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Of the group with increased serum C1$_q$-BA, serial studies in eight patients showed a correlation between increased serum C1$_q$-BA and the occurrence of rejection; with reversal by therapy, serum C1$_q$-BA returned to within normal levels. Complexes from six patients were analyzed by sucrose density gradient ultracentrifugation to have sedimentation coefficients ranging from 15S to 18.4S. After acid dissociation and analysis by double-diffusion techniques, C1$_q$-reactive complexes were shown to contain IgG. Immunofluorescent studies done in five renal biopsies from this group revealed granular deposits of immunoglobulin, and (or) less frequently, of complement in the glomeruli or the tubular basement membranes.

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CORRELATION OF INCREASED ^{125}I-C1q BINDING ACTIVITY WITH ACUTE REJECTION CHARACTERIZED BY FIBRIN DEPOSITION IN THE KIDNEY

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ABSTRACT To assess the role of circulating immune complexes in the pathogenesis of acute rejection, sera were measured for such complexes by the ^{125}I-C1q binding assay in 45 normal subjects, 24 allografted patients undergoing acute rejection, and in 11 allografted patients in a quiescent phase. Increased C1q-binding activity (C1q-BA) was detected in 14 patients with acute rejection, 9 of whom had renal biopsies showing fibrin deposition in the vasculature together with cellular infiltrates in the tubulo-interstitial structures; renal histology was not available in the other 5 patients. The other 10 patients with acute rejection, whose biopsies showed only cellular infiltrates, and the 11 patients in a quiescent phase posttransplantation did not have increased levels of serum C1q-BA.

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The findings suggest that circulating immune complexes may mediate the type of acute rejection characterized by fibrin deposition in the kidney. The role of circulating immune complexes arising from the recipient's original kidney disease could be excluded in 10 patients with humoral rejection, inasmuch as the underlying renal pathology was of a "nonimmunologic" nature; this was corroborated by sequential studies in six patients in whom circulating immune complexes could not be demonstrated before rejection. The participation of administered antilymphocyte globulin (ALG) as an antigen also appears to be excluded in four patients, two who were not given ALG, and in two of whom episodes of rejection occurred unrelated temporally to ALG administration.

INTRODUCTION

Most patients with renal allografts undergo one or more clinical episodes of acute rejection. The pathobiology of the rejection reaction is complex, and there is abundant evidence that it involves both cell-mediated and humoral mechanisms (1). The latter mechanism may include the deposition of antibodies or of soluble antigen–antibody complexes. Evidence for the role of immune complexes stems from immunofluorescence observations showing the presence of granular deposits of immunoglobulin and (or) complement in the glomerulus (2–4). A recent study also describes the detection of circulating immune complexes after renal transplantation but

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does not define the relationship of such complexes to the rejection phenomena (5).

The present communication reports the detection of circulating immune complexes, as determined by a ^{125}I-C1q binding assay, in relation to a type of acute rejection distinguished morphologically by the deposition of fibrin in the renal microvasculature; it analyzes some of the characteristics of these complexes and their relationship to serum levels of the various components of complement.

METHODS

Definitions

In this paper, humoral rejection refers to episodes in which circulating immune complexes were demonstrable by the ^{125}I-C1q binding assay. Cellular rejection refers to instances in which clinical and laboratory features of rejection occurred but in which circulating immune complexes were not detectable by the same assay.

Sera

Sera were obtained from the following populations: (*a*) normal healthy subjects (45 samples); (*b*) renal allografted patients undergoing clinical acute rejection, 24 sera being obtained from different patients at the time of rejection (days 5–235 posttransplantation); in addition, 8 of the 14 patients with humoral rejection and 4 of the 10 patients with cellular rejection were studied sequentially, with another 54 sera being analyzed; and (*c*) allografted patients with stable renal function attending the Transplant Clinic (11 samples). All sera were stored in 1–2-ml aliquots at −70°C, and were thawed only once, when ready to be used.

Clinical data of 24 patients undergoing rejection

The original diseases giving rise to end-stage renal failure in the patients were the following: (*a*) patients with humoral rejection—three with diabetic glomerulosclerosis, one with hereditary nephritis, one with polycystic kidneys, two with hypertensive nephrosclerosis, two with probable chronic glomerulonephritis, two with reflux pyelonephritis, and three with renal disease of undetermined etiology; and (*b*) patients with cellular rejection—two with diabetic glomerulosclerosis, two with chronic interstitial nephritis, one with hypertensive nephrosclerosis, two with probable chronic glomerulonephritis, and three with renal disease of undetermined etiology.

