Heparin-Activated Clearing Factor

Standardized Test, Agewise Application, and Clinical Observations

By Saul P. Baker, M.D.

A possible relationship may exist between the concentration of heparin-activated plasma clearing factor and alterations in lipid metabolism associated with advancing age, atherosclerosis, and other diseases. To test this hypothesis, 121 men were evaluated in the development and application of a standardized in vitro method for the determination of clearing factor response to minimal heparin stimulation. Eighty-four men, ranging from 21 to 93 years, selected from this group, were evaluated in an age-wise study. Patients with diagnoses of recent myocardial infarction (presumptive evidence of coronary atherosclerosis), cirrhosis of the liver, and chronic alcoholism were also tested.

DURING the past 6 years, many studies of lipid metabolism and its derangements have been concerned with a clearing factor evoked in blood plasma by the in vivo injection of heparin. Since the observation that heparin administration clears the turbidity of alimentary lipemic plasma,1 many investigators have sought to elucidate its mechanisms.2-7 Clearing factor has been identified as a plasma lipase, now called "lipoprotein lipase,"8,9 which hydrolyzes the triglyceride of lipoproteins or fat emulsions, reducing it to free fatty acid and glycerol.3,5 A linear relationship has been observed during the initial in vitro clearing of a standard fat emulsion by heparin-activated plasma and the release of free fatty acid.3,4 Furthermore, it has been shown that the rate of in vitro clearing of a fat emulsion is proportional to the concentration of clearing factor present, and that the in vitro clearing activity of postheparin plasma may be quantitatively accounted for on the basis of its lipolytic activity.3,4

In view of the possible relationship of plasma clearing factor concentration to atherosclerosis and other diseases associated with alterations in lipid metabolism, clearing factor response has been extensively investigated in man.10-14 Similarity between possible alterations in lipid metabolism with advancing age and atherosclerosis has been suggested by experiments in which standard fat meals were administered to young and old subjects15,16 and to patients with coronary atherosclerosis.10,11,13 Both the old subjects and those with atherosclerosis demonstrated prolonged high levels of alimentary chylomicronemia15 and lipemia.13,16 Heparin injection after a standard fat meal, in one study, demonstrated a decreased clearing response in patients with coronary atherosclerosis,10 and in another study, no decrease in clearing response in atherosclerotic subjects11 as compared to controls. In a third report,13 heparin was found to accelerate the clearing of alimentary lipemia in normal subjects and to initiate the clearing response in subjects with coronary atherosclerosis after a standard fat meal.

When fasting subjects with coronary atherosclerosis, the nephrotic syndrome, and idio-pathic hyperlipemia were evaluated with respect to their clearing factor response,12 the normal control subjects demonstrated the greatest clearing; the patients with atherosclerosis slightly less; and the nephrotic and idio-pathic hyperlipemic subjects considerably less clearing. Another observer14 found a variable clearing factor response in atherosclerotic patients, normal or elevated clearing in young subjects, and decreased or absent clearing in old subjects or those suffering from preocuous senility. Although one study17 demonstrated significantly lower blood lipase levels in older people than in the young, these same investigators18 found no appreciable difference in the disappearance rate of chylomicrons following
intravenous injection of fatty plasma in old subjects as compared to young subjects.

Inasmuch as no uniform technic or heparin dosage was employed to determine clearing factor response to heparin stimulation in the various studies cited above, results of these investigations cannot be compared with each other. The research reported here was designed to analyze various factors in the development of a standardized in vitro assay method for the evaluation of clearing factor response to minimal heparin stimulation in man and to apply this method to a study of possible age differences in clearing factor response in men. Observations were also made on patients with recent myocardial infarction and cirrhosis of the liver.

Material and Methods

One hundred and twenty-one men were selected from the population of the Infirmary Division (Old Peoples’ Home) and from convalescent patients of the Baltimore City Hospitals. These were utilized in the analysis of various factors in the development of a standardized in vitro assay method for the determination of clearing factor response to minimal heparin stimulation. From this group, 84 men, ranging from 21 to 93 years of age, were selected for an evaluation of possible age differences in clearing factor response. All subjects were afebrile, free from evident acute infection, and on ward diet for at least 1 week prior to test. None had evidence of recent myocardial infarction, congestive heart failure, clinical jaundice, uncontrolled diabetes mellitus, the nephrotic syndrome, or had received heparin within 1 week prior to study. No attempt was made to identify subjects with or without atherosclerosis, in view of the improbability of completely excluding this disease in any subject in the age group studied. Tests were also performed on 7 subjects with a diagnosis of recent myocardial infarction and 6 subjects with a diagnosis of cirrhosis of the liver.

