Complement-Mediated Vasoconstriction and Graft Rejection

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ABSTRACT

The hyperacute rejection of renal allografts has much in common with pig-to-dog renal xenograft rejections. An analysis of the xenograft model revealed new functional aspects of graft rejection that appear to be an integral part of the humoral immune injury. Anaphylatoxin or C5a, a cleavage product of the fifth complement component was shown to play a major role in the rejection of isolated kidneys; it was generated in pig and dog serum by activation of the complement system and caused severe vasoconstriction in the renal vasculature. It is suggested that histocompatibility antibodies and complement may affect allograft survival via this potent mediator.

KEY WORDS anaphylatoxin renal blood flow hyperacute rejection preformed histocompatibility antibodies pig-to-dog renal xenografts

The steady yearly increase of kidney transplants performed worldwide allows one to predict that the conditions for acute, humorally mediated graft rejections will be encountered more and more frequently. Many prospective first-time recipients have preformed circulating cytotoxic antibodies against various histocompatibility antigens (1–3). The formation of these antibodies appears to be most frequently induced by blood transfusions and pregnancies. If these antibodies are directed against the lymphocytes of a potential donor, the resulting positive crossmatch is generally acknowledged as an absolute contraindication against the acceptance of that donor. Moreover, it has been shown recently that recipients with preformed antibodies against histocompatibility antigens, even if they are not shared by the donor cells, face greater immunological problems than recipients with no detectable antibodies (1). This situation is not reflected in a positive crossmatch; it can only be recognized if the recipient serum is tested with a large number of cells representing all possible antigens. Whether or not circulating antibodies are detected and the immunological warning is heeded, the number of patients who might reject acutely will increase in a given list of recipients. This is inevitable since such patients will either be selected less frequently as recipients or, if selected, the kidneys will more frequently and more rapidly be rejected, and the patients will again be on the list of recipients, often with antibodies of broader and greater reactivity.

This unfortunate situation raises basic issues regarding the role of the humoral immune response in allograft rejections. A valid assessment depends largely on (1) an improvement of techniques for the detection of histocompatibility antibodies, (2) the recognition of their fluctuation in time, and (3) our ability to predict secondary responses.

Since clinical transplant experience places renewed emphasis on humoral immune factors, it seems to be of paramount importance to advance our understanding of the mechanisms by which these factors take part in the
rejection process. We have attempted to analyze this process with the aid of the pig-to-dog renal xenograft model. The rapid rejection of pig kidneys transplanted to dogs has frequently been compared with hyperacute renal allograft rejections. Naturally occurring dog antibodies react with antigens carried by various pig cells, e.g., erythrocytes, lymphocytes, and the endothelium of the glomerular and intertubular capillaries. Though these antibodies are cytotoxic in the presence of complement we have reason to believe that cytotoxic or cytolytic damage is not the primary or only injury that initiates the rejection process.

It is the purpose of this paper to focus attention on functional vascular disturbances induced by antibody and complement. Recognition and appreciation of complement-mediated vasconstriction in the graft may lead to new preventive and therapeutic measures for the most discouraging rejection episodes; it may furthermore serve to reevaluate the fundamental questions regarding humoral involvement in graft rejection in general.

Methods

METHODS OF PERFUSION

Kidneys were removed from anesthetized pigs (30–60 kg) and perfused with 6% dextran 70 in isotonic saline containing sodium heparin (10 units/ml) at 37°C and 70–90 mm Hg. An average of 500 ml perfused over a 10-minute period usually was sufficient to wash out residual blood. The flushed kidneys were perfused with dog or pig blood, plasma, serum, or other blood components by one of several methods: (1) Kidneys were anastomosed to the recipient’s (greyhound, 23–34 kg) carotid artery and jugular vein by Silastic tubing. (2) Isolated, gravity- and in the pump-perfused kidney (constant flow) resistance changes in the renal vascular bed were included means for temperature control and oxygenation. The flow was manually adjustable. (3) Pump-perfused kidneys were perfused by a recirculating pump system which included means for temperature control and oxygenation. The flow was manually adjustable. Resistance changes in the renal vascular bed were monitored under the first two conditions by measuring the venous flow (constant pressure) and in the pump-perfused kidney (constant flow) by recording the renal arterial pressure. In all perfusion systems, arterial and venous samples were obtained from Y-tubing connectors, which were also used for the introduction of test materials into the renal artery.

