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Attenuation of Anti-Thy1 Glomerulonephritis in the Rat by Anti-Inflammatory Platelet-Inhibiting Agents

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Although both ecto-ADPase and prostacyclin (PGI_2) inhibit platelets and neutrophils, their action in acute glomerulonephritis is unknown. We tested the PGI_2 analog Iloprost and 2chloro-adenosine (2Cl-ADO), an analog of adenosine, the end product of nucleotidase activities, during anti-Thy1 nephritis. Rats received anti-Thy1 immunoglobulin G (5 mg/kg body weight, intravenously) and subsequently one subcutaneous injection of either 2Cl-ADO (10 mg/kg body weight; (n = 6) or Iloprost (1 mg/kg body weight; n = 6). Control rats received anti-Thy1 immunoglobulin G with saline (n = 6) or saline alone (n = 6). After 24 hours, kidneys were processed for light-microscopical evaluation. Proteinuria was studied in additional rats. Results showed that both drugs inhibited intraglomerular platelet activation ($P < 0.005$). 2Cl-ADO also reduced intraglomerular O_2^- production of neutrophils ($P < 0.05$), in contrast to Iloprost. Intraglomerular immunoglobulin G deposition, complement activation, neutrophil influx, and myeloperoxidase release were not affected by 2Cl-ADO or Iloprost. However, proteinuria was completely prevented by both drugs. It is concluded that PGI_2 and nucleotidases are potentially able to attenuate this form of nephritis by inhibiting platelet activity, whereas nucleotidases also inhibit neutrophil activity in vivo. (Am J Pathol 1993, 142:441–450)

Endothelial prostacyclin (PGI_2) production has been considered as an important platelet-inhibiting mechanism,[1–3] whereas PGI_2 also may have an anti-inflammatory role.[4–6] In addition, blood vessel walls possess ADPase activity[7–9] with potent antithrombotic activity as demonstrated in the kidney microvasculature *in vitro*[10,11] and *in vivo*.[12,13]

Like PGI_2, this ectonucleotidase may also display anti-inflammatory activity because ADP and ATP potentiate neutrophil responses.[14–16] Therefore, removal of extracellular adenine nucleotides by nucleotidases[17,18] may be of significance, in particular because, in conjunction with 5′-nucleotidase, adenosine (ADO) can be generated, which is a potent inhibitor of inflammatory cells.[15,19]

Although *in vitro* studies indicate that both ADO and PGI_2 inhibit platelets and inflammatory cell responses, the activities of nucleotidases and PGI_2, to our knowledge, never have been studied *in vivo* during acute inflammatory processes.

Therefore, the activities of PGI_2 and ADO are studied during anti-Thy1 nephritis, a model of acute glomerulonephritis (GN) characterized by intraglomerular platelet accumulation and neutrophil influx.[12] Because PGI_2 and ADO both have a short half-life *in vivo*, the stable analogs Iloprost and 2-chloro-adenosine (2Cl-ADO) are applied in the present study.

Results show that these analogs strongly inhibit intraglomerular platelet activation *in vivo*. 2Cl-ADO also reduces *in situ* O_2^- production of neutrophils, but neither 2Cl-ADO nor Iloprost affects other parameters of inflammation. Nevertheless, proteinuria of nephritic rats is completely prevented by both agents.

The inhibitory activity of both analogs upon the inflammatory process *in vivo* demonstrates the protective potential of PGI_2 production and ADO generation in glomeruli, although ADO seems a more effective anti-inflammatory agent as compared with PGI_2. Furthermore, the data support the notion that activated platelets play an important role in the development of proteinuria during anti-Thy1 GN,

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which indicates the necessity of an intact platelet inhibiting mechanism in rat glomeruli.

Materials and Methods

Animals

Female inbred PVG rats (200 to 220 g) fed on standard chow (Hope Farms, Woerden, The Netherlands) and 3 months of age were used in the present study. Peritoneal exudate cells (PEC) were elicited in Lewis rats (300 g) because more cells could be harvested using this strain.

Preparation of Neutrophil Suspensions

PEC were obtained 16 hours after an intraperitoneal injection of 7 ml of 10% proteose pepton (Difco, Detroit,MI) with 0.8% heart infusion broth (Difco) as decribed previously.[12] Suspensions were prepared according to standard procedures[12] and volumes were adjusted to a cell concentration of 7.0×10^5 cells per ml Hanks balanced salt solution.

Biochemical Assay of Oxygen Free Radical Production

O_2^- production of PEC was assayed biochemically by measuring superoxide dismutase (Sigma Chemical Co, St. Louis, MO) inhibitable cytochrome-*c* reduction according to standard methods.[16] Cells were stimulated with 5 ng/ml phorbol myristate acetate (PMA) or with 0.5 mg/ml serum-treated zymosan (STZ), prepared by adding 10 mg of boiled zymosan (Sigma) per ml Lewis pool serum according to standard procedures.[14] 2Cl-ADO (Sigma) and Iloprost (a gift from Schering AG, Berlin, FRG) were added to the incubation media in final concentrations of 0.2 and 2 µmol/L.

Histochemistry

Tissue Processing

Kidneys were removed under ether anesthesia and specimens were snap frozen in isopentane (−80 C). Subsequently, cryostat sections (4 µmol/L) were cut at −20 C and stained for intraglomerular antibody deposition, platelet aggregation, and inflammatory cell influx. O_2^- production *in situ* and myeloperoxidase depositions were also demonstrated histochemically.

Intraglomerular Antibody Deposition and Complement Activation

Air-dried cryostat sections were stained with flourescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin (Ig) G or with FITC-conjugated goat IgG directed against rat C3b (Nordic, Tilburg, The Netherlands) according to standard procedures and examined with a fluorescence microscope (E. Leitz Inc. Rockleigh, NJ).

