Neutrophil Function in an Experimental Model of Hemolytic Uremic Syndrome

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ABSTRACT. To understand the role of neutrophil leukocytosis in hemolytic uremic syndrome, we studied the changes in neutrophil function in the modified generalized Schwartzman reaction in rabbits. This model resembles hemolytic uremic syndrome associated with endotoxemia. At the end of an endotoxin infusion, we observed leukopenia, thrombocytopenia, and a decrease in hematocrit associated with schistocytosis. Plasma B-glucuronidase levels increased and this was associated with a decrease in neutrophil content of the enzyme. The chemotactic index and neutrophil aggregation to zymosan-activated serum were impaired compared to controls. The neutrophil procoagulant content increased after endotoxin infusion. The serum creatinine concentration and proteinuria increased in the endotoxin-treated animals. The changes returned to normal by 48 h. Renal cortical malondialdehyde, a reflection of lipid peroxidation, was higher in the endotoxin-treated animals than in the controls. We have shown enzyme release by neutrophils, impairment of chemotaxis and aggregation, increased procoagulant content in neutrophils, and evidence of lipid peroxidation in renal cortical tissue in this model. These observations raise the possibility that leukocytes may have a role in the pathogenesis of the hemolytic uremic syndrome. (*Pediatr Res* 21: 252-256, 1987)

Abbreviations

HUS, hemolytic uremic syndrome
MGSR, modified generalized Schwartzman reaction
ZAS, zymosan-activated serum
MDA, malondialdehyde
PMN, polymorphonuclear
HBSS, Hanks' balanced salt solution
MEM, minimal essential medium
MSB, Martius yellow scarlet and soluble blue stain
PMN, polymorphonuclear cells

The HUS is an acute illness mainly of childhood, characterized by acute hemolytic anemia with typical fragmented red blood cells, thrombocytopenia, and nephropathy (1, 2). A neutrophil leukocytosis has been documented in many cases (3-5) including those of Gasser *et al.* (6). The occurrence of a neutrophil leukocytosis is not surprising because many cases are thought to follow infections with agents such as *Escherichia coli* (7), shigella (5, 8),

salmonella (9), or pneumococci (10). It is surprising, however, that there have been no reports of the functional state of these cells because neutrophils have been shown to cause endothelial injury experimentally by release of enzymes, by the production of oxygen intermediates, and by alterations in prostaglandins. Endotoxin-induced renal injury in the generalized Schwartzman reaction has been prevented by depletion of neutrophils before administration of endotoxin (11). Similarly, Butler *et al.* (12) have shown that busulphan-treated leukopenic rabbits did not develop renal cortical necrosis after injection of lipopolysaccharide derived from *Shigella* species. Glomerular endothelial cells are generally accepted as the targets of injury in HUS (1, 2, 13), but the studies reported herein have focussed on changes in neutrophil function because of the difficulty of studying neutrophil-endothelial interactions. We have previously demonstrated that platelet function is impaired in a modified model of the MGSR (14). This model which has similar features to the form of HUS associated with shigellosis (5, 8) has been studied extensively in our laboratory (14). The purpose of the present study was to determine whether circulating neutrophils were functionally impaired as a result of prolonged infusion of endotoxin in this model.

MATERIALS AND METHODS

Male New Zealand White rabbits weighing 1.5 kg were purchased from Ferme Cunicole (St. Scholastique, Quebec, Canada) kept in an animal facility for 2-3 days before use and fed a standard food. The MGSR was produced by infusing endotoxin in a dose of 40 µg/kg/h for 5 h (14). The endotoxin was dissolved in 5 ml of a 0.9% sodium chloride solution and was a Boivin preparation of *E. coli*, the biological characteristics of which have been described previously (15). Control animals were given infusions of pyrogen-free saline in the same volume. Blood samples were drawn from ear arteries at 0, 3, 5, 24, and 48 h. Measurement of white blood cell count, neutrophil count, platelet count, hematocrit, serum creatinine concentration, and the examination of peripheral blood smears were done by standard laboratory methods.