Patients were treated with a standard immunosuppressive regimen, consisting of prednisone, azathioprine, and antilymphocytic globulin (ALG),[1] the last being given during the first 3 wk posttransplantation. In four patients undergoing "humoral" rejection, ALG was omitted in two patients, and in two others, the episodes of rejection studied occurred 39 and 80 days after cessation of the ALG therapy.

The clinical diagnosis of rejection was made independently by the physicians and surgeons managing the patients. Clinical and laboratory criteria for the diagnosis of rejection consisted of combinations of the following: (*a*) tenderness, with enlargement of the graft; (*b*) oliguria; (*c*) fever; (*d*) increase in level of serum creatinine by >0.2 mg/100 ml or failure of predicted decline in serum creatinine; (*e*) concomitant change in blood urea nitrogen (BUN); and (*f*) decreased urinary sodium. When indicated, renal scan, renal arteriography, and retrograde pyelography were done to exclude the presence of other conditions responsible for renal functional deterioration. Renal biopsies, done in 19 of the 24 rejection episodes, confirmed the diagnosis in all instances.

Methods of histologic evaluation

Light microscopy. Renal tissue, obtained by percutaneous biopsy in 19 patients, was fixed in Helly's solution, and sections were stained with hematoxylin and eosin, periodic acid-Schiff, Masson's trichrome, silver methanamine, and Lendrum. Fibrin was identified on light microscopy by the presence of material staining with the Lendrum reagent.

The histopathologic changes were assessed semiquantitatively by two observers independently, and without knowledge of the results of the assay for circulating immune complexes. Particular attention was paid to the presence of fibrin deposition in the microvasculature.

Immunofluorescence microscopy. In a few cases, a portion of renal biopsy material was used for immunofluorescence studies, employing a previously described technique (6). The antisera were directed against IgG (γ-chain), IgA (α-chain), IgM (μ-chain), β_1C globulin, and fibrinogen. Each antiserum gave a single precipitin arc when tested by immunoelectrophoresis against normal human serum. Antisera were conjugated with fluorescein isothiocyanate (7), and a single layering technique was used. Appropriate blocking experiments were also performed.

Isolation and labeling of C1q

C1q was isolated from human serum by the method of Yonemasu and Stroud (8). The purity of the preparation was established by the demonstration of a single precipitin line on double immunodiffusion against a highly polyvalent goat antiserum to whole human serum. There was no evidence of contamination with IgG, IgA, or IgM by radial immunodiffusion using monospecific antibody to these proteins. The isolated protein was labeled with ^{125}I (Radiochemical Centre, Amersham, England) according to a modified lactoperoxidase technique (9), and the specific radioactivity was 0.08–0.2 μCi/μg C1q. The labeled material produced a single peak of radioactivity by cellulose acetate electrophoretic analysis, and by sucrose density gradient analysis.

The ^{125}I-C1q was stored in 0.2-ml aliquots at −70°C until used. Immediately before use, the preparation was rapidly thawed in a 37°C water bath and centrifuged at 25,000 *g* for 30 min to remove aggregates.

Detection of immune complexes by C1q binding assay

The method for detection of circulating immune complexes was the assay described by Nydegger et al. (10) with slight modification. Before use, the serum to be analyzed was heated to 56°C for 30 min and immediately cooled in an ice-water bath. To 0.1 ml of the heated test serum was added 0.1 ml of Veronal-buffered saline, pH 7.4, contain-

[1] *Abbreviations used in this paper*: ALG, antilymphocytic globulin; C1q-BA, binding activity of C1q; HSA, human serum albumin.