Blood was collected individually from the jugular vein of fasted greyhounds or the femoral vein of pigs. Except when serum was to be obtained, the animals were heparinized and plastic or siliconized containers were used for the collection of the blood.

IMMUNOLOGICAL METHODS

Buffers and Diluents.—Isotonic veronal buffers pH 7.4–7.6 containing 1% gelatin were used as diluents. Gelatin veronal buffer (GVB2+) contained 0.00015M CaCl2 and 0.0005M MgCl2. Dextrose gelatin veronal buffer (DGVB2+) was made by diluting GVB2+ containing twice the concentration of gelatin, CaCl2 and MgCl2 with an equal volume of 5% dextrose in water to give an isosmotic buffer with reduced ionic strength. GVB2- contained no CaCl2 or MgCl2 (4).

Pig Erythrocytes.—Blood from individual pigs was collected in modified Alsever’s solution (5) containing penicillin C (1 mg/ml) and streptomycin sulfate (0.2 mg/ml) and stored at 4°C. Erythrocytes were thoroughly washed with saline followed by buffer. Cells were standardized to the appropriate concentration by correlating cell counts with spectrophotometrically measured oxyhemoglobin concentration for each batch of cells.

Rabbit Antibody-Sensitized Sheep Erythrocytes.—Sheep erythrocytes were optimally sensitized with immunoelectrophoretically pure IgG fractions from sera of rabbits immunized with sheep erythrocytes.

Dog Antibody Titration by Lytic Technique.—One volume of serum or plasma dilutions was incubated with 2 volumes of GVB2+, 1 volume guinea pig complement 1/15 (pooled guinea pig serum, Hyland Laboratories) and 1 volume pig erythrocytes (1 x 108/ml) for 60 or 90 minutes at 37°C.

Dog Antibody Titration by Agglutination Technique.—One volume of dilutions of serum was incubated with 2 volumes 0.02M EDTA-saline and 1 volume pig erythrocytes (1 x 108/ml) in U-bottom microtiter plates on vibrators for 10 minutes at 30°C. The plates were then allowed to settle at room temperature.

Dog Complement Titration.—One volume of serum or plasma dilutions was incubated with 3 volumes of DGVB2+ and 1 volume antibody-sensitized sheep erythrocytes (1 x 108/ml) for 60 or 90 minutes at 37°C.

Alternatively, a modification of the standard C titration (5) was used with a reaction volume of...
7.5 ml including 1 ml antibody-sensitized sheep erythrocytes (1 x 10^8/ml) and varying amounts of a suitable dilution of serum.

**Erythrocyte Stroma and Stroma Absorption.**—Saline-washed erythrocytes were lysed by osmotic shock in 20–30 volumes of 0.005M phosphate buffer pH 7.4 containing 0.005M EDTA and stirring in the cold for 24–48 hours. The stroma were collected by centrifugation and washed with phosphate buffer and then with saline until the supernatant fluid was colorless. During washing, any clumped stroma or unlysed cells were discarded. The stroma were resuspended in saline, counted by phase microscopy, and stored at −20°C.

For in vitro absorption studies, a well-drained stroma pellet was resuspended in serum. After incubation, stroma were removed by centrifugation and the absorbed serum compared to controls incubated without the addition of stroma.

**Chemotaxis Assays.**—Chemotaxis assays were performed according to Snyderman et al. (6), except that the cells were obtained from the peritoneal cavity of greyhounds 4 hours after the injection of sterile glycogen and that 5.0μ μmipore filters (Brinkmann) were used.

**Lymphocytotoxic Assays.**—Pig lymphocyte suspensions were prepared according to Thorsby and Bratlie (7). Lymphocytotoxic activity in dog serum was measured according to Amos et al. (8).

