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Normal human immunoglobulins for intravenous use (IVIg) delay hyperacute xenograft rejection through $F(ab')_2$-mediated anti-complement activity

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SUMMARY

Xenotransplantation between discordant species leads to a hyperacute rejection mediated by natural antibodies, both of the IgG and IgM isotypes, activation of complement and endothelial cell activation. The combination of these mechanisms leads to a transplant survival of minutes to a few hours. Polyclonal human immunoglobulins for intravenous use (IVIg) from normal donors have proved effective in a number of antibody-mediated disorders, as well as in inflammatory disorders. We demonstrate that administration of IVIg in a guinea pig to rat model of cardiac xenografting can effectively delay hyperacute rejection. This effect is mediated by the $F(ab')_2$ fragments of IVIg, and is correlated to an anti-complementary activity.

Keywords xenotransplantation complement IVIg

INTRODUCTION

Xenotransplantation between discordant species leads to a hyperacute rejection mediated by natural antibodies, mainly of the IgM isotype, activation of complement and endothelial cell activation [1]. The combination of these mechanisms leads to a transplant survival of minutes to a few hours. Complement activation is triggered through both the classical antibody-dependent, and alternative pathways [2]. In the guinea pig to rat xenotransplantation model, complement activation via the alternative pathway was reported to be the major mechanism of graft destruction [3].

Polyclonal human immunoglobulins for intravenous use (IVIg) from normal donors have proved effective in a number of antibody-mediated disorders [4]. The proposed mechanisms of action include anti-idiotypic activity on circulating autoantibodies [5], blockade and saturation of FcR sites on reticuloendothelial system cells [6], and anti-complement activity [7]. In the present study, we investigated the potential effectiveness of IVIg on the hyperacute rejection of a guinea pig heart transplanted in a Lewis rat recipient, with special emphasis on the modulation of the alternate complement pathway.

MATERIALS AND METHODS

Reagents

IVIg (Gamma-PEG; Institut Merieux, Marcy l'Etoile, France) and Fc fragments of IVIg were a kind gift from Dr M. C. Bonnet (Institut Merieux, France). $F(ab')_2$ fragments were prepared from IVIg by pepsin digestion (2% w/w) (Sigma Chemical Co., St Louis, MO) in sodium acetate buffer 0·2 M pH 4·1 for 18 h at 37°C followed by chromatography on protein A-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). $F(ab')_2$ fragments were free of detectable Fc fragments and IgG as assessed by SDS–PAGE and ELISA using goat anti-human Fcγ antibodies (Jackson Immunoresearch, West Grove, PA).

Plasma collected from four healthy blood donors were a kind gift of our local blood bank (CTS Broussais). The IgG fraction was prepared from plasma by chromatography on protein A-Sepharose (Pharmacia). The purity of IgG was assessed by SDS–PAGE. Purified IgG was concentrated at 100 mg/ml, and sterilized through a non-pyrogenic sterile filter (0·22 μm).

Animals

Male inbred Lewis rats, 250–300 g (RT1 l) (Charles River, Cléon, France) were used as recipients and female Hartley guinea pigs, 300–350 g, as heart donors for xenografting and as a source for target erythrocytes for determination of haemolytic complement activity.

Pre-transplant treatment

Prior to transplantation, in a first set of experiments, animals received either no pretreatment, saline solution, human albumin (Centre National de Transfusion Sanguine, Paris, France) or IVIg (Gamma-PEG), at a dose of 1 g/kg body weight. In a second set of experiments, animals were pretreated with $F(ab')_2$ or Fc fragments

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of IVIg, either in equimolar quantities to IVIg or at 1 g/kg body weight, or purified human IgG prepared from single donors. All solutions were adjusted to a concentration of 100 mg/ml and administered through a catheter inserted into the right internal jugular vein at a rate of 2 ml/h. In animals receiving IVIg, some transplanted hearts were harvested before rejection at 60 min in order to study histopathology and complement activation.

Surgical procedure

Heart intra-abdominal xenografting was performed according to Ono & Lindsey [8]. Survival time of the transplant was expressed in minutes and defined by the existence of ventricular contractions. For the first 10 min the existence of ventricular contractions was ascertained by visual inspection. After abdominal closure, survival time was determined by palpation every 10 min. Transplant rejection was confirmed by opening the abdomen and removing the transplanted heart for histological studies, except in one group where the transplanted hearts were removed at 60 min while beating.