ing 0.005 M Veronal (Winthrop Laboratories, Sterling Drug Co., New York) and 0.1 M NaCl, followed by 100 ng ^{125}I-C1q. This mixture was incubated at room temperature for 60 min and then at 4°C for another 60 min. At the end of this incubation, polyethylene glycol (average mol wt 6,000) was added to a final volume of 2 ml in a final concentration of 2.5%. Incubation was at 4°C for another 2 h. The tubes were then centrifuged at 2,000 *g* for 20 min. An equal volume of the upper half of the supernate (*s* fraction) was carefully transferred off, leaving precipitate and the remainder of the supernate (*r* fraction). The radioactivity of both fractions was counted in an automatic gamma counter. C1q-binding activity (C1q-BA) was calculated as percent of ^{125}I-C1q precipitated according to the formula $(r - s)/(r + s) \times 100$. All samples were tested in duplicate. Serum specimens from five normal subjects and two sera containing 100 and 500 μg/ml of aggregated human gamma globulin were included in each test run. The coefficient of variation of the technique was 4.2 and 4.9% for specimens with mean values of C1q-BA of 25 and 48%, respectively, 25 replicate samples of each specimen being assayed.

The procedure was also performed, reacting ^{125}I-C1q with varying amounts of heat-aggregated human gamma globulin. Aggregated human globulin was prepared from IgG purified by DEAE-chromatography and aggregated at 63°C for 20 min. The lower limit of sensitivy of the assay in terms of heat-aggregated human IgG was ≅75 μg/ml. Additionally, to determine if DNA would interfere with the test, native DNA and single-stranded DNA were added in concentrations of 0.5–200 μg/ml to normal human serum with repetition of the assay. At those concentrations, increased ^{125}I-C1q precipitation was not observed. Single-stranded DNA was prepared by heating DNA (calf thymus—Worthington Biochemical Corp., Freehold, N. J.) to 100°C for 10 min, followed by immediate cooling to 0°C in an ice-water bath.

Sucrose density ultracentrifugation of C1q-reactive material

For this procedure, a 0.2-ml aliquot of the reaction mixture between ^{125}I-C1q and test sera prepared as above, and without the addition of polyethylene glycol, was layered on a 10–40% linear sucrose density gradient in Veronal-buffered saline, and centrifuged in a Spinco ultracentrifuge, model L2 (Beckman Instrument, Inc., Spinco Div., Palo Alto, Calif.), with an SW 50.1 rotor at 35,000 rpm for 24 h at 4°C. Fractions collected through a hole in the bottom of the tube were counted for radioactivity. Markers employed were human IgG (6.8S), human IgM (19S), human serum albumin (HSA, 4.6S), and catalase (11.2S). IgG, IgM, and HSA were measured by radial immunodiffusion and catalase by the disappearance of peroxide as measured spectrophotometrically at 240 nm.

Analysis of C1q binding complexes isolated by sucrose gradient analysis

Corresponding fractions obtained by repeated sucrose gradient analyses on a single serum specimen were pooled, dialyzed at 4°C against 0.05 M ammonium formate buffer, pH 7.4, lyophilized, and stored at −70°C until used. By this method, the complexes binding ^{125}I-C1q contained in 1.2–1.5 ml of serum could be isolated. For analysis, the lyophilized pooled fractions were restored to a volume of 0.5 ml with neutral phosphate-buffered saline and dialyzed against 0.2 M glycine buffer, pH 3.2. All fractions were tested for the presence of IgG, IgM, and IgA by double diffusion or by radial immunodiffusion using monospecific antiserum to each immunoglobulin. The protein concentration of the pooled fractions containing complexes was 1–2 mg/ml.

Measurement of levels of complement components

Serum levels of C1q, C4, C3, properdin, and factor B were measured by radial immunodiffusion. The production of monospecific antiserum to each complement component has been previously described (11).

RESULTS

Classification of patients by histological analysis

Light microscopy. Of 19 renal biopsies evaluated, nine were found to have focal and segmental fibrin deposition in the glomeruli and (or) arterioles. In all instances, infiltration of tubulointerstitial struc-

