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INTRAGLOMERULAR UPTAKE OF PLATELETS IN UNILATERAL MASUGI NEPHRITIS IN THE RABBIT

BY

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ABSTRACT

The pathogenetic roles of platelets were studied in unilateral progressive Masugi nephritis in the rabbit. On fourteenth day after the injection of anti-kidney serum, peripheral platelet counts, platelet aggregation and platelet aggregation rate were not changed statistically compared with those before the initial injection. The presence of platelet antigen within the glomeruli of the unclamped nephritic kidney suggests the participation in the pathogenesis of glomerular lesion. No significant difference was observed in the intrarenal uptake of ^{111}Indium-labeled platelets between clamped and unclamped kidneys. This is probably due to the possibility of the participation of the very small amount of platelets or the possible decreased renal blood flow in the nephritic side in this model, or the active uptake of platelet not being found in this progressed stage of this model.

Key words: ^{111}Indium-oxine labeled platelets, anti-platelet anti-serum, unilateral progressive Masugi nephritis, intraglomerular coagulation, platelet aggregation.

INTRODUCTION

It has been shown that platelets are important in the development and progression of human and experimental glomerulonephritis (Bang *et al.* [1], Clark *et al.* [2], Huang *et al.* [3], Mustard [4], Cameron [5], Remuzzi *et al.* [6], Woo *et al.* [7], Duffus *et al.* [8]). Following the deposition of immune complex or anti-kidney antibody, platelets are believed to initiate inflammation by activating complement and releasing inflammatory substances, resulting in proliferation of glomerular mesangial and endothelial cells. It is, however, not yet clear whether platelets are truly involved in glomerular lesions in the development and progression of glomerulonephritis.

To examine this, we tried to demonstrate the accumulation of platelets in the kidney in unilateral Masugi nephritis in the rabbit, using ^{111}Indium(In)-oxine-labeled platelets and immunofluorescent staining with anti-platelet antiserum. We employed unilateral Masugi nephritis in the rabbit because it had progressive nature. Control studies could be performed by comparing the non-nephritic side and severe nephritic side in the same animals. ^{111}In was employed because of its high α-photon emission and labeling efficiency.

MATERIAL AND METHODS

I. Preparation of anti-rabbit kidney serum: Renal cortex was separated from rabbit kidneys which had been perfused

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with heparinized saline and homogenized (Saare and Wirtz [9], Shibata and Kurisu [10], Nagasawa [11]). Ducks were injected intraperitoneally with this homogenate with complete Freund's adjuvant over a period of five weeks. One week after the last injection the ducks were bled after demonstration in the serum of precipitation titers of antibody to rabbit kidney homogenate as high as 1:64. The serum of ten ducks was pooled and decomplemented and then adsorbed with rabbit erythrocytes and plasma. This duck antiserum gave intense linear fluorescence only in the glomerular capillary walls. Preliminary observations showed that intravenous injection of 2 m*l* of this antiserum into rabbits invariably resulted in the production of proliferative glomerulonephritis with occasional cellular crescent formation on fourteenth day.

Preparation of ^{111}In-labeled platelets: The mothod of labeling was similar to that described previously (Thakur *et al.* [12], Sheffel and McIntyre [13], Yui *et al.* [14]). ^{111}In-oxine (Nippon Amersham Co., Japan) 300 μ Ci was added to the platelet suspension which was made from blood of a rabbit. Greater than 85% labeling efficiency was achieved with this procedure. Radioactivity of ^{111}In was measured with the auto 800C spectrometer (Packard, Inc.).

Preparation of anti-rabbit platelet antiserum (Miller, Dresner and Michael [15]): This was obtained from guinea pigs (n=10) by injecting a rabbit platelet suspension (Zimmerman [16]) with complete Freund's adjuvant at intervals of two weeks over a period of twelve weeks. Pooled anti-platelet serum was decomplemented and sequentially adsorbed with erythrocytes and plasma. The antiserum obtained was shown to react with the surface of platelets by use of an indirect immunofluorescence technique. This was done by using the anti-platelet serum followed by fluroescein-isothiocyanate-labeled (FITC) goat anti-rabbit IgG (Cappel Laboratories, Inc., Cochranville, Pennsylvania) on peripheral blood smears, fixed in acetone for ten minutes.