Histopathological studies

For conventional histology, tissues were formalin-fixed, paraffin-embedded and tissue sections were stained with haematoxylin–eosin.

For immunohistochemistry, cryostat sections were cut from snap-frozen cardiac tissue. They were incubated with fluorescein-labelled antibody directed against rat C3 (Institut Pasteur Production, Paris, France) or with anti-rat IgG or IgM polyclonal antibodies (Institut Pasteur) revealed with alkaline phosphatase-anti-alkaline phosphatase technique.

To assess histopathological involvement of the graft myocardium, a blinded semiquantitative analysis grading from 0 to 4 for each of the following parameters was used: oedema; congestion; haemorrhagic interstitial infiltrates; microthrombi.

For immunohistochemistry, the intensity of fluorescence and the number of immune deposits were assessed as well as the size of the vessels involved (i.e. micro- or macrocirculation).

Complement activation study

Activation of the complement alternative pathway was determined as described by Miyagawa [9], with minor modifications. Briefly, variable amounts of rat plasma were incubated for 60 min at 37°C with 100 μl of a solution of 10^8 guinea pig erythrocytes/ml in a GVB-Mg-EGTA buffer (gelatin 0·1%, Mg 2·8 mmol/*l*, EGTA 80 mmol/*l*). Haemolysis was quantified by the presence of haemoglobin in the supernatants and determined by optical density (OD) at 410 nm. Controls included: OD for 100% haemolysis in pure water, OD for spontaneous haemolysis (ODsh) in buffer without plasma, and OD due to plasma colour, named OD diluted sera (ODds).

Haemolysis (OD) was expressed as:

$$OD = OD_{observed} - ODsh - ODds$$

The amount of plasma allowing haemolysis of 50% of guinea pig erythrocytes was determined and converted to arbitrary units (AU). For each sample, results were expressed as AU/ml [10].

When the functional activity of complement was present but too low to allow calculation, a value of 5 AU was attributed, and when no haemolysis occurred, a value of 1 AU was attributed.

The results for each animal were expressed as a percentage of the pretreatment value, so that each animal served as its own control.

Immunoblotting

Rat plasma diluted at 1:200 in Laemmlis 1X buffer without β-mercaptoethanol were subjected to SDS–PAGE electrophoresis using a 7·5% polyacrylamide gel.

Proteins were transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) using a semi-dry electroblotter A (Ancos, Denmark APS, Hojby, Denmark). After blocking with Tris-buffered saline–bovine serum albumin (TBS–BSA) 1% for 2 h at room temperature, a goat IgG antibody to rat C_3 (The Binding Site, Birmingham, UK) diluted 1:2000 in TBS was incubated with the membranes for 2 h at room temperature.

After washing with TBS–Tween, bound antibody was revealed using alkaline phosphatase-conjugated rabbit antibody to goat IgG (Cappel, West Chester, PA) diluted 1:4000 in TBS and incubated 1 h. Membranes were then washed with NaCl 0·15 M, Tris 0·05 M pH 7·5, and nitroblue tetrazolium and bromo-chloroindolylphosphate (Sigma) were used as revealing substrates. As a positive control of complement activation, we used human serum incubated with zymosan. Briefly, zymosan A (Sigma) was boiled at 2 mg/ml with NaCl 0·15 M for 30 min. The solution was rinsed with NaCl 0·15 M until cleared. One volume of this solution was incubated with one volume of human serum for 45 min at 37°C. After centrifugation, the serum was frozen at −80°C until use.

Statistical analysis

Graft survival data were expressed in minutes as mean ± s.d. Statistical analysis between the different groups was performed by the ANOVA test, comparison of the AP50 values was performed by the Mann–Whitney test, and correlation between complement levels and graft survival was determined by simple regression analysis (Spearman's test; Statview II Software, Abacus Concepts Inc., Berkley, CA).

