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Application of Prastat ELISA in the determination of anti-HLA specificity for immunized patients awaiting kidney transplant: five years' experience

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Abstract Three hundred sixty-five patients who underwent cadaver donor kidney transplantation between 1993 and 1998 were divided into four groups: 40 immunized patients with at least one peak panel-reactive antibody (PRA) value more than 50%, 11 hyperimmunized patients with more than three peak PRA values over 50%, 10 retransplanted patients and 304 control patients. Before transplantation, we ascertained the antibody specificities against individual HLA antigens (Prastat Sangstat ELISA method for HLA typing of first donor, husbands of multiparous women and potential donors against whom candidates gave positive cross-matches); thus, patients underwent transplantation excluding the presence of the HLA antigens previously detected and looking for high HLA (class I and II) compatibility. Actuarial graft survival after 12 months was satisfactory in all groups: 87% immunized, 81% hyperimmunized and 80% retransplanted vs 92% controls. Renal function at the end of the first year was similar and the number of rejection episodes in the first 3 months did not significantly differ.

Key words Pre-transplant immunization · Anti-HLA antibodies · Prastat · Kidney graft

Introduction

One of the still unsolved questions of renal transplantation is the immunized patient. More and more of these have been found on waiting lists with the passing of time – in some caseloads as many as 30–40% of transplant candidates [1–3]. The immunized patient is generally defined as one who has serum antibodies (IgG and IgM) reacting against HLA class I and II antigens in a percentage ranging from 30% to 70%; the hyperimmunized patient reacts to a percentage varying from 50% to 100% [4, 5]. The degree of immunization among patients awaiting renal transplantation is usually assessed by antibody screening against a panel of separate lymphocytes, harvested from donor blood (panel reactive antibody, PRA), via complement-dependent lymphocytotoxicity testing.

The presence of immunization lowers the chances of receiving a graft, so that such patients accumulate on waiting lists; it is likewise a cause of lower graft survival than occurs with the non-immunized patient [6, 7].

Immunization or sensitization set in three ways: pregnancy, previous transplantation or blood transfusion. The likelihood of the condition increases with number of pregnancies; multiparous women are also more prone to develop reactive antibodies after blood transfusion [8]. The formation of antibodies following transplantation is chiefly, though not exclusively, found as a response to episodes of acute or chronic rejection [9, 10]. It would appear that the immunogenicity of blood transfusions depends acutely on the number of HLA antigens shared by blood donor and recipient [11, 12]. The impact of this problem, which is common to Europe and North America, calls for specific treatment and allocation sytems if either the timespan of a transplant is to be kept reasonable or the success of its outcome ensured. Such strategies over the years have aimed to reduce the degree of immunization by pre-

transplant therapy, or else to optimize the choice of donor by seeking the utmost possible compatibility.

To remove cytotoxic antibodies from some patients, the therapy protocol chosen has been cyclophosphamide combined with cycles of plasmapheresis [13]. The results of this treatment are controversial when it comes to the real possibility of transplanting the patients so treated [14]; what is more, it has proved to carry a high risk owing to the frequency of grave, even mortal, infectious complications in certain patients. Repeated administration of polyclonal immunoglobulins is another treatment that has been proposed [15–17], though its results are not yet fully satisfactory. Another approach used since 1986 to remove anti-HLA antibodies is extracorporeal immunoadsorption on columns of staphylococcal protein A [18–20]. Although such treatment removes the antibodies effectively, it is often followed by a rapid high rebound of anti-HLA antibodies, even when combined with massive immunosuppression by cyclophosphamide and steroids in high doses [21]. While the method is useful in removing antibodies and enabling patients to undergo transplantation [18, 22], its results vary in terms of graft survival: despite removing large quantities of antibodies, it is clearly unable to interfere with the immune memory [23].