Experimental design: Albino rabbits of either sex were used. Anti-kidney antiserum was injected in doses of 2.0 m*l* into the central ear vein. The clamp with artery forceps to the pedicle of the left kidney was released after 20 minutes. Animals were divided into two groups by the method of renal examination; the first group was employed for radiological study and the second group for immunohistological study.

The animals in the first group (n=11) were infused intravenously with a suspension of rabbit ^{111}In-oxine platelets on the twelfth day after the injection of anti-kidney antiserum. Both kidneys were removed immediately at 48 hours following the injection of labeled platelets. Renal cortex was separated from rabbit kidneys after the intrarenal blood was washed out by perfusion with heparinized saline from renal artery to vein.

Renal cortex was weighed and measured for radioactivity. Renal accumulation of platelets was expressed as radioactive counts per gram (Ci/g). Corrected radioactivities of both kidneys (i.e., the left clamped and right non-clamped) from each animal were expressed as a ratio (Table 2).

On the fourteenth day after the initial injection, tissues of the second group (n=8) were taken from both kidneys for light and fluorescence microscopy.

Albuminuria was measured semiquantitatively at the time of necropsy with 3% sulphosalicylic acid.

The blood samples for platelet studies

were first obtained from the vein of the rabbit ear before the injection of anti-kidney serum and again on the fourteenth day after the injection.

Platelet counts were performed electronically in a Coulter counter (Coulter Counter Electronics, Inc., Hialeah, Fla.). The quantitative detection of platelet aggregates was made using the method of Wu-Hoak [17]. Platelet aggregation was measured in a Sienco aggregometer (Model DP-247E, Sienco Co., Morrison, Colo.). Adenosine 5′-diphosphate (ADP) and collagen were obtained from Sigma Chemical Co., St. Louis, Mo. and Collagenreagent hormon Hormon-Chemie, FRG, respectively.

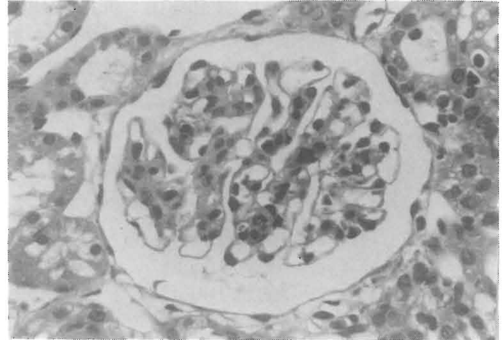
Light microscopic studies were performed on kidney specimens fixed in buffered formalin and stained with hematoxylin-eosin, PAS, Masson's trichrome and PAM stains.

For immunofluorescent studies, sections were fixed in acetone and stained with FITC-labeled sera specific for rabbit IgG, C3, fibrinogen, albumin (Cappel Laboratories, Inc., Cochranville, Pennsylvania) and anti-rabbit platelet anti-guinea pig serum followed by FITC-labeled goat anti-guinea pig IgG. The intensity of glomerular staining was scored as follows: 0–negative, 1–mild, 2–moderate and 3–severe.

Statistical evaluation was performed using paired and unpaired t-tests and the Wilcoxon two sample test (Mann-Whitney test).

Results

On day 14, the clamped kidney showed almost normal glomeruli. In contrast, there were severe cell proliferation, increase in mesangial matrix and occasional cellular crescent formation in the unclamped kidney (Fig. 1, a, b). Albuminuria was found invariably at the time of necropsy in the group treated with the anti-kidney antiserum.



