CHAPTER 5

INTRAGLOMERULAR THROMBOTIC TENDENCY AND GLOMERULAR ADPase
Unilateral impairment of ADPase elicits a proaggregatory microenvironment in experimental glomerulonephritis

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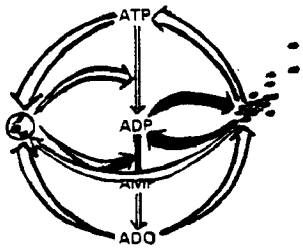

SUMMARY

It has been proposed, predominantly from ex vivo studies, that glomerular ADPase may function as an antithrombotic principle within the rat kidney. Therefore, intraglomerular platelet aggregation was studied in vivo in rats following impairment of glomerular ADPase activity using local X-irradiation (20 Gy). Biochemical assays in suspensions of glomeruli obtained from rats 24 hr after local X-irradiation (group I) demonstrated a significant reduction in ADPase activity as compared to sham treated rats (group II; $p < 0.01$). Cytochemical observations at the ultrastructural level showed that this reduction in glomerular enzyme activity represents in particular ADPase activity detectable in the basement membrane. Following X-irradiation, intraglomerular platelet aggregation was quantitatively studied in two groups of rats. Both groups received X-irradiation of the left kidney (20 Gy). 24 hr after X-irradiation, animals received an i.v. injection of either 0.5 ml saline (group III;$n=6$) or 0.5 ml heterologous nephrotoxic serum (NTS; group IV; $n=6$). Subsequently, 24 hr following this injection, platelet aggregation in left kidneys was compared to aggregation in contralateral non-X-irradiated kidneys.

The results showed that while X-irradiation per sé did not induce intraglomerular platelet aggregation as compared to the contralateral kidney ($0.20 \pm 0.08\%$ versus $0.17 \pm 0.06\%$ platelet aggregation/glomerulus), a significant increase in platelet aggregation could be demonstrated in X-irradiated kidneys in the early phase of NTS nephritis as compared to the contralateral nephritic kidney ($2.45 \pm 0.66\%$ vs $1.37 \pm 0.35\%$ platelet aggregation per glomerulus; $p < 0.005$). A potential effect of altered influx of inflammatory cells following X-irradiation could be excluded since no difference in H_2O_2 producing cells was observed between left and right kidneys. Thus, while ADPase impairment by X-irradiation does not induce platelet aggregation per sé, it is clear that in proaggregatory conditions, like in NTS nephritis, the thrombotic tendency, due to decreased glomerular ADPase, is enhanced.

These results demonstrate the functional significance of glomerular ADPase activity as an antithrombotic principle following platelet activation in vivo.

INTRODUCTION

The intriguing role of activated platelets during various inflammatory reactions has been widely recognized during the last decades (8,26). For instance, procoagulatory, chemotactic and vasoactive effects of platelet products have been described as well as stimulation of neutrophil responses i.e. the production of oxygen free radicals (33). To counteract vascular damage due to proinflammatory reactions, potent anti-aggregatory mechanisms associated with the vessel wall seem warranted.

Since ADP is a major aggregatory stimulus (5,17), it is highly likely that vessel wall associated ADP degrading enzymes fullfill such an antithrombotic function (14,22). Even in extracellular sites, i.e. the glomerular basement membrane (GBM) of the rat kidney where the blood stream comes into close contact with collagen of the GBM through the fenestrated endothelium, abundant ADPase activity has been demonstrated at the ultrastructural level