**Results**

We have previously shown that pig kidneys were equally rapidly rejected by whole heparinized dog blood if perfused by any one of the three techniques described here (9, 10). Isolated organ perfusion with a limited and well-defined blood volume allowed careful monitoring of antibody and complement uptake from the perfusate as it passed through the kidney. More important, plasma or serum with and without added formed blood elements could be perfused in attempts to analyze the rejection mechanism. Whether the kidney was perfused with whole dog blood, plasma, or serum, a significant rise in flow resistance was consistently observed; at the same time antibody and complement were depleted (Figs. 1 and 2). Heat-inactivated

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![Graph](http://circres.ahajournals.org/)

**Figure 1**

Decrease of flow, depletion of natural dog anti-pig erythrocyte antibody and dog complement during gravity perfusion of an isolated pig kidney with normal greyhound serum. Antibody was measured as percent lysis of pig erythrocytes by a 1:25 dilution, and complement as percent lysis of a 1:75 dilution of venous samples obtained at various times of perfusion. Pressure and flow were monitored on the arterial and venous side, respectively.

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Flow profiles of pig kidneys perfused with heparinized dog blood (anastomosis and gravity, see Methods) and with dog plasma (gravity).

(30 minutes at 56°C) dog serum, on the other hand, proved to be an excellent perfusion fluid for pig kidneys. Although antibody was also depleted, no increase in flow resistance was observed. In a typical experiment the flow rate of gravity-perfused heat-inactivated dog serum remained constant for 7 minutes (45 ml/min) while the lytically measured antibody titer dropped by 40% at the third minute.

It followed from these experiments that the increased intrarenal vascular resistance could not possibly be due to mechanical obstruction by aggregated cellular elements or platelets. We therefore suspected functional vascular changes caused by pharmacologically reactive mediators generated by the interaction of natural dog antibody, pig kidney antigens, and dog complement.

To investigate this possibility, we initially decided to use a homologous rather than the more complex heterologous system. We reasoned that activation of the complement system in pig serum or plasma should also lead to the generation of vasoactive mediators that would cause increased flow resistance in the homologous kidney. As a possible mediator, anaphylatoxin (AT) was considered. An established technique for the generation of AT is activation of serum or plasma by incubation with the polydextran Sephadex (11).

One gram of Sephadex G-75 was suspended in 100 ml of normal pig plasma and the suspension was incubated for 60 minutes at 37°C in a waterbath shaker. Subsequently, the Sephadex was removed by centrifugation. Whole complement was lowered by 35% during this activation procedure, as determined by comparison of the complement titer of the activated plasma with that of the same plasma incubated without Sephadex. When the Sephadex-treated plasma was gravity-perfused through a pig kidney, the flow rate decreased dramatically to less than 10% of its original value within a few minutes. This effect was reversed when perfusion was continued with untreated pig plasma or with the flush solution.

When pig serum activated by the same procedure was fractionated by gel filtration, a heat-resistant material with a molecular weight of about 15,000 was isolated (Fig. 3). This material had the following properties: (a) It caused increased renal vascular resistance if injected into the renal artery of a pump-perfused pig kidney (Fig. 4). (b) It caused contraction of the isolated guinea pig ileum followed by tachyphylaxis (11). (c) It was chemotactic for rabbit peritoneal polymorphonuclear leukocytes. (d) It caused increased vascular permeability in the guinea pig skin (12).

Figure 3 shows the fractionation profile. The position of the chemotactic, smooth muscle contracting and vasoactive fractions are superimposed in the area where materials of a molecular weight of 15,000 eluted from the previously calibrated column. The delineated characteristics are precisely those described for the classical anaphylatoxin or C5a (11). This pharmacologically highly reactive material is cleaved from the fifth complement component, C5, during activation of the complement system; it is an important mediator in immune-inflammatory processes. The profound effect of C5a on the renal vascular
Molecular sieve chromatography of activated pig serum. Pig serum was activated at 37°C for 60 minutes with Sephadex G-75 (10 mg/ml), centrifuged to remove the Sephadex, heated at 56°C for 30 minutes and cleared by centrifugation. 5.5 ml of this material was applied to a 2.5 x 100 cm column of Sephadex G-100, equilibrated with phosphate-buffered saline, pH 7.5. The column was previously calibrated with molecular weight markers. 5.0 ml fractions were collected. The crosshatched areas indicate chemotactic activity; the horizontal bars, increased kidney resistance (K.R.), smooth muscle constriction (S.M.) and increased vascular permeability (V.P.); circles, OD_{280}

bed is a new property previously not recognized.