Heparin Sodium, 3 mg. in 5 ml. of isotonic saline* injected intravenously, was used as the clearing factor stimulus. Control and 8-minute postheparin blood samples (25 ml.) were obtained from all subjects after an overnight fast (at least 12 hours). Blood samples were immediately mixed with dried sodium oxalate (1 mg./ml. blood) and cooled to 0 C. in an ice bath. The plasma was separated by centrifugation at 0 C. It was subsequently kept at 0 C. or frozen until used. Substrate consisted of a 1 per cent emulsion of coconut oil (Ediol)† freshly prepared with distilled water. Three tenths of a milliliter of this emulsion was added to and thoroughly mixed with a 4.5 ml. aliquot of postheparin plasma, which had been previously incubated for 10 minutes in a water bath at 30 ± 1 C. This proportion, in general, gave an initial optical density reading between 0.600 and 0.700 against distilled water on the Beckman Model DU Spectrophotometer, with microcuvettes (3 by 10 by 25 mm.) at 6500 Å. The postheparin plasma-fat emulsion mixture was kept in the 30 C. water bath for 1 hour. During this time, optical densities were read every 5 minutes on 0.3 ml. aliquots of the mixture. Optical densities of control plasma and control plasma-fat emulsion mixtures were similarly determined. A graph of optical density versus time was then plotted for each sample. To this plot, a straight line was visually fitted at the section demonstrating the greatest rate of change in optical density. The negative slope of this line, k, (decrease in optical density per hour), was taken to represent the rate of clearing of postheparin plasma.

In preliminary studies, heparin dosage, state of alimentation, time of postheparin blood sample, effect of temperature on rate of clearing, type of substrate used, and the effect of room temperature on clearing factor activity were evaluated. An analysis of the method presented here included its reproducibility, proportionality between k and clearing factor concentration, effect of freezing and storing at 0 C. on clearing factor activity, and biological variability. Reproducibility was determined by simultaneous duplicate runs on aliquots of 52 postheparin plasmas. Proportionality between k and clearing factor concentration was tested by determining calculated versus observed k's for 57 mixtures of postheparin plasmas and 28 mixtures of postheparin and control plasmas. Effects of freezing and storing at 0 C. on clearing factor activity were tested in 3 categories. Forty postheparin plasma samples were frozen and stored for 1 day to 2 weeks, k first being determined on the fresh plasma. Fifty-three mixtures of postheparin plasmas and 66 mixtures of postheparin plasma with control plasma were similarly tested after freezing and storing for 1 day to 8 weeks. Biological variability was determined by repeat runs, at least 1 week apart, on 34 subjects.

Results

Figure 1 illustrates the graphic determination of k for high, intermediate, and low k’s. In testing reproducibility of the method, simultaneous duplicate runs on aliquots of 52 postheparin plasmas yielded a standard error of the mean $k = \pm 0.05$, $r = 0.98$. A scatter plot of

* The author wishes to acknowledge the courtesy of Dr. E. A. Hawk of the Upjohn Company in supplying the heparin for this investigation.

† Ediol was kindly supplied by Mr. Stanley J. Schapiro of Schenley Laboratories, Inc.
duplicate runs is shown in figure 2. Observations on the proportionality between \( k \) and clearing factor concentration for 57 mixtures of postheparin plasmas indicated a standard error of the mean \( k = \pm 0.10, r = 0.95 \), when calculated \( k \)'s were compared with observed \( k \)'s. Twenty-eight mixtures of postheparin plasma and control plasma similarly evaluated yielded a standard error of the mean \( k = \pm 0.08, r = 0.94 \). Effects of freezing and storing at 0 C. on clearing factor activity were as follows: 40 postheparin plasma samples frozen and stored for 1 day to 2 weeks gave a standard error of the mean \( k = \pm 0.14, r = 0.93 \); 53 mixtures of postheparin plasma frozen and stored for 1 day to 8 weeks yielded a standard error of the mean \( k = \pm 0.11, r = 0.93 \); and 66 mixtures of postheparin plasma with control plasma similarly tested after freezing and storing gave a standard error of the mean \( k = \pm 0.09, r = 0.90 \). Biological variability in 34 subjects tested by repeat runs at least 1 week apart yielded a standard error of the mean \( k = \pm 0.13, r = 0.93 \). A scatter plot of repeat runs is shown in figure 3.

