IMMUNOFLUORESCENT EXAMINATION OF BIOPSIES FROM LONG-TERM RENAL ALLOGRAFTS*

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Abstract Immunofluorescent examination of open renal biopsies revealed clear-cut glomerular localization of immunoglobulins not related clearly to the quality of donor-recipient histocompatibility in 19 of 34 renal allografts. The biopsies were obtained 18 to 31 months after transplantations primarily from related donors with a variable quality of histocompatibility match. IgG was the predominant immunoglobulin class fixed in 13 biopsies, and IgM in six. The pattern of immunoglobulin deposition was linear, connoting anti-GBM antibody in four of the 19; it was granular and discontinuous, connoting antigen-antibody-complex deposits, in 13. An immune process may affect glomeruli of renal allografts by mechanisms comparable to those that cause glomerulonephritis in native kidneys. The transplant glomerulonephritis may represent a persistence of the same disease that originally destroyed the host kidneys or the consequence of a new humoral antibody response to allograft antigens.

THE occurrence of glomerular lesions in viable renal allografts has been well documented by light and electron microscopy of biopsy material.[1-4] Understanding of the pathogenesis for the morphologic changes, however, is not clear. In particular, uncertainty exists concerning the role of well defined mechanisms[5,6] known to mediate glomerulonephritis in native kidneys in causing glomerular disease in the transplanted kidney.

It is the purpose of this report to present results of immunofluorescent examinations of 34 human renal allografts. These observations are derived from a series of renal grafts, functional 18 to 31 months after implantation. All but three of the kidneys were from living, related donors, and all the recipients were treated initially with antilymphocyte globulin (ALG).[7]

The results indicate considerable focal or generalized immunoglobulin deposits in glomeruli of over half the allograft biopsies studied, and document the occurrence of antiglomerular basement-membrane (anti-GBM) antibodies in 20 per cent of the glomeruli showing such fixation. Although immunoglobulin M (IgM) occasionally was found in the absence of immunoglobulin G (IgG), IgM deposits were usually in the company of IgG deposits, often of a different distribution and distinctly less extensive.

The data are compatible with the hypothesis that the glomerular lesions observed are the result of conventional mechanisms known to cause glomerulonephritis in native kidneys. Such a hypothesis suggests that the glomerular injury in the allografts is either a result of the same antibody responsible for the pre-existing processes that originally destroyed the patient's own kidneys or a de novo humoral-antibody response to the alien antigens of the new organ.

Materials and Methods

Thirty-five patients receiving renal allografts at Colorado University Medical Center between June 21, 1966, and August 25, 1967, were readmitted to the Center in January, 1969, for routine re-evaluation and biopsy of the transplants. An additional patient with cystinosis who had had a transplant only six months before was also included. Open surgical biopsies were performed under local anesthesia, and tissue prepared promptly for light, electron and immunofluorescent microscopy; results of the light and electron microscopy are detailed elsewhere.[8] Biopsies for immunofluorescent study were frozen in liquid nitrogen and stored at −20°C until examined. Sections 6 μ in thickness were cut in a Harris cryostat, and immunofluorescent testing was done by the technic of Coons and Kaplan[9] as previously described.[10] Tissue from two of the 36 patients biopsied was insufficient for all examinations; hence, data presented include only the 34 with satisfactory studies. Reagents used were antiserums made in rabbits to 7S human IgG, IgM (μ-chain specific), β_{1C} component of complement (C′), fibrinogen and albumin. Rabbit antiequine globulin was obtained commercially,† as was rabbit anti-IgA (α-chain specific).‡ Specificity of antiserums was assured by analyses by double diffusion in agarose and immunoelectrophoresis. Before labeling with fluorescein, IgG fractions of antiserums were isolated by fractionation with neutral ammonium sulfate at half saturation in the cold followed by chromatography at pH 6.5 on diethylaminoethyl (DEAE) cellulose columns equilibrated with phosphate buffer, 0.0175 M. Conjugation of proteins with fluorescein

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isothiocyanate was done by the dialysis method of Clark and Shepard[11]; subsequently, they were rechromatographed on DEAE, and the conjugate eluting at 0.05 M phosphate buffer, pH 7.4, was used after suitable concentration. Antiserum against alpha chains of IgA was used by indirect immunofluorescence, fluorescein-conjugated sheep anti-rabbit IgG being used as the final reagent. Specificity of immunofluorescent observations was confirmed by absorption of labeled antiserums with specific antigens, blocking of positive reactions with unconjugated antiserums and use of an antihuman serum albumin control. Intensity of immunofluorescence observed was graded from 0 to 3+.