(16). Within the rat kidney, this antithrombotic role for ADPase has been demonstrated in perfusion studies ex vivo showing impaired glomerular ADPase activity in association with increased thrombotic tendency in rat kidneys within 48 hr following local X-irradiation or adriamycin treatment (3).
However, in X-irradiated or adriamycin treated rats, intra-glomerular platelet aggregation in vivo is not a prominent finding within 48 hr. Apparently, decreased glomerular ADPase in vivo does not necessarily lead to thrombus formation.
Therefore, we now conducted experiments which enabled us to study intraglomerular antithrombotic activity, with and without induction of thrombus formation in vivo. Thus, intraglomerular platelet aggregation was studied in rats, taking advantage of the possibility to impair ADPase activity by local X-irradiation of one kidney, allowing the contralateral kidney to serve as an internal control. Aggregation within kidneys of these rats was quantitatively measured in conditions without additional stimulation of platelet aggregation and during proaggregatory conditions, that is, in the early phase of nephrotoxic serum (NTS) nephritis (21, 29).
Another objective of the present study is to confirm and quantify the enzymecytochemical data described at the ultrastructural level (4), by biochemical assays in suspensions of glomeruli obtained from rats with normal versus reduced glomerular ADPase activity.
The results demonstrate a significant decrease in glomerular ADPase activity following local X-irradiation as detected biochemically, which is in aggreement with the present and previous (4) ultrastrastructural observations showing ADPase impairment within the GBM. Moreover, the results also show that in the present model intraglomerular platelet aggregation only occurs if two conditions are present i.e. reduced ADPase activity and an appropriate proaggregatory stimulus to initiate platelet aggregation.
These results clearly demonstrate the functional role of glomerular ADPase in vivo as an antithrombotic mechanism within the rat kidney in particular during inflammatory conditions.

EXPERIMENTAL DESIGN

Experiments were performed to examine the relationship between glomerular ADPase activity cytochemically as well as biochemically on the one hand (first set of experiments), and intra-glomerular thrombotic tendency in vivo on the other hand (second set of experiments).

Glomerular ADPase activity.

Rats were divided into two groups; group I received X-irradiation of both kidneys (20 Gy), whereas group II received sham treatment. 24 hr after X-irradiation or sham treatment, kidneys were either perfused ex vivo and processed for cytochemical demonstration of ADPase activity (n=5) or kidneys were prepared for biochemical demonstration of glomerular ADPase activity. These biochemical phosphatase assays were performed in suspensions of glomeruli. Each suspension was obtained from four kidneys and suspensions of group I and II were assayed in parallel experiments. Results are expressed as arithmetic means ($\pm$ SD) of five experiments.

Intraglomerular platelet aggregation.

Intraglomerular platelet aggregation was examined in two groups of rats (III and IV),

receiving both unilateral X-irradiation of the left kidney (20 Gy). 24 hr after X-irradiation, at a time interval when reduced ADPase activity has been demonstrated (4), rats of group III (n=6) received a single i.v. injection of 0.5 ml saline and rats of group IV (n=6) received a single i.v. injection of 0.5 ml heterologous rabbit serum against rat GBM (kindley provided by Dr.E.de Heer, Leiden) to induce NTS nephritis. Subsequently, 24 hr after the saline or NTS injection, left and right kidneys were removed and processed for histochemical examination of platelet aggregation and infiltrating cells. Intraglomerular platelet aggregation in left kidneys of both groups was compared with aggregation in the contralateral kidney using image analyzing techniques. The inflammatory response was assessed by counting the number of peroxidase positive cells producing oxygen free radicals.

RESULTS AND DISCUSSION

Glomerular ADPase activity.

Using the cerium-based method, reaction product could be demonstrated in normal rat kidneys along plasmamembranes of endothelial and epithelial cells as well as throughout the glomerular basement membrane (GBM) (figure 1a). In contrast, a decreased amount of reaction product within the entire glomerular capillary wall was observed 24 hr after local X-irradiation of rat kidneys (fig.1b). In the mesangial area however, a similar amount of reaction product could be seen as compared to untreated kidneys. The results confirm ultrastructural observations of Bakker et al (4), describing a reduced glomerular ADPase activity 24 hr after local X-irradiation of rat kidneys.

