

Erythrocyte Aggregation in Relation to Diabetic Retinopathy

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Summary. Erythrocyte aggregation, plasma fibrinogen concentrations and serum protein fractions have been studied in 19 diabetic patients without retinopathy, 18 patients with retinopathy and 41 non-diabetic controls. — Significant differences in the parameters were observed between diabetics and controls. The differences between the diabetics with and without retinopathy were not

significant. — The role of abnormal erythrocyte aggregation in the development of diabetic retinopathy is discussed. — Striking parallels were found with similar findings concerning platelet aggregation in diabetics.

Key words: Diabetes, retinopathy, erythrocyte aggregation, fibrinogen.

Introduction

Ditzel and Moinat (1959) observed cell aggregates in the conjunctival vessels in diabetics. They found that the tendency to aggregation was due to a coating of the erythrocytes related to elevated plasma fibrinogen and globulin levels in their patients. Skovborg *et al.* (1966) found increased blood viscosity in long term diabetics, which they ascribed to the formation of erythrocyte aggregates, caused by interaction of fibrinogen and globulins at high concentration with the erythrocytes. Since erythrocyte aggregation or blood sludging may be a pathogenetic factor in diabetic retinopathy, we investigated erythrocyte aggregation in groups of controls and diabetics with and without retinopathy.

early in life in diabetic patients, interfering with both the results of erythrocyte aggregation measurement and the evaluation of the fundus lesions. Two groups of diabetic patients were selected.

Group A consisted of 19 diabetics without retinopathy, 6 males and 13 females. Their average age was 24 years (range 12–38 years).

Group B consisted of 18 diabetics with retinopathy, 12 males and 6 females. Their average age was 32 years (range 19–47 years).

The two groups of patients were considered to be comparable because previous studies had shown that the results of erythrocyte aggregation measurements are unaffected by differences in sex or age. The presence or absence of diabetic retinopathy was established both from ophthalmoscopic observation and from a fluorescein-angiographic study of pictures of the macular and perimacular areas of the retina. In diabetic patients determination of erythrocyte aggregation was performed twice, with an interval of 6–12 months, to find out if any

Table 1. *Syllectometry values and plasma protein concentrations in controls and diabetics*

	Controls	Group A	Diabetics		after 6–12 mo
			Group B	after 6–12 mo	
Number	41	19	19	18	18
Rate (sec ⁻¹)	0.21 ± 0.01	0.26 ± 0.02	0.25 ± 0.02	0.29 ± 0.02	0.26 ± 0.02
Corrected rate	0.22 ± 0.01	0.27 ± 0.02	0.26 ± 0.02	0.31 ± 0.02	0.29 ± 0.02
Defibrinated rate	0.07 ± 0.005	0.09 ± 0.01	0.08 ± 0.01	0.10 ± 0.01	0.10 ± 0.01
Fibrinogen rate	0.15 ± 0.005	0.18 ± 0.01	0.18 ± 0.01	0.21 ± 0.01	0.19 ± 0.01
Haemacel rate	0.29 ± 0.01		0.28 ± 0.01		0.27 ± 0.01
Fibrinogen g/l	2.8 ± 0.1	3.7 ± 0.1	3.6 ± 0.1	3.6 ± 0.2	3.6 ± 0.2

Clinical material

Controls consisted of 41 non-diabetic volunteers, 19 males and 22 females, with an average age of 29 years (range 17–49 years). From the diabetes Out-patient Department of Leyden University Hospital patients were selected with the juvenile onset type of diabetes. Selection of this material seemed reasonable if we were to prevent senile vascular changes, which may develop relatively

change in aggregation tendency had occurred within this period.

Methods

Blood was withdrawn by venepuncture. One sample was anticoagulated with lithium-E.D.T.A. (0.8 mg/ml blood). Another sample was defibrinated by shaking with glass beads for 10 min.

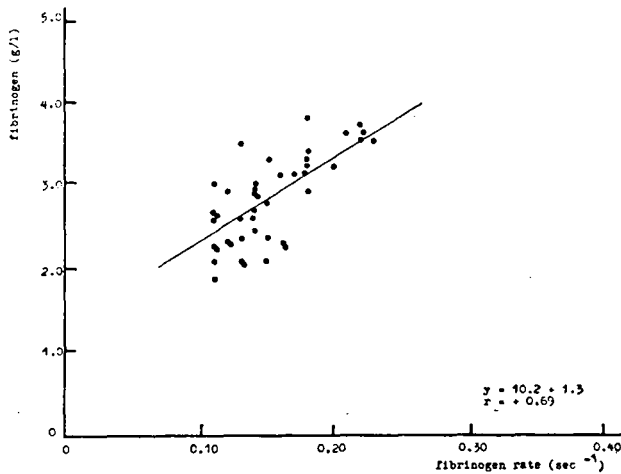


Fig. 1a. Scattergram of the relation between fibrinogen concentration and fibrinogen rate in the controls. Circles and broken line indicate values after 6–12 mo.