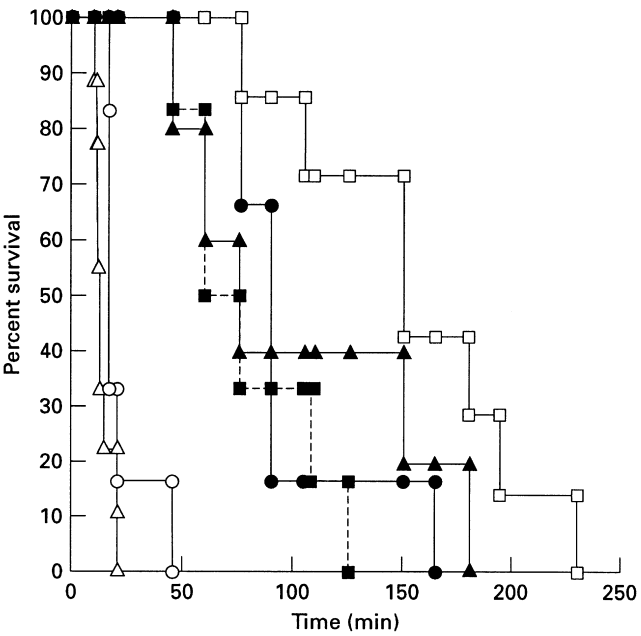

Fig. 1. Survival of cardiac xenografts after pretreatment of recipient with IVIg, F(ab′)$_2$ or Fc fragments of IVIg. △, Albumin; ●, IVIG (1 g); □, F(ab′)$_2$ (1 g); ▲, Fc (1 g); ■, F(ab′)$_2$ (iso); ○, Fc (iso).

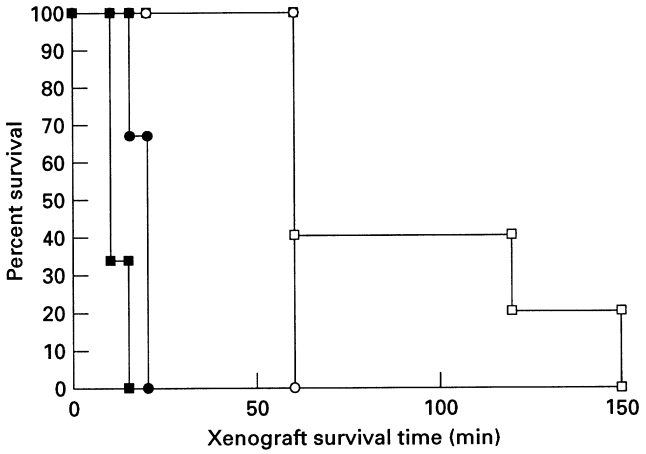

Fig. 2. Survival of cardiac xenografts after pretreatment of recipient with four single-donor preparations of human IgG. ■, 1; ●, 2; □, 3; ○, 4.

RESULTS

Survival time

Infusion of IVIg prior to cardiac xenotransplantation in Lewis rats led to a prolongation of graft survival (Fig. 1). All grafts survived for more than 1 h with a mean time of 97·5 min, in striking contrast with control (8 min), saline- (13·3 min) or albumin-infused groups (13·8 min). The ANOVA test shows a significant difference between the IVIg-treated group and control groups ($P < 0{\cdot}001$ *versus* control, saline- and albumin-infused groups). Survival time in the albumin-infused animals was not significantly prolonged compared with saline or control.

In order to gain further insight into the mechanisms involved, fragments of IVIg as well as single-donor preparations of human IgG were also used. $F(ab')_2$ fragments, used either at 1 g/kg body weight or at equimolar quantities to IVIg, led to similar prolongation of graft survival, whereas Fc fragments only prolonged survival at 1 g/kg body weight, but not when used at equimolar quantities to IVIg (Fig. 1). Single-donor IgG preparations resulted in contrasting results, with no prolongation of survival with two preparations and a strong effect with two others (Fig. 2).

Histopathology

Immunofluorescence studies revealed massive deposits of fibrin and C3 in the control group. These deposits were sparse and faint in the IVIg group (Fig. 3A,B).