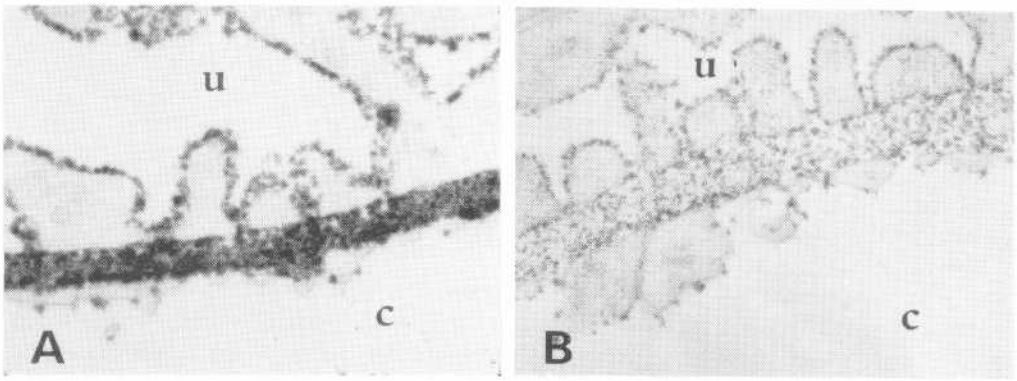

Fig. 1: Photomicrographs of glomerular staining for ADPase activity using the cerium-based cytochemical method in a normal kidney (A) and 24 hr after local X-irradiation of the kidney (B). Reaction product (dark staining) can be demonstrated along plasma membranes of endothelial and epithelial cells as well as throughout the GBM in normal kidneys (A) but reduced staining is observed following X-irradiation (B). Abbreviations: C=capillary lumen, U=urinary space. Magnification 34,000x.

These cytochemical observations could be confirmed in parallel biochemical studies in suspensions of glomeruli, isolated from kidneys of X-irradiated or sham treated rats. The biochemical assays demonstrate a reduction in glomerular phosphatase activity of approximately 30% after local X-irradiation of rat kidneys as compared to sham treated kidneys, which is significant at all time intervals tested (5, 10 and 30 minutes; $p<0.01$, fig.2). As the partial reduction of glomerular ADPase activity following X-irradiation is confined mainly to the GBM localized enzyme as is shown by cytochemistry at the ultrastructural level (fig.1) it is highly likely that also the partial reduction detected biochemically may represent in particular the GBM bound ADPase.

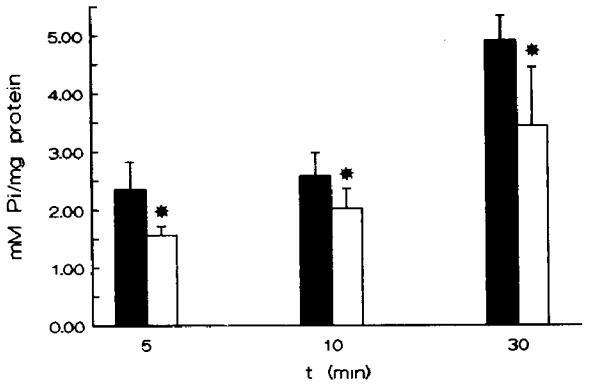

Fig. 2: ADPase activity in suspensions of glomeruli obtained from sham treated rats (dark columns) and X-irradiated kidneys (open columns). Columns represent arithmetic means (± SD) of biochemical phosphate assays (n=5) measured after several incubation times as indicated. Phosphate release following incubation with ADP is expressed per mg protein. It can be seen that phosphate release by glomeruli from X-irradiated kidneys is significantly reduced following a 5, 10 or 30 min. incubation period as compared to normal glomeruli (= $p<0.01$).*

Intraglomerular platelet aggregation.

Since it has been suggested that ADP degradation by glomerular phosphatase activity may function as an antithrombotic principle within the rat kidney (3), a decrease in glomerular ADPase activity is expected to enhance intraglomerular thrombotic tendency in vivo. Therefore, intraglomerular platelet aggregation was evaluated in rats receiving unilateral X-irradiation of the left kidney followed by a systemic injection of either saline or a platelet activating stimulus. Since NTS nephritis is characterized by intraglomerular platelet aggregation within 24 hr (21,29) nephrotoxic serum was used as the platelet activating stimulus. This has the advantage of stimulating platelet aggregation specifically within glomeruli with no trapping of emboli formed elsewhere which may be the case following injection of a general platelet activating stimulus (6).

