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ORIGINAL ARTICLE

Effect of IVIG administration on complement activation and HLA antibody levels

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Summary

The objective of the present study was to determine if there are changes on complement (C) activation and concentration of HLA antibodies (Abs) in patients treated with intravenous immunoglobulin (IVIG). The patients evaluated were given IVIG as treatment of Ab-mediated rejection or desensitization. The patients' sera obtained before and after IVIG administration were tested for their effects on the deposition of both IgG (HLA Abs) and C3b (C activation) as measured by flow cytometry on T cells. IVIG consistently inhibited C activation when measured shortly after IVIG infusion but returned to the initial levels at 2–4 weeks, when total serum IgG also returned to pre-infusion levels. C inhibition was more pronounced with higher IVIG doses and the degree of inhibition was inversely proportional to the HLA Ab concentrations. IVIG did not block the binding of HLA Abs immediately after administration, although levels were slightly but consistently lower after several monthly IVIG infusions. The data show that C inhibition by IVIG is short-lived and that IVIG induces only a mild reduction of HLA Abs, seen not immediately but after months of treatment. These results may explain the inconsistent results of IVIG to achieve desensitization.

Intravenous immunoglobulin (IVIG) is increasingly used in transplantation, its main indications being desensitization of patients with high alloantibody levels and antibody (Ab)-mediated rejection (AMR). High dose IVIG (2 g/kg) given monthly for 4 months has been reported to allow transplantation of more highly sensitized patients than patients who were not treated [1]. Lower doses of IVIG in conjunction with plasma exchanges and additional immunosuppression have also been used with encouraging results [2]. Similarly, IVIG appears to be effective in the treatment of AMR, either alone or, more effectively, as part of a multi-pronged strategy [3]. However, as the use of IVIG expands, questions remain regarding optimal doses, frequency of administration, need of concomitant therapies and, in fact, overall degree of effectiveness.

Proposed mechanisms of action for IVIG include stimulation of the inhibitory FcγIIB receptors on B cells, blocking of other Fcγ receptors, modulation of cytokines, inhibition of lymphocyte stimulation and apoptosis of T and B lymphocytes [4]. Most of these mechanisms have been studied *in vitro* or in experimental animals and it is not known if there is any mechanism that predominates over the others, or if they play a significant role in desensitization or AMR treatment. Another well-documented action of IVIG is inhibition of complement (C) activation [5]. As AMR involves C activation, as suggested by the frequent deposition of C4d in graft biopsies, the C inhibitory activity of IVIG may be a significant mechanism of its possible therapeutic effects. In addition, C also plays a significant role in the induction of Ab production [6]. By

recognizing bacterial molecular patterns, C components help discriminate nonpathogenic foreign molecules that may not induce an immune response from those, like bacteria, that can be harmful and engage the innate and adaptive immune systems [7]. Thus, antigen plus C3 in bacteria or immune complexes, including those containing HLA Abs, promote the binding of C3d to CD21 on B cells, which, by interacting with CD19 and the antigen receptor, lower the B-cell threshold for activation by several orders of magnitude [7].

Intravenous immunoglobulin appears to inhibit C activation by sequestering C components from the circulation, especially C3 and C4 [8]. In a previous study, we determined that this effect of IVIG is observed in an *in vitro* system where C activation is induced by HLA Abs [9]. These observations highlighted the need to determine if inhibition of C activation also occurs when IVIG is given to patients [10]. To address this issue, in the present study, we examined C activation in the sera of patients obtained before and after receiving IVIG. We also measured HLA Ab levels, as the effect of IVIG on this parameter is not clear from reports using C-dependent Ab tests and whose end-point is the transplantation rate of sensitized patients [1].

Material and methods

Serum samples

Blood specimens were obtained from patients before and after treatment with IVIG. IVIG was given to eight patients for desensitization purposes as the sole agent, and to six patients to treat AMR together with other immunosuppressive drugs (but not anti-thymocyte globulin or CD20 Abs). Doses given were 2, 1 and 0.4 g/kg over the course of 1–4 days. Patients received Gamunex (Talecris Biotherapeutics, Research Triangle Park, NC, USA), Flebogamma (Grifols Biologicals, Los Angeles, CA, USA), or Gammagard (Baxter, Deerfield, IL, USA). Panel reactive Abs (PRA) were measured by Luminex i.d. kits (One Lambda, Canoga Park, CA, USA). Heat-inactivated (56 °C for 30 min) serum from a broadly HLA-sensitized patient was used as the source of HLA Abs in some experiments. Normal C activity was provided by fresh serum from a normal unsensitized individual. Negative controls for the C3b binding experiments were obtained using the patients' fresh sera in the absence of HLA Abs or by using normal serum only. The term fresh serum implies that the serum was frozen at −80 °C within a few hours after collection.