Results

IgG

Nineteen of the 34 biopsies showed no remarkable glomerular fixation of IgG. Fifteen biopsies, however, demonstrated IgG deposits of varying intensity and localization (Table 1). Four of the 15 disclosed linear fixation of IgG to glomeruli, characteristic of anti-GBM antibodies (Fig. 1); nine had discontinuous, granular deposits, typical of antigen-antibody-complex nephritis (Fig. 2), and two had faint fluorescence of an indistinct, focal, lobular pattern (Table 1). Two of the nine biopsies with granular-type patterns of IgG deposition were distinctly focal in distribution, with some parts of glomeruli and, often, whole glomeruli spared completely (Fig. 3). Although almost all biopsies showing a granular pattern had some mesangial as well as peripheral capillary-loop deposits, one (Case 17) had a predominantly mesangial localization, with relatively few deposits along capillary walls.

FIGURE 1. *Photomicrograph of Immunofluorescence of Typical Linear Fixation of IgG on Glomerular Walls of Case 2.*

IgM

Deposits of IgM occurred in glomeruli in association with IgG (seven of 15 biopsies) and in the ab-

TABLE 1. *Renal Allografts in Which Immunoglobulins in Glomeruli Were Localized by Fluorescent Microscopy.*

Case No.	Immunofluorescent Intensity of Glomerular Deposits					Donor	Tissue-Typing Match*	Interval between Operation & Biopsy	Original Disease†	Blood Urea Nitrogen	Protein Excretion
	IgG	*IgM*	*IgA*	β_{1c}	*fibrinogen*			*mo*		*mg/100 ml*	*gm/24 hr*
Linear pattern of immunofluorescence:											
1	3+	1+(F)‡	TR§	1-2+	TR	Brother	A	20	CGN	20	3.9
2	3+	–	–	2-3+	–	Brother	B	23	CPN; ?CGN	24	0.3
3	1+	–	–	1+		Brother	B	23	CGN	12	0
4	TR	TR	–	TR	TR	Brother	C	30	CGN	25	0.07
Granular pattern, generalized:											
5	3+	2+	–	3+	TR	Sister	C–	30	CGN	23	2.2
6	3+	–	–	TR		Brother	B	30	CGN	28	2.7
7	2+	–	–	TR	–	Brother	C	24	CGN	40	6.5
8	2+	–	–	TR	–	Sister	A	21	CGN	62	1.7
9	2+(F)‡	2+	–	TR	TR	Brother	C	30	CGN	22	0
10	TR	2+	–	TR	–	Cadaver	F	18	CGN	37	2.8
11	1+	1+	TR	TR	–	Mother	C	20	CGN	40	0.2
12	–	TR	–	1+	–	Cadaver	D–	6	Cystinosis	43	0
13	–	TR	–	2+	–	Brother	C–	19	CGN	22	0.12
Granular pattern, focal:											
14	1+	–	–	–	–	Brother	A	18	CGN	49	1.6
15	–	1+	–	1-2+	–	Father	A	25	CGN	33	0
16	–	TR	–	TR	–	Brother	A	30	CGN	23	0
Granular pattern, primarily mesangial:											
17	2+	1+	–	1+	–	Sister	D	25	CGN	23	0.7
Indeterminate pattern:											
18	TR	–	–	–	TR	Father	D	18	CGN	46	0.8
19	TR	–	–	–		Sister	B	22	CGN	22	0.06

*A indicates histocompatibility identity, B no overt mismatches of known HLA alleles (but with incompatibilities of unclassified antiserums), C direct mismatch of usually 1 & sometimes 2 HLA antigens, D 2 or more HLA mismatches, & F transplantation in face of preformed lymphocytotoxic antibodies.

