

Model for Disseminated Intravascular Coagulation: Bacterial Sepsis in Rhesus Monkeys<sup>1,2</sup>

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4-5 days. In the group of monkeys developing rash, activation of coagulation was suggested by an 80% decrease in platelet count and 20-30% increases in prothrombin and activated partial thromboplastin times. Fibrinolytic system activation was indicated by the appearance of fibrin degradation products. Kinin system activation was evidenced in both prekallikrein and kininogen. Changes in laboratory tests suggestive of subclinical DIC were also noted in infected monkeys which did not develop a rash. Pathological evidence of DIC was obtained through observation of numerous fibrin thrombi in the kidneys of the only monkey which died in the course of infection. Occurrence of DIC in association with this experimental infection in rhesus monkeys was established on the basis of clinical, laboratory, and pathologic criteria. Expression of the syndrome on day 1-2 following infection correlated with the period of increasing bacteremia.

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## INTRODUCTION

The syndrome of disseminated intravascular coagulation (DIC)<sup>1</sup> is an acquired hemorrhagic disorder characterized by the apparent simultaneous activation of blood coagulation (1), fibrinolysis (2), and kinin generation (3). It is manifested as a bleeding diathesis resulting from loss of platelets and consumption of clotting factors (1) together with organ ischemia caused by deposition of fibrin in the microcirculation (4). DIC is a well-described clinical problem (5-8), and has been identified as a serious complication of an ever increasing number of infectious diseases (6, 8, 9), especially in Gram-negative bacterial sepsis (9-13).

The pathogenesis of DIC has been inferred from clinical and pathologic data combined with the results of in vitro studies of the activation mechanisms for coagulation, fibrinolysis, and kinin generation (5-9). Certain aspects of the pathophysiology of DIC have been investigated in animal models (14, 15) based on infusion of endotoxin, thromboplastin, or thrombin. Our objective was to develop an animal model of DIC based upon an actual infection, in which the appearance and progress of the syndrome could be related to the entire course of the disease process as assessed by laboratory methods. This model would parallel, under controlled conditions, the clinical situation of a patient who develops DIC during the course of an illness such as bacterial sepsis. Using this model, events signaling or predisposing to clinical expression of DIC could then be identified.

Specific criteria for the diagnosis of DIC vary among reports in the literature, however, it is generally accepted that there are

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<sup>1</sup> Abbreviations used in this paper: DIC, disseminated intravascular coagulation; FDP, fibrin degradation products; PT, prothrombin time; APTT, activated partial thromboplastin time; EDTA, ethylenediaminetetracetic acid.

clinical, laboratory, and pathologic manifestations of the syndrome (8). Each manifestation is a reflection of the presumed pathophysiology of the DIC syndrome, simultaneous activation of coagulation, fibrinolysis, and kinin generation. This report describes a model for DIC in rhesus monkeys with experimental Salmonella typhimurium sepsis which satisfies the clinical, laboratory, and pathologic criteria for this syndrome.

## METHODS

Animal procedures. Healthy, well conditioned male rhesus monkeys (Macaca mulatta) weighing 4-6 kg were housed in individual cages with free access to both food and water. All clinical procedures were carried out under ketamine anesthesia (Ketaset, Bristol Labs, Syracuse, N. Y.; 5 mg/kg, intramuscularly). Blood samples were taken by venipuncture from either femoral vein using small "Vacutainers" (Becton-Dickenson, Rutherford, N. J.), and the small Vacutainer holder equipped with a 22-gauge needle. Rectal temperatures were measured daily using an electronic thermometer (Yellow Springs Instrument Co., Yellow Springs, Ohio).

Experimental infections. Eight separate studies were conducted. Groups of 3-4 monkeys were experimentally infected with S. typhimurium by intravenous (i.v.) injection into the saphenous vein of  $10^9$  live organisms in 1 ml of tryptose saline while 2-4 control monkeys received an injection of saline. The clinical course was followed for 7-10 days.

Organisms. S. typhimurium, the etiologic agent used in these studies, was from a culture maintained at this Institute for several years, which was originally obtained as a gift from Dr. Paul Newborne at the Massachusetts Institute of Technology. Organisms were grown on brain heart infusion agar at 37°C, then suspended at a concentration of  $10^9$  organisms/ml in tryptose saline.

Laboratory tests. Quantitative cultures for bacteria from the blood of infected monkeys were performed using serial dilutions of heparinized blood on sheep blood agar plates. White blood cells (16) and platelets (17) were counted with a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.). Prothrombin time (PT) and (celite) activated partial

thromboplastin time (APTT) were measured by standard procedures (18-19) on a Fibrinometer (Bioquest Divison of Becton-Dickenson, Rutherford, N. J.) using commercial reagents (General Diagnostics, Divison of Warner-Lambert Company, Morris Plains, N. J.). Microhematocrit was determined by a standard method. Fibrinogen was measured by the technique of Ratnoff and Menzies (20), and fibrin degradation products by electroimmunoassay (21) in agar containing specific antimonkey fibrinogen antisera (Cappel Laboratories, Cochranville, Pa.). Plasma prekallikrein was measured using the method of Colman et al. (22) based on hydrolysis of tosylarginine methyl ester (TAME) following kaolin activation. Plasma kininogen was determined by bioassay on guinea pig ileum using the method of Prado et al. (23) as modified by Webster and Pierce (24) and Pierce and Guimaraes (25).

Representative tissues were taken from one monkey which died during the course of illness, fixed in 10% buffered neutral formalin, processed through paraffin in a routine manner, and sectioned at 6  $\mu$ m. Tissues were stained with hematoxylin and eosin; selected sections were stained with phosphotungstic acid hematoxylin (PTAH) for the demonstration of fibrin (26).