Flow cytometry

Lymphocytes from lymph nodes or peripheral blood isolated by Ficoll–Hypaque centrifugation from transplant donors were used as target cells. The basic experiment consisted in incubating target cells (2×10^5) with 20 μl of patient's serum or appropriate controls for 15 min at 37 °C [9]. After washing, cells were stained with $F(ab)'_2$ fragments of fluorescein-conjugated goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA, USA) or goat anti-C3c (Dako Corporation, Carpinteria, CA, USA). Abs to C3c, a soluble degradation product of C3b, detect C3b attached to target cells. Cells were also stained with a CD3 Ab conjugated to phycoerythrin and, when peripheral blood lymphocytes were used, a CD20 Ab (both from BD Biosciences, San Jose, CA, USA) conjugated to peridinin chlorophyll to distinguish T from B cells. The fluorescence intensity of 10^4 cells was measured using a FacScan (Becton Dickinson, San Jose, CA, USA) immediately after staining. The median of logarithmically acquired data were expressed as linear values either as such or as a ratio of the patient's values divided by the values of the normal serum control. Deposition of IgG and C3 was evaluated only on T cells because B cells normally show C3 deposition through the activation of the alternative pathway by CD21 [11].

Experimental design

Most experiments compared the C activity and HLA Ab reactivity of sera collected before and after IVIG infusion. The basic assay consisted of mixing the patient's serum (as a source of HLA Abs and/or C) with random donor lymphocytes, measuring IgG and C3b deposition by indirect immunofluorescence and flow cytometry as described above. If the patient's serum (pre-IVIG) induced IgG and C3 deposition, a full experiment was set up with all serum specimens available. If the patient did not have IgG reactivity against the target cells, serum from a broadly sensitized patient was used as the source of HLA Abs. In this case, the basic assay was modified by adding 20 μl of a heat-inactivated serum with broadly reactive HLA Abs to the target cells for 15 min at room temperature, washed, and the patient's serum was added as the source of C in a second incubation, followed by fluorescein-labeled anti-IgG or anti-C3c. There were some instances in which the patients' sera lost their C activity in storage. All these patients had HLA Abs reacting with the target cells, so that the experiments were set up adding 20 μl of patient's serum plus 2 or 4 μl of normal fresh human serum before incubation with the target cells to restore C reactivity.

Results

Patients evaluated

The patients evaluated were treated with IVIG for AMR or sensitization (Table 1). The criteria for diagnosis of

Table 1. Patients evaluated.

Patient	Organ	Indication	Initial PRA class I/class II	Outcome
Post-transplant IVIG treatment				
A	Heart	AMR (C4d)*	46/57	Partial response
B	Kidney	AMR (PTC)*	62/59	Lost graft
C	Kidney	AMR (PTC)	0/20	Liver cancer
D	Heart	AMR (C4d)	4/6	Responded
L	Kidney	AMR (C4d)	0/10	Responded
M	Kidney	AMR (PTC)	0/0	Responded
Pretransplant IVIG treatment				
E	Kidney	Sensitization	60/80	No response*
F	Kidney	Sensitization	91/37	No response
G	Kidney	Sensitization	64/34	No response
H	Kidney	Sensitization	100/3	No response
I	Kidney	Sensitization	62/97	No response
J	Kidney	Sensitization	76/97	No response
K	Kidney	Sensitization	84/97	No response
O	Kidney	Sensitization	95/94	No response†

C4d, peritubular deposition of C4d; PTC, peritubular capillaritis.
*No change in PRA in multiple determinations after completion of the IVIG protocol.
†This patient received a compatible graft after 8 months having had no change in the PRA.

AMR included graft dysfunction and peritubular capilaritis or C4d deposition in biopsy specimens. Four patients with AMR had a full or partial (persistent C4d deposition) response and their grafts are functioning at 1 year. All patients treated for sensitization were highly sensitized and had no significant PRA changes after four or more monthly IVIG infusions. Seven patients are on dialysis 12–30 months after starting the protocol and one patient was transplanted. Sera from all patients except J, K and O were available before and within 3 days of IVIG administration for C and Ab studies. The IVIG protocol for patient O was discontinued after three infusions because of access issues and the patient received a crossmatch negative transplant 8 months later that is functioning well at 13 months. No specimen at the dates needed was available from patient O for the C and Ab studies.

