8	Cytotox. IgG Tx-serum/ HLA-A31	A31	A31	—	A31	18/3	15/3	0/0 ^a	32.6%/6.5%	15 (7 unknown) A19, 30, 32, 33; B60	12 (4 unknown) A19, 30, 32, 33; B60	0	26.1% A19
10	Non-cytotox. IgG/ HLA-B44 (xA1)	—	B44	B44	B44	10/0	16/5	5/4	26%/8.7%	10 unknown	11 (6 unknown) A1; B12	1	17.3% B12, 13
23	Non-cytotox. IgG/ HLA-B44 (xA1)	—	B44	B44	B44	0/0	16/5	4/4	26%/8.7%	0	11 (6 unknown) A1; B12	0	17.3% B12, 13
19	Cytotox. IgG Tx-serum/ HLA-B7 (xA3)	B7	B7	B7	—	21/14 ^b	24/14 ^b	1/4 ^b	0%/6.5%	8 (4 unknown) B35	11 (4 unknown) A3; B35	0	0
2	Non-cytotox. IgG/ HLA-DR Except DR7	—	—	—	—	0/#	49/#	8/#	89%/#	0	#	#	#

^aNo HLA-A31 cell in the test panel.^bIn each assay false negative reactions: 1 each in CDC and IPI, 3 in IPIp and 6.5% in PRA-STAT.

—, no specificity detected; #, not possible to specify.

for sera 16, 21, and 24, which were initially described as polyspecific. Serum 16 was shown to have a specificity for HLA B60 in each of the assays. The cytotoxic polyspecific HLA-antiserum no. 21 was positive exclusively in the CDC, exhibiting clear HLA-B8 and -B18 specificity. The noncytotoxic serum no. 24 showed an HLA-A1 specificity with the complement-independent assays, while eight reactivities with unknown specificity in the CDC remain unclear.

Test Sensitivity

The sensitivity of the different assays was evaluated using dilution series of the mabs BRO11F6 and SN607D8, and of one cytotoxic polyspecific HLA alloantiserum (no. 12 in Table 3). For this part of the study we did not use encrypted samples. Test cells were chosen to correspond with a known antibody specificity. Dilution series tests with the alloantiserum gave the following results when the previously described cutoff values were used for each assay: the CDC and IPIp were positive with the undiluted sample. Both the IPIIm and PRA-STAT recognized the 1:2 diluted sample (Table 4).

The mab SN607D8 with specificity for HLA-A2 and cross-reactivity with HLA-A28 was detectable in the CDC, IPIIm, PRA-STAT, and IPIp up to dilutions of 1:45, 1:40, 1:38, and 1:36, respectively (Table 4).

The order of the sensitivity of the assays in the detection of dilutions of the mab BRO11F6 was similar to that of SN607D8 (Table 4). The CDC (detection limit 1:90) and both IPI methods (1:50) were superior to the PRA-STAT (1:10).

DISCUSSION

The detection and characterization of PRA in patients awaiting renal transplantation is of major interest for transplant

survival. Because the standard cytotoxicity testing exhibits some disadvantages, we compared three alternative methods to the CDC with respect to antibody specificity and sensitivity. The IPIIm, IPIp, and PRA-STAT equally detected cytotoxic as well as noncytotoxic antibodies, while the CDC depends on complement fixation and therefore was not able to detect noncytotoxic antibodies. These noncytotoxic antibodies, however, may also be brought into context with acute rejection episodes and graft loss, as was demonstrated impressively by different studies (7,10,15–17).