Blood collection. Blood samples were taken on days -4 and -1 prior to beginning the experiment (infection), 4 hr after infection (day 0), and daily thereafter. Samples for quantitative blood cultures and hematologic measurements were drawn into heparin or EDTA using 2- and 4-ml Vacutainers (Becton-Dickenson), respectively. For coagulation and prekallikrein tests blood was drawn into siliconized Vacutainers containing 1 part 3.8% sodium citrate to 9 parts whole blood, and transferred after mixing to plastic tubes. Samples for kininogen

determinations was drawn into similar tubes containing 1 part 3.8% sodium citrate, 0.01 M benzamidine hydrochloride, and 0.1% hexadimethrine bromide (Aldrich, Milwaukee, Wis.) to 9 parts blood. Plasma was separated by centrifugation for 20 min at 1500 x  $g$  at 4°C, and aliquots were frozen in methanol-dry ice then stored at -70°C until time of assay.

Calculations. Data from laboratory tests on individual monkeys were tabulated either as absolute values (temperature, bacteremia, fibrin degradation products) or as changes from that monkey's "normal value" (leukocyte and platelet counts, prothrombin and activated partial thromboplastin times, fibrinogen and prekallikrein levels). Normal values were calculated for each monkey as the average of that monkey's values from two separate days (day -4 and -1) prior to infection. Data from the individual monkeys in each of the three clinical groups: noninfected controls, infected without petechial rash, and infected with petechial rash, were then combined, and the mean and standard error of the mean are reported throughout the experiment for each clinical group. Tests to identify statistically significant changes from the pooled normal values within each group of monkeys were performed by analysis of variance using a repeated measurements design (27). Data from each day of the experiment for each group of monkeys was compared with the normal value of that laboratory parameter for that group. Tests to identify significant differences between individual means were computed using Student's  $t$  test (28).

## RESULTS

Baseline values for the laboratory tests performed on each monkey were calculated as the average of determinations done on two separate days prior to infection (days -4 and -1). The collected mean "normal" values from all of the monkeys of these tests are presented in Table I.

Infected monkeys became lethargic, and developed fever and a significant, though transitory leukopenia (Fig. 1). Their illness was most severe 1-3 days after infection and lasted 6-8 days. Ten of 23 infected monkeys developed a petechial rash, suggesting DIC, at some point during their illness. The rash appeared 1-2 days after infection and lasted an average of 4-5 days. Petechiae, when present usually first appeared in the axillary areas and often spread over the entire body. A hemorrhagic diathesis was indicated by marked prolongation of bleeding from venipuncture sites in monkeys with the petechial rash. However, epistaxis, bleeding at the gums, or bloody stools were not observed. Those monkeys which developed rash appeared to be more severely ill than infected animals without rash. This impression was supported by the finding that those infected monkeys which developed rash had a 10-fold higher average titer of bacteremia than infected monkeys without rash (Fig. 2 upper panel). Appearance of rash was taken as clinical evidence of DIC, and provided the basis for useful comparisons between infected monkeys; those which developed petechial rash, and those which did not.

Activation of the coagulation system in infected monkeys was suggested by significant changes in several laboratory parameters. Infected monkeys developing rash experienced severe thrombocytopenia (Fig. 2, lower panel), with significant depression ( $P < 0.001$ ) for several days. The lowest average platelet count for this group was observed on day three following

infection, and was only  $37.0 \times 10^9$  per liter. Platelet counts for the group of infected monkeys who did not develop petechial rash also fell significantly ( $P < 0.01$ ) (on days 2 to 3 following infection), but the decrease was much less than for the group with rash. Following this comparatively mild depression, platelet levels in this group rose markedly, and became significantly elevated ( $P < 0.001$ ) during recovery. Platelet counts in the non-infected control group of monkeys did not change significantly.

Prolonged prothrombin times (PT) were observed ( $P < 0.01$ ) in infected monkeys which developed rash, coinciding with the height of illness (Fig. 3 upper panel). In the group of infected monkeys which did not develop rash, PT were also prolonged significantly ( $P < 0.01$ ) for 2 days following infection. The PTs of the group of noninfected control monkeys became 2-3 seconds shorter than their control value, presumably due to the stress of daily venipunctures.

Activated partial thromboplastin times (APTT) were significantly prolonged for infected monkeys whether they did or did not develop petechial rash (Fig. 3, lower panel). APTTs were prolonged ( $P < 0.001$ ) for the group of infected monkeys without rash between days 2 and 3 of the infection, at the height of illness. The APTTs of the group of infected monkeys which developed rash was shortened on day 0 (4 h after infection), then became prolonged, reaching a maximum on day 3 of the infection before returning to normal. In contrast, APTTs for noninfected control monkeys were shortened, relative to their normal values throughout the study, as had been observed for the PT values of the control group.

The fibrinogen concentration in the plasma of noninfected control monkeys rose slightly, peaking on day 2 of the experiment (Fig. 4,

upper panel). Plasma fibrinogen concentration in infected monkeys which did not develop rash rose to significantly elevated values ( $P < 0.001$ ), as might be expected since fibrinogen is an acute-phase reactant (29). Fibrinogen concentrations of infected monkeys developing rash rose to essentially the same value as those of monkeys without rash, but did so more slowly. During the period in which the rash was appearing at the height of illness (day 1), fibrinogen values in the group of monkeys developing rash were relatively lower than for those monkeys who did not develop rash.