C activity immediately after IVIG administration

Experiments were performed in several patients using sera before and within 3 days after beginning the IVIG infusion. As HLA Ab reactivity and the C activation it produced were variable with different target cells, multiple repeat experiments were performed to include a range of Ab and C activities. Table 2 shows results of C3b and IgG deposition on T cells as measured by flow cytometry. Patient A, treated for AMR of a heart transplant, had HLA Abs of restricted specificity that did not react with most of the target cells used. C activation was induced *in vitro* with serum from a broadly sensitized patient, whereas the patient A's own serum before and after IVIG was used as the source of C. The results show that C inhibition was seen in all 10 tests. IgG deposition on target cells was evaluated in seven of the 10 experiments: five in which third party HLA Abs had been added, and two in which the patient's own HLA Abs reacted with the target cells. The average of the first five cases is shown to note that there was not any significant change in IgG deposition to explain the C inhibition by IVIG, whereas the other two experiments denote no change in patient A's Ab binding after IVIG administration.

Patient B was treated for AMR of a kidney transplant and results were similar to patient A. Patients C, L and M were also treated for AMR of a kidney transplant. Patients E through I were treated for desensitization with a protocol consisting only of monthly IVIG for four or more months. Specimens were collected at different times but Table 2 shows only the results when there was a specimen drawn right before and soon after IVIG infusion. Sera from patients E–H were stored at −20 °C and lost C activity, but all of them had broadly reactive HLA Abs that reacted with the target cells. By restoring C reactivity with a small amount of normal fresh serum as described in methods, C inhibition after IVIG was seen to a degree comparable with other patients. In addition, little or no *in vivo* inhibition of HLA Ab binding was observed. Patient I did have preserved C activity and Ab reactivity against the target cells used and results again were similar to the other patients. Results are also shown on patients A and C, who received additional infusions at 1 g/kg, and on patient D who received 0.4 g/kg.

In general, C inhibition after IVIG infusion was observed in all patients, in most cases with statistically significant differences between the pre- and postinfusion values. There was little or no inhibition of Ab binding.

Relationship of HLA Ab strength with C inhibition

We next examined the possibility that the C inhibitory effect of IVIG might be different according to the degree of C activation (which in turn is influenced by Ab strength). This was addressed with the data from patients A, B, C, I, L and M who were tested with their own serum as the source of C. Figure 1 shows the magnitude of C3b deposition before IVIG administration plotted against the inhibition of C activation after IVIG administration. The coefficient of correlation was −0.46, which is suggestive of a negative correlation between the two parameters.

This issue was further explored with different amounts of HLA Abs in three separate experiments (Table 3). Two patients who did not have Abs against the target cells

Table 2. Effect of complement and antibody deposition immediately after administration of intravenous immunoglobulin (IVIG).

Patient*	IVIG dose (g/kg)	C3b deposition			IgG deposition		
		N	% Inhibition†	*P*-value	*N*	% Inhibition†	*P*-value
A	2	10	65 ± 24	<0.009‡	5	−16 ± 15	<0.17
					2§	−18 ± 16	
B	2	6	67 ± 23	<0.049	5	5.1 ± 18	<0.63
					3§	18.6 ± 2.2	
C	2	3	49 ± 13		1	−0.9	
E	2	4	68 ± 6.4	<0.001	2	−25 ± 20	
F	2	4	69 ± 23	<0.13	2	1.1 ± 16	
G	2	4	54 ± 14	<0.001	2	20 ± 3.5	
H	2	4	96 ± 1.1	<0.007	2	−11 ± 8.4	
I	2	4	74 ± 15	<0.069	4§	−8.7 ± 13	<0.249
L	2	2	95 ± 2		2	−11.4 ± 4.3	
M	2	2	93 ± 2		2	−13.4 ± 1.4	
A	1	5	40 ± 16	<0.09	2	−3 ± 3	
C	1	1	36		1	−18	
D	0.4	10	40 ± 15	<0.005	1	−1.8	

N, Number of experiments performed.

*Patients A, B, C, D, L, M: Target cells were incubated with heat-inactivated serum from a patient with broadly reactive HLA antibodies and washed. Patients' A, B, C, D, L, M fresh sera were added and C3b and IgG binding were measured by flow cytometry.

Patients E, F, G, H: Target cells were incubated patients' E, F, G, H sera and 2 or 4μl of fresh normal serum. C3b and IgG binding were measured by flow cytometry.