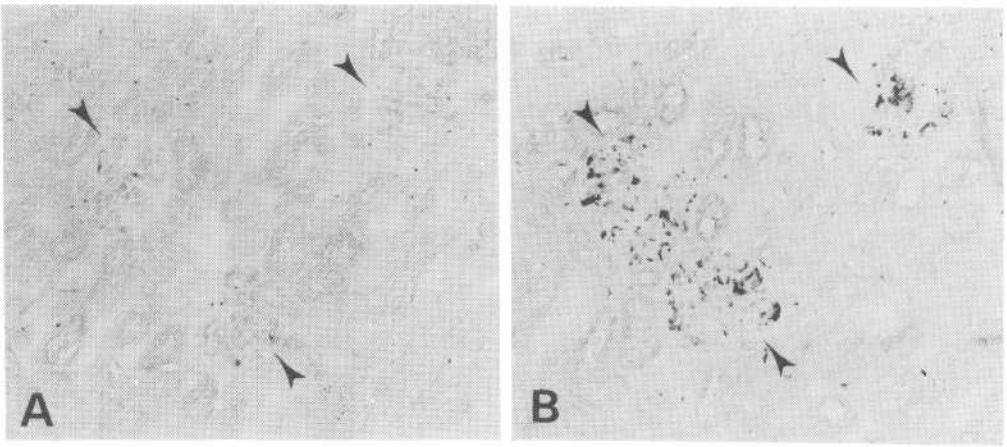

Fig. 3: Platelet aggregation within glomeruli (arrows), as detected by immunohistochemical staining with PL1, in X-irradiated kidney following saline injection (A) and in non-X-irradiated kidney 24 hr after NTS injection (B). Intraglomerular platelet aggregation can be demonstrated in NTS injected rats (B) but not in X-irradiated rats receiving subsequent injections with saline (A). Magnification 140x.

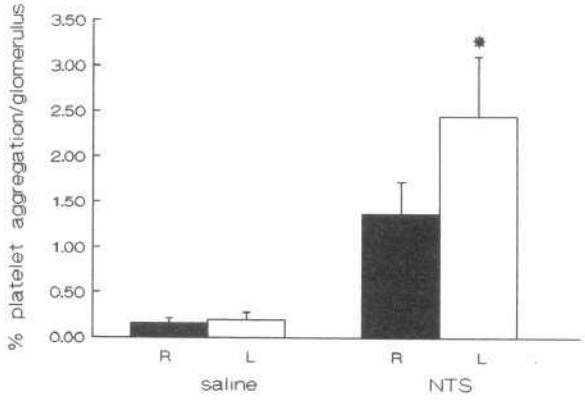

Fig. 4: Mean amount of intraglomerular platelet aggregation in rats following local X-irradiation of their left kidneys and subsequent i.v. injection of either saline (left set of columns) or NTS (right set of columns). Results are expressed as arithmetic means (± SD) of percentage of glomerular area stained for platelet aggregates, in six rats per group. No aggregation is observed in X-irradiated kidneys as compared to control kidneys in rats receiving saline (left set of columns), but a significant increase is observed in X-irradiated kidneys following induction of NTS nephritis as compared to the contralateral kidney (= $p < 0.005$). Abbreviations: R =right kidney; L =left kidney.*

24 hr after the saline injection, no platelet aggregation was observed within X-irradiated kidneys as detected by immunochemistry using the antiplatelet antibody PL-1 (figure 3). In contrast, 24 hr after NTS injection, abundant platelet aggregation was observed within glomeruli in particular in X-irradiated kidneys. Quantitative evaluation of this PL-1 staining in different experimental groups (figure 4) demonstrates no significant increase in platelet aggregation in X-irradiated kidneys as compared to the contralateral control kidney in rats receiving saline after X-irradiation (0.20 ± 0.08 % vs 0.17 ± 0.06 % platelet aggregation /glomerulus), indicating that X-irradiation per sé does not induce platelet aggregation within glomeruli. However, in the NTS injected rats, X-irradiated kidneys displayed significantly more platelet aggregation as compared to the contralateral kidney receiving no X-irradiation (2.45 ± 0.66 versus 1.37 ± 0.35 % platelet aggregation/glomerulus respectively; $p < 0.005$). These results show that no platelet activation is observed following ADPase impairment by local X-irradiation as such. However, the increased intraglomerular thrombotic tendency, as induced by X-irradiation, is only revealed after induction of NTS nephritis. Therefore, it can be concluded that $_{(a)}$ glomerular ADPase is effective as an anti-thrombotic mechanism in vivo after platelet aggregation has been promoted in the local inflammatory environment during the induction of nephritis and $_{(b)}$ glomerular ADPase inactivation per sé is not able to induce platelet aggregation in the present model.
In a previous report we also noted a strong association between increased intraglomerular platelet aggregation and a reduced glomerular ADPase activity in vitro (3) as well as in vivo after induction of NTS nephritis (29). The present report not only strengthens this notion of a causal relationship between these intraglomerular events but also demonstrates the functional significance of this enzyme in vivo.

