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AN IMPROVED QUANTITATIVE METHOD FOR THE STUDY OF FIBRINOLYSIS IN THE SKIN AS EXEMPLIFIED IN PATIENTS WITH LEUKOCYTOCLASTIC VASCULITIS AND ERYSIPELAS

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Abstract. The activity of plasminogen activator has been measured in tissue sections with the aid of a modified Todd technique. Frozen skin biopsies were sectioned and the tissue covered with fibrin–plasminogen film. After incubation at 37°C fibrinolysis was studied for a period of 30 min and was graded into six exponentially increasing steps. During this period, grades were linear with the logarithm of time of incubation. The rate of fibrinolysis is a measure of the activity of the plasminogen activator and is expressed by the slope of the linear regression of grades versus the logarithm of time; the value of the slope is proportional to the common logarithm of the activity of the plasminogen activator present in the section. Using urokinase as a specific plasminogen activator, the same linear expression was shown with time, indicating the validity of our grading and experimental system. Eleven healthy subjects served as controls to 6 patients with leukocytoclastic vasculitis and 4 patients with erysipelas and 4 with necrotizing fasciitis. The controls showed values similar to the non-involved skin in the patients. The activity was higher in the arm than in the thigh sites. The activity in the thighs was lower in women than in men. A decrease in the plasminogen activator activity was found in the three disorders.

Key words: Skin, plasminogen activator activity; Fibrinolysis; Method of measurement of; Leukocytoclastic vasculitis; Erysipelas; Necrotizing fasciitis

In recent years fibrinolysis in the dermis in various skin disorders has been evaluated (for references, see Cunliffe, (2)). The methodology of Todd (10) has been the basis for the histochemical visualization of fibrinolysis used in these studies. This technique has been modified, mainly by alterations in the composition of the reagents needed for making the fibrin film applied to the skin sections and in the way in which lysis zones are evaluated. Dodman et al. (3) measured the area of fibrinolysis as a percentage of the overall area of the section after a constant incubation period. However, there is a need for a more accurate estimation of the fibrinolytic activity in tissues. In the present investigation the progress of fibrinolysis was studied at intervals during the period of incubation and the degree of lysis graded in order to obtain more accurately the fibrinolytic activity in the sample. Its relevance and the results obtained in healthy subjects and patients with inflammatory skin reactions are reported.

MATERIAL AND METHODS

Clinical material

Eleven healthy subjects (4 females, 7 males), mean age 25±5 (S.D.) years, were used as controls and for the assessment and validation of the test procedure used. Eight patients (6 females, 2 males), mean age 55±16 (S.D.) years, having leukocytoclastic vasculitis as defined by Sams et al. (9) and 8 patients having erysipelas or necrotizing fasciitis as recently described (4) were also studied. Table 1 summarizes the clinical data of the patients.

Reagents

Human fibrinogen, fraction 1–4, 97–100% clottable, (1) was obtained from ImCo Co, Stockholm. The fibrinogen was dissolved to a concentration of 20 g/l in a saline phosphate buffer, pH 7.3, containing 0.3 M NaCl and 0.02 M sodium phosphate. It was passed through a lysine-Sepharose column to remove contaminating plasminogen according to Matsuda et al. (6). The purified fibrinogen was recovered and diluted to 10 g/l in 0.03 M sodium phosphate buffer, final pH 7.8, and stored until use at −70°C.

Plasminogen (11) was obtained either from Dr Per Wallén, University of Umeå, or from Kabi AB, Stockholm. The first preparation was dissolved in 0.03 M sodium phosphate buffer, pH 7.8 and stored at a concentration of 2 g/l in aliquots of 0.1 ml at −70°C. The latter plasminogen was kept dry and immediately before use dissolved in distilled water to the same concentration.