Eighty-four men, evaluated as an agewise sample (21 to 93 years) for clearing factor response, demonstrated a mean \( k \) of 0.74, \( \sigma_d = 0.29 \). Mean age was 61.3 years, \( \sigma_d = 17.8 \). Range of \( k \) in this group was from 0.13 to 1.40. Eight subjects demonstrated \( k \) greater than 1.10. The 3 youngest of these (32, 42, and 51) all had diagnoses of chronic alcoholism. Of the remaining 5 subjects (68, 77, 78, 80, and 89), none had been diagnosed as a chronic alcoholic. Incidence of alcoholism in the 76 subjects with \( k \leq 1.10 \) was not determined. It is interesting to note that the 68-year-old subject \( (k = 1.22) \) developed an acute myocardial infarction 8 weeks after being tested for clearing factor response.

Table 1 presents the mean \( k \) for each decade.

\[ k = \text{rate of optical density per hour} \]

\[ \text{FIG. 1. Relation between optical density and time expressed as rate of clearing, } k \text{, (decrease in optical density per hour) of mixtures of postheparin plasma and fat emulsion. Readings of optical density have been corrected for control plasma in each case (Control O.D. = 0.082, 0.080, and 0.063, respectively). High (A), intermediate (B), and low (C) } k \text{'s are illustrated ( } k = 1.12, 0.66, \text{ and } 0.14, \text{ respectively).} \]

\[ \text{FIG. 2. Reproducibility of the method. Simultaneous duplicate runs on aliquots of 52 postheparin plasmas. Standard error of mean } k = \pm 0.05, r = 0.98. \]

\[ \text{FIG. 3. Biological variability. Repeat runs on 34 subjects at least 1 week apart. Standard error of mean } k = \pm 0.13, r = 0.93. \]
TABLE 1.—Agewise Distribution of Clearing Factor Response by Decades

<table>
<thead>
<tr>
<th>Age group</th>
<th>Number of subjects</th>
<th>Mean age</th>
<th>Mean $k$</th>
<th>$s_k$</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-29</td>
<td>3</td>
<td>21.0</td>
<td>0.58</td>
<td>0.53-0.66 (range)</td>
</tr>
<tr>
<td>30-39</td>
<td>7</td>
<td>33.4</td>
<td>0.64</td>
<td>0.35</td>
</tr>
<tr>
<td>40-49</td>
<td>11</td>
<td>44.5</td>
<td>0.75</td>
<td>0.25</td>
</tr>
<tr>
<td>50-59</td>
<td>15</td>
<td>53.7</td>
<td>0.72</td>
<td>0.25</td>
</tr>
<tr>
<td>60-69</td>
<td>18</td>
<td>64.9</td>
<td>0.70</td>
<td>0.25</td>
</tr>
<tr>
<td>70-79</td>
<td>14</td>
<td>73.5</td>
<td>0.80</td>
<td>0.31</td>
</tr>
<tr>
<td>80-89</td>
<td>14</td>
<td>84.1</td>
<td>0.85</td>
<td>0.25</td>
</tr>
<tr>
<td>90-99</td>
<td>2</td>
<td>92.5</td>
<td>0.56</td>
<td>0.28-0.34 (range)</td>
</tr>
<tr>
<td>Entire Group (21 to 93 years)</td>
<td>84</td>
<td>61.3</td>
<td>0.74</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Except for the 2 subjects over 90 years, there appears to be a slight over-all increase of mean $k$ with age in this sample. Figure 4 shows the individual $k$’s as a scatter plot versus age. Agewise regression lines have been drawn with and without including 4 young subjects (32, 42, 51, and 53) with diagnoses of chronic alcoholism. The apparent increase of $k$ with age is not statistically significant when the alcoholics are included. When they are omitted, however, the regression of $k$ on age becomes significant at $p < 0.02$. The mean increase of $k$ per decade in this instance is 0.04.