Fibrinolytic system activation during the experimental sepsis, as assessed by determination of fibrin degradation products (FDP) was not observed at any time in the serum of the group of noninfected control monkeys (Fig. 4, lower panel). They were, however, observed in the serum of infected monkeys, whether or not the monkeys developed rash. Infected monkeys which developed rash, had much higher values of FDPs, especially at the height of illness, than the group of infected monkeys without rash.

Evidence for activation of the kinin system was obtained by measuring plasma prekallikrein activity and plasma kininogen (Fig. 5). Prekallikrein activities of all infected animals fell significantly ( $P < 0.001$ ); however, the lowest value observed in the group of infected monkeys which developed rash (-57.6 units/ml on day 2 of the infection) was much more depressed than the lowest value observed in the group of infected monkeys without rash (-32.5 units/ml). Further evidence of kinin system activation came from determination of plasma kininogen concentrations (Table II). Infected monkeys with rash had a lower average kininogen value at the height of illness, compared to monkeys without rash and noninfected controls.

Pathologic evidence that DIC occurred in association with this experimental infection was obtained by examination of the tissues of one monkey which expired at the height of illness. Gross examination at necropsy showed extensive petechial rash, evidence of hemorrhage in the small intestine, and hemorrhagic congestion of the spleen. Microscopic examination of the several organs above confirmed the gross observations of multifocal hemorrhage. In addition, multiple fibrin thrombi were found in glomerular tufts of both kidneys (Fig. 6), and other tissues as well. All other monkeys recovered spontaneously (within 7-10 days) from their infection.

## DISCUSSION

Only one monkey of twenty-three infected with S. typhimurium (10 with petechial rash) died. This low mortality is in contrast to data reported for human patients with sepsis associated DIC (8). The prognosis for survival of monkeys following experimental sepsis with Salmonella is probably dependent on the size and age of the animal, and the presence or absence of additional stress. Kastello and Spertzel (30), investigating the response of rhesus monkeys to Salmonella sepsis, reported a 40% mortality rate using a similar dose of infecting organisms. However, their monkeys (2-4 kg) were somewhat smaller than those used in this study, and in addition, their monkeys were restrained in chairs rather than caged.

According to the proposed scheme of pathophysiology of DIC (8, 10), activation of the coagulation pathway is initiated as a result of tissue injury caused by the underlying disease process. In Gram-negative sepsis, activation of coagulation is proposed to be by way of the intrinsic system through activation of Hageman factor (Factor XII) either by endotoxin (31) or by injury to endothelial cells, exposing the underlying collagen (32). Activated Hageman factor also plays a central role in kinin generation, (a) activating plasma prekallikrein to kallikrein (33), (b) in fibrinolysis by (34) activating plasminogen either directly (35), or (c) through the plasma plasminogen activator, which may be plasma kallikrein (36, 37). Thus the expression of DIC is a result of the simultaneous activation of these three systems, combined with the pathological consequences of fibrin deposition in the microcirculation (5).

The laboratory data from the infected monkeys with rash satisfy the criteria for positive diagnosis of DIC established by Minna et al.

(8), which are representative of those in current practice. Together with the clinical evidence of an increased bleeding tendency and petechial rash, and the pathological findings of fibrin deposition in the kidneys and internal hemorrhage; there can be no doubt but that DIC is associated with this experimental infection. Interestingly, laboratory evidence of DIC in the absence of clinical indications of bleeding were observed in the group of infected monkeys which did not develop rash. This group of monkeys had significantly prolonged ( $P < 0.001$ ) APTT for several days, FDP in amounts consistent with levels found in humans with DIC secondary to sepsis (38) and lowered prekallikrein levels ( $P < 0.001$ ). This suggests that the processes which give rise to the expression of DIC are at work in infected monkeys even in the absence of clinical manifestations of the syndrome. Similar observations have been made by Hawley et al. (39) in rhesus monkeys infected with S. pneumoniae. Subclinical expression of DIC in humans has been recognized (40, 41), and anticoagulant therapy has even been advocated (8) in these patients.

It is interesting to correlate the observed laboratory changes to the time course of the disease process. Intravenous injection of  $10^9$  S. typhimurium into a monkey produces a titer of bacteremia of  $10^4$ - $10^5$  organisms/ml immediately following the injection.<sup>2</sup> Within 4 h, the titer of bacteremia falls to  $10^1$ - $10^2$  organisms/ml and a transitory leukopenia develops (Fig. 1, lower panel).

At the 4 hr postinoculation time, the only laboratory parameters which were significantly changed were increased PT ( $P < 0.01$ ) in all infected monkeys and a decreased prekallikrein value in the group of

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<sup>2</sup>D. Wing and T. Yamada, unpublished observation.

monkeys which subsequently developed rash. Peak bacteremia occurred on day 1 following infection and remained elevated until about day 4, while fever in the infected monkeys peaked on day 2, then gradually returned to normal.