These findings strongly suggested that a similar mechanism might be the cause for the increased flow resistance observed during perfusion of the pig kidney with dog plasma or serum.

The depletion of antibody and the consumption of complement observed in the perfusion experiments could also be demonstrated by in vitro treatment of dog serum with pig erythrocyte stroma: typically, absorption of 1 ml of fresh normal dog serum with $10^{10}$ pig erythrocyte stroma resulted in 90% depletion of anti-pig erythrocyte antibody, 75% depletion of the anti-pig lymphocyte antibody (as determined by lymphocytotoxicity assays) and 30% depletion of whole complement activity.

If our assumption was correct, this in vitro activation of the dog immune system should also result in the formation of AT.

Though we have not so far been able to isolate anaphylatoxin from activated dog serum, the following experiment indicates that the in vitro interaction of dog antibody and pig antigens in the presence of complement resulted indeed in the formation of a biologically active material with similar characteristics.

A dog was immunized with pig antigens by intravenous injection of a total of $10^8$ pig erythrocyte stroma administered over a 3-week period. A serum sample taken a week later showed a fivefold increase in the lytic antibody titer (from 1/64 to 1/320). This immune serum and a normal serum sample were incubated for 60 minutes at 37°C with pig erythrocyte stroma, centrifuged at high speed and then tested in an in vitro chemotactic assay with dog peritoneal polymorphonuclear leukocytes. The experimental details and the results are listed in Table 1.
Pressure responses of constant flow pump-perfused pig kidney. The perfusion fluid was 6% dextran in saline. Test materials were injected into the tubing close to the renal artery. The sharp pressure spikes indicate the time of injection. Fr = fraction numbers (Fig. 3); boldface numbers = volumes (ml) injected; SM = starting material of column (Fig. 3). The bottom tracing shows that control serum (Co), heated control serum (Co act.) and heated control serum treated with Sephadex (act.) had no effect. Treatment with Sephadex (act.) generated the vasoactive material which was heat resistant (act. a) and caused tachyphylaxis.

It is apparent that chemotactic activity was generated in normal greyhound serum by incubation with pig erythrocyte stroma. It is not surprising that the immune serum with its higher antibody titer generated more activity than the normal serum with its limited amount of natural antibody.

The major chemotactic activity generated in mammalian serum by complement activation is a property of anaphylatoxin or C5a (12). It is therefore more than likely that C5a is also responsible for the increased flow resistance in the xenografted kidney.

We are not certain at the present time where the flow resistance originates and what part of the renal vascular system responds to the vasoactive material. Preliminary angiographic experiments show that perfusion with activated plasma is followed by fading of the distal interlobar arteries, resulting in the appearance of a severely "pruned" arterial tree and that the perfusion of the cortex becomes irregular and patchy (Fig. 5). Sometimes

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**TABLE 1**

<table>
<thead>
<tr>
<th>Reagent*</th>
<th>Final Dilution</th>
<th>Chemotactic Activity†</th>
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<tbody>
<tr>
<td>Normal serum</td>
<td>1:8</td>
<td>62</td>
</tr>
<tr>
<td>Normal serum plus</td>
<td>1:16</td>
<td>65</td>
</tr>
<tr>
<td>pig stroma</td>
<td>1:8</td>
<td>84</td>
</tr>
<tr>
<td>Immune serum</td>
<td>1:16</td>
<td>79</td>
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<tr>
<td>Immune serum plus</td>
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<td>65</td>
</tr>
<tr>
<td>pig stroma</td>
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<td>4</td>
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<tr>
<td>Pig stroma control</td>
<td>2.5 × 10⁸</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1.25 × 10⁸</td>
<td>0</td>
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</tbody>
</table>