Inflammatory Cell Influx

Monocytes were demonstrated by staining of kidney sections for nonspecific esterase activity according to standard methods with α-naphthylacetate as the substrate.[20]

Presence of neutrophils was demonstrated using a monoclonal antibody against rat granulocytes (HIS48[21]). In view of the presence of mouse antibodies within glomeruli of nephritic rats (ie, anti-Thy1 IgG), we labeled the mouse monoclonal antibody against granulocytes (HIS48) with biotin (Dakopatts, Glostrup, Denmark). Cells were subsequently demonstrated using the avidin-biotin complex (Dakopatts), according to standard procedures[12] with 3,3′-diaminobenzidin (DAB; Sigma) in the final step.

Intraglomerular Activity of Neutrophils

Activity of neutrophils in glomeruli *in situ* was assessed by histochemical demonstration of superoxide anion production and by immunohistochemical detection of myeloperoxidase (MPO) deposits along capillary walls.

Superoxide anion production by activated polymorphonuclear neutrophils was demonstrated in kidney cryostat sections using Mn^{++} and DAB as decribed previously.[12] Inhibition of staining by addition of superoxide dismutase to the incubation media (300 U/ml) confirms the demonstration of O_2^- production by this method.

MPO deposits were demonstrated using a rabbit polyclonal antibody against rat MPO. Antibodies were prepared by immunizing rabbits with purified MPO that was isolated from rats according to the method of Meuil.[22] Staining for MPO upon acetonfixed cryostat sections was performed using standard indirect fluorescence methods with anti-MPO serum in the first and FITC-conjugated goat anti-rabbit IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) in the second step. Sections were examined with a fluorescence microscope (E. Leitz Inc.).

Platelet Aggregation

Intraglomerular platelet aggregation was demonstrated using a mouse monoclonal antibody against rat platelets (PL-1[12]). This antibody was also labeled with biotin for the reason mentioned above. Staining for platelets was performed as decribed above for neutrophils.

Platelet Factor 4 (PF4) Depositions

PF4 was demonstrated in air-dried cryostat sections using standard indirect immunofluorescence methods with goat antiserum against PF4 (Atlantic Antibodies, Stillwater, MN) and subsequently FITC-conjugated rabbit anti-goat IgG (Kirkegaard & Perry). To remove background staining in tubular brushborders, the antiserum against PF4 was absorbed (30 minutes) using rat tubular brushborder suspensions (1 mg/ml), prepared according to standard methods.

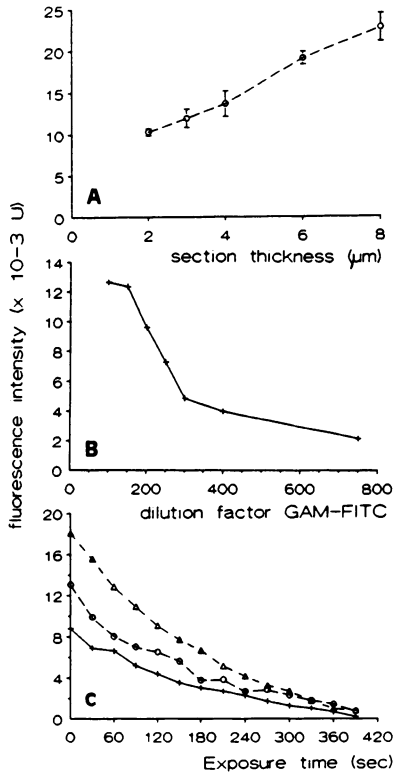

Figure 1. *Glomerular fluorescence intensity, as measured using a light exposure meter, in kidney sections of rats with anti-Thy1 nephritis stained with GAM-FITC. Section thickness* (A), *GAM-FITC concentration* (B), *and FITC intensity* (C) *are varied to assess sensitivity of the method for measurement of fluorescence staining. Data of* A and B *represent arithmetic means of 50 GCS per kidney section and results of* A *are the mean (± SD) of three sections. Extinction of FITC intensity during light exposure* (C) *is demonstrated in three different glomeruli as indicated by three separate curves. It is shown that variations in fluorescence intensity are readily detectable using this method.*

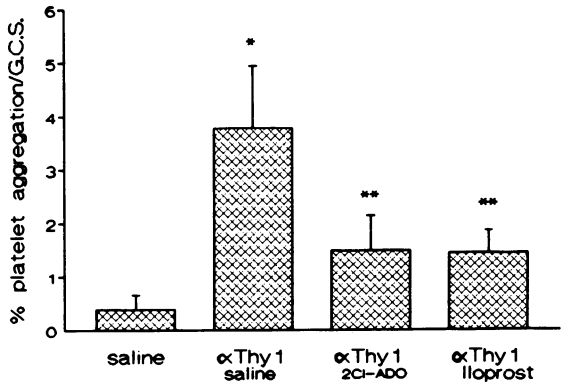

Figure 2. *Intraglomerular platelet aggregation in rats injected with saline or anti-Thy1 IgG. Immunohistological staining was quantitatively evaluated using computerized image analyzing techniques in 6 rats per group. Results are expressed as mean percent platelet aggregation per GCS (± SD) which represents the mean glomerular area stained with PL1. Significant platelet accumulation can be observed in rats with anti-Thy1 GN (*= P < 0.005 as compared with saline, Wilcoxon's test), which is reduced in rats treated with 2Cl-ADO or Iloprost (**= P < 0.005 as compared with untreated anti-Thy1 nephritis). Abbreviation: αThy1= anti-Thy1 IgG.*

Proteinuria

Urine was collected by keeping rats in metabolic cages. Protein excretion per 24 hours was subsequently measured using pyrogallol-red (Merck, Darmstadt, FRG) according to standard methods.[23]

Quantitative Analysis of Tissue Sections and Statistical Evaluation

Intensity of fluorescence staining for anti-Thy1 IgG and complement C3b in each single glomerular cross-section (GCS) was measured using an exposure meter (Leitz Orthomat E, automatic microscope camera). Using spot measurements, light exposure time (seconds) upon 400 ASA film for each GCS was measured. At magnification ×160, the complete GCS can be covered by the spot. The size of the spot is kept constant at the maximal glomerular diameter during all measurements. Variations in the radii of GCS do not significantly interfere with the measurements at lower magnifications because the chance to hit a glomerulus is proportionally to its volume and consequently the maximal radius is by far the most frequent.[24] In a minority of measurements, fluorescence intensity is underestimated because a small portion of the negatively stained surrounding tissue is covered by the spot. However, when the mean glomerular volume is not different among experimental groups, this underestimation is constant in all sections. Fluorescence intensity is inversely proportional to the exposure time. Per kidney section, 50 GCS are measured and the mean staining intensity