†CGN indicates chronic glomerulonephritis, CPN chronic pyelonephritis & CRD unclassified chronic renal disease.

‡(F) = focal.

§TR = focal.

FIGURE 2. *Photomicrograph of Immunofluorescence of Typical Discontinuous, Granular Deposits of IgG Found on Glomerular Capillaries in the Biopsy of Case 5.*

FIGURE 3. *Photomicrograph of Immunofluorescence of Focal Deposits of IgG as Visualized in Glomeruli of Case 9 (the Same Biopsy Was Stained for Presence of IgM and Showed a Typical Diffuse Granular Pattern).*

sence of concomitant IgG (four cases). Where deposits of the different immunoglobulin classes occurred in the same glomerulus, IgG was usually more extensive; in addition, IgM deposits were smaller and more often in the mesangium in such circumstances. However, in three biopsies (Cases 9, 10 and 11) IgM granular deposits were clearly more extensive and of the predominant immunoglobulin class. One of the four biopsies exhibiting IgM deposits in the absence of IgG had rare focal deposits; another had faint deposits. The remaining two, though not severely involved, had more important capillary-loop deposits. Linear IgM localization was seen only in Cases 1 and 4, in which anti-IgM reagent gave a patchy, focal, linear stain, whereas IgG was fixed to all glomerular capillary basement membranes in a typical, continuously linear manner. In addition, foci of IgM deposition were seen rarely in two biopsies (Cases 6 and 11) in walls of small arteries; in Case 11 localization appeared to be in afferent or efferent arterioles.

IgA

IgA was detected in glomerular deposits only twice, both times in the company of more extensive IgG and IgM fixation.

β_{1C} was detected by fluoresceinated antiserums more frequently than immunoglobulins or fibrinogen. Only nine of 34 specimens failed to show some β_{1C} fixation (Tables 1 and 2). The principal site of fixation in 14 of the 25 positive biopsies was to the glomeruli, in association with detectable IgG and IgM. Three (Cases 20, 21 and 22) biopsies showed

TABLE 2. *Renal Allografts in Which Immunoglobulins Were Not Localized in Glomeruli by Immunofluorescence.*

Case No.	Immunofluorescent Intensity*		Donor	Tissue-Typing Match†	Interval between Operation & Biopsy	Original Disease†	Blood Urea Nitrogen	Protein Excretion
	β_{1C}	fibrinogen			mo		mg/100 ml	gm/24 hr
20	1+(G,A)	–	Sister	A	30	CGN	16	1.8
21	TR(G,T)	–	Brother	A–	24	CGN	61	0.7
22	TR(G)	TR	Mother	B	28	CGN	18	0
23	2+(A)	–	Brother	B–	21	CGN	27	0
24	2+(A)		Brother	B	27	CRD	27	0.06
25	1+(A)	–	Brother	C	22	CGN	32	3.8
26	TR(A)	–	Brother	C–	28	CRD	20	0
27	TR(A)	–	Brother	B	31	CGN	13	0
28	TR(T)	1+	Cousin	A–	24	CGN	24	0
29	–	–	Mother	B+	26	CGN	27	0.1
30	–	–	Sister	B+	24	CGN	14	0
31	–	TR	Brother	C	19	CRD	57	3.7
32	–	–	Father	B–	27	Medullary cystic disease	24	0.4
33	–		Sister	B	21	CGN	26	0
34	–	–	Father	C	18	CPN	24	0
35	N		Mother	C	30	CGN	23	0
36	N		Uncle	B	27	CGN	17	0.2

*G indicates glomerular, A arteriolar, T peritubular, & N insufficient tissue for determination of intensity.

†See Table 1.

focal, granular localization of β_{1C} to mesangium or glomerular capillary walls in the absence of immunoglobulin deposition. Fluorescent demonstration of β_{1C} fixation to subendothelial areas and the medial coat of arterioles was frequently encountered, almost always in the absence of detectable immunoglobulins. In some cases, such fixation was extensive; rarely, was it detected in the medial coat of larger vessels, although such vessels were often included in the biopsy. Focal peritubular localization of β_{1C} was seen in six biopsies (Cases 1, 12, 18, 21, 24 and 28); only Case 1 had concomitant fixation of IgG to tubular basement membrane.

