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Original Citation:

Donor-specific anti-HLA antibodies in Huntington's disease recipients of human fetal striatal grafts / B Porfirio; M Paganini; B Mazzanti; S Bagnoli; S Bucciantini; E Ghelli; B Nacmias; AL Putignano; G Rombolà; R Saccardi; L Lombardini; N Di Lorenzo; GB Vannelli; P Gallina. - In: CELL TRANSPLANTATION. - ISSN 0963-6897. - STAMPA. - 24:(2015), pp. 811-817. [10.3727/096368913X676222]

Availability:

This version is available at: 2158/827540 since: 2018-03-28T11:18:11Z

Published version:

DOI: 10.3727/096368913X676222

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Donor-Specific Anti-HLA Antibodies in Huntington's Disease Recipients of Human Fetal Striatal Grafts

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Fetal grafting in a human diseased brain was thought to be less immunogenic than other solid organ transplants, hence the minor impact on the efficacy of the transplant. How much prophylactic immune protection is required for neural allotransplantation is also debated. High-sensitive anti-HLA antibody screening in this field has never been reported. Sixteen patients with Huntington's disease underwent human fetal striatal transplantation in the frame of an open-label observational trial, which is being carried out at Florence University. All patients had both brain hemispheres grafted in two separate robotic–stereotactic procedures. The trial started in February 2006 with the first graft to the first patient (R1). R16 was given his second graft on March 2011. All patients received triple immunosuppressive treatment. Pre- and posttransplant sera were analyzed for the presence of anti-HLA antibodies using the multiplexed microsphere-based suspension array Luminex xMAP technology. Median follow-up was 38.5 months (range 13–85). Six patients developed anti-HLA antibodies, which turned out to be donor specific. Alloimmunization occurred in a time window of 0–49 months after the first neurosurgical procedure. The immunogenic determinants were non-self-epitopes from mismatched HLA antigens. These determinants were both public epitopes shared by two or more HLA molecules and private epitopes unique to individual HLA molecules. One patient had non-donor-specific anti-HLA antibodies in her pretransplant serum sample, possibly due to previous sensitization events. Although the clinical significance of donor-specific antibodies is far from being established, particularly in the setting of neuronal transplantation, these findings underline the need of careful pre- and posttransplant immunogenetic evaluation of patients with intracerebral grafts.

Key words: Donor-specific antibodies; Fetal striatal tissue; Huntington's disease; Neural transplantation

INTRODUCTION

Huntington's disease (HD) is an incurable autosomal dominant genetic disorder caused by increased cytosine–adenine–guanine (CAG; codes for glutamine) repeats on the huntingtin gene (36). The presence of mutant huntingtin ultimately results in neuronal degeneration (32). Onset is typically in middle age when motor, cognitive, and psychiatric impairments start and progressively lead to death within 15–20 years. The medium spiny projection neurons of the caudate and putamen are the first to be lost, preceding degeneration in other areas. Fetal-derived cell transplantation is an experimental therapy aimed at replacing degenerated neurons, thereby preventing or retarding disease

progression (12,38). The Italian experience with intracerebral transplantation of human striatal primordia has been reported (11,13,14). This procedure was thought to be less immunogenic than other solid organ transplants, hence of minor impact on the efficacy of the transplant. How much prophylactic immune protection is required for neural allotransplantation is also a matter of debate. Attention to histocompatibility has been high since the beginning in our framework, with preliminary data made available (28). Whereas several studies have demonstrated that the presence of donor-specific antibodies (DSA) and/or non-DSA significantly correlates with lower graft survival after solid organ transplantation (18,39), the occurrence of anti-human