Thrombin of bovine origin was either prepared at the Department of Chemistry, Karolinska Institutet in prin-

Table I. *Patient material. Lesions were located on calves or thighs*

Diagnosis	No.	Age
Leukocytoclastic vasculitis		
♂	2	50, 63
♀	6	22, 50, 54, 65, 68, 72
Erysipelas		
♂	3	23, 53, 64
♀	1	72
Necrotizing fasciitis		
♂	0	–
♀	4	50, 81, 84, 86

ciple according to Magnusson (5) or else was purchased from Roche AG (Thrombin Roche). The thrombin was dissolved in 0.15 M saline to 100 NIH units/ml and stored in 0.2 ml portions at −70°C until use when it was further diluted with saline to 20 NIH units/ml. Urokinase (Leo laboratories, Copenhagen) was used as plasminogen activator. A dilution series was prepared to give a final concentration of 0.1 to 2 units/ml buffer which consisted of 0.03 M sodium phosphate, pH 7.8 in 0.15 M saline. Gelatin, 2.5 mg/ml, was added to the buffer for stabilization purposes.

Studies on plasminogen activator in tissue specimens

Punch biopsies were obtained from the skin of the extensor aspect of the forearm and the thigh in controls and from the same areas of non-involved skin in the patients. Biopsy samples were also collected from fresh lesions on the dorsal side of the calves in some patients. The tissue samples were immediately frozen in cold isopentane (−80°C) and kept in a deep freezer (−90°C) until sectioned at 10 μm on a cryostat. Six to ten sections were then placed on a microscope slide without thawing. The slides were kept in a deep freezer until further processed.

To analyse the skin sections for plasminogen activator a modification of the Todd-Pandolfi method (10, 8) was used. To 1.0 ml of human fibrinogen 0.05 ml of plasminogen was added. After mixing, an aliquot of 0.06 ml was placed together with 0.01 ml of thrombin on top of the sections on the slide. The solutions were quickly mixed and spread evenly over an area of about 25 mm×40 mm to cover the tissue specimens. The slides were preincubated at room temperature in moist chambers (Petri dishes) for 30 min in order to stabilize the fibrin film obtained. Subsequently, the chambers were placed in an incubator at 37°C and the slides were withdrawn at various time points from 0 to 30 min. The fibrin-covered sections were fixed in 4% formalin to interrupt the fibrinolytic reaction and subsequently stained in hematoxylin–eosin. They were read in a microscope at low power (30–100×).

The grading of fibrinolysis was performed separately for subpapillary and reticular dermis. It was made in six increasing steps fom 0 in the following way (Fig. 1 and Table II).

Grade 0: no lysis zones

Grade 1: partial lysis of the fibrin film is present just above a vessel. The area of the section affected is about 1% or less.

Grade 2: one or several small but complete holes in the film are present above dermal vessels. The average area of the holes is between 1–3% of the total section area

Grade 3: the holes are both larger and smaller but are not confluent; their area comprises about 5%.

Grade 4: consists of confluent holes in subpapillary or/and reticular dermis. The lysis zones account for about 10–15% of the film covering the section.

Grade 5: large confluent holes whose area is more than 25–30% of the total section area.

Studies on plasminogen activator (urokinase) on fibrin plates

The fibrin plates were prepared essentially according to Norén et al. (7). 1 g of agarose was dissolved in 100 ml of 0.01 M sodium phosphate buffer, pH 7.8, in 0.15 M NaCl solution in a boiling water bath and then allowed to cool down to about 40°C. Fibrinogen was diluted with the agarose solution to a final concentration of 1 g/l in a prewarmed glass tube (20 ml). 0.2 ml plasminogen solution was added per 10 ml of agarose fibrinogen solution followed by the addition of 0.1 ml thrombin (150 NIH units/ml) and the mixture was quickly poured into a Petri dish, mixed and placed on a levelling table to case the fibrin plates. After cooling at room temperature for 5 to 10 min, the plates were stored at 4°C, the lid of the Petri dish being made watertight with tape to avoid moistening.

Six 3 mm holes were drilled into plates and filled with 7 μl of the five different urokinase solutions or with saline–phosphate buffer. The plates were incubated at 37°C up to 24 h. The lysis zones were photographed at 6, 9, 12, 18, 21 and 24 h and the area of lysis calculated after measurement of the diameters of the zones from the pictures obtained.