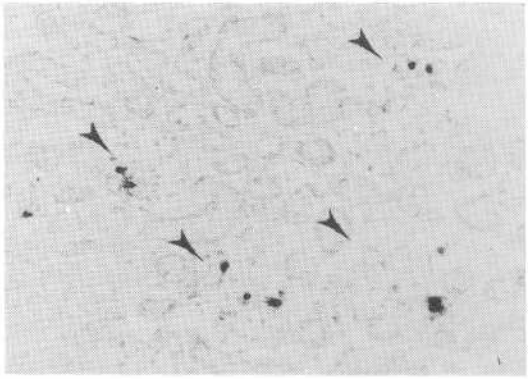

Fig. 5: Oxygen free radical producing cells within glomeruli (indicated by arrows), 24 hr after injection of NTS, as demonstrated by staining for peroxidase activity and concomitant H_2O_2 production using DAB. Number of H_2O_2 producing cells (dark staining) within glomeruli can be easily counted (see figure 6). Magnification 140x.

Thrombus formation during glomerulonephritis has shown to be a common phenomenon (10) due to a variety of platelet aggregatory and coagulatory stimuli released from the vascular wall and inflammatory cells (reviewed in 35). A change in these proaggregatory stimuli

induced by X-irradiation might also increase platelet aggregation in X-irradiated kidneys. Therefore, other parameters of inflammation were also studied.
No difference in immunofluorescence pattern or intensity between left and right kidneys was observed in NTS injected rats after staining of kidney cryostat sections with FITC-conjugated goat-anti-rabbit IgG. Moreover, following local X-irradiation of kidneys no influx of inflammatory cells, as detected by histochemical staining for oxygen free radical producing cells (fig.5), within glomeruli was observed, nor an increased influx of monocytes and macrophages as detected by a monoclonal antibody specific for these cells (ED1; reference 11) could be observed.

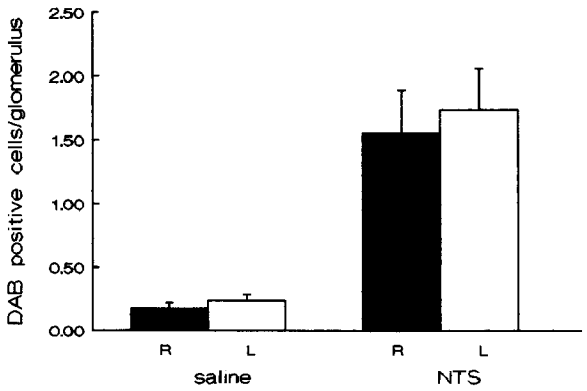

Fig. 6: Number of H_2O_2 producing cells present in glomeruli of rats following unilateral X-irradiation of the left kidney and subsequent i.v. injection of saline (left set of columns) or NTS (right set of columns). Columns represent arithmetic means (± SD) of DAB positive cells per glomerulus in six rats per group. No difference is observed between X-irradiated left kidneys and contralateral kidneys receiving no X-irradiation. Abbreviations see fig.4.