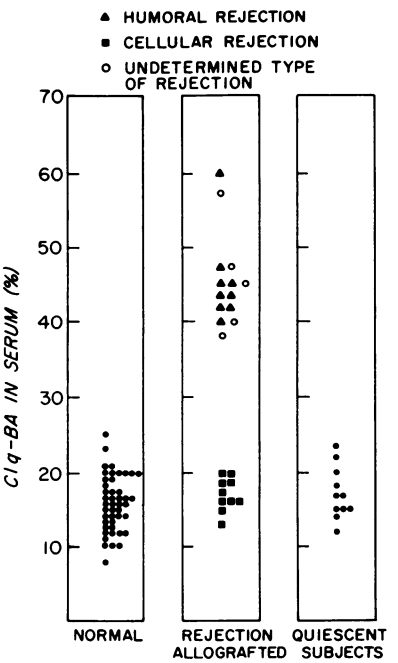

FIGURE 1 C1q-BA of normal sera and sera from patients after renal allografting. Values of C1q-BA for normal sera are 16.4±7.2 (mean±2 SD). Humoral rejection refers to episodes in which renal biopsies disclosed fibrin deposition in the renal microvasculature, together with cellular infiltrates. Cellular rejection refers to episodes in which renal biopsies showed only cellular infiltrates, no fibrin being visualized. Undetermined type of rejection refers to episodes diagnosed clinically, no renal histology being available. Note that increased C1q-BA was observed in nine sera from patients with humoral rejection, and in five from the patients of the undetermined category.

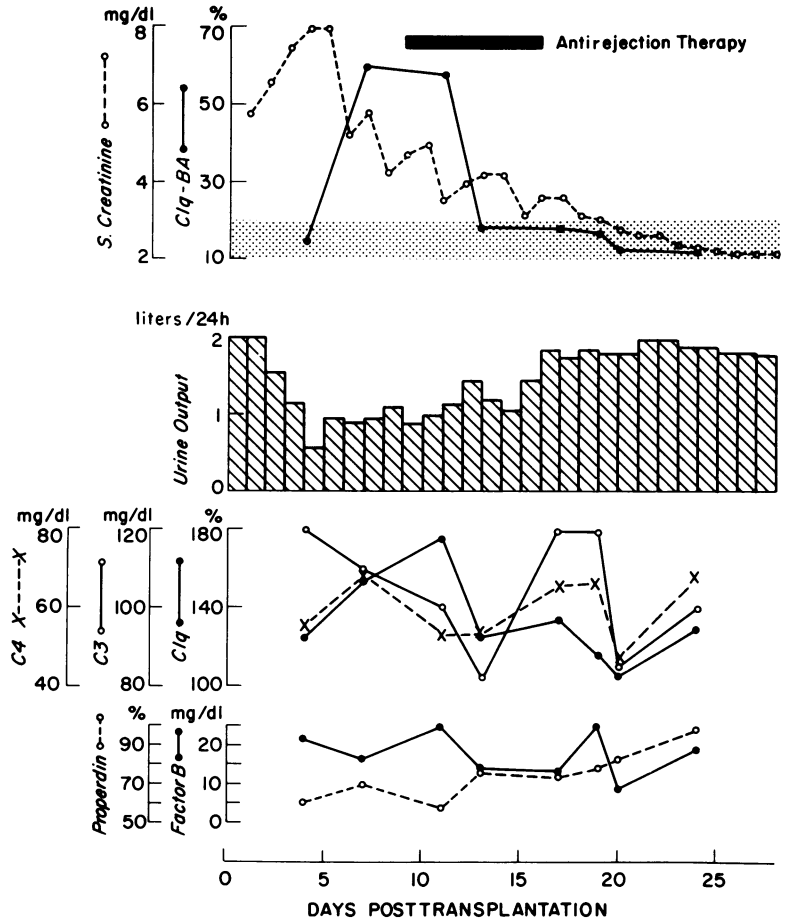

FIGURE 2 Relationship between levels of C1q-BA and clinical and laboratory parameters of rejection of patient T. V. Clinical rejection was diagnosed on day 9, and therapy was instituted. Increased serum C1q-BA (shaded areas in uppermost panel represent normal range) was detected on days 7 and 11, and rapidly returned to normal, coincident with clinical resolution. Serum C3 levels showed a definite fall at the time of rejection. Other measured levels of complement components displayed variable fluctuations. (Normal values—mean±2 SD—were: C1q-BA 16.4±7.2%, C1q 94–130% of normal; C4 26–72 mg/dl; C3 97–268 mg/dl; and properdin 67–126% of normal; factor B 15.5–33 mg/dl.)

tures by mononuclear cells was also present from mild to moderate degree. Renal histological examination of the other 10 biopsies revealed only cellular infiltration of the tubulointerstitial structures, no fibrin in the vasculature being detected.