Histological studies showed minor lesions in the IVIg-infused group compared with the albumin-infused group, with mean scores, respectively, of 0·42 and 2·14 for oedema, 0·28 and 1·14 for congestion, 0 and 0·14 for haemorrhagic suffusions, and 0 and 0·14 for microthrombosis (Fig. 3C,D). Native heart, kidneys,

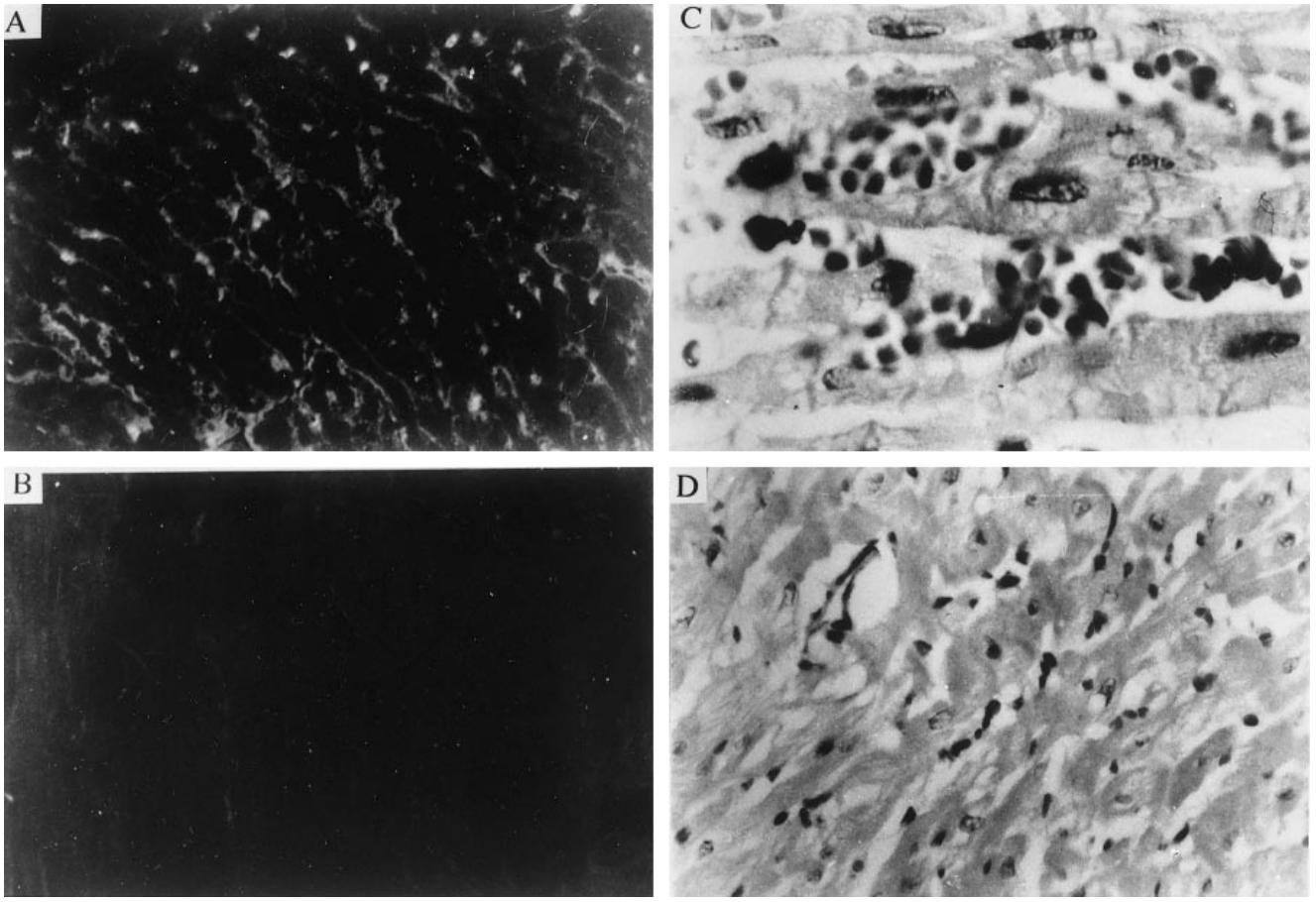

Fig. 3. Histopathology of control and IVIg-treated xenografts: immunofluorescence using anti-fibrin antibody in controls (A) and IVIg-treated (B) animals, conventional histology in controls (C) and IVIg-treated (D) animals.

Table 1. Complement results. Complement activity is expressed as a percentage of initial value before treatment, as described in Materials and Methods.