In addition, influx of inflammatory cells was similar in left and right kidneys after induction of NTS nephritis; 1.74 ± 0.32 DAB positive cells/glomerulus can be demonstrated in left kidneys and 1.55 ± 0.34 DAB positive cells/glomerulus in contralateral kidneys (not statistically significant, figure 6). Therefore, the increased aggregation points towards an impaired antithrombotic mechanism within X-irradiated left kidneys; proaggregatory stimuli derived from inflammatory cells in left and right kidneys are similar.
Other vascular wall associated antithrombotic mechanisms, i.e. the fibrinolytic system and heparan sulfate proteoglycanes (19), have not shown to be sensitive for 20 Gy radiation as detected by the Todd slide technique and PEI1800 staining respectively (3). Conflicting data exist concerning the effects of X-irradiation upon prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) production in different organs (12,13,31,34). In the kidney however, local production of TXA_2 is reported not to be increased (increased urinary TXB_2 excretion is shown to be of extrarenal origin), whereas PGI_2 and PGE_2 production is increased following local X-

irradiation with a dosis of 20 Gy (32), as in the present study. Therefore, increased intraglomerular platelet aggregation cannot be attributed to a decreased PGI_2/TXA_2 ratio in the kidney, whereas systemic changes in this ratio i.e. in circulating platelets (23) or in the abdominal vascular walls (32) cannot account for the observed difference in aggregation between both kidneys.
It has been suggested that ADPase impairment, observed after local X-irradiation of kidneys, is mediated by oxygen free radicals (4,27). Radical formation is an abundant phenomenon during X-irradiation (12) and a protective effect of the superoxide anion scavenger, superoxide dismutase, upon glomerular ADPase has been demonstrated in this model (27). In addition to ADPase impairment during acute glomerulonephritis and adriamycin nephrosis, during which also a protective effect of radical scavengers has been demonstrated in vivo (28,29), oxygen free radicals may be responsible for the ADPase impairment within glomeruli in this model. In this way, radical production may be a final common pathway, leading to increased thrombotic tendency within glomeruli. The ADPase impairment within the glomerular filtration barrier does not directly increase glomerular permeability for plasma proteins since X-irradiation in the present short term experiment does not lead to proteinuria or hematuria, as has been observed previously (4). However, it may facilitate further deterioration of glomerular permeability during nephritis since platelet aggregation can induce direct damage to vascular walls (10), or indirect damage by release of a variety proinflammatory platelet products (33,35). The significant inhibition of proteinuria by platelet depletion in an other experimental model of immune complex nephritis illustrates this role of platelets during acute inflammation (20).
In summary, histochemical as well as biochemical evidence was obtained showing decreased glomerular ADPase activity in kidneys of X-irradiated rats as compared to normal rats. In addition, application of the unilateral kidney X-irradiation model enabled us to study altered versus normal ADPase activity in individual rats in relation with platelet aggregation following a proaggregatory stimulus. ADPase impairment was shown to be associated with an increased intraglomerular thrombotic tendency, which is revealed after induction of NTS nephritis. These observations demonstrate the role of ADPase activity within the GBM of the rat kidney in vivo as an important antithrombotic mechanism following platelet activation.

METHODS

ANIMALS

Female PVG/c rats (200-220 g) ad libitum fed on standard chow (Hope Farms, Woerden, The Netherlands) were used throughout the study.

LOCAL X-IRRADIATION

Rats were anaesthesized with hypnorm (0.1 ml/100 g b.w.) and bodies were shielded with lead, leaving either both or the left kidney (see "Experimental design") unprotected. Rats received 20 Gray during a 21 minutes radiation period using a Philips Müller MG 301 X-ray machine which was operated at 200 KV and 15 mA. In parallel experiments rats received

sham treatment, that is, the same anaesthesia and handling without turning the machine on.

ENZYME CYTOCHEMISTRY

Kidneys of rats, anaesthesized with halothane and O_2/N_2O, were fixed by perfusion. After removal of the blood with saline, kidneys were perfused with 2% glutaraldehyde in 0.1 M cacodylate buffer (PH 7.38) during 1 min, and subsequently with 0.1 M cacodylate buffer with 6.8% sucrose (16). Immediately after perfusion, 30 μm vibratome sections were cut and incubated for demonstration of phosphatase activity according to the cerium-based method as described previously (16,18). Subsequently, sections were postfixed with 1% OsO_4 in 2% $K_4[Fe(CN)_6]$ and embedded in Epon 812 according to standard procedures (18).