Immunofluorescence microscopy. Nine renal biopsies, containing fewer glomeruli then were present in sections examined by light microscopy, were available for immunofluorescence studies. Five of these biopsies were from the group in which fibrin was demonstrated by light microscopy. In this group, in addition to fibrinogen, focal granular deposits of immunoglobulin and/or β_1C globulin were detected in glomerular capillary cells in four biopsies; in the fifth, granular deposits of β_1C globulin only were present in tubular basement membrane. Deposits in the glomerular capillary cells consisted of IgG (two biopsies), IgM (one biopsy), IgG and IgM (one biopsy), and IgG, IgM, and β_1C globulin (one biopsy). The other four biopsies were from the group in which no fibrin was visualized in the microvasculature. Immunohistochemical studies did not reveal the presence of any deposited plasma proteins.

C1q-BA in serum samples

The C1q-BA of sera from normal subjects, patients undergoing rejection, and allografted patients in the quiescent period are shown in Fig. 1. The values for C1q-BA of 45 normal sera were 16.4±7.2 (mean±2 SD). A level of serum C1q-BA exceeding 23.6, greater than mean±2 SD, was considered indicative of in-

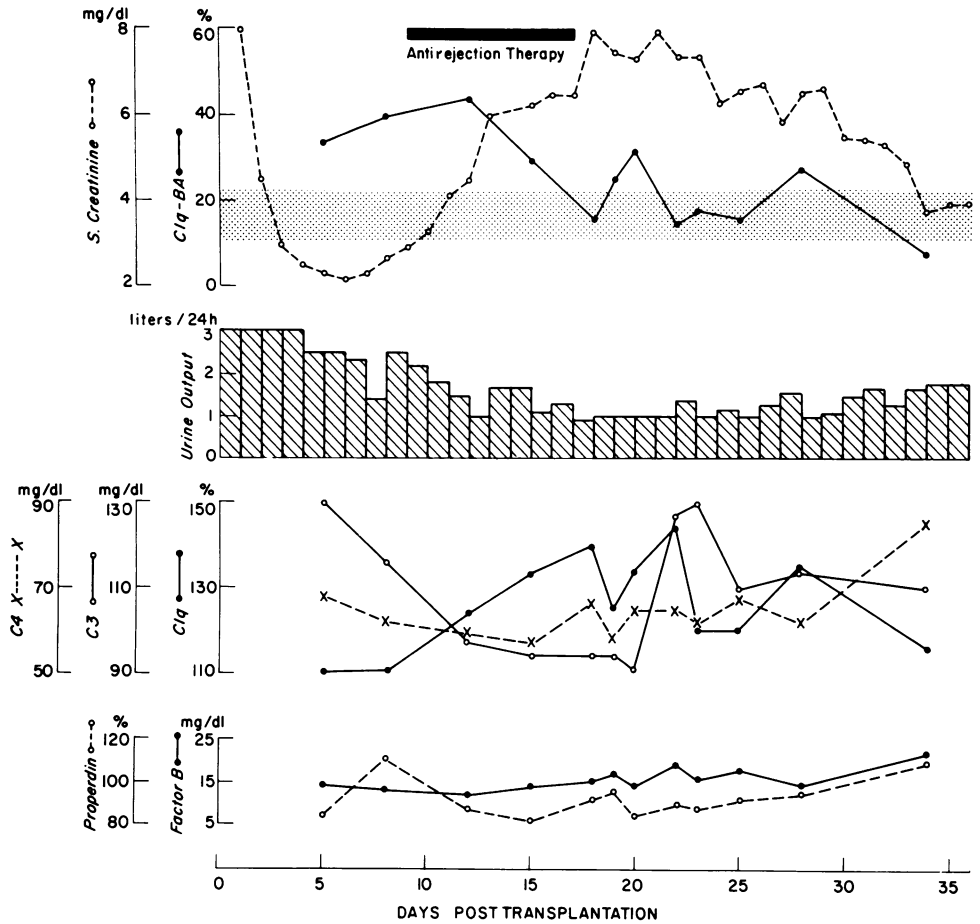

FIGURE 3 Relationship of serum C1q-BA to clinical and laboratory events of a more protracted rejection episode in patient B. G. Clinical rejection was diagnosed on day 9 (confirmed by biopsies on days 11 and 19), with resolution beginning to occur on day 30. Elevated serum C1q-BA (shaded areas in uppermost panel represent normal range) was observed on days 5, 8, 12, and 15; thereafter, fluctuating levels were detected. Serum C3 levels fell during the period of rejection. Levels of C1q, C4, properdin, and factor B were more variable. (Normal values of C1q-BA are 16.4±7.2—mean ±2 SD.)