Isolation of neutrophils. Neutrophils were isolated from citrated blood by a modified Herrson's procedure (16). Ten ml of blood were collected with acid citrate dextrose and spun at 550 g for 20 min at room temperature. The plasma, buffy coat, and top 2 mm of erythrocytes and leukocytes were removed and the tube was filled with 2.5% gelatin in normal saline. The blood gelatin mixture was drawn up in 10-ml syringes, which were inverted and placed in an incubator at 37° C for 45 min. The supernatant containing the granulocytes was ejected through a bent 18-gauge needle into polypropylene tubes and centrifuged at 400 × g for 10 min at 22° C. The button of cells was resuspended in 0.2% saline and held for 30 s, followed by addition of an equal volume of 1.8% saline solution to restore tonicity. The tube was centrifuged at 400 × g for 10 min at 4° C. The cells at the bottom were washed in HBSS, containing

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Hepes buffer (pH 7.2) and resuspended in it to the desired cell concentration.

B-glucuronidase assay. The B-glucuronidase content in plasma and of sonicated neutrophil suspensions was measured with Sigma Kit no. 325 (Sigma Chemicals, St. Louis, MO). The activity of B-glucuronidase contained in the neutrophils was expressed per 1000 μg of the DNA content of each sample. DNA was measured by the indole method (17).

Neutrophil chemotaxis. Chemotaxis was assayed using the agarose method (18). ZAS (activated C5) was used as a chemoattractant. The gel was prepared by dissolving 0.8 g/100 ml agarose (type I, Low EED, A-6013, Sigma Chemicals). Eagle's MEM and decomplexed rabbit serum were added to achieve a final concentration of 10%. About 8.5 ml of this mixture was poured onto 15-mm tissue culture plates (Corning, no. 3002/60 Corning Glass Works, Corning, NY) allowed to gel, and stored at 4° C until use. Wells were punched into the gel using a template with configuration as described (18). Chemoattractant, zymosan, and activated serum (activated C5), were placed into the outermost wells. Fifteen- μl neutrophil suspensions (5×10^7 cells/ml) were placed in the middle two wells and 15 μl of HBSS with hepes buffer were placed in the central well. The plates were incubated at 37° C in an atmosphere of 5% carbon dioxide for 6 h. After the incubation, the plates were flooded with a 1% glutaraldehyde solution for 10 min. The gel was gently flipped out and discarded. The plates were then washed in distilled water stained with Wright's stain. The distances moved by the outermost edge of neutrophils were measured using a loupe. The chemotactic index was calculated as follows: distance moved toward chemoattractant divided by the distance moved as a result of random migration (toward the buffer).

Neutrophil aggregation. Neutrophil aggregation was measured using a Chronolog Aggregometer (Chronolog Corporation, Havertown, PA) coupled to a Fisher Recordall (Fisher Scientific Co., Montreal, Quebec, Canada). The machine was calibrated using the neutrophil suspension for the baseline light transmission and the buffer (HBSS with hepes) for the peak light transmission. Four hundred μl (1×10^6 /ml) neutrophil suspension were placed in a cuvette containing a stirring bar. After obtaining a steady baseline for 1 min, 100 μl of ZAS were added and the changes in light transmission were recorded until a steady record was obtained for 1 min. The aggregation response was measured as the maximum increase in light transmission of the specimen expressed as a percentage of the difference in light transmission between the neutrophil suspension and the buffer.

Neutrophil procoagulant activity. Neutrophil procoagulant activity was measured by a one stage test as follows (20): 0.1 ml of rabbit plasma and 0.1 ml of the neutrophil lysate suspension were mixed and the clotting time was measured after addition of 0.1 ml g 0.025 M CaCl_2 in a MLA Electra Coagulation Timer E650. The procoagulant activity was expressed as the time taken

to clot the plasma by a unit number of PMNs containing 1,000 μg of DNA.

Fibrin deposition in glomeruli. This was assessed by hemotoxylin and eosin stain and MSB in paraffin-embedded sections of kidneys.

Renal cortical MDA content. The animal was anesthetized, the abdomen was opened, a cannula was inserted into the aorta, the aorta was ligated above the level of the renal arteries, a renal vein was incised, and the kidneys were flushed with sterile normal saline. After removal of the capsule about 1 g of cortex was placed in 5 ml of HBSS and homogenized with butylated hydroxytoluene in 0.1 N HCl. Protein was precipitated out by adding 0.5 ml 60% trichloroacetic acid. An aliquot was removed for the determination of the protein concentration. The MDA was determined in the supernatant by the thiobarbituric acid method (19) in the kidneys of four control and four experimental animals.