Clinical and laboratory evidence of the onset of DIC appeared between days 1 and 2 of infection for the group of monkeys developing rash, and included a significant drop in platelets ( $P < 0.001$ ), continued prolongation of the PT, prolongation of the APTT ( $P < 0.01$ ), appearance of substantial amounts of FDP, depression of the plasma kininogen concentration, and a further decrease in prekallikrein activities. The group of infected monkeys which did not develop rash had a more moderate decrease in the number of circulating platelets, which was barely significant at  $P < 0.01$ . They had prolonged APTT but the PT for the group returned to normal by day 3. Their decrease in prekallikrein activity was less pronounced than for the group with rash, and FDP in their sera rose slowly, peaking at days 4-5 of infection at a value less than half of the peak mean concentration of the group which developed rash. Plasma fibrinogen concentrations for both infected groups of monkeys rose to significantly increased values ( $P < 0.01$ ) reaching essentially the same value by day 3 after infection. However, as noted previously, the increase in mean fibrinogen concentration of the group of infected monkeys developing rash was delayed relative to that of the infected monkeys not developing rash. Even though fibrinogen values rose in the infected monkeys they experienced an increased rate of fibrinogen metabolism, based on the turnover of labeled fibrinogen (42), with those monkeys developing rash having a faster rate than those not developing rash. Thus it would seem that the onset of the DIC syndrome

can be correlated with a period of rapidly progressing disease reflected in an increasing bacteremia. Clinical manifestation of the syndrome seems to depend on the severity of the infection as indicated by the titers of bacteremia attained.

DIC has been described in association with a number of naturally occurring diseases in animals (43); however, reported experimental models for DIC (14, 15) are based on injection of endotoxin, thromboplastin, or thrombin. While infusion of these substances has been helpful in identifying pathologic changes resulting from DIC and elucidating aspects of the pathophysiology of the syndrome, they are not strictly analogous to the situation presented by the patient with sepsis. An animal model in which one could follow appearance and development of the syndrome in relation to the entire course of a disease process offers the advantage of being more closely analogous to real clinical problems. We have shown S. typhimurium sepsis in rhesus monkeys to be associated with a high incidence of DIC by clinical, laboratory, and pathologic evidence. A model for DIC associated with gram-negative sepsis in nonhuman primates may more closely parallel disease in humans than models in lower species, and presents relatively less risk to the investigator than other proposed infectious disease models (43). This present model offers the opportunity to investigate the biochemical mechanism by which episodes of DIC are triggered, and to evaluate the relative efficacy of various therapeutic regimens.

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## FIGURE LEGENDS

FIGURE 1 Temperature and leukocyte counts. Average daily rectal temperatures are presented (mean  $\pm$  SE) for the groups of noninfected control monkeys ( $\circ$ ), and infected monkeys with ( $\blacktriangle$ ) or without ( $\blacksquare$ ) petechial rash. The mean temperature of all monkeys in the three groups averaged from days -4 and -1,  $37.9 \pm 0.8$  (mean  $\pm$  ISD,  $n = 63$ ) is represented by the horizontal line. Statistical analysis to identify significant deviations from the normal value was carried out by one-way analysis of variance within each group's data. The point of infection is noted by the vertical arrow. (lower panel) Leukocyte counts for the three groups of monkeys are presented as mean differences from the average of day -4 and day -1 values for each monkey to normalize the data between individual monkeys. Statistical analysis to detect significant changes in leukocyte count from the postinfection samples was done by analysis of variance within each group. The point of infection is noted by the vertical arrow.

FIGURE 2 Bacteremia and platelet levels. Bacteremia in monkeys was measured by quantitative blood culture from samples of heparinized blood, and is presented as  $\log_{10}$  bacteria/ml observed each day (mean  $\pm$  1 SE) for the noninfected controls ( $\circ$ ), infected monkeys without rash ( $\blacksquare$ ), and infected monkeys with rash ( $\blacktriangle$ ). The vertical arrow notes the time of infection (lower panel). The number of circulating platelets, determined by electronic counting, were normalized for each monkey as described in FIGURE 1. Mean values ( $\pm$  1 SE) for the difference of counts compared to the average of day -4 and -1 values are presented for the control ( $\circ$ ), infected without rash ( $\blacksquare$ ), and infected with

rash (▲) monkeys. Statistical analysis to identify significant changes in platelet counts were computed as described for the leukocytes in FIGURE 1.

FIGURE 3 Prothrombin and activated partial thromboplastin times. Prothrombin times were determined on citrated plasma samples, then each monkey's times were normalized relative to the mean of his day -4 and -1 values by computing differences from this "normal" value. Mean differences in seconds are presented (mean  $\pm$  1 SE) for the noninfected control (○), infected without rash (■), and infected with rash (▲) monkeys. Statistical analysis to detect significant changes was done by analysis of variance as described in FIGURE 1. The point of infection is noted by the arrow. (lower panel) Activated partial thromboplastin times were determined on citrated plasma samples using celite activation. The data were handled as described above for the prothrombin time. Mean changes in APTT are shown for the control (○), infected without rash (■), and infected with rash (▲) groups of monkeys. The point of infection is noted by the arrow.

FIGURE 4 Plasma fibrinogen and fibrin degradation products. Plasma fibrinogen concentration, determined as thrombin-clottable protein, was normalized for each monkey relative to his average concentration between days -4 and -1 by computing the difference on each day from that mean. Fibrinogen levels are presented (mean  $\pm$  1 SE) as the mean difference for the noninfected control (○), infected without rash (■),

and infected with rash ( $\blacktriangle$ ) groups of monkeys. Statistical analysis of the data from each group to identify significant changes in fibrinogen concentration were computed as described in FIGURE 1. The point of infection is noted by the vertical arrow (lower panel). Fibrin degradation products were measured by electroimmunoassay through an agarose gel containing specific antimonkey fibrinogen antibody. Mean FDP concentrations in  $\mu\text{g/ml}$  ( $\pm 1$  SE) are shown for the noninfected control ( $\circ$ ), infected without rash ( $\blacksquare$ ), and infected with rash ( $\blacktriangle$ ) groups of monkeys. The point of infection is noted by the arrow.