Better results have come from the HIT project (*h*ighly *i*mmunized *t*ray) conducted by the Collaborative Transplant Study between 1986 and 1996, which consisted in seeking high donor–recipient compatibility [24, 25]. This led to an approach based on extensive analysis of antibody specificities, the aim being to establish the so-called *acceptable incompatibilities* at a pretransplant stage. Patient sera are routinely tested against panels of HLA-typed donor blood, establishing the individual HLA antigens against which the patient has not formed antibodies. Knowledge of these acceptable antigens (or *epitopes* shared by different antigens) comes in useful when choosing future organ donors [26, 27]. Via centrally coordinated organ allocation one is thus able to select all the HLA-compatible kidney donors who will presumably give a negative cross-match to all the sera of a given immunized patient. The advantages over other programmes are clear: first, it is not necessary to distribute patient's sera around other transplant centres; second, selection is based on a predictable negative cross-match instead of cross-matching with all available donors, most of whom would be positive; lastly, selection of potential donors rests on the data and experience of the centre responsible for the immunized would-be recipient, where all the information is held regarding the patient's immune background (transfusions, specific alloantibodies, autoantibodies, etc.), and not on a negative cross-match performed at another transplant centre. On the other hand, the main drawback of the scheme is the great amount of laboratory work it requires [28, 29].

In the light of the foregoing experience, our present study aimed to establish whether a programme based on identifying anti-HLA class I and II antibody specificities in the transplant candidate, and on seeking high compatibility at the time of surgery, makes it possible, first, to perform transplants, and second, to have an acceptable clinical course.

Material and methods

For our purposes we took waiting-list patients subsequently given a renal graft at the Nephrology Department of St. Orsola University Hospital over the 5 years of the study observation period (1993–1998), and assessed them by the following methods:

Before transplantation

1. Genome typing of HLA antigens (DNA typing), an almost 100% reliable way of establishing even those antigens that serological methods fail to identify.
2. The search for antibody specificities against class I and II HLA antigens, via a new ELISA (Prastat Sangstat) test [30–32]. Obtaining the HLA typing of the first donor in the case of a second- or third-time transplantee, that of the husband of multiparous women, and the potential donor's typing wherever candidates had had a positive cross-match at a previous opportunity for transplantation.

At transplantation

After ruling out the existence of previously identified HLA antigens, we set about ensuring maximum HLA compatibility: typing was by the genome technique, even on the donor; donor–recipient cross-match was assessed by the standard NIH method, with both light microscopy and cytofluorograph on three sera: the two most recent and the highest historical (so-called *peak*) serum.

Immediately after transplantation

From 1993 to 1998, 61 immunized patients underwent renal transplantation from cadaver donor by this programme. Forty had a medium degree of immunization (at least one pre-transplant PRA above 50%); 11 were hyperimmunized (more than 3 PRAs above 50%); 10 patients were undergoing a second transplantation (Table 1).

For hyperimmunized patients and second transplantations the standard therapy regimen (steroids and cyclosporine at normal doses) was boosted for the first 10 days by antilymphocyte serum (ATG/ALG), following which a third immunosuppressant (azathioprine) was added [33]. In the other groups the enhancement therapy was added only if steroid-resistant rejection was present. Plasmapheresis was used if a positive cross-match against donor frozen cells was demonstrated, while OKT3 was limited to the second rejection.

To check for group homogeneity and gather statistical data, we compared the patient's personal and clinical details to those of a control group of 304 patients, matched by sex, age, blood group, number of pregnancies, age gap from donor, and cold ischaemia and post-transplant function recovery times (Tables 2–4) according to the

Table 1 Cytotoxic antibodies against anti-HLA antigens determined by complement-dependent cytotoxicity (panel-reactive antibody) and ELISA (Prastat)

	Hyperimmune group ($n = 11$)	Retransplant group ($n = 10$)	Immune group ($n = 40$)	Control group ($n = 304$)
Mean PRA (%)	33.2 ± 11.2*	19.5 ± 21.3*	15.2 ± 6.1*	6.5 ± 5.0
Peak PRA (%)	84.4 ± 10.4*	47.7 ± 38.7*	61.8 ± 14.9*	19.0 ± 12.7
Last PRA (%)	31.6 ± 28.4*	8.3 ± 14.8	14.8 ± 24.2*	6.2 ± 9.4
Mean Prastat (%)	12.8 ± 18.2*	20.3 ± 18.7*	2.0 ± 5.5	1.7 ± 3.9
Anti-HLA specificities (Prastat)	7/4* (64%)	8/2* (80%)	4/36 (10%)	23/281 (8%)

