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Importance of immunoenzyme histochemical reaction in diagnosis of disseminated intravascular coagulation in human and animal material.

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Abstract

Renal tissues from 208 human necropsies were observed histologically for disseminated intravascular coagulation (DIC). The tissues were stained with hematoxylin-eosin, Mallory's phosphotungstic acid hematoxylin (PTAH) and cationic ferric hydroxide colloid stabilized with cacodylate (Fe-Cac), and tested by immunoenzyme histochemical (IEH) reaction for fibrin-related materials (FRMs). The use of the IEH method increased FRM recognition, and FRMs were detected in a total of 80 cases (38.5%). In 26 cases diagnosed clinically as DIC, FRMs were shown in 23 of the cases (88.5%). Thus, 57 patients with FRMs were clinically asymptomatic. In rats with DIC induced by endotoxin injection, glomerulus FRM was effluxed into the tubulus through the Bowman's capsule and was excreted into urine. The electric charge was reduced on the endothelial surface of the glomerular capillaries in both human and rat DIC. Under the scanning electron microscopy, the endothelial surface appeared coarse in the glomerular capillary and fibrin degradation was present. Our conclusions are: (a) PTAH is non-specific for FRMs, (b) IEH aids the pathohistological diagnosis of DIC, especially in asymptomatic forms including the compensated DIC state, (c) FRMs in tubuli suggest DIC, and (d) DIC is possibly initiated by a reduction in the capillary electric surface charge.

KEYWORDS: disseminated intravascular coagulation, immunoenzyme-histochemistry, fibrin-related materials

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Importance of Immunoenzyme Histochemical Reaction in Diagnosis of Disseminated Intravascular Coagulation in Human and Animal Material^a

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Renal tissues from 208 human necropsies were observed histologically for disseminated intravascular coagulation (DIC). The tissues were stained with hematoxylin-eosin, Mallory's phosphotungstic acid hematoxylin (PTAH) and cationic ferric hydroxide colloid stabilized with cacodylate (Fe-Cac), and tested by immunoenzyme histochemical (IEH) reaction for fibrin-related materials (FRMs). The use of the IEH method increased FRM recognition, and FRMs were detected in a total of 80 cases (38.5%). In 26 cases diagnosed clinically as DIC, FRMs were shown in 23 of the cases (88.5%). Thus, 57 patients with FRMs were clinically asymptomatic. In rats with DIC induced by endotoxin injection, glomerulus FRM was effluxed into the tubulus through the Bowman's capsule and was excreted into urine. The electric charge was reduced on the endothelial surface of the glomerular capillaries in both human and rat DIC. Under the scanning electron microscopy, the endothelial surface appeared coarse in the glomerular capillary and fibrin degradation was present. Our conclusions are: (a) PTAH is non-specific for FRMs, (b) IEH aids the pathohistological diagnosis of DIC, especially in asymptomatic forms including the compensated DIC state, (c) FRMs in tubuli suggest DIC, and (d) DIC is possibly initiated by a reduction in the capillary electric surface charge.

Key words : disseminated intravascular coagulation, immunoenzyme-histochemistry, fibrin-related materials

Disseminated intravascular coagulation (DIC) is a clinical entity histologically characterized by microcirculation of fibrin thrombi, but its diagnosis is a controversial issue between clinicians and pathologists (1-11). Fibrin microthrombi have not been

found in 45% to 75% of cases diagnosed clinically as DIC (4, 10, 11). On the other hand, 70% to 87% of cases with microthrombi did not show DIC symptoms (4, 10, 11). These discrepancies are probably due to the effect of different primary diseases and differing mechanisms of abnormal clotting and fibrinolysis, and to the ill-defined criteria of DIC (4). Fibrin thrombi are unstable following treatment with anticoagu-

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lants and are quickly altered to fibrin degradation products (FDPs). The severity and continuity of the DIC state and the time lapse between death and necropsy may also contribute to the discrepancy. Special stains, such as phosphotungstic acid hematoxylin (PTAH), Lendrum's Martius Scarlet Blue (MSB) (6) and Weigert for fibrin thrombus, ordinarily used in pathohistology, are only sensitive to mature fibrin thrombus and non-specific to fibrin or FDPs.