Patient I: Target cells were incubated with fresh serum from patient I and C3b and IgG binding was measured by flow cytometry.

†Average ± SD. Percent inhibition was calculated using the following formula: 100 – (value after IVIG × 100/value before IVIG).

‡Paired *t*-test comparing C3b or IgG antibody deposition before and 1–3 days after IVIG administration. *P* values are shown only for series consisting of four or more tests.

§These were cases in which the patients' HLA Abs reacted with the target cells, so that no other source of HLA antibodies was needed.

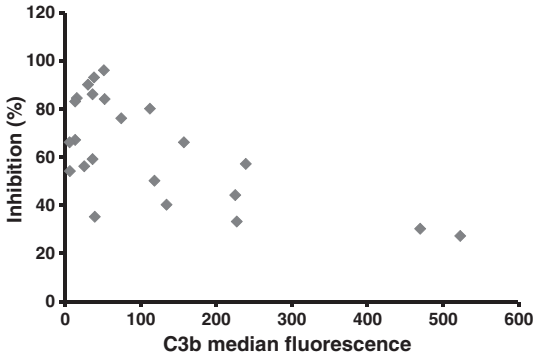

Figure 1 Correlation of complement deposition with complement inhibition by intravenous immunoglobulin (IVIG). Each point represents individual tests of patients A, B, C, I, L, M with their own serum as source of complement. C3b median fluorescence values were obtained before IVIG administration and % inhibition represents the effect of IVIG on C3b deposition immediately after administration.

were chosen and their own C was activated by adding a broadly reactive serum, diluted and undiluted to achieve different HLA Ab concentrations. In the first experiment

Table 3. Inhibition of complement activation by intravenous immunoglobulin (IVIG) at different HLA antibody concentrations.

Patient	IVIG (g/kg)	Serum dilution*	IgG†	C3†	% C inhibition	*P*-value
A	0	None	588	2503		
	2	None	835	1762	30	
	0	1:5	370	1370		
	2	1:5		599	56	<0.001‡
A	0	None	610	2788		
	1	None	615	2072	26	
	0	1:5		1263		
	1	1:5	389	805	36	<0.004
B	0	None	79	259		
	2	None		168	35	
	0	1:2	53	44		
	2	1:2		20	54	<0.21

*Target cells were incubated with heat inactivated serum from a patient with broadly reactive HLA antibodies diluted and undiluted. Patients' A and B fresh sera before and after IVIG infusion were added and IgG and C3b binding was measured by flow cytometry.

†Values denote median fluorescence intensity.

‡Chi-square test.

shown, patient A produced a 30% C inhibition 2 days after IVIG administration (2 g/kg) compared with the C activity before IVIG, but the inhibition of C activation was almost double when diluted Ab was used and less C activation was induced. The patient subsequently was given IVIG at 1 g/kg, again showing more inhibition at lower levels of HLA Abs and C deposition. Similar results were seen with patient B, although in this case, the statistical significance was not reached.

Effect of IVIG on C added *in vitro*

We next wanted to find out if IVIG, after interacting with C components *in vivo*, still had the ability to inhibit *in vitro* C activity from normal human serum. To this end, sera from patients A and B, fresh and heat inactivated, before and after IVIG administration, were added to target cells coated with HLA Abs as explained in Table 4. Serum from patient A had normal C activity, which was inhibited after administration of IVIG. Heating at 56 °C for 30 min completely prevented C activation; adding normal serum in limiting amounts restored C activity which in turn was inhibited by the heat-inactivated patient' serum after receiving IVIG. The results of both patients were similar. These experiments provide evidence that IVIG can inhibit the patient's own C or another source of C. They also show that this effect can be measured in patients' specimens that lost C activity through storage or other means.

Duration of IVIG effect

Figure 2 shows the C activity of patient A (top left) and patient D (top right) before and right after each of several IVIG infusions. The graphs at the bottom show the serum IgG levels at the same time points for each patient. Patient A received 2 mg/kg the first time and 1 g/kg subsequently (the first two doses were given about 2 months apart because of tolerability issues). Patient D received 0.4 g/ml every month. The time of IVIG infusion is easily seen by a sharp drop in C activity and a sharp rise in IgG levels. Of interest, both C activation and total IgG levels were back to baseline before the next infusion. Experiments with other patients' sera (not shown) suggested that C activity recovers after the second week: at 7 days, there was still significant inhibition but at 14 and 20 days, the activity was close to the values before the IVIG infusion.