Measurement of proteinuria. Urine protein was determined by Lowry's method in a urine sample collected from the bladder at the time of laparotomy. The urine creatinine concentration was measured by a modified Jaffe's method in the same sample. Values were expressed as mg protein per mg of creatinine in the urine.

Statistics. Statistical significance was determined by the unpaired Student's *t* test. the Wilcoxon rank sum test was used for non-parametric data. Values were expressed as the mean $\pm$ SEM.

RESULTS

Seventeen rabbits were given endotoxin infusions and 12 served as controls. A control rabbit was studied with each (or pair) of the experimental rabbits. Each study could not be done in every rabbit as some died or sufficient blood could not be obtained from others. All control animals were killed at 48 h. Two endotoxin-treated animals died during the infusion, six died between 5 and 24 h, and three died between 24 and 48 h. Six rabbits survived for 48 h. Endotoxin-treated rabbits developed signs of endotoxemia: fever, lethargy, and conjunctival inflammation. Anemia (Table 1), thrombocytopenia (Fig. 1), and a rise in the serum creatinine concentration (Table 2), occurred in all endotoxin-treated animals.

Leukocyte count. The leukocyte counts (Table 3) in peripheral blood in experimental animals showed an initial reduction in number at 3 h, and a further decrease by 5 h. This was followed by an increase in number at 24 h and then a drop at 48 h. Over the same time intervals, control animals' counts were almost unchanged. The differences between the means of the leukocyte counts of control and endotoxin-treated animals were statistically significant at 3, 5, 24, and 48 h. A differential count was done in six experimental rabbits at 5 h. The percentage of PMN was 12 ± 6 and no band forms were seen.

Table 1. Hematocrit and reticulocyte count

Hematocrit (%)	0	3	5	24	48
H					
Control	35 $\pm$ 1*	34 $\pm$ 1	33 $\pm$ 1	34 $\pm$ 2	36 $\pm$ 1
	(12)†	(6)	(12)	(12)	(12)
Experimental	34 $\pm$ 1	27 $\pm$ 2	24 $\pm$ 1	24 $\pm$ 2	29 $\pm$ 1
	(17)	(8)	(15)	(9)	(6)
<i>p</i>		NS	<0.001	<0.05	<0.05
Reticulocyte count (%)	0	5	24	48	
H					
Control	1.0 $\pm$ 0.3	1.3 $\pm$ 0.4	2.6 $\pm$ 1.2	3.8 $\pm$ 1.6	
	(12)	(12)	(12)	(12)	
Experimental	1.2 $\pm$ 0.4	1.6 $\pm$ 0.6	8.2 $\pm$ 0.6	9.0 $\pm$ 1.2	
	(17)	(15)	(9)	(6)	
<i>p</i>		NS	<0.01	NS	

* Mean $\pm$ SEM.

† Number of animals studied.

Table 2. Serum creatinine

Sample	Control (mg/100 ml)	Experimental (mg/100 ml)	<i>p</i>
0 H	0.80 ± 0.07* (12)†	0.82 ± 0.06 (17)	
3 H	0.80 ± 0.07 (5)	1.34 ± 0.13 (8)	<0.05
5 H	0.88 ± 0.10 (12)	1.56 ± 0.16 (17)	<0.02
24 H	1.03 ± 0.12 (9)	1.77 ± 0.81 (9)	NS
48 H	1.08 ± 0.13 (12)	0.82 ± 0.08 (6)	NS

* Mean ± SEM.

† Number of animals studied.

Fig. 1. Platelet count in peripheral blood in control and experimental animals.

Table 3. Leukocyte counts in control and endotoxin-infused rabbits

Time (h)	Leukocyte count (/cmm)	
	Control	Experimental
0	5391 ± 263	5276 ± 296
3	6100 ± 870	1300 ± 207*
5	7750 ± 774	1153 ± 112*
24	6837 ± 622	17088 ± 2856†
48	6428 ± 1761	11480 ± 1012‡

Controls versus experimental: * $p < 0.001$; † $p < 0.01$; ‡ $p < 0.02$.