Table 1. *Mean Number (± SD) of granulocytes (HIS48), Monocytes (Nonspecific Esterase Positive Cells) and IgG- or C3b-Staining (Fluorescence) per Glomerulus in Different Groups, 24 Hours After Injection of Anti-Thy-1 IgG or Saline*

Group	HIS48*	Nonspecific Esterase*	Fluorescence Intensity ($\times 10^{-3}$ Units)*	
			IgG	C3b
Saline	0.20 ± 0.08	0.15 ± 0.04	0.30 ± 1.5	0.17 ± 2.2
Anti-Thy-1 + saline	2.55 ± 0.84†	0.09 ± 0.09	14.4 ± 1.9†	2.18 ± 1.9†
Anti-Thy-1 + 2Cl-ADO	2.56 ± 0.25†	0.08 ± 0.04	15.2 ± 3.3†	2.59 ± 1.7†
Anti-Thy-1 + Iloprost	2.16 ± 0.74†	0.14 ± 0.06	13.6 ± 4.1†	3.93 ± 1.8†

* 50 glomeruli per kidney section were evaluated.
† = $P < 0.005$ as compared to saline (ANOVA).

of control sections of saline-injected rats is subtracted from the mean staining intensity of nephritic kidneys. Studies with kidney sections of 2-, 3-, 4-, 6-, and 8-μm thickness demonstrate a linear relationship between section thickness (T), ie, intraglomerular IgG deposits and fluorescence intensity (I) measured using this method (Figure 1A, empirical equation: $I = 2.2 \times 10^{-3}T + 5.4 \times 10^{-3}$; $r = 0.9923$). Furthermore, results of studies with goat anti-mouse (GAM)-FITC dilutions and measurements of FITC extinction during light exposure, demonstrate that differences in fluorescence intensity can be measured using this method (Figure 1, B and C). Results are expressed in units ($seconds^{-1}$).

To study inflammatory response upon nephritogenic antibody in rats of different experimental groups, inflammatory cell influx, which can be considered as the resultant of antibody deposition and chemotactic activity, was quantified. Therefore, the number of HIS48 and nonspecific esterase positive cells per 50 GCS were counted in each individual animal.

Intraglomerular neutrophil activity was assessed by counting the number of oxygen free radical-producing cells in 50 GCS. The total number of O_2^- positive cells per kidney section was related to the number of HIS48 positive cells per 50 GCS.

Glomerular fluorescence intensity for PF4 and MPO staining was quantified using the light exposure meter as described above.

Intraglomerular platelet aggregation *in vivo* as detected by staining with PL-1 was quantitatively evaluated using standard computerized image analyzing techniques as described previously.[12] The degree of intraglomerular platelet aggregation is expressed as mean percentage of glomerular area stained positive for platelets.

Results are expressed as arithmetic means (± SD) of six animals per group. Data were analyzed by Wilcoxon's test or analysis of variance (ANOVA) as indicated, and differences were considered significant at $P < 0.05$.

Experimental Design

Six biochemical assays were performed to study effects of Iloprost or 2Cl-ADO upon radical production by PEC *in vitro*. Each assay was performed with a single batch of PEC, obtained from one animal.

For *in vivo* studies, rats received a single intravenous injection of monoclonal anti-Thy1 IgG (5 mg/kg body weight[20]). Rats of group I ($n = 6$) received subsequently 0.3 ml of saline, whereas rats of group II and III received immediately afterwards a subcutaneous injection of 2Cl-ADO ($n = 6$) or Iloprost ($n = 6$), respectively. Pilot studies demonstrated an optimal dose of 10 mg/kg body weight for 2Cl-ADO, whereas Iloprost was administered in a dose of 1 mg/kg body weight.[25] To compare diseased versus nondiseased animals, we used saline-treated rats (group IV; $n = 6$) as control animals. Twenty-four hours after injection of anti-Thy1 IgG or saline, kidneys were removed and processed for histochemical studies as described above.

For longitudinal studies, additional rats received an injection of anti-Thy1 IgG and immediately afterwards a single subcutaneous injection of saline (group V; $n = 6$), 2Cl-ADO (group VI; $n = 6$) or Iloprost (group VII; $n = 6$) in a dose of 10 and 1 mg/kg body weight, respectively. Control rats received an intravenous injection of saline (group VIII; $n = 6$). Urine samples from these rats were collected every other day, starting immediately after the injections (day 1) and finishing at day 11.

Results

Immunohistochemical staining of kidney sections, 24 hours after injection of anti-Thy1 IgG or saline revealed abundant intraglomerular platelet aggregation in rats with anti-Thy1 IgG (Figure 2) and no staining for platelets in glomeruli of rats receiving saline ($P < 0.005$, Wilcoxon's test). Rats treated with 2Cl-ADO or Iloprost after induction of anti-Thy1 GN showed

significantly less intraglomerular platelet aggregation as compared with nephritic rats receiving no treatment ($P < 0.005$, Wilcoxon's test). Both 2Cl-ADO and Iloprost reduced aggregation in nephritic rats to a similar level (Figure 2). A fine granular staining for PF4 could be observed along glomerular capillary walls of rats with anti-Thy1 GN. Although the staining pattern remained unchanged, the staining intensity for PF4 was reduced in nephritic animals treated with 2Cl-ADO or Iloprost; 36.9% (± 18.0) reduction in rats treated with 2Cl-ADO and 26.1% (± 19.9) reduction in Iloprost treated rats, as compared with nephritic animals treated with saline ($P < 0.05$).