* $P < 0.001$

Table 2 Study population: patient data

	Hyperimmune group ($n = 11$)	Retransplant group ($n = 10$)	Immune group ($n = 40$)	Control group ($n = 304$)
Gender (M/F)	5/6 (45%)	6/4 (60%)	21/19 (52%)	149/83 (64%)
Age (years)	40.7 ± 14.4	41.2 ± 5.0	38.5 ± 10.4	42.8 ± 11.2
Time span of renal dialysis (months)	70.2 ± 66.7**	62.5 ± 15.4*	42.2 ± 37.6	32.1 ± 29.7
Nephropathies:				
Glomerular	5 (46%)	5 (50%)	22 (55%)	142 (48%)
Interstitial	2 (18%)	1 (10%)	5 (12%)	41 (13%)
Cystic/hereditary	2 (18%)	2 (20%)	8 (20%)	56 (18%)
Vascular	1 (9%)	1 (10%)	2 (5%)	28 (9%)
Other	1 (9%)	1 (10%)	3 (8%)	37 (12%)

* $P < 0.01$, ** $P < 0.001$

commonest statistical methods (Student's t-test, Yates corrected χ^2). Results were analysed by these tests and also by application of Kaplan-Meier actuarial curves and standard error assessment.

Genomic typing of HLA antigens

To increase accuracy in defining HLA antigens, we used the SSP-PCR technique of DNA typing (Sequence-specific primer-polymerase chain reaction). The principle behind this method is that only primers with a perfectly complementary sequence to the DNA sample being tested for HLA loci can bond to it and give rise to an amplification by the PCR reaction. Non-complementary primers fail to bond to the DNA and no amplification occurs.

Search for anti-HLA antibodies

The search for anti-HLA antibodies in patient sera was conducted both by the standard complement-dependent cytotoxicity technique (CDC) and by ELISA (Prastat Sangstat); IgG anti-HLA antibodies present in the serum may be identified by using soluble HLA antigens (sHLA) adhering to microwells on an ELISA plate with an immunoenzyme method. Adhering to the ELISA plate microwells are 44 different sHLA antigen preparations, deriving from B cellular lines (obtained by transformation with Epstein-Barr virus), with differing HLA phenotypes such as to identify 79 different antigen specificities [34]. Compared to CDC testing, Prastat is a more standardizable method in not requiring cell preparations, while the cell panel used to produce HLA antigens remains stable in its composition over time.

Results

Some points emerge from comparing the four patient groups. Although it is no surprise that the mean and peak percentages of cytotoxic antibodies against HLA (PRA) antigens are decidedly higher than those of controls in all three groups (immune, hyperimmune, and retransplanted), the Prastat tests sharply distinguish which patients are at most risk (hyperimmune and retransplanted: respectively 12.8 ± 18.2% and 20.3 ± 18.7%) as against controls (1.7 ± 3.9%) and – more to the point – patients with a low level of immunization (2.0 ± 5.5%, $P < 0.001$; Table 1). Antigen specificity (against both class I and class II) were identified with high frequency only in retransplanted and hyperimmune patients (80% and 64% respectively; Table 1).

The personal details of the four groups differ only in the time spent awaiting transplantation (defined as dialysis time span). This is, clearly, more than double that of non-immunized patients. Analysis of the nephropathies causing renal failure shows no differences (Table 2).

Of more interest is the basic immunological picture which confirms the role of transfusion and pregnancy: of our immunized patients, the majority have received multiple transfusions (55% of hyperimmune patients 70% of retransplantees and 28% of immune patients, vs 13% of controls).