Fibrinogen and Fibrin

Twenty-one of 29 biopsies tested gave no indication of fibrin or fibrinogen in glomeruli. However, eight biopsies showed some fibrinogen deposits, usually in the glomerular mesangia. When fibrinogen was detected in glomeruli, it was focal and associated with detectable immunoglobulin or β_{1C} fixation. Two biopsies (Cases 6 and 11) disclosed rare, small foci of fibrinogen in arteriolar or arterial walls. The same two biopsies also showed focal fixation of IgM in vascular walls.

Equine Globulin

All biopsies showing granular deposits were tested for the presence of equine globulin and, as expected, all were negative.

Discussion

The present data suggest participation of immunoglobulins in the injury to glomeruli of chronically tolerated renal allografts transplanted from related, living donors. The extent of the immunoglobulin deposits was not related to the quality of the histocompatibility matching.

Previous reports of immunofluorescent observations of human renal allografts have been weakened by three principal qualifications: failure to define the patterns of immunoglobulin deposition and their relative frequency[12]; failure to define the class as well as the intensity of immunoglobulins in the deposit[13]; and confusion introduced by the acute events relating to active or recent surges of allograft immunity as reflected in clinical episodes of rejection.[12,13]

This study sought to establish with clarity immunofluorescent observations in a relatively homogeneous group of long-term renal allografts at a point in time remote from recent rejection episodes. Such an attempt was made here in a large series of patients with surviving allografts, all treated initially with antilymphocyte globulin as well as azathioprine and steroids over their entire clinical course.

The data indicate that appreciable immunoglobulin deposition in glomeruli is found in over half of such allografted kidneys. Moreover, they indicate that linear fixation characteristic of specific anti-GBM antibody occurs, as well as granular, discontinuous deposits bespeaking injury to glomeruli resulting from accidental lodging of phlogistic, soluble, macromolecular nonglomerular basement-membrane antigen-antibody complexes. In this series, in approximately 20 per cent of the specimens in which immunoglobulins were found in glomeruli, they were fixed in the linear pattern characteristic of anti-GBM antibodies.

IgG was the most frequent immunoglobulin class documented to localize in glomeruli of these allografts. Moreover, the relative intensity and extent of localization, where mixed IgG and IgM deposits were found, indicated that IgG was the principal class of antibody fixing to glomeruli. All cases of anti-GBM antibody were of the IgG class, and the most extensive of the granular, complex type of deposits were due to IgG as well. Nonetheless, cases were seen of IgM deposits alone or as extensive as concomitant IgG deposits.

It cannot be said with certainty whether the fixation of IgG as well as IgM to glomeruli in either linear or granular patterns is due to homograft immunity or to a persistence of the pathogenetic mechanism causing the destruction of the patient's native kidneys. In favor of the persistence of active nephritogenic mechanisms affecting the allograft are recurrence of glomerulonephritis in a considerable number of renal isografts,[4] transmission of "one-shot" experimental immune-complex-mediated glomerulonephritis into renal isografts in rats[14] and glomerular disease in allografts of recipients whose immunopathogenetic process can be documented as similar in graft and native kidney.[15] Regarding the latter point, the original kidney that was available for immunofluorescent examinations from one of this group of patients was of special interest (Case 12, Table 1). Although the diagnosis was cystinosis this native kidney showed trace amounts of IgG, IgM and β_{1C} in a granular pattern on badly scarred glomeruli; the allograft showed similar granular localization of IgM and β_{1C}.

In the four allografts with linear localization of host immunoglobulins characteristic of anti-GBM antibodies it is most probable, although not proved, that the organs had been affected by an extension of the same process as the one that destroyed the native kidneys. The precise etiology of the granular, complex deposits is less certain because the specific antigen (or antigens) in the antigen-antibody complex responsible for this kind of lesion has not been identified. Presumably, the same endogenous or exogenous antigen that provoked antibody formation and circulating antigen-antibody complexes before transplantation could have been responsible again. Alternatively, complexes could have formed in the same way as a host response to new antigens presented in the allograft. In either circumstance, the final mechanism of injury would be the same as

in naturally occurring glomerulonephritis, the difference being in the antigens initiating the immune response. Unfortunately, no inferences could be made about this distinction in the presently reported series since the original disease in the vast majority of cases was glomerulonephritis. In the future, it will be of great interest to learn how often "homograft glomerulonephritis" develops in uncomplicated cystinosis and in other renal disease of nonimmunologic origin.