Robboy *et al.* (8) stated that even a single thrombus found in a glomerulus is presumptive evidence of DIC, and that the kidneys are the best organ for a histological diagnosis. There is, however, no report on the histology of fibrin-related material (FRM) in the renal tubuli. Recently, the usefulness of immunoenzyme histochemical (IEH) reaction for detecting FRMs has been suggested (12, 13). In the present study, we report a histological study of DIC using IEH in renal tissues of 208 human necropsied cases (14) and in rats with DIC induced with endotoxin (15).

Materials and Methods

Human materials. Renal tissues were obtained from 220 consecutive cases necropsied between April 1977 and May 1985 at the Himeji Red Cross Hospital. Of these cases, 208 did not have renal disease as a complication and were selected for observation. The age of the 208 patients ranged from a newborn infant to 84 years. There were 113 cases of malignant tumor, and the remainders were mostly liver diseases, infectious diseases and heart diseases. Cases of hemorrhagic diathesis accompanying hematological abnormalities, such as thrombocytopenia, prolonged prothrombin times, high serum FDP levels and low fibrinogen levels, were regarded as being in a clinical DIC state (14).

Animals. Male Wistar rats weighing 280–320 g were used. They were intravenously drip-infused with 100 ml of saline (20 ml/h) for 5 h

and injected with endotoxin (0.1 mg/kg body weight of *E. coli* 026 with B6 lipopolysaccharide B, Difco Lab., MO, USA). Controls were infused with saline. Prior to sacrifice, blood was collected from the abdominal aorta and used for platelet count and for measurement of FDP and fibrinogen, and the excreted urine was stored for 1 or 2 h and tested for FDP by the latex aggregation reaction (FDPL test U, Teikoku Hormone Mfg. Co., Tokyo, Japan) (15). The four animals in each experimental subgroup and its two control animals were killed at intervals of 1, 2, 3, 4, 5, 6, 12, 24, 36, 48 and 72 h.

Light microscopy. The human and rat renal tissues were fixed in 10% buffered formalin, and serial sections were prepared and stained with hematoxylin-eosin (H-E) and PTAH. IEH was performed to identify FRMs (fibrinogen, FDP-D and FDP-E) according to the PAP (Sternberger (16)) and ABC (Hsu *et al.* (17)) methods. Histological DIC was assumed to be present when there were FRMs in the glomerulus and tubulus of PTAH-prepared material or IEH-prepared material (14, 15). In this study, a new staining procedure with cationic ferric hydroxide colloid stabilized with cocadylate (Fe-Cac) (8, 19) was applied to some cases.

Scanning electron microscopy. Renal tissues of animals after one injection of endotoxin (0.3 mg/kg body weight) were used. Briefly, renal tissues were fixed by perfusion with 1% glutaraldehyde in phosphate buffer (pH 7.2, 100 ml). Dissected tissues were postfixed in 2% osmium tetroxide in 0.1M phosphate buffer (pH 7.2), washed with phosphate buffer, and fixed again in 2% tannic acid solution. After dehydrated with a graded series of ethanol (20–100%), specimens were freeze-cracked in liquid nitrogen, transferred to isoamyl acetate, processed by critical point drying with CO₂, coated with gold in vacuum evaporator, and observed using a scanning electron microscope (Hitachi 430) (20).

Results

Human materials. Among 208 necropsied cases, 26 cases (12.5%) were diagnosed as clinical DIC, and 23 of them (88.5%) showed FRMs histologically (Table 1).

Table 1 Positive staining of fibrin-related material (FRM) in cases with clinical and histological disseminated intravascular coagulation (DIC)^a

Diagnosis	Site	PTAH	IEH	Both PTAH and IEH	Total
Clinical cases (N=26)	Glomerulus	0	0	1	1
	Tubulus	0	1	5	6
	Both glomerulus and tubulus	3	3	10	16
	Total	3	4	16	23
Histological cases (N=80)	Glomerulus	5	14	5	24
	Tubulus	4	14	13	31
	Both glomerulus and tubulus	3	8	14	25
	Total	12	36	32	80

a: Staining response of FRMs in the glomerulus and tubulus. Note the staining differences among hematoxylin-eosin (H-E), phosphotungstic acid hematoxin (PTAH) and immunoenzyme histochemical reaction (IEH). FRMs hardly recognized under PTAH can be demonstrated under IEH, especially in histological cases.