Group	*n*	Post-treatment	Post-transplant
Saline	4	88·2 ± 5·1	65·1 ± 6·4
Albumin	9	47·1 ± 5·5	34·7 ± 9·4
IVIg	7	21·6 ± 1·3	7·1 ± 5·7
$F(ab')_2$ 1 g/kg	7	19 ± 6·5	11·4 ± 8
Fc 1 g/kg	3	64·9 ± 4·9	23·5 ± 16·2
$F(ab')_2$ 0·66 g/kg	6	40·2 ± 8·2	20
Fc 0·33 g/kg	4	70·8 ± 4·8	46·1 ± 6·7

bowel, lungs, aorta, brain and liver from animals infused with IVIg were studied, and no lesions were found.

Complement (Table 1)

Xenotransplantation led to a significant decrease of residual complement activity from 143·5 AU to 128·5 AU, a 10% decrease from pre-transplant values. Regarding complement variation in pretreated animals, saline infusion led to a minor decrease of 12%, whereas albumin infusion led to a significant decrease of 43%. Pre-transplant IVIg infusion led to a major decrease of complement activity, below the detection limits in most animals. $F(ab')_2$ infusion, either in equimolar quantities to IVIg or at 1 g/kg body weight, led to a significant decrease in residual complement activity and to an undetectable level after xenografting. Fc infusion either in equimolar quantities to IVIg, or at 1 g/kg body weight, induced only a minor decrease in complement activity, similar to albumin. Single donor IgG infusion led to contrasting results, with a major decrease in complement activity with two preparations, and minor changes in two others. All animals perfused with the same IgG preparation showed similar complement changes. Moreover, there was a strong correlation ($P = 0{\cdot}016$) between residual complement activity and graft survival (Fig. 4). A similar correlation ($P = 0{\cdot}023$) was observed for the $F(ab')_2$ group alone.

Immunoblotting

Immunoblotting with untreated normal sera showed a single band of 180 kD corresponding to intact C3 and C3b (Fig. 5).

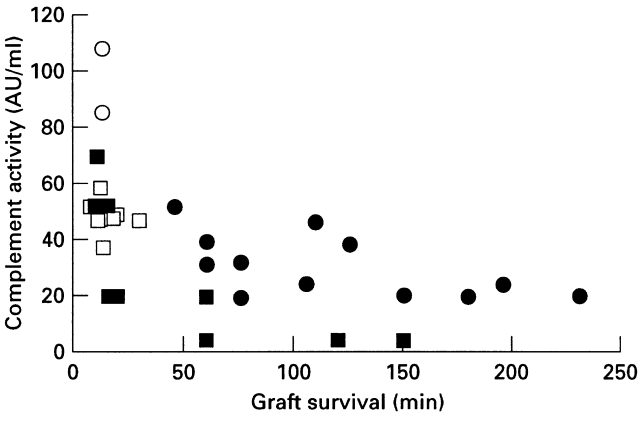

Fig. 4. Correlation between complement depletion and graft survival. ●, $F(ab')_2$; □, albumin; ■, single donor; ○, saline.

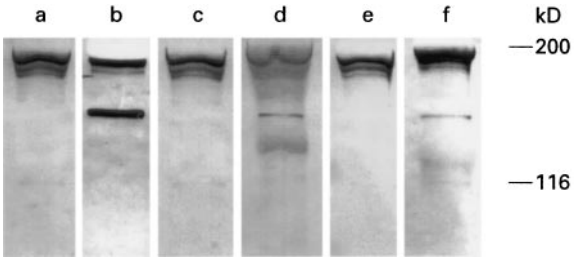

Fig. 5. Immunoblotting of untreated (a) and zymosan-treated sera (b), and of sera from animals perfused with albumin (c), IVIg (d), Fc fragments of IVIg (e), and $F(ab')_2$ fragments of IVIg (f).

With zymosan-treated plasma, a 140-kD band, corresponding to the C3c fragment resulting from C3b cleavage, was seen. A similar 140-kD band appeared after treatment of plasma with IVIg but not with albumin. Plasma treated with $F(ab')_2$ fragments showed again a similar 140-kD band, whereas in plasma treated with Fc fragments only the 180-kD band was seen, as in untreated or albumin-treated plasma. Thus IVIg and $F(ab')_2$ fragments of IVIg, but not Fc fragments of IVIg, induced complement activation.