Anti-Thy1 GN was also characterized by a profound influx of neutrophils (Table 1). No significant influx of monocytes, as reflected by nonspecific esterase staining in glomeruli, could be observed in nephritic rats (Table 1). This inflammatory cell influx was not affected by 2Cl-ADO or Iloprost: neither HIS48 staining nor the number of nonspecific esterase positive cells was significantly changed in nephritic rats treated with 2Cl-ADO or Iloprost as compared with rats with anti-Thy1 GN receiving no treatment (Table 1). Also, no change in deposition of anti-Thy1 IgG or complement C3b could be observed in glomeruli of different groups of rats with anti-Thy1 nephritis (Table 1).

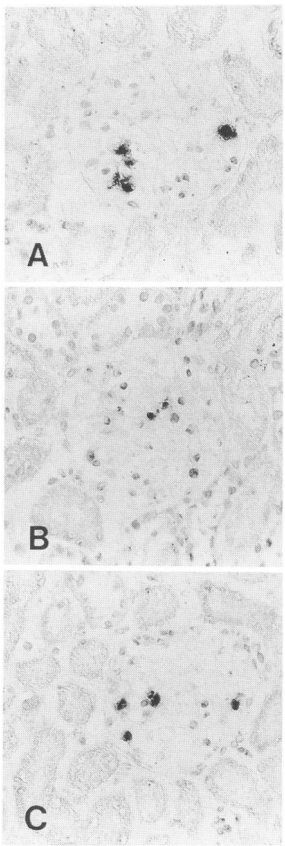

Figure 3. *Micrographs of glomeruli stained for O_2^--producing activity (dark staining), as detected histochemically in cryostat sections, 24 hours after induction of anti-Thy1 GN. Rats were treated with either saline* (**A**), *2Cl-ADO* (**B**), *or Iloprost* (**C**) *after induction of nephritis. Reduced staining for O_2^--producing activity can be observed in rats treated with 2Cl-ADO after injection of anti-Thy1 IgG. Magnification, ×350.*

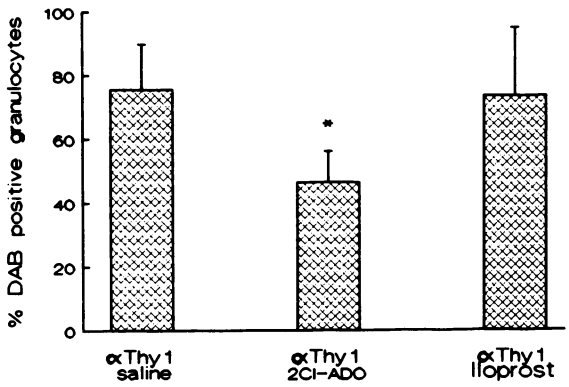

Figure 4. *Mean percentage (± SD) of granulocytes (HIS48 positive cells) producing O_2^- per 50 GCS in different groups of rats with anti-Thy1 GN* (n = 6). *The percentage of granulocytes producing O_2^- in situ, 24 hours after induction of anti-Thy1 GN, is significantly reduced after treatment with 2Cl-ADO but not after treatment with Iloprost (*=$P < 0.05$ as compared with rats receiving anti-Thy1 IgG alone, Wilcoxon's test). Abbreviation as in Figure 2.*

Staining of kidney sections for O_2^--producing activity showed abundant presence of O_2^--producing cells in glomeruli of nephritic rats, whereas virtually no O_2^--producing cells were found in glomeruli of rats injected with saline. Staining intensity per cell for O_2^- production was reduced in rats receiving 2Cl-ADO after the anti-Thy1 injection as compared with staining intensity per cell in nephritic rats treated with saline (Figure 3). Treatment with Iloprost did not influence cellular staining intensity for O_2^-.

Quantitative evaluation of the number of O_2^--producing cells in glomeruli showed that in nephritic rats with anti-Thy1 GN, 1.89 ± 0.65 DAB positive cells per GCS could be detected, whereas in glomeruli of saline injected rats 0.1 ± 0.04 DAB positive cells per GCS were found ($P < 0.005$, Wilcoxon's test). When this number of radical producing cells was related to the influx of HIS48 positive cells, 75.6% ± 14.3 of the HIS48 positive cells were also positive for O_2^- (Figure 4). Treatment of nephritic rats with 2Cl-ADO reduced this percentage to 50.7% ± 4.8 ($P < 0.05$ as compared with rats recieving anti-Thy1 IgG alone). However, treatment with Iloprost caused no reduction in the percentage of O_2^- positive granulocytes (73.2% ± 21.4).

In vitro, both 2Cl-ADO and Iloprost inhibited O_2^- production of activated PEC significantly in a dose-dependent manner (Figure 5). This inhibition

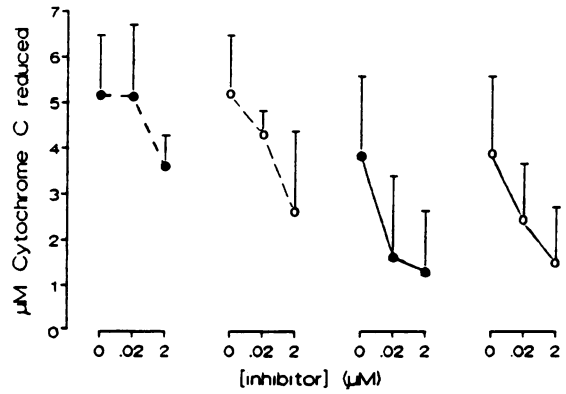

Figure 5. *In vitro O_2^- production of PEC stimulated with either STZ (- - - -) or PMA (——) and incubated in the presence of 2Cl-ADO (closed symbols) or Iloprost (open symbols). It can be seen that both 2Cl-ADO and Iloprost inhibit O_2^- production of neutrophils in vitro upon stimulation with STZ as well as after stimulation with PMA.*

occurred in suspensions of STZ activated as well as in PMA-stimulated PEC.

In glomeruli of rats with anti-Thy1 GN, strong focal immunofluorescence staining for MPO could be detected. Staining seemed to be localized in infiltrating cells and along capillary walls (Figure 6). Intraglomerular MPO depositions were not changed in nephritic rats receiving 2Cl-ADO or Iloprost; distribution as well as staining intensity (Figure 7) was similar to staining in nephritic rats receiving no treatment.