leukocyte antigen (HLA) antibodies in sera of transplanted HD patients has not found obvious correlations with clinical outcome (13,20,21). The detection of DSA in an allograft recipient should always be considered as indicative of an immune system priming toward major histocompatibility complex (MHC) antigens. Principally for logistic reasons, HLA matching is not performed at the time of transplantation. Therefore, the presence of possibly unacceptable antigens and the application of more or less stringent virtual cross-match rules are unfeasible. At the very least, recipients who presented with such antibodies should be considered to be at a higher risk level than negative subjects (15) and have immunosuppression modulated accordingly (13). Since the time between grafting and allogeneic sensitization was shown to be unpredictable, irrespective of immunosuppressive status, the appropriateness of long-term treatment has been cast into doubt (20). More data are needed to assess the significance of circulating antibodies in this setting. This study is auxiliary to the main clinical trial in investigating as systematically as possible posttransplant humoral response to MHC antigens by more sensitive flow-based bead assays in the largest cohort of HD patients receiving neurotransplantation.

MATERIALS AND METHODS

Recipients and Donors

This study was authorized by the Italian National Health Institute, National Transplantation Center (upon approval by Health Ministry, Consiglio Superiore di Sanità, Sessione XLV, Sezione II, 7/21 and 9/22/2005, and acceptance by the National Bioethics Committee). All patients gave written informed consent prior to being enrolled in the study. Patients followed a standard set of neurological, neuropsychological, neuropsychiatric, and imaging assessments at defined intervals over a minimum of 1 year before and up to 7 years after transplantation.

The source of the grafts was tissue dissected from both whole ganglionic eminences of electively aborted fetuses at a gestational age of 9–12 weeks. One randomly assigned fetus for each transplant was needed. The use of human fetal tissue was conducted according to the guidelines of the Italian National Health Institute and approved by the National Bioethics Committee and the Committee for Investigation in Humans of the University of Florence. In particular, items to be fulfilled included (i) distinct separation between the research team and the institution carrying out the pregnancy interruption, (ii) lack of benefits for the transplantation and obstetric teams, and (iii) request of donor's consent after pregnancy interruption. Fetal material was minced into fragments to obtain a tissue suspension suitable for transplantation. A detailed description has previously been provided (11,14).

Cytofluorimetric Characterization

One small aliquot of dissociated cells from striatal fragments was analyzed for the expression of surface antigens and viability using flow cytometry. Cells were incubated with fluorescein isothiocyanate (FITC), mouse anti-human cluster of differentiation 45 (CD45), and FITC mouse anti-human HLA-DR, -DP, -DQ (Miltenyi Biotec, Bergisch Gladbach, Germany). Ten microliters of each antibody have been used in each test tube following the manufacturer's instructions. A volume of 10 μ l of 7-aminoactinomycin D (BD Bioscience Pharmingen, San Diego, CA, USA) was added to identify dead cells. Non-specific fluorescence and morphologic parameters were determined by incubation of the same cell aliquot with isotype-matched mouse monoclonal antibodies (BD Bioscience Pharmingen). Flow cytometric acquisition was performed by collecting 10⁴ events on a FACSCalibur (BD Bioscience, San Jose CA, USA). Data were analyzed on dot-plot biparametric diagrams using Cell Quest software (BD Bioscience).

Surgical Procedure

The transplantation procedure was described previously (11). Briefly, transplantation involved bilateral robotic–stereotactic (Schaerer Mayfield NeuroMate, Lyon, France) caudate–putaminal grafting of striatal fetal tissue in two sessions, 2–7 months apart. Fifty microliters of tissue suspension was injected along each track within 4–6 h from tissue harvesting.

Immunosuppression Protocol

The immunosuppression protocol originally included oral administration of methylprednisolone (40 mg/daily for 3 days after surgery then tapered and discontinued within 2 weeks), azathioprine (3 mg/kg/daily for 12 months), and cyclosporine (CSA; 5 mg/kg/daily for 12 months) starting from 1 day before the first procedure. These drugs were from Hexal, Holzkirchen, Germany. On the basis of the clinical course of R1 (14), we modified the immunosuppression protocol by continuing CSA administration to sensitized patients sine die. CSA serum levels were checked weekly for the first 2 months and bimonthly thereafter.