When 7 patients with recent myocardial infarction (mean $k = 0.74$) were compared with subjects evaluated agewise in the same age decades (40 to 69 years, mean $k = 0.69$), no significant difference was observed.

None of the 84 subjects in the agewise sample had been diagnosed as having cirrhosis of the liver. One patient with clinical jaundice due to hepatic cirrhosis, separately examined, yielded a $k = 1.60$. Consequently, 5 other patients with cirrhosis of the liver, all with clinically manifest jaundice, were separately evaluated. Their mean $k$ was found to be 1.01. When this group was combined with the 4 chronic alcoholics (mean $k = 1.09$), and examined as a group of 10 (mean $k = 1.04$), they were found to differ significantly ($p < 0.01$) from the agewise sample (mean $k = 0.67$) in the same decades (30 to 69 years).

DISCUSSION

In attempting to establish individual differences in clearing factor response in man, a standardized method would appear to be essential. Development of such a method involved the consideration of several parameters. In previous studies, heparin dosage ranged from 3 to 100 mg. or more.\textsuperscript{10-14} Although the heparin dosage falling within the physiologic dose-response curve may vary with the species,\textsuperscript{1, 6, 7, 10} 3 mg. of heparin yields a suitable submaximal physiologic stimulus level in man. Under the standardized conditions of the study discussed here, with 3 mg. of heparin, no relationship was observed between body weight and clearing factor response, although weights varied over a wide range. The state of alimentation also has varied. Some studies\textsuperscript{12} were performed on fasting subjects, while others\textsuperscript{10, 11, 13} were performed after standard fat meals. Variability in postprandial lipemia made it desirable to use subjects after an overnight fast (at least 12 hours). The substrate employed in various investigations has varied from an alimentary lipemic plasma produced by various standard fat meals\textsuperscript{10-12} to standard fat emulsions.\textsuperscript{1, 4, 6}

A standard coconut oil emulsion (Ediol) employed by one group of investigators\textsuperscript{5} was used in this study. Time of postheparin blood
sampling has differed. In studies on plasmas obtained 8, 15, and 30 minutes after heparin administration, the greatest clearing factor response was generally obtained in the 8-minute sample. Lesser clearing activity was observed in the 15- and 30-minute samples; activity decreasing with increasing time after injection. Consequently, an 8-minute postheparin blood sample was chosen to obtain an estimate of the height of clearing factor response. No temperature control is indicated in many previous studies, although the enzymatic nature of the reaction would appear to make this obligatory. Effect of temperature on rate of clearing was investigated and a Q10 of about 1.4 was found. A temperature of 30 ± 1 C. was therefore selected as producing a convenient intermediate rate of clearing within the range of temperature found in the laboratory. Clearing factor activity decreased when postheparin plasma was allowed to stand at room temperature. Thus, after 2 hours at 25 C., clearing factor activity had attenuated approximately 30 per cent. Consequently, all plasma was extracted at 0 C. and kept at 0 C. until used.

Although 25 ml. of blood were used in these studies, several parameters being evaluated on aliquots of the same plasma, the test may be performed with 10 ml. of blood or less, 3.0 ml. of postheparin plasma being required.

In this in vitro test, if the fat emulsion substrate is acted upon by an enzyme system and the optical density at 6500 A. is a measure of the concentration of fat emulsion present, the optical density of the mixture would decrease at a constant rate determined by enzyme concentration as long as excess substrate was present. Under these circumstances, this rate could be determined by noting the change in optical density in a given period of time, or the time required for given change in optical density. The negative slope of a plot of optical density versus time is not constant, but may increase to a maximum in 0 to 60 minutes, and decrease subsequently, or approach an asymptote. The approximately constant maximal rate of change, usually of the midportion of the curve, has been used and is shown to be well reproducible. This is closer to the maximal rate than the mean rate of change for the period of observation, or the time required for a given change in optical density, and provides a wider spread of values than the use of only 2 points.