Longitudinal studies of rats with anti-Thy1 GN showed a transient proteinuria from day 1 till day 11, with a maximum at day 3. In contrast, rats receiving a single subcutaneous injection of 2Cl-ADO or Iloprost after induction of GN displayed no proteinuria in this time interval (Figure 8). In both groups of rats, urinary protein excretion did not increase significantly above control levels between day 3 and 11.

Discussion

In the present study, the antithrombotic and anti-inflammatory activities of Iloprost and 2Cl-ADO were examined *in vivo* in the acute phase of anti-Thy1 nephritis. Both Iloprost and 2Cl-ADO strongly reduced intraglomerular platelet aggregation *in vivo* during anti-Thy1 GN to a similar level (Figure 2). This was associated with a reduced deposition of the platelet product PF4 along glomerular capillary walls of nephritic rats after treatment with 2Cl-ADO or Iloprost. However, despite the strong reduction in platelet aggregation, considerable staining for PF4 was still observed along capillary walls in glomeruli of all groups of nephritic rats, suggesting that complete inhibition of platelet activation is necessary to prevent all PF4 depositions.

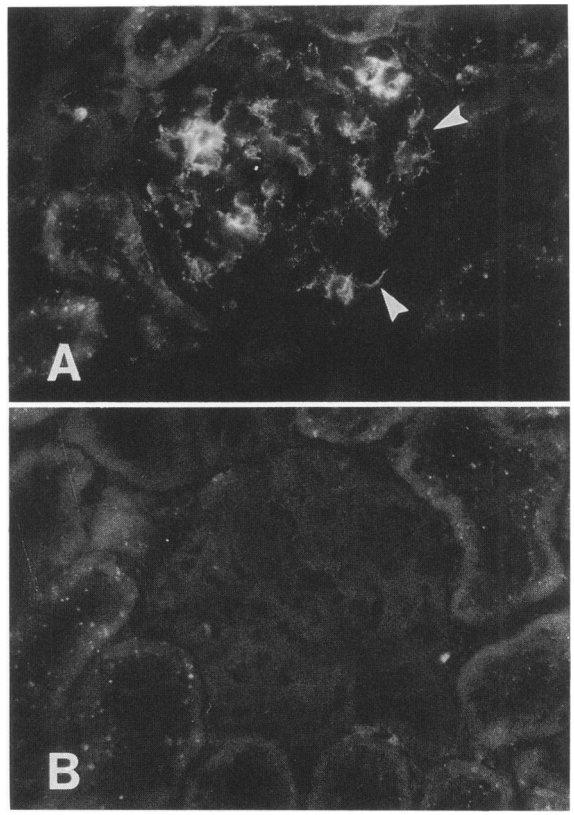

Figure 6. *Intraglomerular staining for MPO, 24 hours after induction of anti-Thy1 GN* (**A**) *or after saline injection* (**B**), *as detected using indirect immunofluorescence methods with rabbit serum against rat MPO. Focal staining for MPO can be observed in infiltrating cells as well as along capillary walls (arrows) in glomeruli of rats with anti-Thy1 GN* (**A**) *but not in control glomeruli* (**B**). *Magnification,* ×*300.*

Potential anti-inflammatory activity of these agents was studied by quantification of several inflammatory parameters and by assessment of *in situ* activity of granulocytes in different groups.

Inasmuch as mesangiolysis during anti-Thy1 nephritis can be induced by the deposition of nephritogenic antibody, complement activation and the influx of inflammatory cells,[26] and because these parameters were not changed by 2Cl-ADO or Iloprost (Table 1), it can be concluded that these agents do not influence this initial part of the disease. Gross histological examination of kidney sections confirmed these immunohistochemical data.

Although application of a light exposure meter does not provide information about the actual amount of PF4, IgG, or C3b depositions in glomeruli, the method is highly sensitive and reproducable for the detection of differences in immunofluorescence between experimental groups, as is demonstrated in Figure 1.

In contrast to inflammatory cell influx as such (Table 1), 2Cl-ADO did influence O_2^- production of

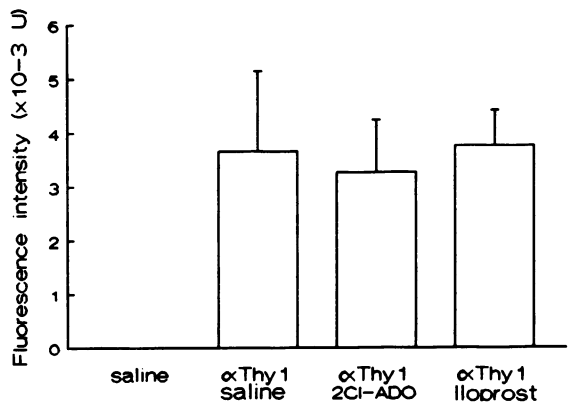

Figure 7. *Mean fluorescence intensity (units) in glomeruli of different experimental rats with anti-Thy1 GN after staining for MPO. Fifty GCS per kidney are evaluated and columns represent arithmetic means (± SD) of six rats per group. Fluorescence staining is related to mean glomerular staining intensity in kidney sections of control rats. A significant increase in staining intensity can be detected in glomeruli of rats with anti-Thy1 GN. No statistical differences in staining intensity for MPO is observed between groups of nephritic rats receiving different treatments (ANOVA). Abbreviation as in Figure 2.*

neutrophils *in situ*. This was reflected by a reduced staining intensity per cell (Figure 3) as well as by a significant reduction in the percentage of neutrophils producing O_2^- *in situ* (Figure 4). After Iloprost treatment, no reduction in staining intensity or in the percentage of activated granulocytes in glomeruli could be detected.

However, *in vitro*, both 2Cl-ADO and Iloprost were able to inhibit oxygen free radical production of activated PEC, irrespective of the neutrophil-activating agent used (Figure 5). Although the reason for this discrepancy between *in vivo* and *in vitro* results is unknown, differences between drugs regarding tissue distribution of agents, half-life *in vivo*, and receptor expression upon target cells may account for this difference.