Utilizing the CDC, IPIIm, and PRA-STAT the cytotoxic human mabs BRO11F6 and SN607D8, in the highest titer tested, recognized both the main specificity as well as the cross-reacting epitope. Both mabs had been previously tested in the cytotoxicity test and the PRA-STAT (19) with comparable results. In our investigation, the IPIp was unable to identify the cross-reactivity of either of the mabs. The mab H6D3 is a human IgM lymphocytotoxin. This mab was positive exclusively in the CDC because both IPI tests specifically detect IgG antibodies, and the PRA-STAT in its standard version uses an anti-IgG conjugated secondary antibody. It is widely accepted that antibodies of the IgM type which are not specific for HLA have no effect on graft survival (5,6,23). However, there is some controversy concerning the influence of IgM HLA-specific antibodies. These may be relevant in first transplantation (8) and retransplantation (24,25), but most authors deny any clinical influence of IgM HLA-specific antibodies (3,5,7). However, if these antibodies do not influence graft survival, exclusion of a potential organ recipient because of a positive cross-match would mean prolonged suffering for the patient. To circumvent this problem, dithiotreitol (DTT) treatment of the sera was introduced to inactivate IgM (26,27) by reducing the disulfide bonds in the IgM tertiary structure, leaving the

TABLE 3. Reactivities of the group III sera with an initial more common description in CDC, IPIIm, IPIp and PRA-STAT

Sample no.	Characteristics/ known specificities	No. or percentage of positive reactions				Detected characteristics or specificities			
		CDC	IPIIm	IPIp	PRA-STAT	CDC	IPIIm	IPIp	PRA-STAT
3	Cytotox. IgG/ Tx-serum broad/ HLA-reactivity	50	50	8	89%	Polyspecific	Polyspecific	Polyspecific	Polyspecific
12	Cytotox. IgG/ Polyspecific HLA	30	31	1	65%	Polyspecific	Polyspecific	Polyspecific	Polyspecific
13	Tx-serum/ LCT neg. IPIp weak pos.	0	0	0	0%	—	—	—	—
15	Tx-serum/ LCT neg. IPIp neg.	0	0	0	0%	—	—	—	—
16	Tx-serum/ Cytotoxic IgG/ Polyspec. HLA	21	28	2	26%	B7, 35, 60; A3, 11	B7, 35, 60; A3, 11	B7, 60	B41, 60
21	Cytotoxic/ Polyspec. HLA	21	0	0	0%	B8, 18	—	—	—
24	Non-cytotox. IgG/ HLA-class I	8	25	4	32, 6%	8 unknown	A1, 28; B12, 44	A1	A1; B12

—, No specificity detected.

TABLE 4. Endpoint titrations of the mabs SN607D8 and BRO11F6 and the cytotoxic polyspecific alloantiserum no. 12 within the four different assays.

Assay	Antibody designation and specificity		
	Serum no. 12 cytotoxic, polyspecific	SN607D8 HLA-A2xA28	BRO11F6 HLA-A11xA3
CDC	1	45	90
IPI _m	2	40	50
IPI _p	1	36	50
PRA-STAT	2	38	10

The reciprocal value of the lowest sample concentration which was still positive in each assay is given.

antibody binding portion unaffected. Under these conditions a positive CDC auto- or donor-cross-match would become negative after DTT treatment if the patient had no further lymphocyte reactive antibodies apart from HLA-specific or non-HLA-specific IgM. An assay that is incapable of detecting IgM antibodies would save the additional effort required to exclude these antibodies.