FIGURE 5 Prekallikrein levels. Prekallikrein levels were determined by measuring the p-tosylargininemethyl ester hydrolyzing activity of citrated plasma following kaolin activation. Activity measurements were normalized for each monkey by computing the difference in activity from that of days -4 and -1, and the net changes in activity are presented (mean  $\pm 1$  SE) for the groups of noninfected control ( $\circ$ ), infected without rash ( $\blacksquare$ ), and infected with rash ( $\blacktriangle$ ) monkeys. Statistical analysis to identify significant changes in prekallikrein activity were computed as described in FIGURE 1. The vertical arrow identifies the point of infection.

FIGURE 6 Fibrin deposition in the kidney of one infected monkey. Fibrin deposits were found upon histological examination of the kidney of one infected monkey who developed petechial rash, and subsequently expired at the height of illness. This photomicrograph shows fibrin deposits in a glomerulus from this monkey. The tissue was stained with phosphotungstic acid. Original magnification, X 320.

TABLE I  
"Normal" Values of Laboratory Findings of Rhesus Monkeys

	*	<u>n</u>
Temperature, °C	37.9 ± 0.8	37
Leukocytes, $\times 10^{-9}/L$	8.4 ± 3.4	59
Platelets, $\times 10^{-9}/L$	29.7 ± 147	64
Hematocrit, %	37.7 ± 4.5	50
Prothrombin time, <u>sec.</u>	14.2 ± 2.0	41
Activated partial thromboplastin time (alite), <u>sec</u>	32.4 ± 8.5	41
Fibrinogen, <u>g/l</u>	2.4 ± 1.0	60
Fibrin degradation products <u>µg/ml</u>	0.1 ± 0.1	62
Plasma prekallikrein, <u>Units/ml</u>	138.5 ± 31.5	68
Plasma kininogen <u>µg/ml</u> kallidin equivalents	0.35 ± 0.10	15

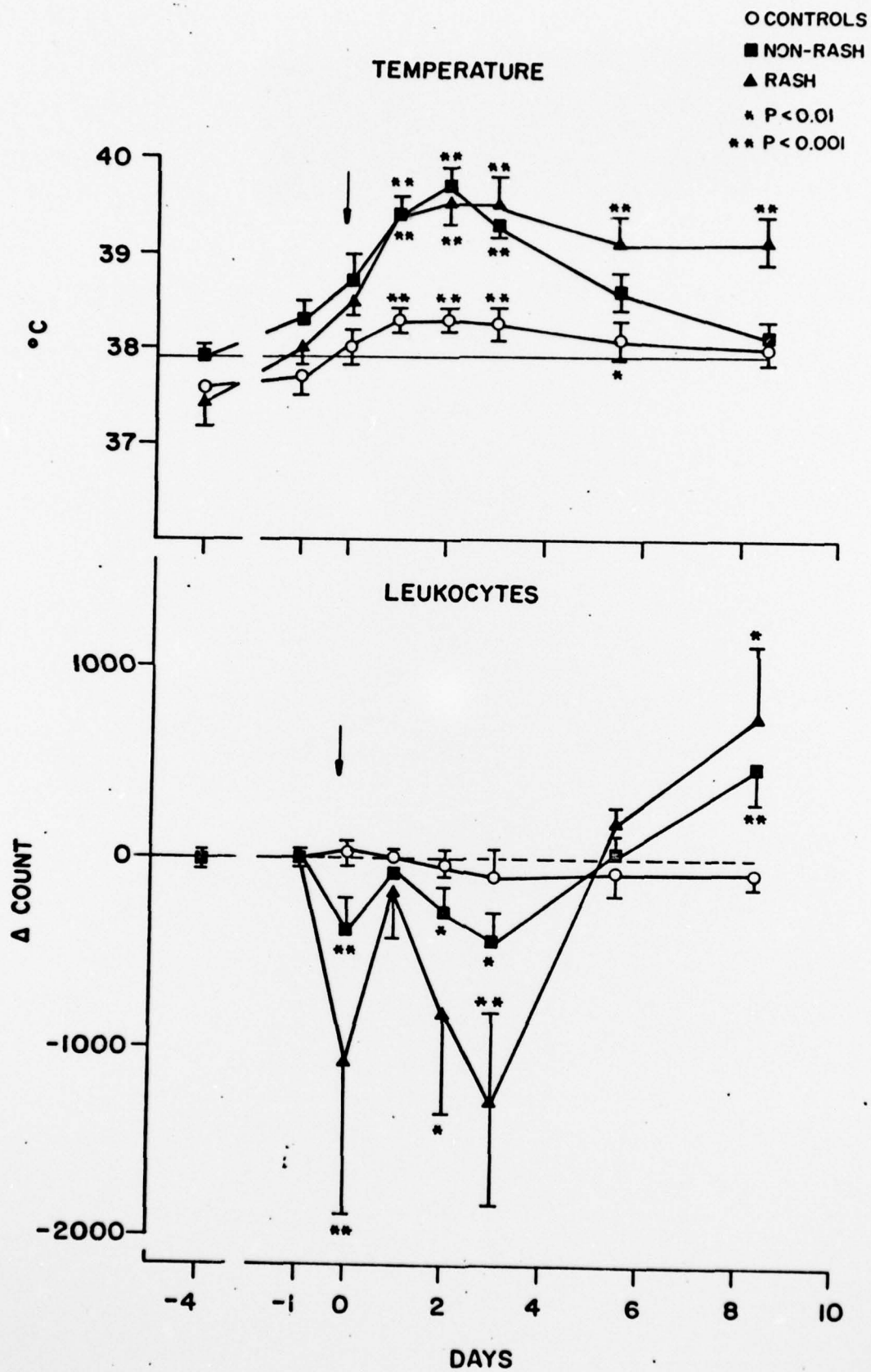
\* Mean ± ISD.