Immunogenetic Assessment and Monitoring

Histocompatibility testing included recipients' and donors' low-resolution HLA-A, -B, -DRB1, -DQB1 typing, and anti-HLA antibody detection and identification by complement-dependent cytotoxicity (CDC) and Luminex™ techniques before transplantation. Each patient was scheduled to be screened for the presence of anti-HLA antibodies at each clinical follow-up evaluation, excluding the first week postsurgery visit. Extra scheduled laboratory checks were performed at every emerging event.

HLA Typing

HLA-A, -B, -DRB1, and -DQB1 typing was performed by using DNA extracted (QIAamp DNA Mini Kit; Qiagen, Hilden, Germany) from peripheral blood mononuclear cells (patients) or wasted tissues (donor fetuses), amplified by polymerase chain reaction, and then hybridized with sequence-specific oligonucleotide probes by using a commercially available (INNO-LiPA, Innogenetics, Gent, Belgium) line probe assay based on the reverse hybridization principle. An Auto-LiPA instrument (Innogenetics, Ghent, Belgium) was used to perform the hybridization. Results were analyzed by using the LiRAS (Innogenetics) interpretation software. The resolution of typing was from low to intermediate level. Throughout the text and tables, the serological equivalents were used, for example, A1 per A*01.

Search for Anti-HLA I and II Antibodies

Pretransplant sera were investigated for the presence of complement-binding anti-HLA class I alloantibodies by CDC test. Panel-reactive lymphocytotoxic antibodies (PRA) were determined in a standard microlymphocytotoxic assay using a cell tray containing a panel of 56 cells (Lymphoscreen HLA-ABC 60; Bio-Rad, Hercules CA, USA). The test was performed according to the manufacturer's instructions. Pre- and posttransplant sera were analyzed for the presence of anti-HLA class I and class II IgG alloantibodies using the multiplexed microsphere-based suspension array Luminex xMAP technology. LABScreen® Mixed and LabScreen® Single Antigen (One Lambda, Canoga Park, CA, USA) color-coded microbeads coated with purified HLA antigens were used for detection and identification, respectively. Any HLA antibodies present in the sera were bound to the LABScreen® surface antigens coating the microbead and were subsequently labeled with R-phycoerythrin (PE)-conjugated goat anti-human IgG. The microbead fluorescent emission of R-PE was then detected and quantified by the LABScan™ 100 flow analyzer (One Lambda). Data analysis was performed with HLA Fusion™ software (One Lambda) according to the manufacturer's guidelines. Positive posttransplant sera were also reassessed by CDC test.

RESULTS

The present study includes 16 HD patients (11 males, 5 females) who received transplantation at the University of Florence. Selected relevant features of the patients and their 32 donors are displayed in Tables 1 and 2. Patients' disease burden (26) ranged from 214 to 761 (mean 430.6; standard deviation 148.4). Mean gestational age of the donor fetuses was 75.0 ± 6.6 days. Viability of cell preparations was $84.3 \pm 6.9\%$ and contained $1.5 \pm 1.5\%$ CD45⁺ cells and 1.6 ± 1.3 class II HLA⁺ cells.

Prospective immunogenetic monitoring spanned 13 to 85 months (median 38.5 months). Only the first patient

(R1) was positive at CDC test. As already reported, R1's posttransplant study showed 18% and 9% PRA for class I and class II HLA antigens, respectively (14,28). Once her two pregnancies were excluded as the immunizing event, identification pointed to DSA (Table 1). Five patients (R2, R4, R7, R8, R12) developed DSA as detected by Luminex xMAP technology (Table 1). Apart from R1, we were able to determine the time window of occurrence of alloimmunization in the other patients' sera collected at follow-up. Anti-HLA antibodies appeared in between 0 and 49 months after the first surgery. Pretransplant serum from R14 resulted positive, which may be due either to the presence of "natural" antibodies (25) or to alloimmunization that occurred with the pregnancies (Table 1). On the other hand, nine patients were still negative at their last serological check 13–63 months from the first graft (Table 2). According to the Kaplan–Meier analysis, half of the patients will develop DSA by 40 months (Fig. 1).