While comparison of the test specificity by use of monoclonal antibodies revealed differences in sensitivity rather than in specificity, the results with human alloantisera were different. Within the group II sera (those with defined specificities), noncytotoxic antibodies could not be detected by the CDC because of the limitations of the test. False-negative results were observed in only a few cases. The IPI_p was unable to demonstrate the anti HLA-A31 in serum no. 8, as no suitable cells were incorporated into the test. Conversely, in each assay additional positive reactions were observed. Usually, the specificity of an assay is defined by the number of false-positive reactions. In our study this would imply a low specificity for each assay tested. Further investigations showed that rather than being false positives, most of these results were indicative of additional specificities. In the case of serum no. 8 the CDC, IPI_m, and PRA-STAT show an additional reaction with HLA-A19, and CDC and IPI_m also indicate HLA-A30, -A32, -A33, and -B60. HLA-A30, -A31, -A32, and -A33 belong to the same cross-reactive group (CREG) (28) and are all splits of HLA-A19. That means that the initial specificity of serum no. 8 as anti HLA-A31 during the study could be defined more precisely by inclusion of the splits of HLA-A19. A noncytotoxic IgG alloantiserum with HLA-B44 specificity and possible cross-reactivity for HLA-A1 was included twice (samples 10 and 23), and the complement-independent assays were able to detect the main specificity, while the anti HLA-A1 could only be confirmed by the IPI methods. The HLA-B12 broad specificity was only demonstrated by the IPI_m and the PRA-STAT, while the other assays only detected the B44 split, again a reflection of increased specificity rather than false positivity. The B7 specificity of serum no. 19 was not detected by the PRA-STAT, a finding that had been previously reported (29,30). The noncytotoxic IgG alloantiserum no. 2, specific for HLA-DR

(except DR7), was negative in the CDC but positive in the other assays. This serum had been described previously in a workshop as IPI positive and CDC negative (serum designated as "no. 24" in Ref. 31). The PRA-STAT in the version we used was described to detect only HLA class I antibodies, but it was also positive with the HLA-DR-specific serum in a high percentage of positive reactions. This effect was demonstrated previously (20,32) and may be explained by the composition of the soluble HLA antigen preparations bound to the ELISA plate. Both IPI methods regularly recognize HLA-DR antibodies because the assays are performed on enriched monocyte preparations and not on total MNC, among which only a subpopulation of cells expresses HLA-DR antigens. Because the influence of HLA-DR antibodies on graft survival should not be underestimated (2,3,17), HLA-antibody detection should be performed by an assay that is able to follow these criteria. In some of the group II sera additional positive reactions with unknown specificities were observed within the CDC, IPI_m, and PRA-STAT, but we were not able to report these as either false positives or genuine additional reactions.

Of the group III sera, nos. 6, 21, and 24 were of special interest, because they were initially described as polyspecific. In our study we were able to demonstrate an HLA-B60 antibody in serum 16 by each of the assays. Interestingly, the B7 specificity was again undetectable by the PRA-STAT, as observed previously in serum no. 19. The combination of a positive CDC and negative IPI and PRA-STAT tests with serum no. 21 indicates that the anti HLA-B8 and -B18 antibodies are of the IgM type. A noncytotoxic serum (serum no. 24) again could not be identified by the CDC; the eight reactions with unknown specificity could be false positives. This series of experiments demonstrated remarkable differences between the assays showing the advantages of using the IPI_m and PRA-STAT. The IPI_p was less specific and demonstrated fewer additional or false positive results than the IPI_m, which is contradictory to previous findings (33). This may be explained by the fact that the cell panel for the IPI_p was significantly smaller, for technical reasons. Furthermore, the samples for the IPI_p were the only ones that underwent transportation, so a possible loss of activity cannot be excluded.

The PRA-STAT has been compared with the standard CDC and with flow cytometry in several publications. The main conclusion is that the ELISA method is a good alternative, with an 80% agreement between the CDC and ELISA (15,19,32,34), and an 80% agreement between flow cytometry and ELISA (30). But the problem of IgM reactivity of the CDC and of nonreactivity with non-complement-fixing antibodies remains. Many studies comparing different assays for the detection of HLA antibodies determine the percentage of PRA in pre- or posttransplant sera (32,35,36). These PRA values then may be correlated with the clinical outcome of the transplantation. Thus, important data for transplantation management strategies may be collected. The predictive values for graft survival generally seem to correspond between