FRMs could not be detected in three cases in which anticoagulants were used intensively. FRMs were detected in one case in only the glomerulus, in 6 cases in only the tubulus, and in 16 cases in both the glomerulus and tubulus. FRMs were found in both PTAH and IEH specimens in 16 cases, in only IEH specimens in 4 cases and in only PTAH specimens in 3 cases.

A total of 80 cases (38.5%) had DIC histologically. In these DIC cases, FRMs were more frequently detected in the tubulus (31 cases) than in the glomerulus (24 cases) or in both the glomerulus and tubulus (25 cases). IEH specimens alone showed a high frequency of positive FRMs (36 cases), followed by both PTAH and IEH (32 cases) methods and then by PTAH stain alone (12 cases).

In both clinically and histologically diagnosed DIC, FRMs were more frequently detected in the tubuli than glomeruli. There was a trend of more positive FRMs in IEH materials than in PTAH-stained materials. In clinically diagnosed DIC, a high rate of positive response was present in both PTAH and IEH specimens and in both the glomeruli

and tubuli. However the same trend did not follow in the histological cases. FRM recognition by PTAH alone or IEH alone was similar in the clinical cases, but differed in the histological cases.

Animal experiments.

Hematological and biochemical analyses of DIC. Fig. 1 summarizes the platelet counts and fibrinogen levels. The platelet counts in the experimental group decreased sharply by 3 h after endotoxin injection, reached a minimum at 36 h, and increased thereafter. No remarkable change was noted in the control group. Fibrinogen values decreased one hour after saline infusion in both the experimental and control rats. In the experimental group the value continued to decrease, reaching a minimum at 5 to 12 h, returning to almost the normal level at 24 h, and thereafter remained in the normal range. In controls, the fibrinogen value began to recover at 12 h, and sharply increased thereafter.

Fig. 2 shows changes in serum and urinary FDP values. FDP was not detected in controls. Serum FDP appeared 4 h after endotoxin injection, then increased and

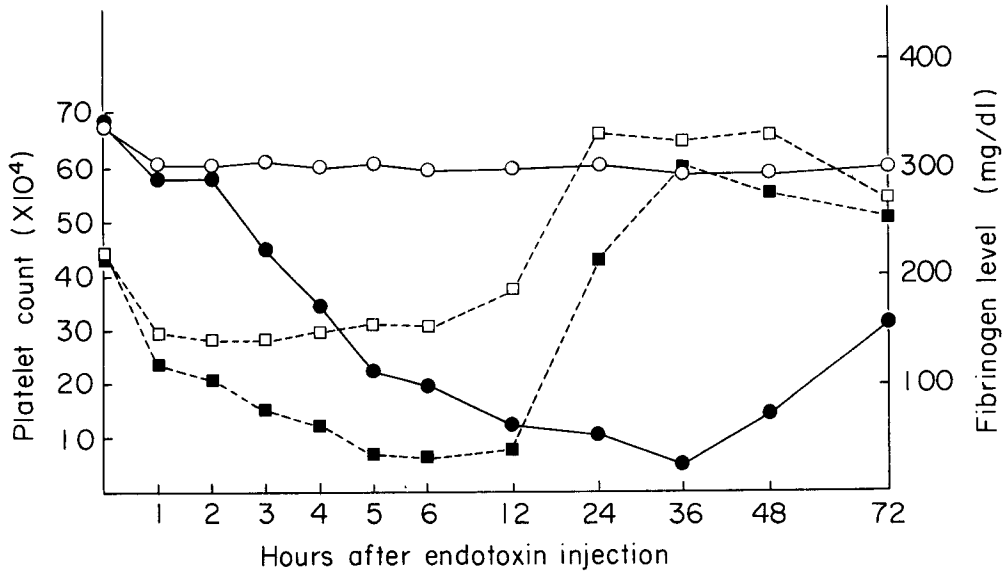


Fig. 1 Rat platelet count and fibrinogen level after endotoxin injection. Platelet count: experiment (N=4), ●—●; control (N=2), ○—○. Fibrinogen level: experiment (N=4), ■—■; control (N=2), □—□.