Calculation of fibrinolytic activity

The fibrinolytic activity in the skin sections was calculated from analysis of linear regression of the grades of lysis present at various time points and given as the rate expressed by the slope (b) of the linear estimate obtained, according to the formula

$$b = \Sigma_t S \lg(t+30) : [\Sigma_t (\lg(t+30)^2 - \lg 15 \Sigma_t \lg(t+30)],$$

where S is the grade according to Fig. 1 and t the time of incubation at 37°C at which the grade was evaluated. The derivation of the expression above was obtained after the following considerations.

Fibrinolysis measured in our system is the result of enzymic degradation of the fibrin film by plasmin formed from the plasminogen added to the plate or slide by the action of the activator urokinase or of the tissue plasminogen activator. Suppose that the activity of the plasminogen activator is i. If the activity of plasmin is j, then the fibrinolysis obtained during time t is $j \cdot t$. When further production of new plasmin molecules by the action of the plasminogen activator occurs, the fibrinolysis should increase

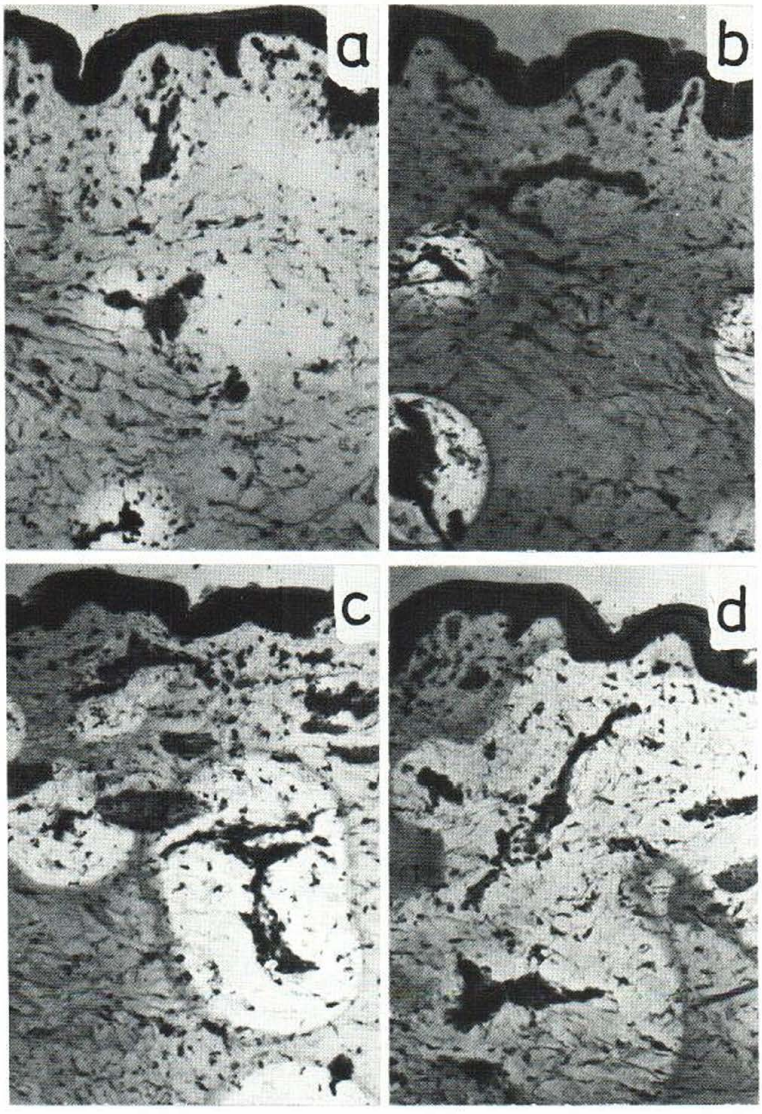

Fig. 1. Fibrinolysis in a specimen from a healthy subject after incubation at 37°C from 0 to 30 min. (*a*) Incipient lysis without formation of a complete hole in fibrin film overlying the section. The grades in subpapillary and reticular dermis are 1 and 1, respectively (1, 1). (*b*) Small lysis zones, grades 2, 3. (*c*) Partially confluent zones in reticular dermis, grades 2, 4. (*d*) Extensive lysis, grades 5, 5.

proportional to $(j \cdot t)^i$. It can be assumed that during an initial period of the reaction the area of the lysis zone

$$A_t = q(j \cdot t)^i,$$

where q is a proportionality fractor and the area at time t is A_t and t is the time from the start of the reaction.