creased binding activity. C1q-BA of sera from patients undergoing allograft rejection represent single sera from 24 different rejection episodes. In 12 patients in whom sequential measurements were made, the C1q-BA of the first serum sample when rejection was unequivocally diagnosed was used for this plot. Sera from 10 patients whose renal histology showed only features of cellular rejection exhibited normal C1q-BA. Sera from nine patients whose renal biopsies showed evidence of fibrin deposition, and from five patients in whom no renal histology was available showed increased levels of C1q-BA ranging from 38 to 60%. These levels of C1q-BA correspond approximately to equivalent concentrations of 500–900 μg/ml of aggregated human IgG. In comparison, the C1q-BA of sera from 11 allografted patients in the quiescent period was within normal limits.

Sequential measurements of serum C1q-BA

42 sera derived serially from eight patients with humoral rejection were also studied. The clinical course, measurements of serum C1q-BA, and various levels of complement components of two of these patients are shown in Fig. 2 (patient T. V.) and in Fig. 3 (patient B. G.). In patient T. V. (Fig. 2) there was an early period (days 1–8) of renal functional impairment attributed to acute tubular necrosis. A serum sample obtained early in this period (day 4) showed normal serum C1q-BA. On day 7, a serum C1q-BA of 55% was detected, antedating the clinical diagnosis of rejection by 2 days. A sample obtained during the rejection period (day 11) continued to show an elevated serum C1q-BA of 50%; with anti-rejection therapy, the levels of serum C1q-BA returned to

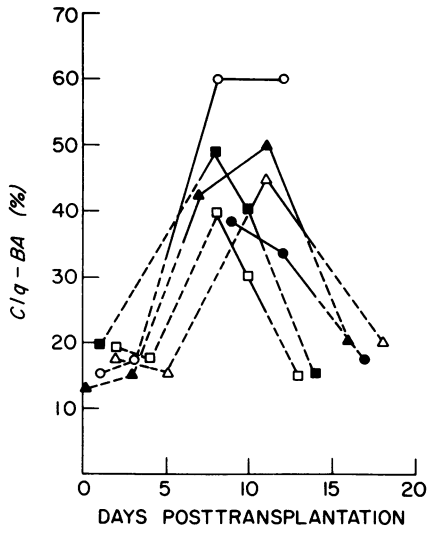

FIGURE 4 Sequential studies of serum C1q-BA of six patients after renal allografting. Normal values of C1q-BA were 16.4±7.2 (mean ±2 SD). The interrupted lines represent periods before or after treated rejection. The continuous lines represent periods when rejection was unequivocally diagnosed and treated. Note the presence of increased serum C1q-BA during rejection episodes, as compared with levels during pre- and post-rejection periods.

normal levels, parallel with the improvement of renal function.

In patient B. G. (Fig. 3), who experienced a protracted rejection episode, abnormal values for serum C1q-BA of 34 and 40% were obtained on days 5 and 8, respectively, the first abnormal value for serum C1q-BA antedating the diagnosis of clinical rejection by 4 days. Thereafter, despite treatment, renal functional impairment persisted, a biopsy during this period showing fibrin deposition in the glomerular capillaries together with mild cellular infiltration. Fluctuations of serum C1q-BA levels were noted, five of nine samples exhibiting increased levels of serum C1q-BA. By the 30th day, resolution of the rejection crisis occurred, concordant with a normal value for serum C1q-BA.

Serial assays of serum C1q-BA in the remaining six patients are shown in Fig. 4. Increased values of serum C1q-BA were recorded in all patients during the period of rejection; with successful therapy, serum C1q-BA returned to within normal limits. Samples obtained from five patients soon after transplantation and before the onset of rejection showed normal serum C1q-BA; early samples were not available for the sixth patient.

Sequential studies of 12 sera from four patients undergoing cellular rejection showed normal C1q-BA for all specimens.