Table 3 Study population: immunological data (*HBV* hepatitis B virus, *HCV* hepatitis C virus)

	Hyperimmune group ($n = 11$)	Retransplant group ($n = 10$)	Immune group ($n = 40$)	Control group ($n = 304$)
Pregnancy (*n*)	2.0 ± 2.1	0.67 ± 0.5	1.4 ± 1.1	1.3 ± 1.5
Multiple transfusions (more than 3)	6/5** (55%)	7/3** (70%)	11/29* (28%)	39/265 (13%)
rHU-EPO	7/4 (64%)	9/10 (90%)	28/12 (70%)	190/114 (63%)
HBV +	1 (9%)	0	0	6 (2.5%)
HCV +	1 (9%)	5 (50%)	3 (7.5%)	23 (7.6%)
Blood group				
A	5 (45%)	4 (40%)	16 (41%)	138 (45%)
B	1 (9%)	1 (10%)	4 (10%)	22 (7%)
AB	1 (9%)	1 (10%)	1 (3%)	10 (3%)
O	4 (36%)	4 (33%)	18 (46%)	134 (44%)

* $P < 0.05$, ** $P < 0.001$ rhu-EPO = recombinant erythropoietin.

Table 4 Study population: clinical data

	Hyperimmune group ($n = 11$)	Retransplant group ($n = 10$)	Immune group ($n = 40$)	Control group ($n = 304$)
Donor/recipient age gap (years)	20.9 ± 17.1	15.9 ± 14.9	16.4 ± 12.6	16.1 ± 13.5
Cold ischaemia (h)	18.1 ± 6.6	17.2 ± 8.6	18.7 ± 8.2	18.5 ± 7.8
Functional recovery				
Transplant unsuccessful	0 (0%)	1 (10%)	1 (3%)	15 (5%)
Severe tubular necrosis	3 (27%)	2 (20%)	9 (23%)	66 (22%)
Slight tubular necrosis	3 (27%)	1 (10%)	8 (20%)	48 (16%)
Good recovery	5 (46%)	6 (60%)	22 (55%)	174 (57%)

The number of pregnancies is much higher among the hyperimmune patients, and lowest in the retransplanted. This seems paradoxical, but, age being equal, it tells a simple story of earlier diagnosed and longer protracted renal impairment (mean of 0.6 pregnancies vs. 1.3 among controls).

No differences were found in either the frequency of liver disease correlated with the presence of hepatitis B or hepatitis C virus (but chronic active hepatitis were excluded by the transplantation program) or in the blood group distribution (Table 3).

The clinical details of transplantation and the early outcome pattern do not differ significantly among the four groups. The cold ischaemia times are similar and the incidence of slight or serious tubular necrosis did not affect any one group in particular (Table 4).

The donor–recipient age gap was different in the hyperimmune group, though not to a statistical degree. This was because the search for maximum compatibility was here seen as more important to graft outcome than the question of age limits (Table 4).

It is of fundamental importance to analyse the compatibility achieved at the moment of choosing the recipient: this, it can be seen, is the only true strategy for ensuring graft success. The overall average is one mismatch on the DR locus and slightly more than one mismatch for class I antigens.

The immune patient group had slightly greater compatibility on being transplanted, but where the difference was most marked is in the hyperimmune and retransplant groups. There the values were almost identical for the DR locus (0.44 ± 0.53 mismatches for the hyperimmune, 0.54 ± 0.5 for the retransplanted, as against 0.96 ± 0.62 for controls), and around two mismatches for class I loci, with a preference for locus B (0.83 ± 0.40 mismatches for the hyperimmune, 0.73 ± 0.98 for the retransplanted, as against 1.47 ± 0.5 for controls). The difference is obvious and also statistically significant, thus confirming the importance of greater antigen exposure, the predictive value of pre-transplant anti-HLA antigen determination and the need for careful selection to exclude the presence of antigen against which pre-formed antibodies have been identified (Table 5).

The long-term clinical results are broadly reassuring. Renal function, seen as serum creatininaemia (mg/dl), is virtually the same in all four groups 1 year after surgery (1.53 ± 0.5 mg/dl in the hyperimmune, 1.43 ± 0.31 mg/dl in the retransplanted, 1.54 ± 0.6 mg/dl in the immune and 1.48 ± 0.4 mg/dl in controls). The mean number of