Methods as described in methods section.

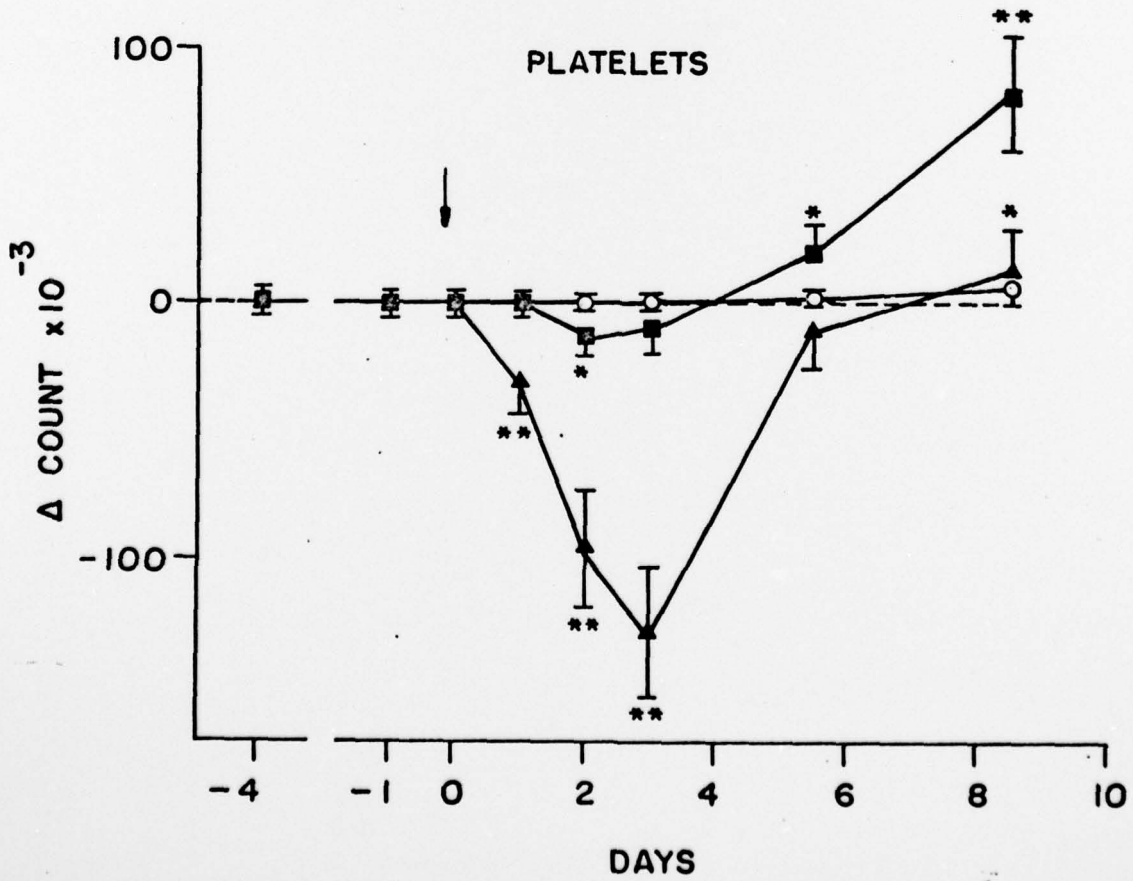
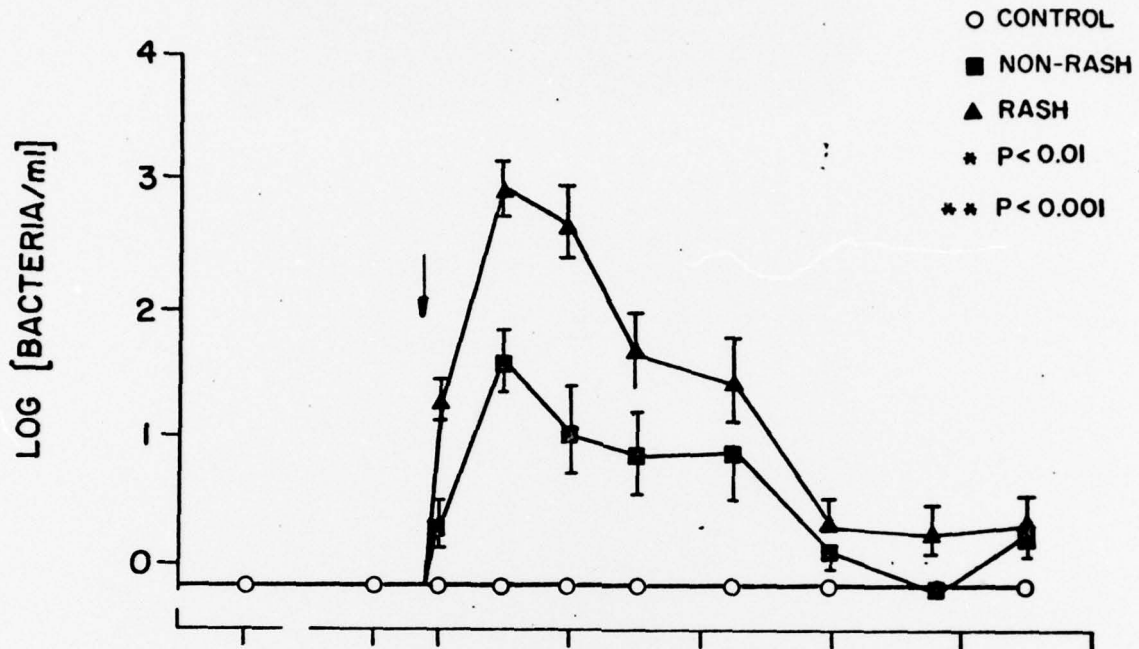
TABLE II  
Kininogen Levels