Complement studies

Serial studies in eight patients showed a relative fall of serum C3 levels at the time of humoral rejection. Less consistent relationships were obtained with serum C1q, C4, properdin, and factor B levels. By regression analysis, significant correlations were not obtained between levels of serum C1q-BA and levels of complement components measured. However, a significant correlation was derived between serum C4 and C3 levels ($r = 0.62$ $P < 0.01$).

Characteristics of C1q-reactive complexes

Sucrose density gradient ultracentrifugation analysis of six C1q-reactive complexes from different patients showed them to have S values of 15S, 15.6S, 16.8S, 17S, 18.1S, and 18.4S. Two representative analyses are depicted in Fig. 5.

Analysis of the composition of the six complexes after acid dissociation (as described in Methods) showed them to contain IgG in all instances.

DISCUSSION

The rejection of a renal allograft involves both cellular and humoral mechanisms; morphologic studies have suggested that each mechanism may result in distinctive histopathologic features (12). In predominantly cellular rejection, the principal change is one of infiltration of the tubulointerstitial structures with lymphocytes and other mononuclear cells; in humoral rejection, the dominant characteristic is the deposition of fibrin and microthrombi formation in the renal microvasculature (12). It is recognized that overlap may frequently occur. The present study produced evidence that in the type of acute rejection characterized by fibrin deposition in the renal microvasculature, the responsible humoral mechanism may be the deposition of the circulating immune complexes. The presence of such complexes in the circulation was shown by a ^{125}I-C1q binding assay, the sensitivity and reproducibility of which has been previously validated in other laboratories in studies of sera from patients with systemic lupus erythematosus, carriers of hepatitis-associated antigen (10), rheumatoid arthritis (13), and by our studies of sera from patients with lupus nephritis, membranoproliferative glomerulonephritis, and other types of nephritides (14). The demonstration that C1q-reactive material, after acid dissociation, contained immunoglobulin suggests that antigen–antibody complexes were detected by this test. Interference by nonspecific binding of C1q to DNA or endotoxin can be effectively ruled out by experiments done in this investigation and by other laboratories (10, 15). The presence of endotoxin is