If the logarithm is taken of the members of the above expression, i can be expressed as a linear function of the logarithm of time. If F is the degree of fibrinolysis and F is continuous throughout t, the following expression should satisfy our theory

$$F = \lg(A_t) = \lg q + i \lg j \cdot t.$$

The grading system used in Fig. 1 is a discontinuous variable of fibrinolysis in order to estimate F. We assume that the grades S over the time interval t are a valid estimate of F based on an analysis of the regression of S versus t.

In the expression

$$S = a + b \lg(t + 30), \qquad (1)$$

where a and b are coefficients of linear regression and t period of incubation at 37°C, as a concequence of symmetry the slope b should be a measure of the plasminogen activator activity (i).

It was observed, however, that in some cases fibrinolysis was high in most of the sections studied, even at zero time of incubation at 37°C. Since this would give falsely low values of the slope of the regression, b, this drawback was circumvented by the designation of a fixed starting point set at $S = 0$ and $t = -15$ min.

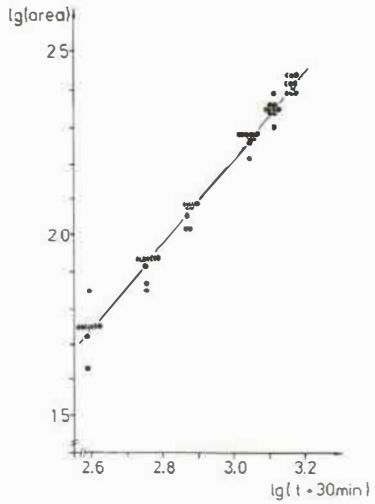

Fig. 2. The effect of urokinase on fibrinolysis in a plasminogen- and fibrin-containing agar plate. The lysis area was recorded at intervals from 6 to 24 h of incubation with 2 units/ml urokinase added to drilled holes in the plate. The plot satisfied the equation $\log(\text{area}) = a + b \lg(t+30)$ with a coefficient of correlation of 0.99.

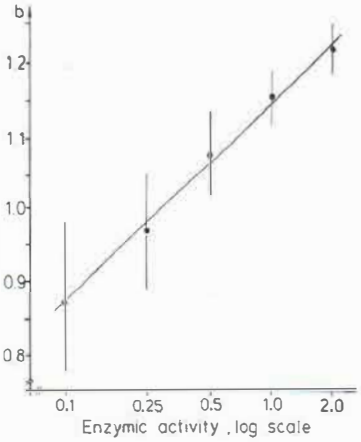

Fig. 3. Relationship between urokinase expressed as the slope b of the fibrinolysis according to eq. (2) obtained after incubation on fibrin agar plates and different concentrations of the enzyme in units/ml added to these plates in ten different experiments. Means and the standard deviations of the slopes are given. The coefficient of correlation was 0.996 between the logarithm of the urokinase activity added and the value of the slope b calculated.

Then

$$S = b\,(\lg(t+30) - \lg 15) \qquad (2)$$

The slope b was obtained from the experimental data by the calculation of least squares according to the partial derivation of eq. (2)

$$\frac{\delta \Sigma (S - b\,(\lg(t+30) - \lg 15))^2}{\delta b} = 0,$$

which resolved in the expression of b given above.

RESULTS

Analysis of the method

The fibrin agar plates were used to study the effect of the plasminogen activator urokinase. A linear relationship similar to eq. (1) between the logarithm of area of the zone of lysis and duration of incubation was obtained for various concentrations of urokinase investigated in 10 different experiments as illustrated by Fig. 2.