The frequency of IgM deposits in these biopsies, as well as in others previously reported,[12] does nothing to answer the question whether IgM antibodies are part of an ongoing nephritic process or new participants evoked solely by allogeneic differences between allograft and recipient. The reported prevalence of rheumatoid factor and heterophil antibodies in recipients after renal transplantation[16] suggests that IgM present in glomeruli may be there as part of macromolecular complexes somehow evoked by the allograft, such as IgM anti-IgG antibodies.[17]

The lack of any apparent correlation between the findings of fibrinogen or fibrin and immunoglobulin in the glomeruli or between the presence of fibrin and abnormal renal function or histoincompatibility raises some question of the role of fibrin formation in the pathogenesis of glomerular injury in these transplants.[18] It may be that in such long standing grafts maintained by obviously successful immunosuppression the immunologic nephritogenic processes are relatively feeble and incapable of inducing the intensity of inflammation usually accompanied by fibrin formation. Certainly, in experimental nephritis only very acute, severe disease induces appreciable fibrin deposition in glomeruli.[19]

The frequency of nonglomerular, vascular localization of β_{1C} component of complement bespeaks some kind of ongoing local, complement-binding process in a few of the transplants, in which immunoglobulins were not obvious participants. Vascular sclerosis is common in human renal allografts and has been considered a hallmark of some kind of immunologically mediated injury.[20-23] Although IgM and fibrinogen were seen only rarely in such vessels, our evidence does not bear against the suggestion. Indeed, β_{1C} fixation in nonglomerular blood vessels is rare in our experience with biopsy material from nephritic patients and from kidneys removed from patients before transplantation.

The importance of these observations is twofold. In the first place, there is a reasonable correlation in this group between glomerular immunoglobulin deposition and proteinuria of any magnitude in excess of 100 mg daily or blood urea nitrogen concentrations exceeding 25 mg per 100 ml. It has been assumed that homograft immunity effected by cell-born mediators poses the paramount threat to functional integrity of allografts. These observations demonstrated, however, that an appreciable proportion of stable, long-term surviving renal allografts contain the hallmarks of recognizable antigen and humoral-antibody mechanisms. They suggest, furthermore, that the proteinuria and azotemia are the result of renal structural damage accruing from this recognizable immunopathologic injury. Although some proteinuric patients gave no evidence of immunoglobulin deposits in glomeruli, there is no assurance that they had not been damaged in this way previously. Similar uncertainty exists in these cases regarding the impact of conventional cellular allograft immune mechanisms. The presence of glomerular immunoglobulin deposits in the absence of proteinuria or azotemia, however, may imply potential risk due to cumulative injury or to a quantitative acceleration of the factors responsible for antigen release and antibody production.

Secondly, these observations indicate a guarded prognosis regarding the indefinite survival of these grafts. The high prevalence of detectable glomerular immunoglobulin localization in long-term allografts indicates a rather satisfactory control of cell-mediated graft-rejection mechanisms, but less than desirable suppression of production of antibodies directed specifically at GBM or the unidentified antigen (or antigens) of the complexes depositing in glomeruli. The lack of correlation between the degree of histocompatibility matching and the immunoglobulin deposition in the glomeruli suggests that close tissue matching may not be effective in preventing this kind of late glomerular injury in transplants. As has been suggested,[15] the most serious long-range threat to such well tolerated grafts may not be classic homograft immunity but rather the persistence or re-creation of a pathogenetic mechanism identical or analogous to that responsible for destroying the native kidneys. It seems reasonable to suggest that better suppression of antibody production, establishment of tolerance to the still unknown antigen (or antigens) or use of beneficial inhibitors of pharmacologic mediators such as histamine, serotonin, or kinins may minimize the long-term risk of the glomerulonephritic process in these transplanted kidneys.

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