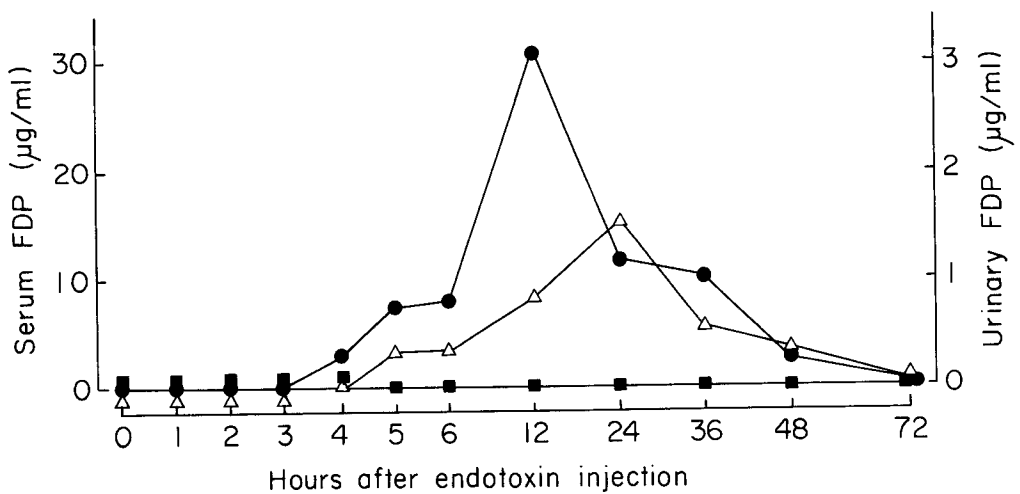


Fig. 2 Rat serum and urinary fibrin degradation products (FDPs) after endotoxin injection. Serum FDP (N=4), ●—●; urinary FDP (N=4), △—△; control (N=2), ■—■.