R1 has generated DSA *anti-B58(17)* and *anti-DR7*. Besides DSA mounted against *DR16*, R2 showed antibodies *anti-DR4* and *anti-DR1*. R4 has produced DSA versus *DQ7(3)*. HLA-DQB1 typing has in fact showed that the *DR4* fetus was *DQ7* as the other *DR11* fetus. This remained valid for R7, who has developed *anti-DQ7* DSA. *Anti-B57(17)* and *anti-DR52* DSA were present in R8. As expected, HLA-DRB3 typing demonstrated that 8D2 fetus was indeed *DR52*. R12 has produced *anti-DR7* DSA, together with *anti-B8*.

Overall, these results indicate that an important proportion of transplanted HD patients display in their sera alloantibodies specific for the mismatched HLA class I and class II antigens from the graft. The immunogenic determinants were the aminoacidic substitutions in the polymorphic positions of the HLA molecules, the epitopes. These determinants were both public epitopes shared by two or more HLA molecules (cross-reactive group, CREG) (30) and, to a lesser extent, private epitopes unique to individual HLA molecules.

DISCUSSION

The brain was once thought to be an immunologically privileged site, owing to absent antigen-presenting cells (APCs) and lymphatic drainage and to the blood–brain barrier (10). Indeed, the central nervous system (CNS) possesses a diverse range of APCs that facilitate immune surveillance. Moreover, the cerebrospinal fluid serves as the equivalent of lymph in the CNS (29). Reactive microglia expressing HLA-DR molecules have long been detected in many neurological conditions including HD (23), and activated T-cells have been shown to traffic through the CNS to exert immune surveillance (17). Furthermore, class I MHC is present in specific subsets of CNS neurons, and a function for class I MHC in neuronal signaling and activity-dependent

Table 1. Recipient/Donor Features of the Seven Positive Huntington's Disease Patients Receiving Intracerebral Fetal Striatum Grafting and Anti-HLA Antibody Monitoring

Patients' Features		Fetal Tissue Characteristics										Immunogenetic Monitoring										
Patient ID	Sex	Disease Burden*	Tissue Typing			Grafting Dates	Fetus ID	Age (days)	Viability (%)	CD45 ⁺ (%)	HLA-DR ⁺ , -DP ⁺ , -DQ ⁺ (%)	Tissue Typing			Pretransplant Anti-HLA Antibodies	Sensitization Time Window (Months)	Posttransplant Anti-HLA Antibodies†	Notes				
			HLA-A	HLA-B	HLA-DR							HLA-DQ	HLA-A	HLA-B					HLA-DR	HLA-DQ		
R1	F	459	2	18	56	11	7	7	0.8	na.†	3	29	13	58	7	13	2	7	0-19	B5, B17, A32, DR7	Two pregnancies; husband's HLA: A2,25;B14,18;DR1,13	
R2	M	374	1	26	35	57	7	8	7	9	na.	11	30	18	55	3	7	2	9	30-49	DR16, DR4, DR1	Deceased by sudden death
R4	M	514	2	26	35	55	1	14	5	-	0.7	2	11	40	52	9	10	5	9	0-14	DQ3	
R7	F	656	2	24	39	52	13	15	6	-	0.7	26	68	18	35	1	11	5	7	0-25	A66, DQ7, 7CREG§	Deceased by sudden death
R8	M	552	24	-	44	-	7	16	2	5	0.7	2	3	62	41	4	-	2	7	18-36	B17, DR52	
R12	M	293	1	32	35	57	4	13	7	-	0.8	32	68	35	51	1	13	5	7	11-17	B8, DR7	
R14	F	338	1	26	35	-	11	12	7	-	0.8	1	68	35	51	7	8	2	4	Not applicable	7CREG	Three pregnancies; husband's HLA: A1,30;B8,42;DR12,13
											4.0	1	68	35	51	1	11	5	7			