Table 5 Mismatches

	Hyperimmune group ($n = 11$)	Retransplant group ($n = 10$)	Immune group ($n = 40$)	Control group ($n = 304$)
HLA locus A	1.0 ± 0.7	1.25 ± 0.59	1.29 ± 0.7	1.3 ± 0.57
HLA locus B	0.83 ± 0.40*	0.73 ± 0.98*	1.32 ± 0.57	1.47 ± 0.5
HLA class I	1.89 ± 0.93	2.06 ± 0.88	2.62 ± 1.02	2.75 ± 0.85
HLA locus DR	0.44 ± 0.53**	0.54 ± 0.5***	0.86 ± 0.53	0.96 ± 0.62

* $P < 0.001$, ** $P < 0.02$, *** $P < 0.05$

Table 6 Rejection episodes, renal function and long-term graft survival

	Hyperimmune group ($n = 11$)	Retransplant group ($n = 10$)	Immune group ($n = 40$)	Control group ($n = 304$)
Rejection episodes in the first 3 months	0.50 ± 0.53	0.63 ± 0.52	0.47 ± 0.49	0.54 ± 0.64
Serum creatinine 1 year after surgery (mg/dl)	1.53 ± 0.5	1.43 ± 0.31	1.54 ± 0.6	1.48 ± 0.4
Graft survival:				
3-month (%)	91	80	94	93
1-year (%)	81	80	87	92
3-year (%)	81	69	87	90
6-year (%)	81	69	87	88

rejection episodes in the first 3 months is less than 0.5 in the immune and hyperimmune (0.54 ± 0.64 among the controls). Only the retransplanted group has an incidence that is higher (0.63 ± 0.52), without being statistically so (Table 6).

The really outstanding finding, however, comes from analysing the long-term actuarial graft survival curves (follow-up over 6 years). Three months from surgery, good graft function (graft survival) accounts for 91% of the hyperimmune group, 80% of the retransplantees, 94% of the moderately immunized and 93% of controls. The same proportions are kept over the 6 years of follow-up (Table 6).

Clearly the group with worst results is that of the retransplanted patients, with a 69% chance of graft survival at 3 years, as compared to 90% among controls and 87% among the immunized. This is probably due not only to the greater immunological difficulties, but

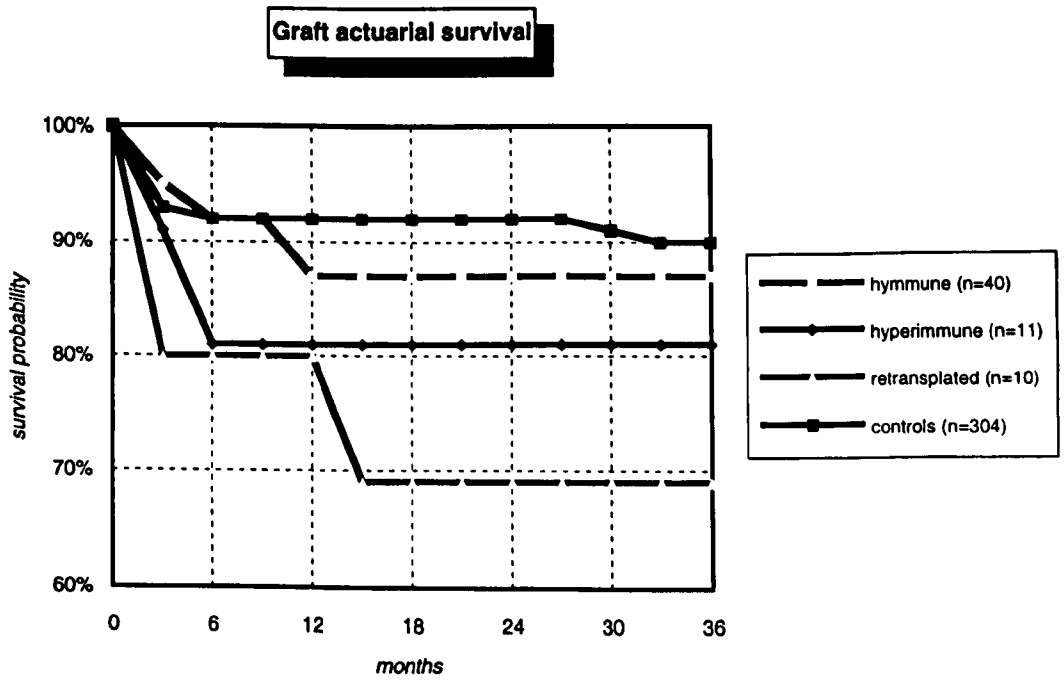

Fig. 1 Actuarial probability of graft survival in the four groups studied

Table 7 Immunosuppressive therapy and relationship with infectious complications in the first 3 months