Slopes b for the different urokinase concentrations were calculated according to eq. (2), in which the logarithm of the area of lysis replaced the grade, S. A linear relationship between the logarithm of urokinase activity and the calculated slopes (b) was present as shown in Fig. 3. The coefficient of correlation in this part was 0.996.

During most of the initial experiments, only minute fibrinolysis was present in the specimens obtained from controls after a preincubation period of 30 min at room temperature. This preincubation period was kept, although later on we found in some control specimens grades up to 3 or 4 after this period.

The 37°C incubation was done at 0, 5, 10, 13, 15, 20, 25, and 30 min in preliminary experiments. When the experimental data in the control subjects were fitted according to eq. (1), correlation coefficients between grade of fibrinolysis and duration of incubation given as means were 0.84 for the values obtained from the arm and 0.56 for the thigh. The lower value of the correlation coefficient obtained from thigh skin data was ascribed to 2 controls with low activator activity combined with a wider scatter in grades. The error calculated after an analysis of covariance for the values of the plasminogen activator activity given as the slope b was 9% for the arm and 15% for the thigh when the standard deviation of the common slope is expressed as a percentage of the mean value of the slope. The larger variation of the thigh skin data was not due to variation between sexes.

It was found that grading combinations for the two dermal parts could be lumped together accord-

Table II. *Degree of fibrinolysis recorded separately for subpapillary and reticular dermis given as pair of grades from 0 to 5 according to Fig. 1*

A condensed grading system is summarized for the outcome of the grading in each section. A dash indicates that the part of the section is not valid or missing

Condensed grading	Individual grades in subpapillary, reticular dermis								
Grade 0	−,0	0,−	0,0						
Grade 1	−,1	−,2	0,1	0,2	1,0	1,1	2,0		
Grade 2	0,3	1,2	2,−	2,1	2,2				
Grade 3	−,3	0,4		1,4	2,3	2,4	3,1	3,2	3,3
	4,1	4,2	1,3						
Grade 4	−,4	1,5	2,5	3,4	4,3	4,4	5,0	5,1	5,2
Grade 5	3,5	4,5	5,3	5,4	5,5	−,5	5,−		

ing to Table II without affecting the accuracy. This was possible since individual plots of grades versus time for subpapillary and dermal dermis gave parallel regression lines, indicating proportionality between grades in the two dermal parts. Correlation between the two gradings was significant, coefficient of correlation being 0.97 (Fig. 4). The grading according to Table II gave straight lines against $\lg(t+30)$ with coefficients of correlation of 0.995 for the values obtained from the arm specimens and of 0.983 for those of the thighs of the control presented in Fig. 4.

The preliminary control material was also used to evaluate whether short cuts could be made without loss of accuracy in the measurement of the plasminogen activator activity. This activity in the thigh given as the slope b in eq. (1) or (2) was therefore calculated from the original control material from which the grade separately obtained for subpapillary and reticular dermis was averaged. These values of the slope were then compared with those obtained by the condensed grading system given in Table II where the regressions calculated were also limited to only three time intervals—0, 15, 30 min.

The slopes according to eqs. (1) and (2) were correlated for the original and the abbreviated material. The correlation between the slopes calculated according to eq. (1) for the calculations on the original and abbreviated material showed a coefficient of correlation of 0.97 and, according to eq. (2), 0.96. The coefficient of correlation calculated for the values obtained in the abbreviated way of calculating the slopes of eqs. (1) and (2) was 0.83.

Then the plasminogen activator activity was calculated in the abbreviated way using the condensed grading of Table II in the 22 control specimens. The error obtained was 16% when expressed as a coefficient of variation.