Effect of IVIG on HLA Ab levels

The results in Table 2 show that HLA Ab binding to target cells remains essentially unchanged when measured

Table 4. Restoration of C activity with normal fresh serum.

	IVIG dose (g/kg)	Median fluorescence	Fluorescence ratio	% Inhibition
Patient A				
Patient's serum*	0	777	157	
Patient's serum	2	267	53	66
Heat inactivated (HI)†	0	5		
Heat inactivated (HI)	2	5		
HI plus 2 μl normal serum‡	0	66	13	
HI plus 2 μl normal serum	2	11	2	83
HI plus 4 μl normal serum	0	176	36	
HI plus 4 μl normal serum	2	24	5	86
Patient B				
Patient's serum*	0	429	112	
Patient's serum	2	89	23	80
Heat inactivated (HI)†	0	4		
Heat inactivated (HI)	2	4		
HI plus 2 μl normal serum‡	0	115	30	
HI plus 2 μl normal serum	2	11	3	90
HI plus 4 μl normal serum	0	198	52	
HI plus 4 μl normal serum	2	31	8	84

*Target cells were incubated with heat-inactivated serum from a patient with broadly reactive HLA antibodies and washed. Patients' A and B fresh sera were added and C3b binding was measured by flow cytometry.

†Same experiment except that patients' A and B sera were heat inactivated.

‡Same experiment except that patients' A and B sera were heat inactivated and fresh normal serum was added.

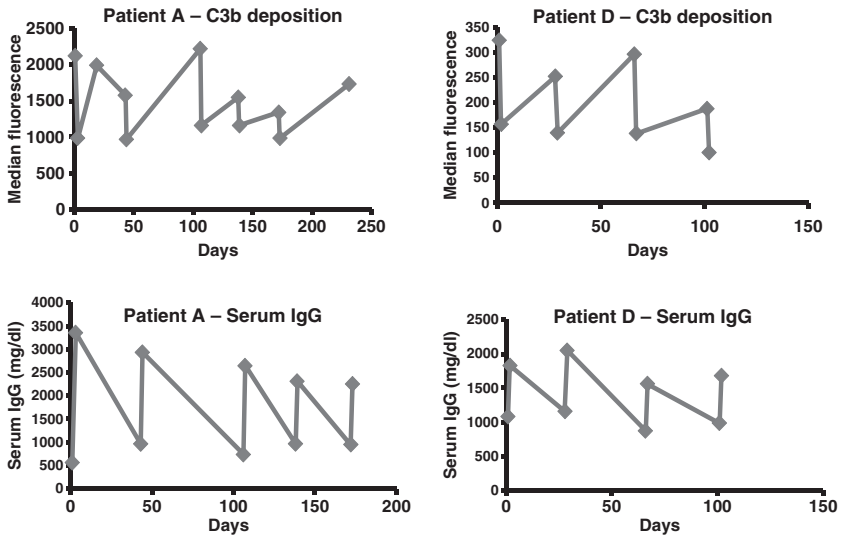

Figure 2 C3b deposition and IgG serum concentrations of patients A and D. Top left: C3b deposition of patient A. Top right: C3b deposition of patient D. Bottom left: IgG serum levels of patient A. Bottom right: IgG serum levels of patient D.

before and immediately after IVIG infusion. We next examined the Ab reactivity of broadly sensitized patients, testing all serial specimens from one patient in the same experiment. We noticed a decreasing trend in all patients, Table 5 showing the results before the initiation of the IVIG protocol and after monthly infusions. The changes were not dramatic: after the 4-month protocol, HLA Ab levels were, on average, 80% of those before treatment (20% decrease). About 6 months after the end of the IVIG protocol, Ab levels were back or closer to the original pre-IVIG values.

Table 5. HLA antibody reactivity before and after the 4-month intravenous immunoglobulin (IVIG) treatment.

Patient*	T-cell ratio†		% Reactivity after protocol‡
	Before	After	
A	11.2	9.1	81.3
E	3.5	3.08	87.4
F	125	106	87.8 ± 12
G	21	13	67.5 ± 14
H	89	67	76.4 ± 8
I	11	8.4	77.2 ± 8.8
J	9.3	7.2	82.0 ± 7
K	42	38	90.1 ± 0.5

*All patients received monthly IVIG at 2g/kg except A who received 2g/kg as a first dose and 1g/kg thereafter.