BIOCHEMISTRY

Glomerular ADPase activity was biochemically assayed in suspensions of glomeruli, isolated from kidneys which were previously snap frozen and stored at -80°C before use. Glomeruli were isolated from the cortex using standard sieving techniques (7) with sieves of respectively 180, 150 and 200 mesh. The isolation procedure was performed in 0.15 M NaCl and at 4°C to preserve viability of glomeruli. Purity of suspensions was tested by light microscopy. Phosphatase activity was assessed by measuring the amount of inorganic phosphate released by glomeruli after incubation with ADP. Samples (50 μl) were incubated at 37°C in 200 μl Tris/HCl buffer (0.015 M, PH 8.2) with 0.015 M 2-amino-2-methyl-1,3-propandiol and 1 mM $MgCl_2$ supplemented with ADP (2.5 mM ;25). Parallel incubations were performed without substrate. Reactions were stopped after different incubation periods, as indicated in "Results", by quickly freezing the mixture at -20°C, rather than to use protein detergentia (25) since this induces ADP hydrolysis. Inorganic phosphate was subsequently measured according to the method of Chandrarajan and Klein (9), which is suitable here since non-enzymatic ADP hydrolysis during the whole procedure is prevented. Phosphate concentrations were related to the protein content of the suspension, measured according to the method of Lowry (24).

EVALUATION OF INTRAGLOMERULAR INFLAMMATION

Intraglomerular antibody deposition was routinely studied in kidney cryostat sections by immunofluorescence with FITC-conjugated goat-anti-rabbit IgG (Nordic, Tilburg, The Netherlands), to ascertain the presence of anti-GBM IgG. The inflammatory response was analyzed after histochemical demonstration of activated neutrophils within glomeruli.

The presence of these neutrophils was demonstrated in air-dried cryostat sections (5 μm) by demonstrating peroxidase (PO) activity characteristic for neutrophils (30), taking advantage of endogenous H_2O_2 production by activated neutrophils (1). Sections were incubated for 20 min. at 37 °C in 0.1 N Tris- HCL buffer (PH 7.2) containing 0.5 mg/ml 3,3-diamino benzidine (DAB; Sigma Chemical Co, St.Louis). PO activity will induce oxidation of DAB in the presence of H_2O_2 (15), the latter being produced by activated neutrophils. Addition of catalase to the incubation medium (0.01 - 0.1 mg/ml) inhibited staining in a dose dependent manner, confirming the involvement of endogenous H_2O_2 production in DAB oxydation. Influx of monocytes and macrophages was immunohistochemically studied in cryostat sections using ED1, a monoclonal antibody against these cells (11), according to standard procedures (11). Sections were routinely stained with hematoxilin for morphological

examination of infiltrating cells.

PLATELET AGGREGATION

Intraglomerular platelet aggregation in experimental and control animals was shown using a mouse monoclonal antibody against rat platelets (PL-1; 2) upon 5 μm cryostat sections fixed in acetone. Histochemical staining was performed according to standard techniques using peroxidase conjugated rabbit anti-mouse IgG (Dakopatts, Glostrup, Denmark) as the second step and subsequently DAB and H_2O_2 (15). Sections were counterstained with hematoxilin.

QUANTITATIVE ANALYSIS OF TISSUE SECTIONS AND STATISTICAL EVALUATION:

To quantify the inflammatory response in different experimental animals the number of oxygen free radical producing cells in glomeruli were counted by scoring the number of DAB positive spots per glomerulus after staining for peroxidase activity. Per rat 50 glomeruli were evaluated.

Intraglomerular platelet aggregation in vivo, as detected by staining with PL-1 was quantitatively evaluated at the light microscopical level using computerized image analyzing techniques (29). This analysis is based upon measuring light absorption by DAB precipitations in glomeruli, correlating with the degree of platelet aggregation. The microscopical field was digitized by a frame grabber (PC-Plus, Imaging Tech.) in 256x256 pixels with possible grey levels from O to 255. Glomeruli were selected by using a digitizing tablet (mm1812, Summagraphics) and total number of pixels minus pixels with background grey value (induced by tissue itself and hematoxilin counterstaining) were counted. Degree of platelet aggregation is expressed as mean percentage of glomerular area with DAB precipitation. Data of biochemical studies and image analyzing techniques were analyzed by the Wilcoxon test and considered significant at $p < 0.05$.

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