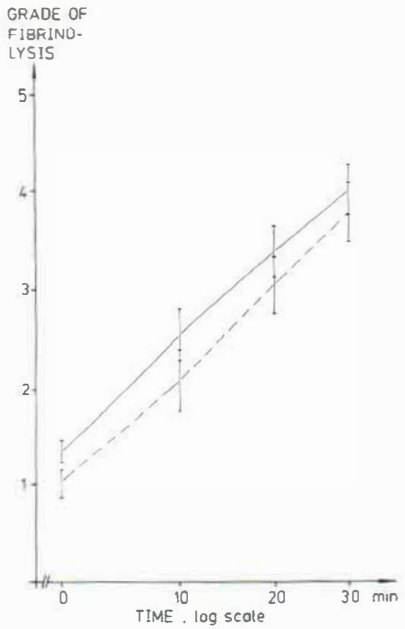

Fig. 4. Fibrinolysis obtained by grading lysis zones. The broken line is obtained by taking the average lysis in subpapillary and reticular dermis in each section for each period of incubation. The continuous line is obtained by the condensed grading system of Table II for the same sections. Both curves fit the general expression $S=a+b\lg(t+30)$, where a and b are coefficients of linear regression and t the duration of incubation at 37°C. S is the grade. Standard errors are indicated together with the means for 9 controls in material obtained from the forearm of healthy subjects.

Results in healthy controls and in patients

The results obtained from the measurements of plasminogen activator activity in healthy controls and in the two patient groups with inflammatory skin disease are summarized in Table III. The shortened form with incubation times 0, 15 and 30 min was used throughout.

In the controls the mean activities in the arm and the thigh were 6.3 and 4.9, respectively. There was a significant difference in the fibrinolytic activity in the unaffected thigh between the two sexes—females have about 40% lower activity than males ($p<0.02$).

In the patients with vasculitis the non-involved

Table III. *Plasminogen activator activity in skin taken from the forearm and the thigh in healthy subjects and in patients with skin diseases*

The activity is given as the rate expressed by the slope of linear regression of graded lysis zones with logarithm of time. Number of specimens, rates and their standard errors are given. The activities did not differ between patients with erysipelas and necrotizing fasciitis, which is the reason for summarizing them

	Controls		Leukocytoklastic vasculitis			Erysipelas and necrotizing fasciitis			
	Arm	Thigh	Arm	Thigh	Lesion (calves)	Arm	Thigh	Lesion (leg)	Near lesion (leg)
Females									
No.	4	4	6	6	2	5	5	5	5
Mean	6.04	3.48	4.83	3.60	2.71	6.49	3.97	1.17	3.10
S.E.M.	0.82	0.37	0.65	0.62	0.47	0.87	1.08	0.55	0.70
Males									
No.	7	7	2	2	–	3	2	3	3
Mean	6.47	5.68	5.10	5.63		4.97	4.27	1.03	4.44
S.E.M.	0.42	0.55	1.87	0.54		2.12	2.50	0.19	1.09
Difference between sexes	–	$p<0.02$	–	–		–	–	–	–
Sum									
No.	11	11	8	8	2	8	7	8	8
Mean	6.31	4.88	4.90	4.11	2.71	5.92	4.05	1.12	3.61
S.E.M.	0.38	0.49	0.60	0.58	0.47	0.91	0.93	0.34	0.67

skin did not differ significantly from controls. In the lesions on the calves 2 patients with vasculitis had a mean activity of 2.7 and 8 patients with erysipelas or necrotizing fasciitis had a mean activity of 1.1. In 3 cases of infection, low activity was present in the normal skin far outside the lesion. They had an activity of 0.1–0.9 in the lesion on the calves. The activities for the thigh were 0.5, 1.8 and 2.7 and for the arm 0.8, 3.5 and 7.9. In the skin near to the lesion the activities were 1.8, 5.2 and 2.6, respectively.

DISCUSSION

To obtain a formal resemblance to that of tissue fibrinolysis, urokinase, a proteolytic enzyme which activates the plasminogen in the fibrin plate, was used according to Norén et al. (7). The experimental evidence presented in Figs. 2–4 shows that our assumptions concerning the proposed exponential expansion of the enzyme reactions are relevant. Furthermore, it is not unusual that proenzymes are substrates in an initial enzyme reaction, as found for instance in the cascade of coagulation and in the activation of kinase kinase and protein kinase activities.