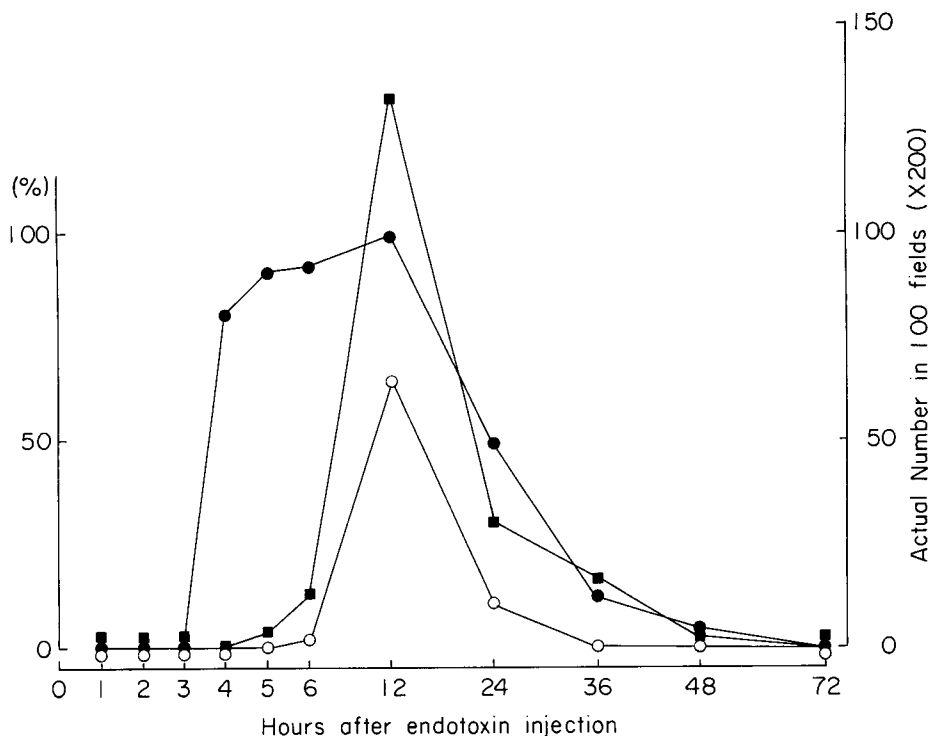


Fig. 3 Fibrinogen thrombus in specimens treated with immunoenzyme histochemical reaction (IEH). Glomerulus (●-●) and Bowman's space (○-○) with fibrin thrombus are indicated by mean percentages on the left vertical axis. Tubulus (■-■) with fibrin thrombus is indicated by the actual mean number on the right vertical axis.

reached a peak at 12 h. Thereafter, the value decreased and was not detected at 72 h. FDP appeared in urine collected 5 h after endotoxin injection, reached a peak in the collection at 24 h, decreased, and disappeared at 72 h.

Light microscopy. Fig. 3 summarizes the sequential changes in the fibrinogen thrombus after endotoxin injection in renal tissues of IEH-stained sections. Fibrinogen was first observed in the glomerulus, and later in Bowman's space and the tubulus. However, the thrombus was observed most frequently at 12 h, and could not be detected at 72 h. The tissue responses of FDP-D and FDP-E were similar (15).

Scanning electron microscopy. Fig. 4 shows

the sequential changes in the glomerular capillaries and FRMs in the lumen after a single injection of endotoxin. The endothelial surface became irregular and coarse (Figs. 4-A and 4-B), and protruded like a sea anemone by 2 h (Fig. 4-C). Four hours after injection, bundle-like FRM thrombi were observed, and thrombi on the endothelial surface of the capillaries became reticular by 8 h (Fig. 4-E) and granular by 12 h (Fig. 4-F). The thrombi almost disappeared and the endothelial surface appeared recovered 12 h later (20) (Fig. 4-G).

Fe-Cac staining of DIC. Fig. 5 shows the human and rat renal glomeruli in normal and DIC states stained with Fe-Cac. Fe-Cac was stained positive in the normal glo-