Plasma and neutrophil B-glucuronidase. The plasma levels of B-glucuronidase rose during the infusion period reaching a peak level at 5 h of endotoxin infusion, followed by a gradual decrease to preinfusion levels by 48 h (Fig. 2). These differences were significantly different at 3 and 5 h. During the infusion of endotoxin, the neutrophil B-glucuronidase content decreased and then started to increase, reaching near normal values at 48

Fig. 2. Plasma B-glucuronidase activity (upper panel) in control and experimental animals expressed as percentage of mean activity of controls at zero time (100% activity = 42.47 Modified sigma units/ml). Neutrophil B-glucuronidase activity (lower panel) in control and experimental groups, expressed as percentage of mean activity of controls at zero time (100% activity = 268.74 modified sigma units/1000 μ g DNA).

h (Fig. 2). The difference in means compared to controls were statistically significant at 3 h ($p < 0.02$) and at 5 h ($p < 0.01$). The decrease in neutrophil content was associated with a rise in the plasma levels of the enzyme ($r = -0.738$).

Neutrophil procoagulant content. The procoagulant content in neutrophils increased by 5 h in endotoxin-treated animals (Table 4). This increase was evidenced by a shorter time required to clot the test plasma. The mean time to form a clot decreased from 312.8 ± 64.6 s/1000 μ g DNA ($100 \pm 27\%$) at zero time to 51.4 ± 22.6 s/1000 μ g DNA ($16 \pm 7\%$) at 5 h. The difference in means compared to the saline-infused animals was significant ($p < 0.01$). At 24 h, the procoagulant activity decreased as reflected by an increase in the time taken to clot 121.3 ± 45.1 s/1000 μ g DNA ($39 \pm 14\%$) ($p < 0.5$). Fibrin deposition in glomerular capillary loops was present in 55% of experimental animals (four of seven) and in none of the controls (none of six).

Neutrophil chemotaxis. Chemotaxis measured as the chemotactic index in response to ZAS was depressed in the endotoxin

infused animals (Table 4). The effect was maximal at the end of infusion (Table 4) (5 h) when it was 1 ± 0.0 compared to 2.1 ± 1.0 at zero time. This was statistically significant compared to controls ($p < 0.05$). At 24 and 48 h, the means of experimental and control animals were not statistically significant.

Neutrophil aggregation. Aggregation of neutrophils in response to ZAS showed a decreased response at the end of 5 h of infusion in the endotoxin-treated animals (Table 4). The percentage of light transmission decreased from $40 \pm 3\%$ (zero) to $14 \pm 4\%$ at 24 h and $28 \pm 7\%$ at 48 h. The values in control animals were $41 \pm 5\%$ (zero), $45 \pm 3\%$ (5 h), $40 \pm 6\%$ (24 h), and $38 \pm 12\%$ (48 h).

Urine protein. Urine protein excretion, measured as protein to urine creatinine ratio, was 9.7 ± 2.4 in the endotoxin-infused animals and 2.0 ± 2.5 in saline controls ($p < 0.01$).

Renal cortical MDA content. The MDA content in renal cortical slices was higher in endotoxin infused animals compared to controls: $98 \mu\text{mol}/100 \text{ mg}$ protein versus $67 \mu\text{mol}/100 \text{ mg}$ protein ($p < 0.05$).

DISCUSSION

Leukocytosis occurs so often in HUS that it is surprising that Gasser *et al.* (6) did not include this finding in their definition of HUS. Attention has focused on the roles of the coagulation system and platelets in the pathogenesis of glomerular endothelial injury and platelet-endothelial interactions have been the focus of treatment aimed at reducing renal injury (1, 2). Leukocytes are more powerful agents of tissue damage and yet few studies have been directed toward defining the role for leukocytes in the pathogenesis of HUS. The presence of neutrophils in the kidney was commented on in early reports of HUS (3, 4, 21). With improvements in survival, few cases now die soon after onset, and biopsies are done weeks after presentation, so that the presence of neutrophils in glomeruli almost has been forgotten. Bolande and Kaplan (22) examined leukocytes in the buffy coats of patients with HUS soon after presentation and noted, in some cases, that there were tactile relationships between leukocyte cell

processes and altered red blood cell walls and that leukocytes had abnormal morphology with cytoplasmic projections.