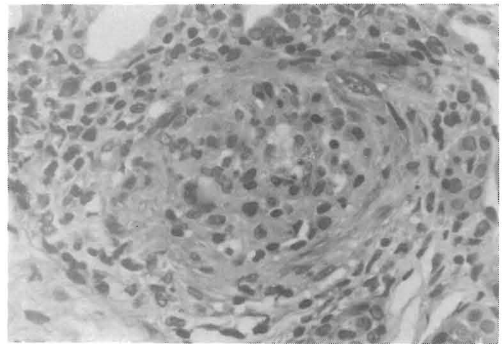


Fig. 1. Histological Findings of Unilateral Masugi Nephritis in the Rabbit.
Light microscopic findings: (a) left clamped kidney shows almost normal glomerulus: (b) Right non-clamped kidney shows severe glomerular cell proliferation and increase of mesangial matrix with cellular crescent. Immunofluorescent microscopic findings: (c) Platelet antigen was present within the glomerulus of non-clamped kidney.

No statistically significant difference was observed in the results of platelet

Table 1. Platelet Studies on Peripheral Blood in Unilateral Masugi Nephritis in the Rabbit

	Control	Nephritis	
Platelet count $\times 10^4/ml$	(n=9) 24.5±6.8	(n=4) 42.5±19.6	n.s.
Platelet aggregation (at 5 min. aggr.)	(n=6)	(n=4)	
ADP, 10 μM	32± 7 mm	33±7 mm	n.s.
ADP, 100 μM	55± 5 mm	51±9 mm	n.s.
Collagen 10 μ/ml	60± 7 mm	67±4 mm	n.s.
Collagen 30 μ/ml	58±10 mm	72±3 mm	n.s.
Platelet Aggregation rate	(n=11) 0.72±0.07	(n=4) 0.63±0.07	n.s.

Values are mean±SEM, n.s. indicates not significant statistically.

Table 2. Renal Cortex Radioactivity of ^{111}In-labeled Platelets in Unilateral Masugi Nephritis in the Rabbit

Left clamped kidney (control) (n=11)	Right non-clamped Kidney (nephritis) (n=11)	Left/right ratio (n=11)
218×10^2 Ci/g	208×10^2 Ci/g	1/0.95
214× 〃	159× 〃	1/0.74
553× 〃	639× 〃	1/1.15
101× 〃	67× 〃	1/0.66
103× 〃	85× 〃	1/0.82
136× 〃	131× 〃	1/0.96
140× 〃	106× 〃	1/0.75
269× 〃	122× 〃	1/0.45
111× 〃	70× 〃	1/0.63
428× 〃	609× 〃	1/1.42
185× 〃	246× 〃	1/1.32
		1/0.89±0.09*

*mean±SEM.

studies on peripheral blood between the unilateral Masugi nephritic stage and the controls with sham operation. However, the tendency toward an increase in the platelet count and platelet aggregation and a decrease in the platelet aggregation rate were noted in the nephritic stage (Table 1).

Renal accumulation of platelets in the unilateral Masugi nephritis in the rabbit (Table 2): Decreased uptake of radioactivity in the renal cortex was observed on the unclamped side in eight out of eleven rabbits with unilateral Masugi nephritis. However, no statistically significant difference was seen between unclamped and clamped sides (clamped kidney/unclamped kidney=1/0.89±0.09 SEM, n=11).

Immunofluorescent study (Table 3): The unclamped kidney was stained intensively for rabbit IgG in a linear pattern along the glomerular capillaries. Platelet antigen was faintly present with-

Table 3. Glomerular Immunofluorescence

	Subject No.								Mean±SD	
	1	2	3	4	5	6	7	8		
Platelet										
Left clamped side	0	0	0	0	0	0	0	0	0	$p<0.05$*
Rt. non-clamped side	1	1	1	1	0	1	0	0	0.62±0.51	
Fibrinogen										
Left clamped side	1	0	0	0	0	0	0	0	0.12±0.35	$p<0.025$*
Rt. non-clamped side	3	3	0	3	0	3	3	3	2.25±1.38	
IgG										
Left clamped side	1	3	0	0	0	1	1	1	0.87±0.99	$p<0.005$*
Rt. non-clamped side	3	3	2	3	2	3	3	3	2.75±0.46	
C3										
Left clamped side	1	3	0	0	0	0	0	1	0.62±1.06	$p<0.01$*
Rt. non-clamped side	3	3	1	3	1	3	3	3	2.50±0.92	
Albumin										
Left clamped side	0	0	0	0	0	0	0	0	0	
Rt. non-clamped side	0	0	0	0	0	0	0	0	0	

Grade of intensity of staining: 0—no staining, 1—mild, 2—moderate, 3—severe; Rt.: right.
*Wilcoxon two sample test.

in the glomeruli on the unclamped side in five out of eight cases (62.5%) (Fig. lc). It was not observed on the clamped side ($p<0.05$). Rabbit fibrinogen, IgG and C3 were stained in almost the same way as platelet antigen ($p<0.025$, $p<0.05$, $p<0.01$, respectively).