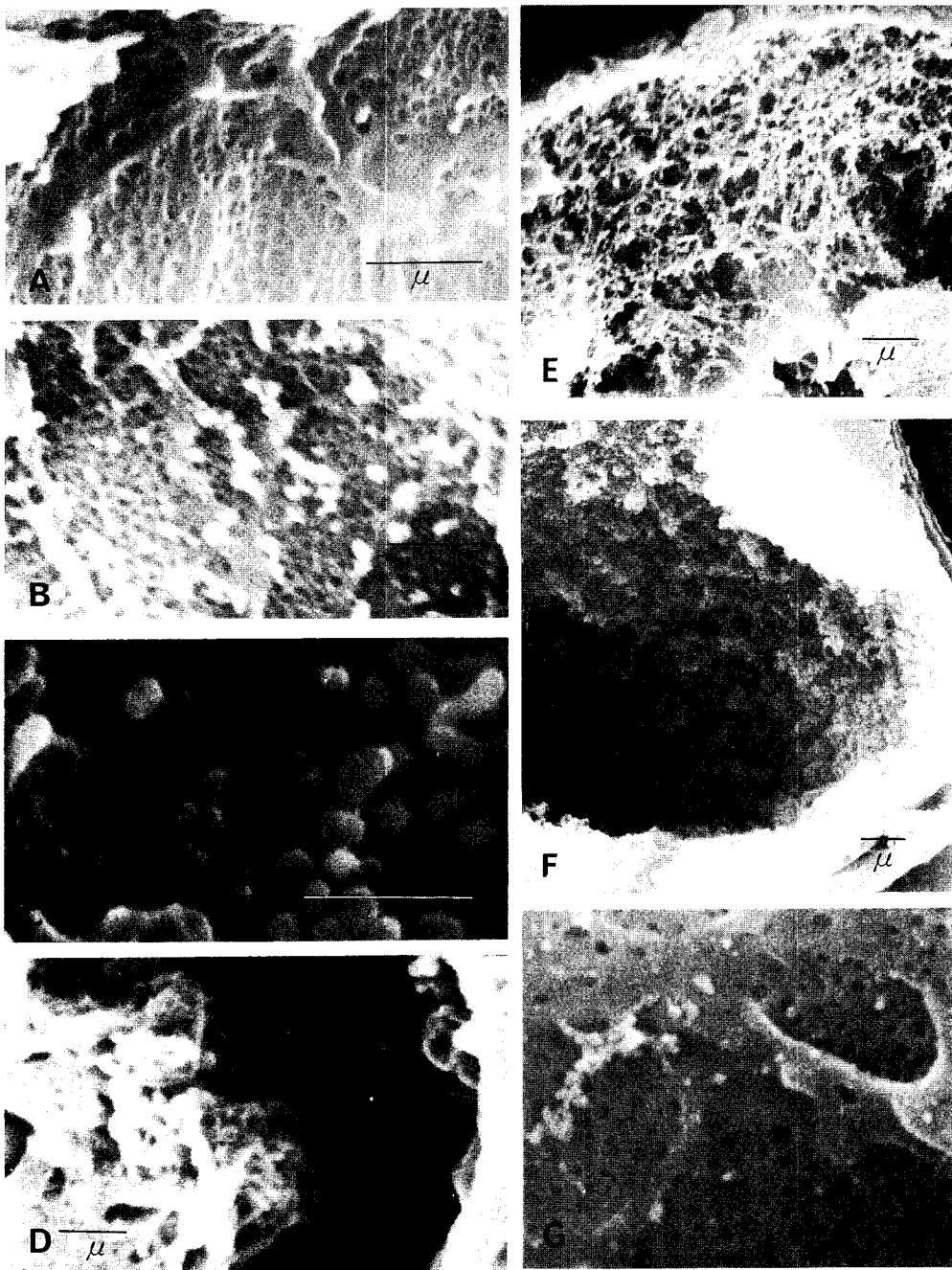


Fig. 4 Rat glomerular capillary surface and fibrin-related materials (FRMs) after endotoxin injection. Scanning electron microscopy. A: Normal rat. B: Irregular surface and attachment of fibrin-like materials, one hour after injection. C: Irregular projections on the surface resembling a sea anemone, 2 h after injection. D: Bundle-like FRM thrombus in the capillary lumen, 4 h after injection. E: Reticular configuration of FRM, 8 h after injection. F: FRM with a granular appearance, 12 h after injection. G: Recovery of the endothelial surface, 16 h after injection.

merular capillaries (Figs. 4-A and 4-C), while it was negative in DIC (Figs. 4-B and 4-D).

Discussion

DIC is a clinical entity characterized by

hemorrhagic diathesis subsequent to an abnormality in clotting and the fibrinolysis system (7, 9, 21-23). However, the mechanism of how DIC develops is still obscure, and criteria for its diagnosis are undefined due to its various pathophysiological states, such as acute, subacute, chronic compensated and local DIC (24-26). On the other hand, his-