HLA, human leukocyte antigen; CD45⁺, cluster of differentiation 45 positive.
 *Disease burden is measured as [(CAG number - 35.5) × age-at-onset] according to Penney and coworkers (26).
 †na., not available.
 ‡Donor-specific antibodies are in bold.
 §Cross-reacting groups (CREG) are defined as in Rokey and coworkers (30).

Table 2. Recipient/Donor Features of the Nine Negative Huntington's Disease Patients Receiving Intracerebral Fetal Striatum Grafting and Anti-HLA Antibody Monitoring

Patients' Features		Fetal Tissue Characteristics										Immunogenetic Monitoring											
Patient ID	Sex	Disease Burden*	Tissue Typing			Grafting Dates	Fetus ID	Age (Days)	Viability (%)	CD45 ⁺ (%)	HLA-DR ⁺ , -DP ⁺ , -DQ ⁺ (%)	Tissue Typing			Pretransplant Anti-HLA Antibodies	Negative Posttransplant Time Span (Months)	Notes						
			HLA-A	HLA-B	HLA-DR							HLA-DQ	HLA-A	HLA-B				HLA-DR	HLA-DQ				
R3	M	550	25	26	8	51	3	11	2	7	0.9	1.1	2	-	51	-	4	9	8	9	22	Deceased from pneumonia	
R5	M	350	2	24	18	51	11	-	7	-	0.8	1.3	23	30	14	18	3	-	2	-	52		
R6	F	214	24	32	63	44	13	-	6	-	1.6	2.0	1	24	49	57	11	13	6	7	63		
R9	M	345	1	25	8	-	3	-	2	-	0.8	0.8	2	-	61	62	8	-	4	-	52		
R10	M	408	3	25	8	44	3	4	2	8	2.1	0.8	1	68	75	18	11	-	5	7	51		
R11	M	361	1	11	7	50	3	13	2	6	1.3	1.3	11	-	13	75	4	15	4	5	15		
R13	M	761	2	3	7	39	4	16	5	8	0.9	na.†	na.	na.	na.	na.	na.	na.	na.	na.	na.	36	
R15	F	472	2	30	58	-	1	16	5	-	1.5	1.8	11	-	40	54	4	15	4	6	37		
R16	M	243	2	31	14	18	1	14	5	-	0.4	na.	na.	na.	na.	na.	na.	na.	na.	na.	na.	13	
											6.0	6.0	24	31	38	78	4	14	7	9			

*Disease burden is measured as [(CAG number - 35.5) × age-at-onset] according to Penney and coworkers (26).
 †na., not available.

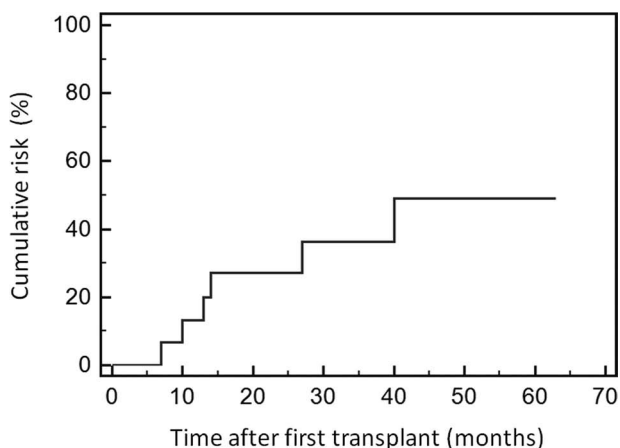


Figure 1. Kaplan–Meier estimate of the cumulative risk of alloimmunization.

synaptic plasticity in the brain both during development and in the adult has been hypothesized (33).