DISCUSSION

It is now well known that hyperacute rejection is mediated through natural or preformed circulating antibodies, complement and endothelial cell activation [1]. IVIg is widely used as an immunomodulatory agent in autoimmune and inflammatory diseases. The multiple mechanisms of action include blockade of Fc receptors of macrophages, such as in idiopathic thrombocytopenic purpura, interactions of variable regions of infused IgG with V regions of patient antibodies as observed in acquired haemophilia due to anti-factor VIII autoantibodies [10], or more recently, with HLA-specific alloantibodies in patients awaiting transplantations [11], as well as an anti-complement activity [12].

Using a highly reproducible heterotopic discordant cardiac xenograft model, we have previously demonstrated that IVIg infusion prior to xenotransplantation significantly delayed hyperacute rejection up to 90 min [13]. This prolongation of graft survival was not observed with saline or human albumin administration, thus ruling out a non-specific effect due to either a haemodilution effect or a heterologous protein load. These results have been confirmed in the same model [14], as well as in the pig to primate model [15].

This prolongation of graft survival is mainly due to the $F(ab')_2$ fraction of IVIg, as this fraction, administered in equivalent molar quantity to whole IVIg, has a similar efficacy, whereas Fc fragments do not prolong graft survival. Thus, the specificity of the immunoglobulins contained in the IVIg preparations seems to be of paramount importance in the prolongation of graft survival. This hypothesis is supported by our finding that single-donor IgG preparations have a variable effect.

The mechanism by which IVIg prolongs graft survival is, at least in part, complement-dependent. The beneficial role of complement depletion has already been reported by other investigators. Total complement consumption, as obtained by repeated injection of low doses of cobra venom factor [16] or sCR1, a soluble form of a complement receptor type I [17], significantly delays hyperacute rejection up to several hours.

Whole IVIg and their $F(ab')_2$ fragments both induce a major

decrease in complement activity and prolong graft survival, whereas Fc fragments of IVIg have little effect on complement levels and do not prolong significantly graft survival if given in equimolar quantity to IVIg. Moreover, there is a strong correlation between prolongation of graft survival and complement depletion. This hypothesis is further strengthened by our histopathologic observations showing the absence of C3 deposition on the xenografts of animals pre-treated with IVIg compared with control animals. A similar inhibition of complement-mediated damage by IVIg has been reported in an antibody-mediated model of pulmonary haemorrhage [7,18].

The diminution of haemolytic activity to 53% of basal value observed after infusion of albumin or Fc fragments of IVIg, but not saline, may be explained by a non-specific adsorption of complement proteins on the infused heterologous proteins, in accordance with the results of the immunoblotting showing no degradation products of C3. This partial decrease in complement activity is insufficient to prolong graft survival. IVIg or their $F(ab')_2$ fragments, however, induce complement activation, as shown by the appearance of C3c, and results in a major depletion of complement.

Thus, the major decrease of haemolytic activity after IVIg infusion is probably due to both adsorption on heterologous protein and to a specific effect of IVIg, mediated through the $F(ab')_2$ region of the immunoglobulin molecules. Similar inhibition of complement activation has been reported in the pig to primate transplant model [15]. This specific effect might be due to the formation of idiotypic–anti-idiotypic immune complexes between IVIg and natural rat antibodies, with subsequent activation of complement.

These results underline the anticomplementary effect, most notably on the alternative pathway, which plays a major role in our model of discordant rejection [3]. In conclusion, pre-transplant infusion of IVIg in this intra-abdominal heterotopic cardiac discordant xenograft model significantly delays hyperacute rejection. This therapy has been proved to be clinically relevant in other situations such as autoimmune diseases or hyperimmunized patients undergoing renal allotransplantation. The mechanisms of action of IVIg in xenotransplantation, mainly due to the $F(ab')_2$ fragments of IVIg, include, but are not limited to, an anti-complementary activity. Other mechanisms such as anti-idiotypic activity or inhibition of endothelial cell activation are under investigation.