It is recognized that clearing factor may act simultaneously upon both an exogenous standard fat emulsion that contributes turbidity and an endogenous nonturbid lipoprotein-linked triglyceride. However, it is suggested that the endogenous lipoprotein-linked triglyceride per se does not exert an appreciable influence upon the initial rate of clearing of fat emulsion. This is postulated in view of the observations that there is a constant ratio (1.4:1.0) between per cent change in optical density to per cent of coconut oil total fatty acid released in free form, and that the amount of free fatty acid released during clearing never reached the total amount of coconut oil fatty acid added to the incubation mixture even when clearing was complete and the optical density of the mixture had returned to control levels. Thus, even the exogenous fat emulsion substrate is probably not completely hydrolyzed during the initial clearing reaction. Under the standardized conditions of the study discussed here, it is suggested that there is excess albumin to act as a fatty acid acceptor, even in cases of hypoalbuminemia. This is postulated in view of the fact that in cirrhotic patients with severe parenchymal liver damage, hypoalbuminemia, and marked inversion of the albumin/globulin ratio, rapid clearing progresses as a linear rate of change of optical density with time. Furthermore, even assuming a hypoalbuminemia of 2 Gm. per 100 ml. of plasma in the in vitro system used in these studies, the exogenous fat emulsion substrate is of the order of 3 mg., while the amount of albumin available as fatty acid acceptor is of the order of 90 mg. In other circumstances, where the hypoalbuminemia is accompanied by marked endogenous hyperlipemia, it is conceivable that conditions for an in vitro clearing reaction may not be optimal. This may be the result of either an initially high optical density of the plasma due to visible endogenous lipemia or to lack of sufficient free fatty acid acceptor or both. In such cases, appropriate modification of the in vitro clearing system might be considered.

No evidence was obtained in this study to in-
dicate a decrease in intravascular clearing factor response, either in old subjects or in patients with recent myocardial infarction. Consequently, it would appear unlikely that the persistent lipemia and chylomicronemia, which have been reported for old subjects and patients with coronary atherosclerosis after a standard fat meal, are due to an intravascular deficiency or absence of clearing factor or its precursors. Clearing factor response can be evoked by minimal intravenous exogenous heparin stimulation in these subjects to at least the same degree as that found in young subjects or subjects without recent myocardial infarction. Evidence indirectly implicating the gastrointestinal tract is found in the equal response of old and young subjects with respect to disappearance rate of chylomicrons following intravenous injection of fatty plasma and the 21 per cent decrease in pancreatic lipase in old versus young men. Further information suggesting gastrointestinal involvement is supplied by the observation that intravenous infusion of a fat emulsion evokes a decrease in serum lipids and changes in their electrophoretic pattern in normal human subjects during alimentary lipemia, in patients with idiopathic hyperlipemia, and in dogs. These alterations appear to be identical with those produced in serum lipids by intravenous heparin injection. Thus, while intravenously administered fatty plasma or fat emulsions may produce adequate clearing and hydrolysis of triglyceride, endogenous production of clearing factor in response to the stimulus of a fatty meal via the gastrointestinal tract may be impaired in old subjects, in patients with coronary atherosclerosis, and perhaps also in patients with idiopathic hyperlipemia.

The increased $k$ obtained in 10 patients with diagnoses of chronic alcoholism or cirrhosis of the liver supports another report of a greatly increased clearing factor response in a subject with ethanolic cirrhosis of the liver. Perfusion experiments in rats wherein citrated plasma was mixed with heparin and perfused through the isolated hind limbs, the lungs, and all of the abdominal viscera drained by the portal vein, demonstrated clearing factor production in each instance. The liver, however, produced no clearing factor upon such perfusion. Furthermore, perfusion of postheparin plasma (containing active clearing factor) through the liver resulted in a decrease in its clearing activity. Also, in rats injected with heparin, blood from the inferior vena cava, portal vein, and aorta had similar clearing activity, while that from the hepatic vein demonstrated less clearing activity than that of the aortic blood. It is, therefore, suggested that one cause of the increase in clearing factor activity found in the postheparin plasma of some subjects may be a decrease in the functional capacity of the liver parenchymal cells to remove or inactivate clearing factor.