†T-cell ratio: median fluorescence intensity of the patient divided by that of the normal control. When the values of all patients were analyzed by the paired *t*-test, the differences between before and after IVIG were statistically significant ($P < 0.037$).

‡Values represent the mean of three or four experiments ± SD except for patient A and E who were tested at these dates once with a cell carrying the appropriate antigens.

Discussion

The main original finding of this study is that IVIG administration induces a short-lived inhibition of C activation and a minor reduction of HLA Ab levels after several treatments. Inhibition of C is maximal immediately after IVIG infusion and fades away in approximately 2 weeks, apparently following the concentrations of IgG serum levels. In accordance with this, inhibition was seen with doses of 2, 1 and 0.4 g/kg, but higher doses were more inhibitory. We also observed that C activation is more difficult to inhibit when HLA Ab levels are higher. When IVIG was given to broadly sensitized patients, HLA Ab reactivity measured before and right after infusion was largely unchanged, confirming that there is no significant anti-idiotypic activity [9]. However, HLA Ab levels were lower after several infusions, suggesting that some of the delayed IVIG effects at the cellular level reduce Ab concentrations, albeit to a limited degree.

Multiple approaches have been used to reduce sensitization, including plasmapheresis [2] and splenectomy [12]. IVIG would represent a less invasive intervention, but the short-term success achieved for AMR in some of our patients was probably also influenced by the clinical presentation and concomitant therapies. None of our patients treated for sensitization showed any reduction of PRA values, including the only patient who was transplanted (this patient could have been counted as a success of IVIG therapy if the only outcome measure was receiving a graft).

The IVIG protocols currently used are largely empirical in terms of dose and frequency of administration. The inhibitory effect of C activation may provide a rationale for the use of IVIG in AMR; however, this effect is

incomplete and transitory. It is possible that, because the inhibition lasts about a couple of weeks, IVIG may be more effective if given more often (e.g. weekly).

The implications for treating desensitization with IVIG are more complex. Patients often have high Ab levels and IVIG is not as effective in producing C inhibition even at high doses. Our results show that HLA Ab levels decrease after several months of therapy, but not in a substantial way. Other studies also reported a modest decrease in panel reactive Abs (PRA) tested by C-dependent cytotoxicity during the months when IVIG was being given, but not after stopping the IVIG infusions [1]. However, these results are likely due to the inhibition of C during the time that the serum IgG levels were increased by IVIG. Similarly, it has been postulated that IVIG blocks HLA Abs *in vitro* in a cytotoxicity assay, an interpretation reinforced by the fact that the inhibition is variable in different patients [13]. Again, an alternative explanation is C inhibition by IVIG, which would be more or less effective according to the Ab levels in different patients. Although the late Ab reduction we showed here may facilitate transplantation in some patients, its magnitude does not seem sufficient for a widely applicable beneficial effect. It remains unknown if continuation of monthly IVIG infusions for longer periods would achieve proportionally higher Ab reductions.

It is also unknown to what degree the C inhibitory activity of IVIG contributes to its clinical effect. In fact, it is not yet clear to what degree IVIG contributes to the treatment of AMR or desensitization. IVIG is rarely used as the only treatment and comparative studies suggest that IVIG in combination with plasma exchanges and other immunosuppressive agents is more effective than IVIG alone [3]. The effectiveness of protocols using high-dose (2 g/kg) IVIG, alone [1] or in combination with rituximab [14], is difficult to judge because success was measured as the transplantation rate of sensitized patients, which depends on multiple variables unrelated to IVIG administration (degree of sensitization, concentration of HLA Abs that turn out to be donor-specific when a donor is selected, crossmatch results, spontaneous versus induced Ab decrease, policies of transplant programmes to transplant across a positive crossmatch, etc).

One limitation of this study is that it included a small number of patients. However, we evaluated each case in depth and the results were consistent and likely to be more widely applicable. Another limitation is that the C tests were performed *in vitro* and therefore do not provide information about mechanisms that may be active *in vivo*.

Even though IVIG has been shown to be clinically useful in a few selected autoimmune diseases, the results presented here question the effect of IVIG on C or HLA Abs as a rationale for using IVIG in transplant patients. Considering this together with the fact that previous reports do not provide clear evidence of a clinical benefit of IVIG, further studies will be required to understand better the advantages and limitations of giving IVIG to transplant patients.

Authorship

TM: performed research, GG: identified study patients, GB: designed patient treatment, FJF: identified study patients, JC: collected data, PR: provided study specimens, H-UM-K: identified study patients, contributed to manuscript, JS: designed study, wrote the paper.

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