Inasmuch as proteases from leukocytes significantly contribute to the induction of glomerular damage,[27] intraglomerular enzyme release by neutrophils was studied as well. Because intraglomerular release of enzymes by neutrophils will be associated by MPO depositions *in situ*,[28] staining intensity for MPO was quantified. Focal staining for MPO deposits along capillary walls could be observed in rats with anti-Thy1 GN (Figure 6). Although these depositions of cationic MPO along the anionic filtration barrier have been proposed,[28] this has up to now never been actually observed during experimental nephritis. Because intraglomerular influx of granulocytes was not different between experimental groups (Table 1), changes in MPO staining will reflect changes in degranulation of granulocytes. Quantitative evaluation of glomerular staining for MPO revealed no differences between nephritic rats of different experimental groups (Figure 7), suggesting that 2Cl-ADO nor Iloprost influences enzyme release by neutrophils in this model.

In view of many reports about the anti-inflammatory activity of Iloprost *in vitro*,[5,29] which we could confirm (Figure 5), the absence of a detecable effect of this agent upon any inflammatory parameter during nephritis *in vivo* may be suprising. Our *in vivo* results obtained with 2Cl-ADO do correspond with *in vitro* studies of other authors.[19]

In summary, from the present data it can be concluded that *in vivo*, both Iloprost and 2Cl-ADO exert potent platelet inhibiting activity during anti-Thy1 GN, whereas 2Cl-ADO displays additional anti-inflammatory activity by inhibiting *in situ* O_2^- production of intraglomerular neutrophils.

Inasmuch as the ultimate glomerular damage induced by the inflammatory process is reflected by proteinuria, the effect of both platelet inhibiting agents upon urinary protein excretion was examined in subsequent experiments. As can be seen in Figure 8, rats with anti-Thy1 GN display a transient proteinuria. Proteinuria of rats, although in this PVG strain not as high as has been described for Wistar rats with anti-Thy1 GN,[26] could be completely inhibited by a single injection of either Iloprost or 2Cl-ADO. Thus, treatment of nephritic rats with Iloprost or 2Cl-ADO significantly protects the kidney from damage in the initial phase of acute GN, which is sufficient to prevent proteinuria in subsequent days.

The mechanism by which both agents inhibit proteinuria is not clear. Both 2Cl-ADO and Iloprost have potent vasodilatory effects[30,31] and influence renal blood flow.[32] Because both agents more or less

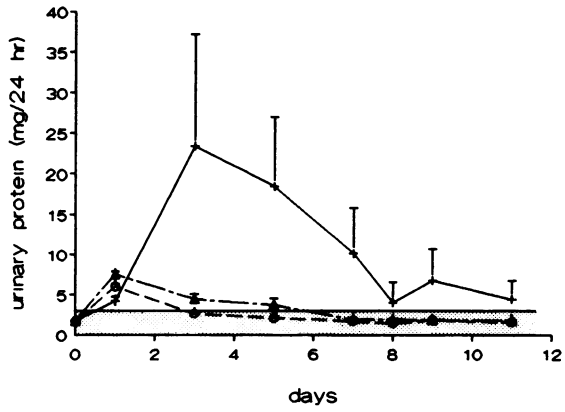

Figure 8. *Mean urinary protein excretion (± SEM) of rats with anti-Thy1 GN (——) and rats with anti-Thy1 GN receiving 2Cl-ADO (- - ○ - -) or Iloprost (— -▲). Shaded area represents range in protein excretion of nonnephritic rats treated with saline.*

reduce glomerular filtration rate,[32] these hemodynamic changes may be responsible for the reduced proteinuria after treatment. However, the complete inhibition of proteinuria after one single injection suggests a diminution of the initial glomerular damage as the causal factor, rather than a transient hemodynamic effect of the agents. Pilot studies from our lab showed reduced platelet responsiveness *in vitro* of 2Cl-ADO and Iloprost treated rats, at day 1 (99.7% and 64.8% reduction of collagen-induced aggregation for 2Cl-ADO and Iloprost, respectively) whereas, at day 2, inhibitory effects were greatly reduced (44.7% and 36.6% reduction in collagen-induced aggregation for 2Cl-ADO and Iloprost, respectively). This suggests that direct effects of the agents are only transient, which is in agreement with *in vivo* studies of other authors, showing that Iloprost levels in plasma are negligible 24 hours after administration of the drug.[33]

Proteinuria in this model does not correspond with intraglomerular oxygen free radical production because Iloprost did not affect *in situ* radical production, whereas proteinuria was completely prevented. This is in agreement with other studies in mice, showing that lysosomal enzyme release by neutrophils, rather than radical production by these cells, contributes to the proteinuria during acute glomerulonephritis.[27]

However, results do suggest a significant role for platelets as a pathogenetic factor for proteinuria inasmuch as the antiplatelet drug Iloprost inhibited proteinuria completely, despite the absence of a detectable effect upon the inflammatory component of anti-Thy1 GN. This notion is supported by results of studies in other models of nephritis, showing reduced proteinuria after platelet depletion.[34,35] The mechanism of this platelet activity is unknown but neutralization of anionic charges by cationic platelet products released from α-granules[36–38] may contribute to proteinuria. However, the small reduction in PF4 staining (a marker for α-granule proteins[38]) observed in 2Cl-ADO or Iloprost treated rats cannot explain the complete inhibition of proteinuria. Release of proteinases from platelets[39] and platelet-neutrophil interactions may also account for this phenomenon.[14,40,41] Inasmuch as platelet depletion as well as neutrophil depletion during acute GN reduces proteinuria,[35] the interaction between platelets and neutrophils seems to be of importance. As a whole, our data suggest that platelets may act as inflammatory cells,[39] which contribute to the proteinuria in more than one way.