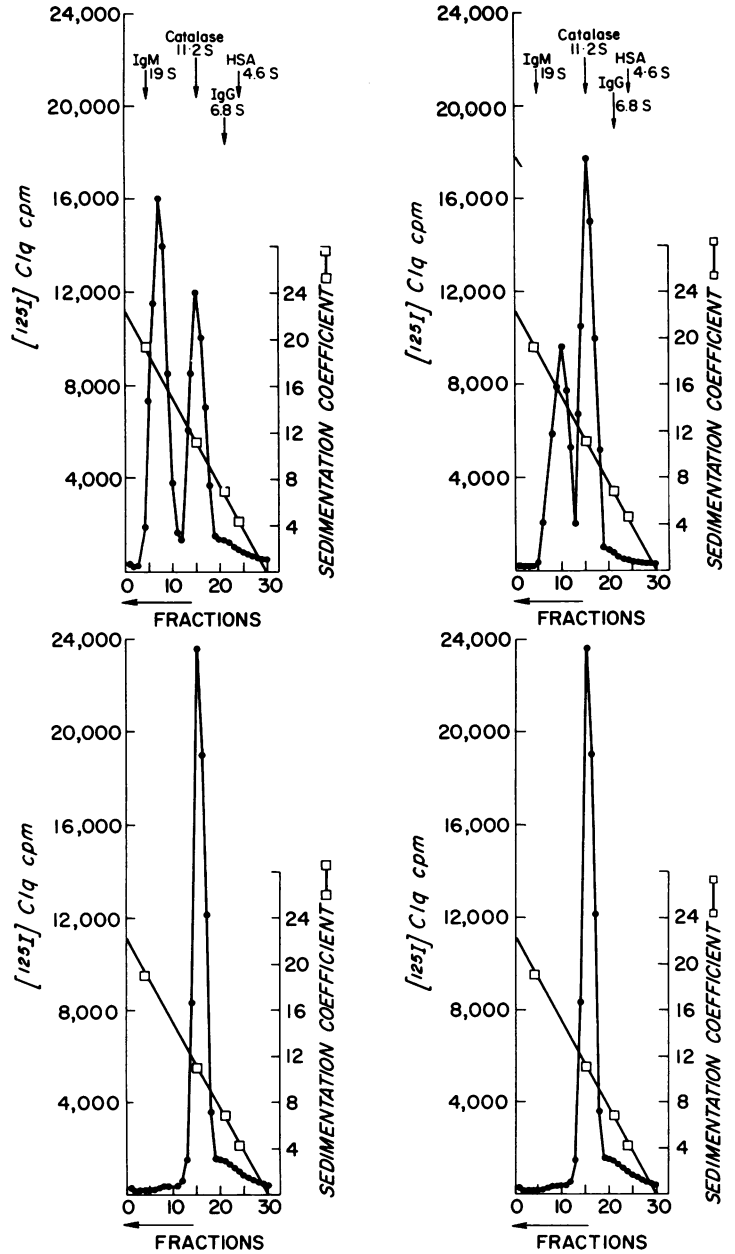

FIGURE 5 Sucrose density ultracentrifugation analysis of two representative ^{125}I-C1q-reactive complexes. Each of the two upper panels (reaction mixture consisting of C1q and transplant sera) shows two peaks, one corresponding to C1q (11S region) and the other to material complexed to C1q, with calculated sedimentation coefficients of 17S (upper left) and 15S (upper right). Each of the lower panels shows only one peak, corresponding to C1q, when this reagent was reacted with normal serum.

also unlikely from clinical consideration, viz., absence of laboratory features of infection, and response to therapy without antibiotics. Such complexes were not demonstrated in the sera of those patients whose biopsies revealed features only of cellular rejection and were absent from sera of allografted patients in a quiescent period. Serial observations in some patients suggest that complexes may be found in the circulation before clinical evidence of rejection, suggesting that it may have diagnostic value as a predictor of this event. In addition, our limited studies suggest that a sustained response to treatment is

accompanied by the rapid disappearance of complexes; by contrast, in a rejection crisis relatively more resistant to treatment , circulating complexes continued to be generated. The sizes of the complexes are consistent with those defined in animal models as capable of producing nephritis (16).

The demonstration by immunofluorescence microscopy of granular deposits of immunoglobulin and (or) less frequently of C3 in the renal biopsies of five of the patients with increased C1q-BA provides morphologic confirmation that soluble immune complexes may mediate glomerular injury in this type of rejection. These findings are supported by previous immunofluorescence studies reporting the presence of such deposits in a high proportion of renal biopsies obtained during a rejection episode (2–4).

Inferential evidence for the occurrence of an antigen–antibody reaction during a rejection crisis comes from measurements of serum levels of various complement components (17–21). Although levels have been found to be unstable and fluctuating, various laboratories have documented a fall of the serum levels of the earlier components of complement, indicating activation of the classical pathway of complement. Our studies conform to this pattern, a fall of serum C3 levels occurring during rejection and a significant correlation being found between serum and C3 and C4 levels.

The induction of fibrin deposition in the microvasculature of the kidney in a process mediated by circulating immune complexes is not unusual, inasmuch as this is a finding in many types of glomerulonephritis. Positive interactions between antigen–antibody complexes, complement, and the coagulation system have been identified and explored in several laboratory and animal models (22). Of special relevance may be the animal model in which infusion of a small amount of antigen into a hyperimmunized animal produced acute cortical necrosis with extensive fibrin deposition (23).

Finally, it is conceded that the current study demonstrates only an association between the presence of circulating immune complexes and the occurrence of rejection characterized by fibrin deposition in the kidney. Alternative theoretical possibilities are that immune complexes may represent bystander phenomena unrelated to the rejection process. It is also conceivable that the appearance of such complexes may be induced consequent to tissue damage by the rejection process or by therapy administered. There is, however, no evidence to suggest that steroid therapy will per se provoke the appearance of immune complexes. The role of ALG as an antigen appears to be excluded in at least four patients, two of whom were not given ALG, and in two others, in whom the episodes of rejection occurred temporally unrelated to ALG administration. The role of preexisting immune complexes also appears to be excluded in 10 patients with humoral rejection, in that their original kidney disease was of a "nonimmunologic" nature; confirmation was obtained from studies in 6 patients in whom assays of C1q-BA in the early period posttransplantation did not reveal the presence of circulating immune complexes. Further studies will be needed to elucidate the nature of the proximate antigen eliciting this sequence of events.

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