The use of allogeneic neural precursor cells as a replacement therapy in neurodegenerative disorders has been proposed (27). Nevertheless, both constitutive MHC gene expression and possible induction of an inflammatory response following implantation need to be taken into consideration since inflammatory cytokines can upregulate MHC expression on transplanted cells (19,22,37), thereby rendering them more susceptible to alloimmunization and eventually to chronic graft rejection even in the presence of immunosuppressive drugs (2). Neurotransplantation clinical trials in HD have definitely documented that the CNS exhibits immunological competence through the processing and the identification of allogeneic stimuli provided by the graft, which in turn elicit antibody production (13,20, 21). The reaction takes place during immunosuppressive treatment and is reminiscent of the humoral response generally detected before renal graft failure in chronic rejection.

In our series, a serum taken from R1 19 months after her first implant, and 7 months after immunosuppression was discontinued, eventually showed positive results for both anti-HLA class I and class II antigens. Identification pointed to *anti-B17* and *anti-DR7* DSA. A serum from R4 14 months after receiving his first graft, and 2 months after CSA discontinuation, also showed DSA. According to a prudence principle, a CSA regimen has been reintroduced for R1 and R4. Since then, immunosuppression was reinstated whenever DSA were detected (R2, R7, R8, R12). The balance of long-term immunosuppression has seen a reduction of antimetabolites, which block the proliferation of lymphocyte precursors, whereas administration of inhibitors of cytokine production has been maintained (1).

This study assessed anti-HLA sensitization by new and more sensitive solid phase assays, in particular flow-based bead assays such as the Luminex™ system (7). Therefore,

we were able to precisely identify both immunogenicity (induction of specific antibodies) and antigenicity (reactivity with antibodies) of epitopes found on the grafted material (8,24). R1 developed DSA versus *B58(17)* and *DR7*. Interestingly, 1D1 and 1D2 had four HLA-A antigens all belonging to 1CREG, two of whom, *A29* and *A30*, belong to the broad specificity *A19*. 1D1 and 1D2 also presented with one 5CREG antigen each, *B58* and *B18*. The sensitization toward *A32* and *B5* may have been triggered by public epitopes shared by those antigens. Since R1 was not studied before grafting, a role for immunogenic epitopes exposed with pregnancies cannot be ruled out. Besides DSA raised against *DR16*, which was present in 2D1, R2 showed antibodies *anti-DR4* and *anti-DR1*. Since 2D2 had *DR10*, it may be argued that the shared epitope (16) between DR antigens of these two donors might have had a role. R4 developed DSA versus *DQ7(3)*. It should not go unnoticed that, as the two donors of R1 share the class II antigen *DR7*, those of R4 are both *DQ7*. Genomic typing has in fact shown that the *DR4* fetus was *DQ7* as the other *DR11* fetus. This also held true for R7, who produced *anti-DQ7* DSA. Noteworthy, 7D1 and 7D2 presented with one 1CREG antigen each, *A3* and *A26(10)*. Sensitization toward *A66(10)* may have been triggered by public epitopes shared by those antigens. In addition, 7D2 had one 7CREG antigen, *B41*, whose immunogenicity might be accounted for the presence of a number of anti-7CREG antibodies (but not *anti-B41* itself) in R7 posttransplantation sera. It is tempting to speculate that both the immunogenic and the antigenic burden brought by the second graft could be faced by memory T-cells able to mediate rapid recall responses after restimulation with antigen. In fact, we documented that our cell preparations from the isolated fetal striatal tissue contained a small fraction of CD45⁺ and/or class II HLA⁺ cells.