	Normal (day -4)	Illness (day +1 or +2)	Convalescent (day +8 to +11)
	<u>µg/ml kallidin equivalents</u> *		
Rash (n = 4)	0.273 ± 0.048	0.134 ± 0.027	0.205 ± 0.041
Nonrash (n = 4)	0.258 ± 0.055	0.248 ± 0.047	0.252 ± 0.034
Controls (n = 2)	0.295 ± 0.005	0.360 ± 0	0.275 ± 0.005

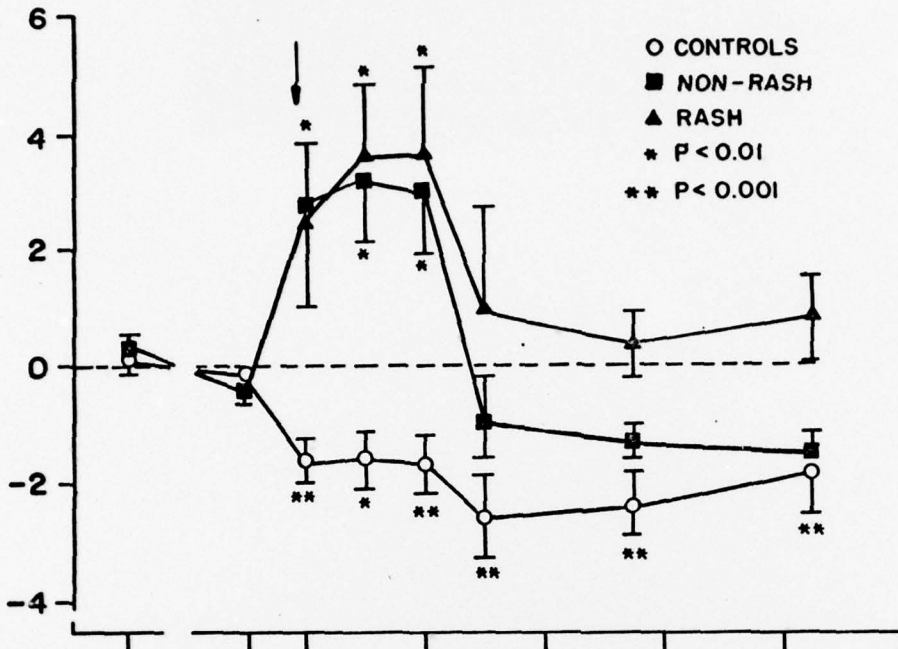
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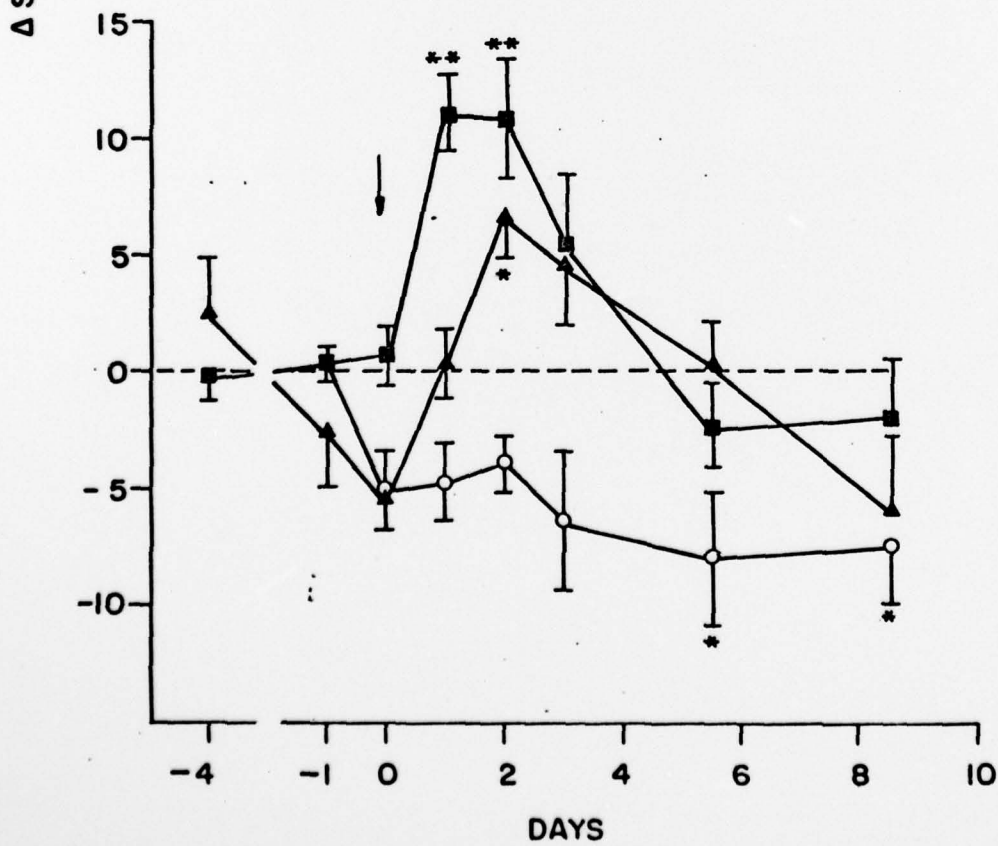
### BACTEREMIA