The effect of 2Cl-A or Iloprost upon mesangial cell proliferation, characteristic for the second stage of anti-Thy1 GN[26] remains to be established. Some studies demonstrated an important role for platelets[42] and also showed that platelet-derived growth factor is involved in the mesangial cell proliferation,[43] whereas others found no effect of platelet depletion upon cell proliferation in this model.[44] It may be that sufficient amounts of platelet-derived growth factor are released by barely detectable aggregates, just like the PF4 depositions in glomeruli of 2Cl-ADO and Iloprost-treated rats in the present study.

In conclusion, the present study demonstrates the importance of platelet-inhibiting mechanisms within the intact glomerulus. Results of the present study implicate that generation of adenosine by glomerular nucleotidases might play a significant role as such a protective mechanism during nephritis in rat kidneys in addition to intraglomerular PGI_2 production. Adenosine can be generated within the inflammatory microenvironment by hydrolysis of extracellular ATP and ADP, released by activated platelets and damaged cells.[45,46] Hydrolysis of this ATP and ADP with proinflammatory activities[14–16] to adenosine, is accomplished by nucleotidases, present in glomeruli[10–13,17] acting in concert with 5′-nucleotidase, present as an ectoenzyme upon neutrophils.[47] In this way, cooperation between the vessel wall and neutrophils may provide the anti-inflammatory activity.

Studies dealing with antithrombotic and anti-inflammatory mechanisms have focused mainly upon arachidonic acid metabolites in the last decades. However, evidence is accumulating that attention to extracellular adenine nucleotide fluxes may provide a new entrance in coping with acute inflammatory processes.

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References

1. Gaetano de G, Berleté V, Cerletti C: Prostaglandins, thromboxanes and platelet function. Prostacyclin and Its Stable Analogue Iloprost. Edited by RJ Gryglewsky and G Stock. Berlin, Springer-Verlag, 1987, pp 25–37
2. Mustard JF, Packham MA, Kinlough-Rathbone RL: Mechanisms in thrombosis. Haemostasis and Thrombosis. Edited by AL Bloom and DP Thomas. New York, Churchill Livingstone, 1987, pp 618–650

3. Radomski MW, Palmer RMJ, Moncada S: The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. Br J Pharmacol 1987, 92:639–646
4. Boxer LA, Allen JM, Schmidt M, Yoder M, Baehner RL: Inhibition of polymorphonuclear leucocyte adhesion by prostacyclin. J Lab Clin Med 1980, 95:672–678
5. Simpson PJ, Mickelson J, Tantone JC, Gallagher KP, Lucchesi BR: Iloprost inhibits neutrophil function *in vitro* and *in vivo* and limits experimental infarct size in canine heart. Circ Res 1987, 60:666–673
6. Haurand M, Flohe L: Leukotriene formation by human polymorphonuclear leukocytes from endogenous arachidonate: physiological triggers and modulation by prostanoids. Biochem Pharmacol 1989, 38:2129–2137
7. Gordon EL, Pearson JD, Slakey LL: The hydrolysis of extracellular adenine nucleotides by cultured endothelial cells from pig aorta: feed forward inhibition of adenosine production at the cell surface. J Biol Chem 1986, 261:15496–15507
8. Glasgow JG, Schade R, Pitlick FA: Evidence that the ADP hydrolysis by human cells is related to thrombogenic potential. Thromb Res 1978, 13:255–266
9. Lieberman DE, Lewis GP, Peters TJ: A membrane-bound enzyme in rabbit aorta capable of inhibiting adenosine-diphosphate-induced platelet aggregation. Lancet 1977, 2:330–332
10. Bakker WW, Willink EJ, Donga J, Hulstaert CE, Hardonk MJ: Antithrombotic activity of glomerular adenosine diphosphatase in the glomerular basement membrane of the rat kidney. J Lab Clin Med 1987, 109:171–177
11. Poelstra K, Baller JFW, Hardonk MJ, Bakker WW: Demonstration of antithrombotic activity of glomerular adenosine diphosphatase. Blood 1991, 78:141–148
12. Poelstra K, Hardonk MJ, Koudstaal J, Bakker WW: Intraglomerular platelet aggregation and experimental glomerulonephritis. Kidney Int 1990, 37:1500–1508
13. Poelstra K, Baller JFW, Hardonk MJ, Bakker WW: Intraglomerular thrombotic tendency and glomerular ADPase: unilateral impairment of ADPase elicits a pro-aggregatory microenvironment in experimental glomerulonephritis. Lab Invest 1991, 64:520–526
14. Ward PA, Cunningham TW, McCulloch KK, Phan SH, Powell J, Johnson KJ: Platelet enhancement of O_2^- responses in stimulated human neutrophils: identification of platelet factor as adenine nucleotide. Lab Invest 1988, 58:37–47
15. Ward PA, Cunningham TW, McCulloch KK, Johnson KJ: Regulatory effects of adenosine and adenine nucleotides on oxygen radical responses of neutrophils. Lab Invest 1988, 58:438–447
16. Kuhns DB, Wright DG, Nath J, Kaplan SS, Basford RE: ATP induces transient elevations of $[Ca^{2+}]_i$ in human neutrophils and primes these cells for enhanced O_2^- generation. Lab Invest 1988, 58:448–453
17. Hardonk MJ, Kalicharan D, Hulstaert CE: Cytochemical demonstration of ATPase activity in the rat kidney basement membrane using the cerium-based method. Acta Histochem 1985, Suppl Band XXXI: S 253–261
18. Bakker WW, Kalichran D, Donga J, Hulstaert CE, Hardonk MJ: Decreased ATPase activity in adriamycin nephrosis is independent of proteinuria. Kidney Int 1987, 31:704–709
19. Cronstein BN, Levin RI, Belanoff J, Weissmann G, Hirschhorn R: Adenosine: an endogenous inhibitor of neutrophil-mediated injury to endothelial cells. J Clin Invest 1986, 78:760–770
20. Bancroft JD: Enzyme histochemistry. Theory and Practice of Histological Techniques. Edited by JD Bancroft and A Steven. New York, Churchill Livingstone 1982, pp 379–405
21. Goor van H, Fidler V, Weening JJ, Grond J: Determinants of focal and segmental glomerulosclerosis in the rat after renal ablation: evidence for involvement of macrophages and lipids. Lab Invest 1991, 64:754–765
22. Meuil DP: Purification of human myeloperoxidase by concavalin A-sepharose affinity chromatography. Prep Biochem 1980, 10:133–139
23. Watanabe N, Kamei S, Ohkubo A, Yamanaka M, Ohsawa S, Makino K, Tokuda K: Urinary protein as measured with pyrogallol-red molybdate complex, manually and in a Hitachi 726 automated analyzer. Clin Chem 1986, 32:1551–1554
24. Damme van B, Koudstaal J: Measuring glomerular diameters in tissue sections. Virchows Arch A Pathol Anat Histopathol 1976, 369:283–291
25. Clark WF, Parbtani A, McDonald JW, Taylor N, Reid BD, Kreeft J: The effects of a thromboxane inhibitor, a prostacyclin analog and PGE1 on the nephritis of the NZB/W F1 mouse. Clin Nephrol 1987, 28:288–294
26. Bagchus WM, Hoedemaeker PhJ, Rozing J, Bakker WW: Acute glomerulonephritis after intravenous injection of monoclonal anti-thymocyte antibodies in the rat. Immunol Lett 1986, 12:109–113
27. Schrijver G, Schalkwijk J, Robben JCM, Assman KJ, Koene RA: Antiglomerular basement membrane nephritis in beige mice: deficiency of leukocytic neutral proteinases prevents the induction of albuminuria in the heterologous phase. J Exp Med 1989, 169:1435–1448
28. Johnson RJ, Couser WG, Chi EY, Adler S, Kiebanoff SJ: New mechanism for glomerular injury: myeloperoxidase-hydrogen peroxide-halide system. J Clin Invest 1987, 79:1379–1387
29. Belch JJF, Saniabadi A, Dickson R, Sturrock RD, Forbes CD: Effect of Iloprost (ZK 36374) on white cell behavior. Prostacyclin and Its Stable Analogue Iloprost. Edited by RJ Gryglewsky and G Stock. Berlin, Springer-Verlag 1987, pp. 97–102
30. Hopwood AM, Lincoln J, Kirkpatrick KA, Burnstock G: Adenosine 5′-triphosphate, adenosine and endothelium-derived relaxing factor in hypoxic vasodilatation of the heart. Eur J Pharmacol 1989, 165:323–326
31. Gerber JG, Payne NA, Murphy RC, Nies AS: Prostacyclin produced by the pregnant uterus in the dog may act as a ciculating vasopressor substance. J Clin Invest 1981, 67:632–636