Although the appearance of anti-HLA antibodies may now be considered the rule, rather than the exception, its clinical significance remains unclear (13,20,21). Krystkowiak and coworkers (21) described a HD patient showing both clinical and neuroimaging signs of graft rejection 14 months after transplantation, which were readily reversed by reintroducing immunosuppression. Capetian and coworkers (4) had the chance to study autoptic material from a HD patient deceased 6 months after grafting. They identified grafted neuronal progenitors within cuffs of CD4⁺ and CD8⁺ T-cells around vessels in the so-called Virchow–Robin space. The influence of the transplantation procedure, that is, the tissue trauma inflicted by the implantation, the preparation of cell suspension, and the site of implantation, together with possible immunomodulatory effects of neural stem cells on microglial activation have also been considered (3). Other authors observed neither important inflammatory/immune reaction nor cuffing in the grafted

area at postmortem examination 18 months from surgery (9). However, the same group when inspecting the brain of HD patients deceased a decade after transplantation found CD8⁺, CD4⁺, and HLA-DR⁺ cells, indicating an ongoing immune response that may have contributed to the disease-like degeneration of the graft observed in those long-term HD patients (6). Unfortunately, none of them have been searched for the presence of DSA. Conversely, we could not perform histological analysis of the brains of the three deceased patients of our series, who died due to common causes in HD (34) and who did not have clinical graft-related events or any imaging sign of aberrant tissue development. As a matter of fact, we have never observed either neovascularization or signs of neighboring tissue infiltration. This holds true also for those patients who showed remodeling of the basal ganglia anatomy as a result of graft development (13,14). Despite all the above, sensitization to HLA alloantigens has appeared so far in 6 of 16 transplanted HD patients without overt signs of rejection. Whether seroconversion will ensue also in the other HD patients of our series remains to be seen at follow-up. Also, the extent to which alloimmunization will be a significant complication of neurotransplantation is still unknown, but will possibly be clarified in the ongoing trial. It should also be recognized that the biological significance of very low antibody levels, or of antibodies that do not bind complement, is yet not completely understood (31,35). Thus, the use of increasingly sensitive methods such as flow-based bead assays for antibody detection may represent a crucial caveat when trying to make correlations with clinical outcome.

The direction, if any, of the clinical outcome associated with the presence of an immune response toward intracerebral fetal striatal grafts is undetermined at present. Interestingly, Krebs and coworkers (20) reported on the improvement of chorea in those grafted HD patients who developed anti-HLA antibodies. However, neuroimaging had a relationship neither with immune reaction nor with clinical response. Thus, both biological and clinical significance of these findings remains uncertain, and further observations are warranted on more patients for a longer time. We showed previously that in four patients of the present series (R1, R4, R6, and R8), the clinical response measured in terms of motor and behavioral indices was associated with an outgrowth of striatal-like imaging features occurring in the left side in R1, in the right side in R4, and bilaterally in R6 and R8 (13). Immunogenetics was considered unlikely to explain the pattern of graft growth in those different recipients. Two of them were positive for the presence of DSA. Noticeably, DSA have in the meanwhile appeared in the serum of R8, that is, one of the two patients who were negative at the time of our previous report (13). R6, who also presented with

those peculiar neuroimaging features, up to now, is indeed negative. However, she is presently under immunosuppression, and it was decided not to discontinue it. Even though more cases, according to a predefined statistical power analysis, and longer posttransplant monitoring are needed to eventually suggest that anti-HLA antibodies could affect transplant function, our findings indicate that donor–recipient histocompatibility issues deserve careful consideration for the bad, the good, and the unknown (5).

ACKNOWLEDGMENTS: *This article is dedicated to the memory of Prof. Domenico Adorno, Transplant Coordinator in Rome, Italy. This study is supported by the University of Florence, Azienda Ospedaliero-Universitaria Careggi, Florence. The study received grants from Ministero Istruzione Università e Ricerca, Italy (PRIN 2008, research program N 2008XN9KLA) and by Ente Cassa di Risparmio, Florence, Italy (2011.1208). The authors declare no conflicts of interest.*

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