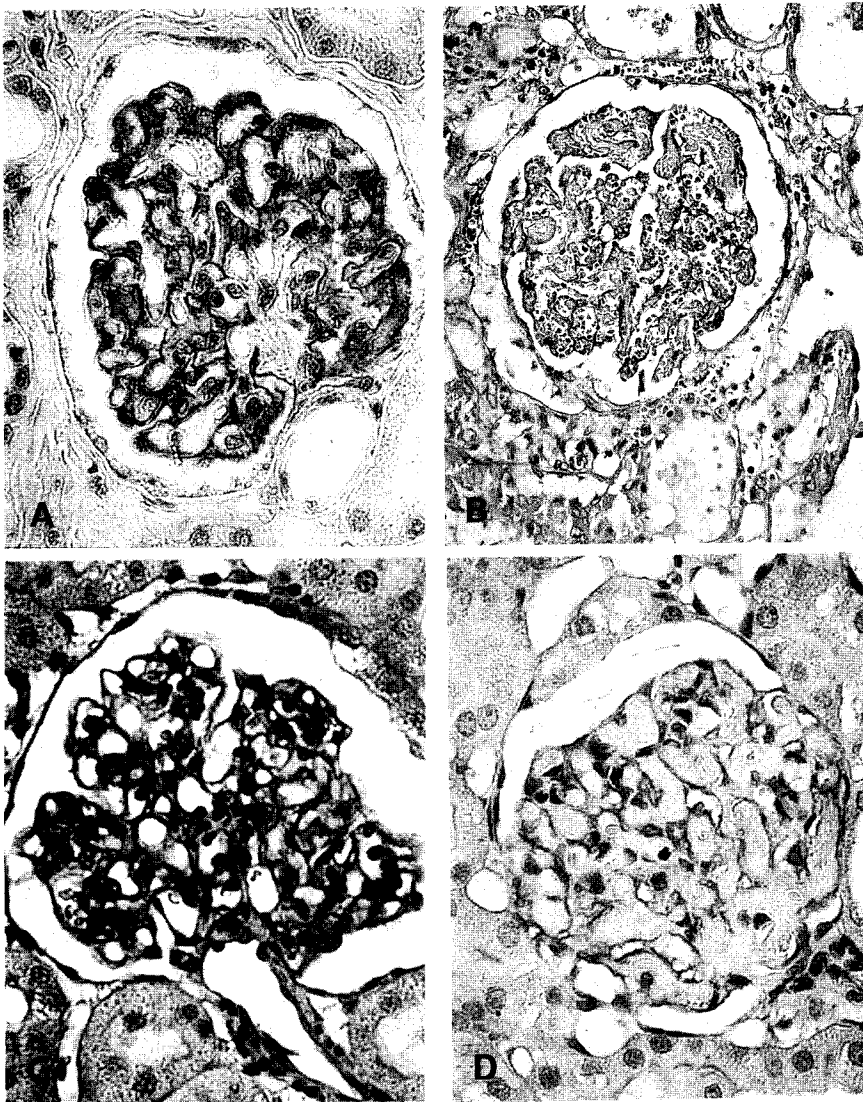


Fig. 5 Renal tissues in disseminated intravascular coagulation (DIC). A marked reduction in Fe-Cac staining in DIC is shown. Human materials (pH 2.0): A, healthy control; B, DIC patient. Rats (pH 4.0): C, healthy control; D, DIC rat.

tological diagnoses of DIC have followed after fibrin thrombi detection in the micro-circulation in multiorgans. The kidneys are the best organ for a histological diagnosis. Robboy *et al.* (8) stated that even a single thrombus in a glomerulus was presumptive evidence of DIC. Histologically, fibrin thrombi are commonly examined in PTAH-stained sections. The staining characteristics are close to those of IEH-reaction products (14, 27), although there are some differences in staining quality between PTAH and IEH (3, 13-15).

In human material, FRMs not recognized after PTAH staining were demonstrated in IEH specimens (Table 1), and the FRM detection rate in the glomerular capillaries of DIC rats was generally lower in PTAH specimens than in IEH specimens (15). The differences were remarkable in FRMs of the tubuli. Almost all eosinophilic IEH-positive casts in the tubuli were weakly positive or negative to PTAH (14, 15). Therefore, PTAH, commonly used to recognize fibrin, is less sensitive than the IEH reaction, and non-specific. As suggested by Ohizumi *et al.* (28), it is only sensitive to polymerized fibrin fibers with a thickness of more than $0.6\text{ }\mu\text{m}$, whereas smaller fibers, fibrinogen and FDPs or those undergoing degradation are not stained with PTAH, and can be detected only by IEH. In DIC rats, the weaker FRM staining, especially in the renal tubuli by IEH, is explained by the fact that the FRMs were diluted by an increased volume of urine due to massively infused saline (100 ml)(15). Except for a few cases, however, no differences in IEH reaction were noted among fibrinogen, FDP-D, and FDP-E (15). The results indicate that FRMs "fibrin thrombus" in the glomerulus and FRMs casts in the tubulus are combined substances of fibrinogen and FDPs.