Although no common diagnosis or pathologic etiology could be discovered for the 5 subjects in the sample of 84 that gave $k$'s less than 0.20 (31, 63, 64, 65, and 74 years old, respectively), a patient separately examined who was suffering from anemia due to gastrointestinal bleeding demonstrated a $k = 0.13$. Furthermore, a patient developing active pulmonary tuberculosis had progressively lower $k$'s on repeat examinations several weeks apart. Another patient with initially low $k$ demonstrated an increase in $k$ upon convalescence from an acute exacerbation of his chronic renal disease. These observations support the report of absence or decrease in clearing factor response in infections, fevers, and anemia, clearing factor response reappearing with convalescence in certain instances.

**SUMMARY AND CONCLUSIONS**

A standardized *in vitro* method for the evaluation of clearing factor response to minimal heparin stimulation in man has been developed. This method supplements, modifies and incorporates various features of tests now in use and critically analyzes some of the factors involved. Reproducibility and biological variability of the method have been determined.

Rate of clearing, $k$, (decrease in optical density per hour) was found to have a standard error on repeat runs on the same subject of $\pm 0.13 k$. Evidence has been presented suggest-
ing that the rate of clearing, $k$, is proportional to the concentration of clearing factor present. Freezing and storage at 0°C produced no appreciable attenuation of clearing factor activity.

Clearing factor response to minimal heparin stimulation was determined in an age-wise study of 84 men, ranging from 21 to 93 years. No statistically significant age-wise regression was found, although a tendency toward an increase in clearing factor response with age was present.

Four young subjects diagnosed as chronic alcoholics had a mean high $k$, 3 of these having the highest $k$'s in the 21–59 age group. Six additional patients with cirrhosis of the liver also had a mean high $k$ value. Liver pathology, either subclinical or clinical, may account for these high $k$ values.

Seven patients with recent myocardial infarction (presumptive evidence of coronary atherosclerosis) demonstrated no lesser clearing factor response to intravenous heparin stimulation than did subjects who were not known to have had a recent myocardial infarction.

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Summario in Interlingua

Esseva disveloppare un metodo standardisate pro le evaluatione in vitru del responsa del factor de clarification al stimulation per heparina in quantitates minimal in subjectos human. Iste metodo supplementa, modifica, e incorpora in se varie aspectos del tests nunc in uso e analyza criticamente alicunes del factores implicate. Le reproducibilite e le variabilitate biologic del metodo ha essite determinate.

Le clarification, exprime como $k$ e definite como reduction del densitate optic per hora, esseva determinabile per le methodo con un error standard, establite per repetitiones del tests pro subjectos individual, de $±0.13\ k$. Es presentate datos que pare provar que le clarification per unitate de tempore es proportional al concentration del factor de clarification. Refrigeration e immagasinage a 0°C non resultava in appreciabile reductiones del activitate del factor de clarification.

Le responsa del factor de clarification al stimulation per heparina in quantitates minimal esseva determinate, con referentia al etate del subjectos, in 84 homines de inter 21 et 93 annos de etate. Esseva trovate nulle statisticamente significative regression con le avantamiento del etate, sed le grupo studiate monstравa un tendentia del responsa del factor de clarification de augmentar se con le avantamiento del etate del subjectos.

Quatro juvenile subjectos, diagnosticate como alcoholicos chronic, habeava alte valores medie pro $k$. Tres de illes habeava le plus alte valores pro $k$ in le grupo de etates de inter 21 et 59 annos. Sex altere patientes con cirrhosis del hepate habeava etiam alte valores medie pro $k$. Morbo hepatic, clinic o subclinico, representa possibilemente un explicatione de iste alte valores pro $k$.

Septe patientes con recente infarcimento myocardial (reguardate como prova presumptional de atherosclerosis coronari) non monstравa plus basse responsas del factor de clarification al stimulation per heparina intravenose que subjectos sin evidencia de recente infarcimentos myocardial.

References


Five patients with myocardial infarction received cortisone within 48 hours of the onset of the attack in doses of 75 mg. orally every 6 hours decreasing to 25 mg. daily after the twelfth day. The usual supportive measures were employed, including pressor agents in 1 patient with shock. Anticoagulants were not used. Electrolyte and water balance measurements were obtained during the course of treatment. Neither morbidity nor mortality was influenced by cortisone therapy in this small group. Death occurred in 2 patients as predicted by the Pathologic Index Rating on admission. The treatment had no stabilizing effect upon blood pressure and did not prevent hypotension. Moderate sodium and water retention occurred in the 2 patients who died, but did not occur in the 3 patients who survived.

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