The parameter used for the expression of the plasminogen activator activity is the slope of the regression of the progressed fibrinolysis by time. It was also found that the value obtained for the slope is proportional to the logarithm of proteolytic activity of this activator. This indicates a wide range of tissue activity, since values can vary from the exponential power of near-zero, up to or exceeding the exponential power of 10, as found in diseased and healthy skin, respectively.

Our preliminary investigation showed in a semiquantitative manner the dermal distribution of the enzyme system operating in vivo. The proteolytic enzyme(s) released from the venous endothelium during the process of fibrinolysis were mostly proportional in subpapillary and reticular dermis, which enabled us to lump together the grades of lysis, as shown in Table II. It seems that the higher recorded activities in the reticular dermis are due to the larger vessels present in this area. It might therefore be true to suggest that data obtained from biopsies of the skin as presented here should correlate with those obtained in the larger veins, as described by Pandolfi et al. (8), provided that regional variations are corrected for.

The abbreviated way of grading as shown in Table II and the abridged form of recording grades at only three points during incubation make the method less cumbersome without unduly reducing

the accuracy of the activator activity so obtained. Our method is more reliable than earlier point estimates published in the literature (2), since the enzymic reaction was studied over a period of time. However, it is still a semiquantitative measure of an activity released from the vein wall, which we hope will be characterized and analysed in the future in a more specific way.

The findings in the patients with vasculitis confirm the results of earlier studies (2). These patients were included as a disease control for the group with bacterial infections. In some of the latter patients the fibrinolytic activity was very low even in the normal skin. This has been observed in patients with vasculitis, as discussed by Cunliffe (2). The reason for the finding is obscure. However, it will be the purpose of further studies to monitor a group of these patients in order to evaluate what position fibrinolysis takes in patients with recurrent infections.

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REFERENCES

1. Blombäck, B. & Blombäck, M.: Purification of human and bovine fibrinogen. Arkiv Kemi *10:* 415–443, 1956.
2. Cunliffe, W. J.: Fibrinolysis and vasculitis. Clin Exp Dermatol *1:* 1–16, 1976.
3. Dodman, B. A., Cunliffe, W. J., Dodman, E. A. & Webster, J. A.: A method of measuring well-defined areas within sections applied to the assessment of tissue fibrinolysis. *In* Methods in Micro-circulation Studies (ed. T. J. Ryan, B. Jolles and G. Lewis Holti), pp. 104–109. London 1972.
4. Hammar, H. & Wanger, L.: Erysipelas and necrotizing fasciitis. Br J Dermatol 96: 409–419, 1977.
5. Magnusson, S.: Preparation of highly purified bovine thrombin and determination of its N-terminal amino acid residues. Arkiv Kemi *24:* 349–358, 1965.
6. Matsuda, M., Iwanaga, S. & Nakamura, S.: A simple large scale method for preparation of plasminogen-free fibrinogen. Thromb Res *1:* 619–630, 1972.
7. Norén, I., Ramström, G. & Wallén, P.: Fibrin plate method with reagents purified by affinity chromatography and its use for determination of fibrinolytic and other proteolytic activity in saliva, bile and plasma. Haemostasis *4:* 110–124, 1975.
8. Pandolfi, M., Bjernstad, A. & Nilsson, I.-M.: Technical remarks on the microscopical demonstration of tissue plasminogen activator. Thromb Diath Haemorrh *27:* 88–98, 1972.
9. Sams, Jr, W. M., Thorne, G., Small, P., Mass, M. P., McIntosh, R. M. & Stanford, R. E.: Leukocytoclastic vasculitis. Arch Dermatol *112:* 219–226, 1976.
10. Todd, A. S.: Histological localization of fibrinolysin activator. J Pathol Bact *78:* 281–283, 1959.
11. Wallén, P. & Wiman, B.: Characterization of human plasminogen. II. Separation and partial characterization of different molecular forms of human plasminogen. Biochim Biophys Acta *257:* 122–134, 1972.

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