REFERENCES

1 Cooper DKC, Kemp E, Reemtsa K, White DJG. Xenotransplantation, Vol. 1. Berlin: Springer-Verlag, 1991.

2 Termignon JL, Calmus Y, Chéreau C, Kahan A, Houssin D, Weill B. *In vitro* analysis of complement activation in xenogeneic hyperacute rejection. Transplant Proc 1992; **24:**443–4.

3 Miyagawa S, Hirose H, Shirakura R *et al.* The mechanism of discordant xenograft rejection. Transplant Proc 1989; **21:**520–1.

4 Dietrich G, Kaveri SV, Kazatchkine MD. Modulation of autoimmunity by intravenous immunoglobulins (IVIg) through interaction with the function of the immune/idiotypic network. Clin Immunol Immunopathol 1992; **62:**S73–S81.

5 Sultan Y, Kazatchkine MD, Maisonneuve P, Nydegger U. Anti-idiotypic suppression of autoantibodies to Factor VIII (anti-haemophilic factor) by high-dose intravenous gammaglobulin. Lancet 1984; **ii:**765–8.

6 Imbach P, Morell A. ITP: immunomodulation by IVIg. Int Rev Immunol 1989; **5:**181–8.

7 Basta M, Kirshbom P, Frank MM, Fries LF. Mechanism of therapeutic effect of high-dose intravenous immunoglobulin. Attenuation of acute, complement-dependent immune damage in a guinea pig model. J Clin Invest 1989; **84:**1974–81.

8 Ono K, Lindsey ES. Improved technique of heart transplantation in rats. J Thorac Cardiovasc Surg 1969; **57:**225.

9 Miyagawa S, Hirose H, Shirakura R *et al.* The mechanism of discordant xenograft rejection. Transplantation 1988; **46:**825–30.

10 Sultan Y, Kazatchkine MD, Nydegger U, Rossi F, Dietrich G, Algiman M. Intravenous immunoglobulin in the treatment of spontaneously acquired factor VIII: C inhibitors. Am J Med 1991; **91** (Suppl. 5A):35–39.

11 Glotz D, Haymann J-P, Sansonetti N, FrancÌois A, Menoyo-Calonge V, Bariety J, Druet P. Suppression of allo-anti-HLA antibodies by high dose intravenous immunoglobulins (IVIg): a potential tool for transplantation of immunized patients. Transplantation 1993; **56:** 335–7.

12 Basta M, Langlois PF, Marques M, Frank MM, Fries LF. High-dose intravenous immunoglobulin modifies complement-mediated *in vivo* clearance. Blood 1989; **74:**326–33.

13 Latremouille C, Haeffner-Cavaillon N, Goussev N, Bariety J, Bruneval P, Carpentier A, Glotz D. Normal human polyclonal immunoglobulins for intravenous use (IVIg) significantly delay hyperacute xenograft rejection. Transplant Proc 1994; **26:**1285.

14 Gautreau C, Kojima T, Woimant G, Cardoso J, Devillier P, Houssin D. Use of intravenous immunoglobulin to delay xenogeneic hyperacute rejection. An *in vivo* and *in vitro* evaluation. Transplantation 1995; **60:**903–7.

15 Magee JC, Collins BH, Harland RC, Lindman BJ, Bollinger RR, Frank MM, Platt JL. Immunoglobulin prevents complement-mediated hyperacute rejection in swine-to-primate xenotransplantation. J Clin Invest 1995; **96:**2004–12.

16 Tavakoli R, Michel A, Cordoso J *et al.* Prolonged survival of guinea pig to rat heart xenografts using repeated low doses of cobra venom factor. Transplant Proc 1993; **25:**407–9.

17 Xia W, Fearon DT, Moore FD, Shoen FJ, Ortiz F, Kirkman RL. Prolongation of guinea pig cardiac xenograft survival in rats by soluble human complement receptor type 1. Transplant Proc 1992; **24:**479–80.

18 Basta M. Modulation of complement-mediated immune damage by intravenous immune globulin. Clin Exp Immunol 1996; **104** (Suppl. 1):21–25.

19 Miletic VD, Hester CG, Frank MM. Regulation of complement activity by immunoglobulin. I. Effect of immunoglobulin isotype on C4 uptake on antibody-sensitized sheep erythrocytes and solid phase immune complexes. J Immunol 1996; **156:**749–57.