the assays, but antibody specificities and test sensitivities vary in detail due to test-specific characteristics (19,29,34,35). The IPI_m detects a higher percentage of panel reactivity in sera from high-risk patients awaiting a kidney than the CDC, and this is in good agreement with graft failure (14). Nevertheless, it is also necessary to determine the specific epitopes against which patient HLA antibodies are directed, in order to obtain the best possible definition of the acceptable donor antigens. In our laboratory (the granulocyte and monocyte reference laboratory of the German Society for Transfusion Medicine and Immune Haematology) we have used the IPI as a routine assay in parallel with the CDC for about 15 years to detect HLA specific alloantibodies. The IPI assay is somewhat more time consuming (1 hr more than the CDC), but this is justified by the results. Screening of pretransplant sera by a combination of two assays (e.g., IPI and CDC, or PRA-STAT and CDC) to detect all possible HLA-specific alloantibodies could lead to graft-recipient compatibility, as shown above (14). The clinical significance of non-complement-fixing HLA-specific antibodies for the outcome of renal transplantation has been demonstrated before (7,10,15–17). However, HLA-antibody testing is also mandatory in some cases of platelet transfusion when the patient is preimmunized against HLA-antigens. Non-complement-fixing HLA antibodies, as well as cytotoxic HLA-antibodies, may provoke platelet refractoriness (37), which is why we perform the IPI_m and CDC in parallel in these cases. Another reason to prefer the FcγR-dependent IPI assay for HLA antibody detection may be that CDC, as well as binding assays like the PRA-STAT, need nonhuman reagents (such as rabbit complement or conjugated antihuman immunoglobulin) for the antibody detection. The action of human complement would be too weak to obtain reliable test results. On the other hand, FcγR-dependent assays such as the IPI, antibody-dependent cellular cytotoxicity (ADCC) (38), or the chemiluminescence inhibition test (39) stick much closer to the *in vivo* action of HLA antibodies, so the results of these assays may better predict the interaction between patient HLA antibodies and donor cells.

In our study, the experiments designed to determine the specificity of the assays in some cases also gave hints to the sensitivity of the assays, because the recognition of CREG antigens and additional antigens probably can be attributed to a higher test sensitivity. Furthermore, within the specificity section, the mabs BRO11F6 and SN607D8 as coded samples were tested in different dilutions. The IPI_m was the only assay that was positive with each tested titer of the mabs in this section of experiments. Titration of the same antibodies in a dilution series revealed comparable antibody titers within both IPI assays. This agrees with the reported correlation coefficient of $r > 0.85$ between both methods (33). Previous reports confirm a modest advantage of the IPI_p over the CDC with respect to sensitivity (12). In contrast, in this series of experiments the CDC was slightly superior to the IPI methods, depending on the test sample. The results of the ti-

tration experiments with the PRA-STAT varied. When titrating the mab BRO11F6, the CDC was slightly superior to the other methods, while the PRA-STAT exhibited the lowest antibody titer. These results contradict those of another study comparing the sensitivity of ELISA, CDC, and flow cytometry (35). In that study, they found that their HLA antigen screening ELISA, which is based on bound HLA antigen preparations, was generally more sensitive than the CDC, with an advantage of some serial dilution steps. Buelow et al. (22) compared the PRA-STAT, CDC, and flow cytometry in dilution studies and found no significant differences. Sensitivity of the PRA-STAT may depend on the composition of the soluble HLA-antigen preparation, so some antigen specificities may be underrepresented within the plate. Sensitivity of each assay depends on the cutoff values. For this study we chose cutoff values as defined by international agreements (CDC) or as recommended by the manufacturer (PRA-STAT). The cutoff values of both IPI methods were chosen according to experience obtained from earlier series of experiments. Lower cutoff values could enhance sensitivity of each assay, but would impair specificity.

In conclusion, these data show that the CDC, IPI_m, IPI_p, and PRA-STAT differ from each other in some instances with respect to test specificity and sensitivity. In this study, the IPI_m was the most reliable assay for the determination of transplantation-relevant HLA antibodies. Since the photometric evaluation of IPI is a new technique, some technical modifications are currently being tested to improve its sensitivity and specificity.

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