*2.0 ml of undiluted fresh dog serum was incubated for 60 minutes at 37°C with 2 × 10¹⁰ pig erythrocyte stroma contained in 2.0 ml GVB⁺ or with 2.0 ml GVB⁺ alone. After removal of the stroma by centrifugation the supernatant fluids were diluted with Gey's balanced salt solution and then tested for chemotactic activity. Heat treatment of the supernatant fluids (60°C for 30 minutes) did not affect their chemotactic activities.

†Expressed as number of cells per high power field.

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FIGURE 5

Effect of anaphylatoxin on the vasculature of a gravity-perfused pig kidney. Top left: Perfusion with 6% dextran 70 in saline; flow 70 ml/min. Top right and bottom: Pictures taken at 10-second intervals beginning 4 minutes after start of perfusion with activated pig plasma; flow 6 ml/min.

Constrictions could be seen in the large, early branches of the renal artery. All these changes appeared to be reversible when the active material was washed out of the circulation. Whether, where, and to what extent the microcirculation is involved remains to be investigated.

Discussion

The dramatic rejection of renal xenografts between widely divergent species has in the past been viewed as being predominantly due to mechanical vascular obstruction by aggregated platelets and polymorphonuclear leukocytes (13). It has also been implied that the damage to the glomerular endothelium is mainly caused by neutrophils phagocytizing the antigen-antibody-complement complexes. We have previously shown, however, that the capillary endothelium is destroyed by fresh, cell-free heterologous serum and that antibody and complement alone can cause the damage (10). Our present data indicate that this early structural damage is preceded by functional vascular disturbances. This suggests that an initial forceful increase in flow resistance is followed by endothelial damage setting the stage for complete blockage of the microcirculation with secondary involvement of the cellular elements of the blood.

With regard to the pathogenesis of renal allograft rejection, it seems obvious that circulating histocompatibility antibodies detectable by complement-dependent assay procedures will react with the corresponding antigens and thus activate the complement system. This situation exists in the sensitized recipient and results in hyperacute graft rejection. On the other hand, even very low antibody levels resulting from the humoral immune response to the grafted tissues in an unsensitized recipient might cause functional and structural disturbances leading to the typical vascular pathology of chronic rejection of organs. The fate of the graft will be influenced by the variability of the individual responsiveness of the recipient, differences in antigenic stimulation and the possible formation of non-complement-fixing (enhancing) antibodies. These same factors can also be expected to influence the effectiveness of immunosuppressive therapy. Successful prevention of hyperacute allograft rejection depends largely on the detection of existing cytotoxic antibodies and the recognition of presensitization in a potential recipient.

Therapeutically, little if anything can be expected from the currently used immunosuppressive drugs which primarily affect the cellular immune response and have no influence on existing antibodies (14). But there are some promising, though at present strictly experimental, possibilities that have been put to test in the xenograft model. Specific inactivation of the complement system can prevent complement-dependent immune injury (15) and can also result in significant prolongation of xenograft survival times (16).
COMPLEMENT AND GRAFT REJECTIONS

17. Even more effective and of greater clinical potential is the use of citrate (as ACD solution) which in our hands has under certain conditions completely prevented xenograft rejections (18) and has also been used successfully in experimental hyperacute allograft rejections (19). The effect of citrate is apparently primarily due to its anticomplementary action, which prevents effective and massive activation of the early complement components (Jensen, unpublished).

We would finally like to point out that it is the purpose of this paper to somewhat overemphasize the role of humoral factors in graft rejections and to suggest that they can join and aggravate the cellular responses. The recent emergence of discouraging data showing a lack of correlation between tissue match grade and kidney graft survival in unrelated recipients (20) might not reflect the true value of tissue typing. Any significant number of presensitized recipients who have the potential for a secondary response or have antibodies that are not detected because of their quality or quantity may suffice to completely abolish a correlation between match grade and clinical function that would exist in immunologically virgin recipients.

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References


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