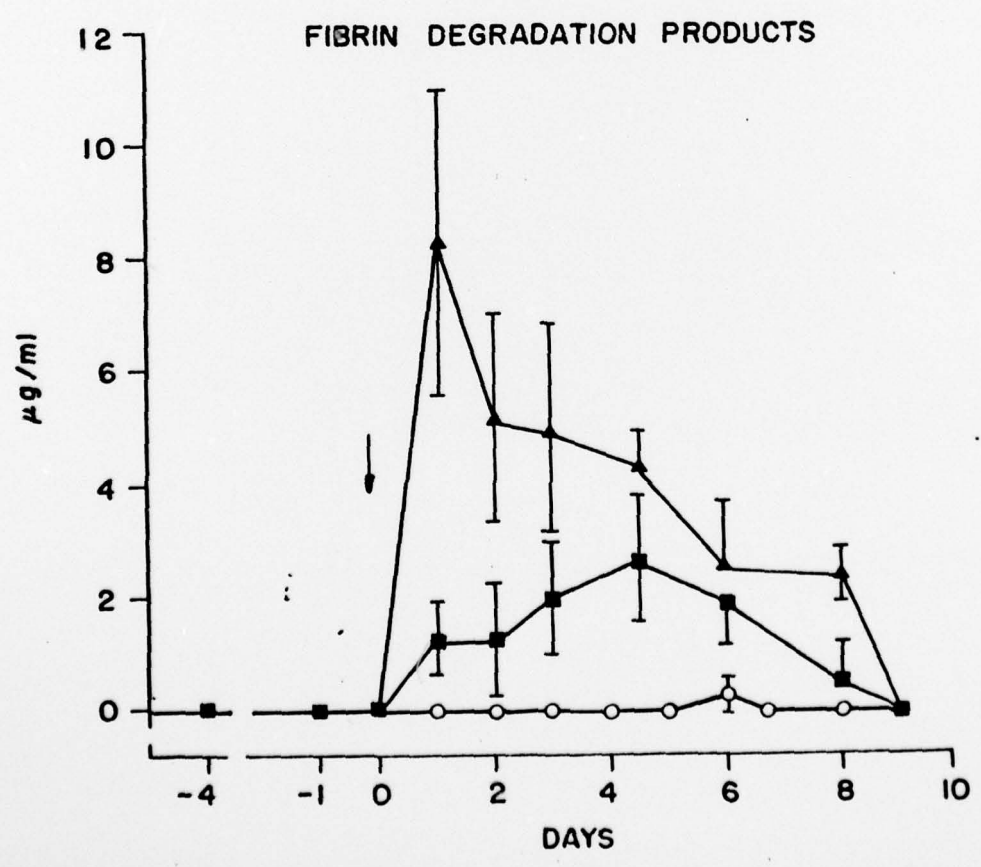
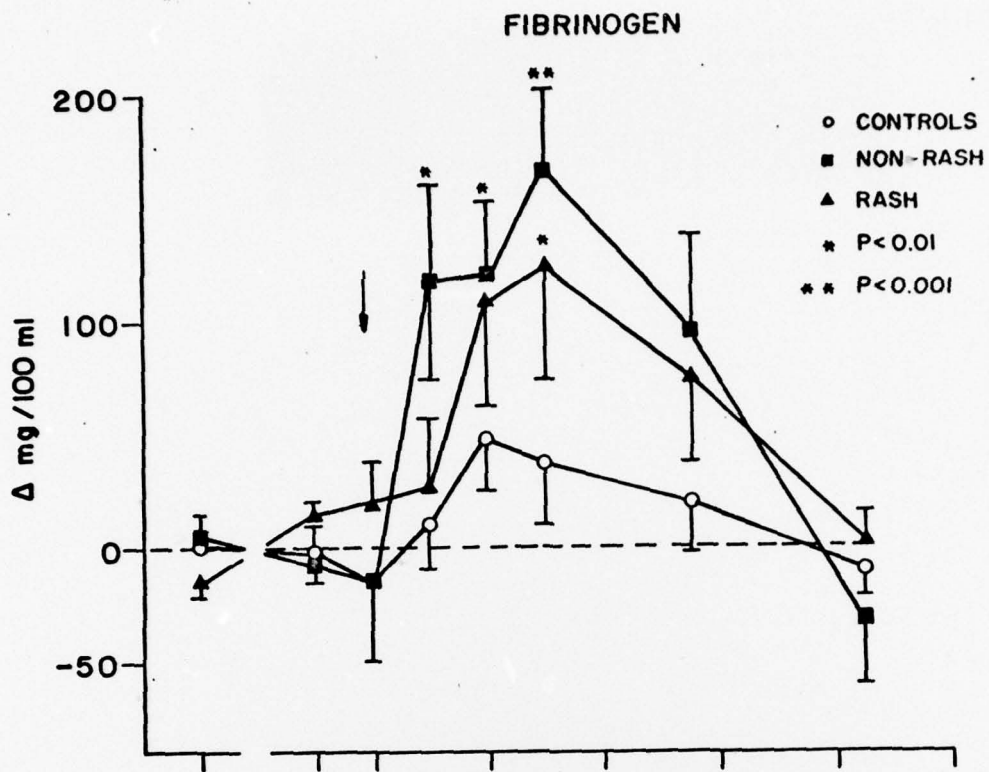


### PROTHROMBIN TIME



### ACTIVATED PARTIAL THROMBOPLASTIN TIME





PREKALLIKREIN

- CONTROLS
- NON-RASH
- ▲ RASH
- \* P < 0.01
- \*\* P < 0.001