32. Arendshorst WJ, Navar LG: Renal circulation and glomerular hemodynamics. Diseases of the Kidney. Edited by RW Schrier and CW Gottschalk. Boston, Little Brown, 1988, pp 65–117
33. Krause W, Schubert M: Pharmacokinetics and biotransformation of the prostacyclin analogue, ZK 36 374. II. Blood and plasma levels and passage of the blood/brain barrier in the rat. Eur J Drug Metab Pharmacokinet 1983, 8:145–150
34. Johnson RJ, Alpers CE, Pritzl P, Schultze M, Baker PJ, Pruchno C, Couser WG: Platelets mediate neutrophil dependent immune complex nephritis in the rat. J Clin Invest 1988, 82:1225–1235
35. Johnson RJ, Alpers CE, Pruchno C, Schultze M, Baker PJ, Pritzl P, Couser WG: Mechanisms and kinetics for platelet and neutrophil localization in immune complex nephritis. Kidney Int 1989, 36:780–789
36. Camussi G, Tetta C, Coda R, Segoloni GP, Vercellone A: Platelet-activating factor-induced loss of glomerular anionic charges. Kidney Int 1984, 25:73–81
37. Tetta C, Coda R, Camussi G: Human platelet cationic proteins bind to rat glomeruli, induce loss of anionic charges and increase glomerular permeability. Agents Actions 1985, 16:24–26
38. Barnes JL, Levine SP, Venkatachalam MA: Binding of platelet factor four (PF4) to glomerular polyanion. Kidney Int 1984, 25:759–765
39. Nachman RL, Weksler BB: The platelet as an inflammatory cell. Handbook of Inflammation, vol 2. Edited by LE Glynn, JC Houck, and G Weissmann. Amsterdam, Elsevier 1980, pp 145–162
40. Margaretten W, McKay DG: The requirement for platelets in the active arthus reaction. Am J Pathol 1971, 64:257–270
41. Ward PA, Macconi D, Sulavik MC, Till GO, Warren J, Johnson KJ, Powell J: Rat neutrophil-platelet interactions in oxygen radical-mediated lung injury. Oxy-Radicals in Molecular Biology and Pathology. Edited by P Cerutti, I Fridovich, and JM McCord. New York, Alan R Liss, 1988, pp 83–98
42. Johnson RJ, Garcia RL, Pritzl P, Alpers CE: Platelets mediate glomerular cell proliferation in immune complex nephritis induced by anti-mesangial antibodies in the rat. Am J Pathol 1990, 136:369–374
43. Johnson RJ, Raines EW, Floege J, Yoshimura A, Pritzl P, Alpers C, Ross R: Inhibition of mesangial cell proliferation and matrix expansion in glomerulonephritis in the rat by antibody to platelet-derived growth factor. J Exp Med 1992, 175:1413–1416
44. Elema JD, Jeunink MF, Bagchus WM: Platelets and mesangial cell proliferation. Kidney Int 1989, 35:345
45. Gordon JL: Extracellular ATP: effects, source and fate. Biochem J 1986, 233:309–319
46. Varani J, Phan S, Gibbs DF, Ryan US, Ward P: H_2O_2-Mediated cytotoxicity of rat pulmonary endothelial cells: changes in adenosine triphosphate and purine products and effects of protective interventions. Lab Invest 1990, 63:683–689
47. Waeg van G, Berghe van den G: Purine catabolism in polymorphonuclear neutrophils: phorbol myristate acetate-induced accumulation of adenosine owing to inactivation of extracellular released adenosine deaminase. J Clin Invest 1991, 87:305–312