Leukocyte interactions with vessel walls can be studied in a series of stages *in vitro* (23), but more simpler whole animal experiments can be performed. We have studied aspects of neutrophil function in an experimental model, the modified generalized Schwartzman reaction, induced by prolonged endotoxin infusion in rabbits and describe the sequential changes in neutrophil number and function after a 5-h period of endotoxin infusion. At the end of the endotoxin infusion, the following significant changes were found: a low leukocyte count, an increase in plasma B-glucuronidase, and a decrease in the intracellular content of this enzyme. The plasma enzyme appears to have been derived from the neutrophils as there was a significant negative correlation ($R = -0.738$) between plasma and neutrophil B-glucuronidase. It is possible that other tissues could have been the source of this enzyme (24). Chemotaxis was depressed, the aggregation response was reduced, and the neutrophil procoagulant content was increased. These findings indicate that neutrophil activation and exhaustion occurred. Preferential margination of an active subpopulation of neutrophils and intravascular enrichment of functionally active neutrophils cannot be ruled out (25).

Neutrophils are required to produce the classical Schwartzman reaction, as animals made neutropenic do not develop this reaction (26). In this model, the serum creatinine concentration, an indicator of renal function, was highest when the plasma levels of B-glucuronidase were at their peak and perturbation of neutrophil functions were most apparent. This raises the possibility that neutrophil activation may be one of the mechanisms by which endotoxin mediates renal injury in this model.

Renal cortical MDA was increased significantly in the animals that received endotoxin. These changes occurred at 5 h (*i.e.* at the end of infusion) which corresponds to peak neutrophil activation in these animals. MDA is the stable end product formed during peroxidation of lipids. Oxygen radicals generated by neutrophils cause lipid peroxidation, and this has been shown in viscera of animals following endotoxin treatment (27, 28). Oxygen radicals from PMN have been shown to be a mechanism by

Table 4. Neutrophil procoagulant activity, chemotactic response to ZAS and aggregation response to ZAS

	0 H	5 H	24 H		
Neutrophil procoagulant activity (%)					
Control	$100 \pm 11^*$ (8)†	138 ± 46 (8) ‡	100 ± 12 (8)	NS	
Experimental	100 ± 27 (10)	16 ± 7 (10)	39 ± 14 (6)		
	0 H	5 H	24 H	48 H	
Chemotactic response to ZAS (chemotactic index)					
Control	2.02 ± 0.90 (6)	3.67 ± 2.08 (6) §	1.89 ± 0.50 (6)	NS	2.42 ± 1.53 (6) NS
Experimental	2.10 ± 1.01 (6)	1.00 ± 0.03 (6)	1.19 ± 0.29 (5)		2.68 ± 1.87 (4)
	0 H	5 H	24 H	48 H	
Aggregation response to ZAS (% maximum light transmission)					
Control	41 ± 5 (6)	45 ± 3 (6) ‡	40 ± 6 (6)	NS	38 ± 12 (6) NS
Experimental	40 ± 3 (6)	14 ± 4 (6)	23 ± 9 (5)		28 ± 7 (4)

* Mean $\pm$ SEM.

† Number of animals studied.

‡ $p < 0.01$. (Student's *t* test).

§ $p < 0.05$ (Student's *t* test).

which PMN cause endothelial injury in glomeruli (29). The increase in MDA in cortical tissue of experimental animals indicates that lipid peroxidation occurred possibly as a result of superoxide generation by activated neutrophils.

Endotoxin-treated animals had fibrin deposits in glomerular capillary loops 5 h after starting the experiment and also had an increase in the neutrophil procoagulant content. Endotoxin increases the neutrophil procoagulant content (21, 30) and this can activate the coagulation cascade (30). These findings suggest but do not prove a sequence in which activated neutrophils may be augmenting a localized coagulation process.

We have demonstrated activation of neutrophils and concomitant renal injury following endotoxin infusion. Although these animals developed features of HUS, the endotoxin dose used was large and for a well-defined period of time, which seldom occurs in clinical settings. We also recognize that this model is not absolutely representative of HUS but such a model does not exist. Activation of neutrophils coincided with evidence of renal injury and we therefore speculate that neutrophils may contribute to the pathogenesis of the renal damage in this model and in patients with HUS associated with endotoxemia. Additional observations in patients with HUS are in accord with the possibility that renal cortical injury may be the result of many factors that include a role of endotoxin (5), complement (31, 32), and platelets (14, 33) and neutrophils. A sequence of events is thereby envisaged that is in many respects analogous to that postulated for the pathogenesis of lung injury associated with bacterial endotoxins (34). In HUS, however, renovascular endothelial cells bear the brunt of the damage.

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