	Hyperimmune group ($n = 11$)	Retransplant group ($n = 10$)	Immune group ($n = 40$)	Control group ($n = 304$)
Steroid pulses *(n)*	1.44 ± 1.1	1.7 ± 1.1	1.25 ± 0.5	1.42 ± 0.6
ATG/GAL	10 (91%)	7 (70%)	17 (43%)	98 (32%)
OKT3	2 (18%)	2 (20%)	1 (2%)	29 (9%)
Plasmapheresis	1 (11%)	2 (20%)	2 (5%)	12 (3%)
Bacterial infections[a]	2/9 (18%)	2/8 (20%)*	5/35 (13%)	25/279 (8%)
Viral infections[a]	4/7 (36%)	4/6 (40%)	9/31 (23%)	60/244 (20%)

[a] Data are given as follows: "2/9 (18%)" means that two patients suffered a total of nine infections, and these patients constituted 18% of their group. * $p < 0.05$

to the fact that such patients generally have a worse clinical status, given their long history of dialysis, transplantation and return to dialysis; they thus tolerate any boosting of immunosuppressive therapy worse.

When one analyses the survival figures of hyperimmune patients, who have an only slightly worse expectancy of graft survival than the non-immunized, the validity of the method seems further confirmed (Fig. 1).

On analysing the cross data on induction therapy and supplementary steroid boluses given during the first 3 months, one sees that the two highest immune risk groups received the same doses of steroids, but higher doses of antilymphocyte globulins or monoclonal antibodies. This is explained by the nature of the treatment protocol used, which involved preventive induction, and by the virtually identical number of rejection episodes in all four groups (Table 7).

While boosting the therapy led to acceptable graft survival, it did expose patients to a higher number of infectious complications, above all viral (Table 7). In particular, there were two deaths from infection among the highest immunological risk patients: one from pneumonia due to cytomegalovirus and one from pneumonia caused by *Pneumocystis carinii*. Among the controls there was one death from bacterial pneumonia and one kidney was lost through pyelonephritic infection (Table 7).

Conclusions

These data suggest some general and some specific conclusions.

In general it may be said that even hyperimmunized patients may receive transplants successfully. Careful and technologically advanced immune screening is required before transplantation, as well as high compatibility with the donor. In terms of either organ survival or graft function, the results are scarcely worse than with less immunologically activated patients. In practice, if compatibility is high and one does not run any risk with a clearly jeopardized immunological match, the immunized patient stands as good a chance of success as others.

Some more specific points also emerge from our experience.

1. Patients with a medium to low degree of immunization (only one PRA peak above 50%) may have double the mean PRA values of controls but have a low pre-transplantation Prastat and good organ function expectancy even in the long term (in this case the difference between PRA and Prastat values would have to be put down to IgM antibodies or the like). It is thus evident that, even where there is sensitization, by knowing the antigens the patient will react to and eliminating them at the moment of choosing the donor, one can transplant without any additional immunological risk. For these patients there are no reasons in favour of supplementary induction immunosuppressive therapy.
2. Unfortunately, patients undergoing retransplantation do not encounter the same destiny. Their clinical course is distinctly worse, and their long and complex clinical history is not enough in itself to explain the difference. The pre-transplantation Prastat values in this group are in fact higher. In future, with the steady build-up of such patients on waiting lists, only greater organizational integration will improve their chances as candidates. In these patients induction immunosuppressive therapy is advisable.
3. Analysis of patient clinical outcome is encouraging where sensitization is high (more than three PRA determinations above 50%). It is with these patients that the method we advocate gives best results. Those patients who managed to have a transplant have a good graft function expectancy, only slightly inferior to that of controls. Such patients call, however, for preventive induction immunosuppression, and it is wise to take extra care in post-transplantation immunological monitoring, in view of the more aggressive immunosuppressive therapy.

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