FRMs in the glomerular capillaries are morphologically fibrillar or homogenous in

humans (14), whereas they are from fine to thick fibers and finally become homogenous clots in DIC rats (15). The degradation process is clearly demonstrated under the scanning electron microscopy (Fig. 4). In our study, about 45% (36/80 cases) of histologically diagnosed DIC cases and 17% (4/23 cases) of clinically diagnosed DIC cases were positive to only IEH reactions. This indicates that these cases would have been overlooked under ordinary staining, such as PTAH. Contrary to this, 15% (12/80 cases) of histological DIC cases and 13% (3/23 cases) of clinical DIC had material positive to PTAH and negative to IEH preparations in the glomeruli and tubuli. This incompatibility may be mainly due to the non-specificity of PTAH for fibrin, although we cannot rule out the possibility that the immunoenzyme was disturbed by other substances in blood or urine.

According to Barnes *et al.* (29) and Jones *et al.* (30), FRMs easily effuse into Bowman's capsules and the tubuli by focal spreading of podocytes. In autopsied cases, FRMs were observed in both the glomeruli and tubuli of PTAH and IEH material, which suggests that FRMs in the glomerular capillaries were effused into the tubuli via Bowman's capsules, and finally excreted into urine. This process was clearly demonstrated in DIC rats. In this DIC model, the hemato-biochemical and histological changes with time showed abnormal clotting and lysis, and demonstrated that FRMs in the glomerular capillaries at 4 h after injection were excreted into the urine via Bowman's spaces and the tubuli (Figs. 1-3).

About 39% (31/80 cases) of histologically diagnosed DIC and 26% (6/23 cases) of clinically diagnosed DIC cases had FRMs only in the tubules; the remaining cases would not have been diagnosed as DIC by conventional staining techniques. We regarded the presence of PTAH- or IEH-

positive substances in glomeruli or tubuli as representing DIC. In the evaluation of tubular FRMs, except for acute DIC, the possibility of subacute, chronic, local, or compensated DIC (24-26) would be considered.

The discrepancies in DIC diagnosis between clinicians and pathologists are probably due to the cited time of DIC occurrence, the continuity of DIC, the influence of anticoagulants, and the time lapse between death and autopsy. Fibrin thrombi could not be confirmed by only histology in 45% (8) to 75% of autopsied cases (4, 10, 11). All our cases were autopsied within 3 h of death, and 3 of 26 cases of clinically diagnosed DIC received an intensive administration of anticoagulants and lacked PTAH- or IEH-positive substances in the glomerulus or tubulus. Except for these three cases, FRMs were recognized by the PTAH method, IEH method or both methods in the glomeruli, tubuli or both regions, indicating a high rate of clinically diagnosed DIC cases with FRMs. On the other hand, 70 to 87% of cases with microthrombi lacked DIC symptoms clinically (10, 11). In renal tissues of the 80 cases with FRMs presented herein, only 23 cases showed clinical symptoms. In other words, 57 cases (71%) were asymptomatic according to traditional DIC criteria. Thus, mild forms of acute, subacute, chronic, local or compensated DIC (24-26) were possibly included.

Recently, we observed that the staining of glomerular capillaries by Fe-Cac was abnormal in DIC (Figs. 5 A-D). This phenomenon may be due to a reduction in the negative charge at the capillary surface of the renal glomeruli. Staining differences vary in renal tissue sections even in the glomerulus of DIC patients and rats (Fig. 5 A-D), indicating that a reduction in the staining of capillaries may be parallel to the severity of DIC. This concept suggests that DIC is initially induced by a reduction in

the electric surface charge of the glomerular capillaries. Fe-Cac staining may be useful for demonstrating mild forms of DIC.

In conclusion, this study showed that: (a) PTAH is non-specific and not sensitive enough to detect FRMs; (b) the presence of FRMs cast in the tubulus can be an indicator of the patho-physiological state of DIC; (c) discrepancies in the clinical DIC diagnoses can be reduced using both PTAH and IEH in the glomeruli and tubuli; (d) mild forms of DIC can be demonstrated by Fe-Cac staining; and (e) DIC may possibly be initiated by a reduction in the surface charge of the capillaries.

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