Discussion

Recently, platelets have come to be considered important mediators of tissue development and damage in glomerulonephritis. Platelets may stimulate intraglomerular cell proliferation (George *et al.* [18], Nakashima *et al.* [19]). Focal glomerulonephritis was produced in rabbits infused with ADP to induce platelet aggregation (Jφrgensen *et al.* [20]).

In human glomerulonephritis, the subendothelial fibrin-platelet aggregates in glomeruli have been observed in minimal change nephrotic syndrome (Duffy *et al.* [21]). The correlation between the shortening of platelet survival and the degree of intraglomerular cellularity and narrowing of capillary lumen has been demonstrated in both chronic glomerulonephritis (George *et al.* [22]) and proliferative lupus glomerulonephritis (Clark *et al.* [23]).

Several findings that show the activation of platelets in glomerulonephritis have been reported. Serotonin-depleted platelets are present and plasma β-thromboglobulin and platelet factor 4 show an increase (Parbtani *et al.* [24], Tomura *et al.* [25]). Platelet-aggregating material is present in glomerulonephritis (Kasai *et al.* [26]) and thrombocytopenia is seen (Floyd *et al.* [27]).

However, few studies have investigated the role of the platelet in the localized area of the kidney in glomerulonephritis. Platelet antigen has been demonstrated within glomeruli and vessels in various forms of human glomerulonephritis

(Miller *et al.*[15]). The presence of platelet-related antigens, platelet factor 4 and β-thromboglobulin has been shown in glomerulonephritis (Duffus *et al.* [8]). The binding of platelet factor 4 to glomerular polyanion has been reported (Barnes *et al.* [28]). We noticed the severe cell and matrix proliferation with cellular crescent formation only in the unclamped kidney in the unilateral Masugi nephritis which was shown to be protected by the administration of heparin (Nakamoto *et al.*[29]).

There were no significant changes in peripheral platelet counts, platelet aggregation and platelet aggregation rate between control and unilateral Masugi nephritic stage. This is probably due to the reason that glomerulonephritis develops only in unilateral kidney. Platelet activation in the nephritic side is probably not strong enough to affect the function of circulating platelets.

The evidence that platelet antigen as well as the fibrinogen were present only within the glomeruli of the nephritic kidney suggests that platelets may participate in accordance with fibrin in the pathogenesis of glomerular lesion (Miller *et al.* [15]). It is unlikely that immunofluorescent staining of platelet antigen in the glomeruli is non-specific, because no albumin was detected in the glomeruli. Moreover, it seemed improbable that intrarenal deposition of platelet antigen was non-specific due to the anti-rabbit platelet contaminated in the anti-rabbit kidney serum. This conclusion resulted from the fact that the deposition was detected only in the nephritic side and the antiserum was previously adsorbed with rabbit erythrocyte and plasma.

Several reasons are considered in the finding that there is no difference between clamped and unclamped side in the uptake of ^{111}In-labeled platelets in the unilateral Masugi nephritis in the rabbit. Among the reasons; First, deposition of platelet in the glomeruli on the nephritic side is not large enough to show the increased intraglomerular uptake of ^{111}In-labeled platelet; Second, increased intraglomerular uptake of ^{111}In-labeled platelet might be masked by the possible decreased renal blood flow on the nephritic side; Third, since ^{111}In-labeled platelet uptake study was performed in fairly progressed stage of glomerulonephritis, active uptake of the isotope might not be found.

To clarify the reason for conflicting results examined by the two methods, it is necessary to measure the renal blood flow on both the nephritic side and non-nephritic side and to do the ^{111}In-labeled platelet uptake study during the earlier stage of glomerulonephritis.

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