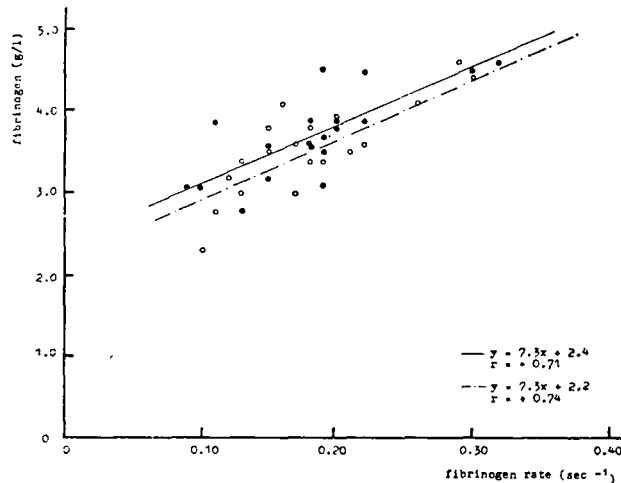


Fig. 1b. Scattergram of the relation between fibrinogen concentration and fibrinogen rate in group A. Circles and broken line indicate values after 6–12 mo.

reflecting surface area of the erythrocytes is then diminished by the formation of rouleaux.

The intensity of the reflected light is measured by a photo-electric cell and the whole process of reflection decrease is recorded. From the curve thus obtained the half-time of reflection decrease is calculated and this is then used as a measure of the rate of rouleaux formation. The method has been described in detail elsewhere (Van Haeringen and Glasius, 1970).

The rate of rouleaux formation is expressed in the reciprocal value of the half-time (sec⁻¹).

The corrected rate is the rate in blood after correction of the haemoglobin concentration to 15 g percent.

The defibrinated rate is the corrected rate of defibrinated blood.

The fibrinogen rate is the rate obtained by subtraction of the defibrinated rate from the corrected rate. It is the theoretical value of the rate of rouleaux formation caused by the presence of fibrinogen alone.

The "Haemacel" rate is the rate of a 40 percent erythrocyte suspension in Haemacel. Haemacel is a commercially available plasma expander containing 3.5 percent of a modified gelatin.

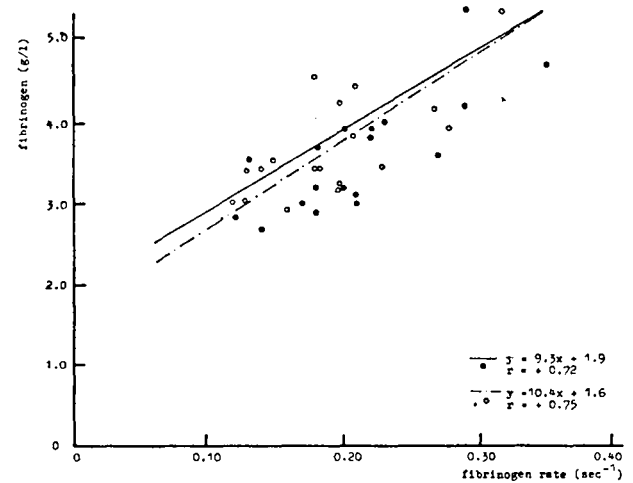


Fig. 1c. Scattergram of the relation between fibrinogen concentration and fibrinogen rate in group B. Circles and broken line indicate values after 6–12 mo.

Table 2. *Syllectometry values and serum protein concentrations in controls and diabetics*

	Controls	Group A	Diabetics after 6–12 mo	Group B	after 6–12 mo
Number	41	19	19	18	18
Defibrinated rate (sec ⁻¹)	0.07 ± 0.005	0.09 ± 0.01	0.08 ± 0.01	0.10 ± 0.01	0.10 ± 0.01
Serum protein (g/l)	73.6 ± 1.5	72.5 ± 1.2	72.0 ± 1.0	68.3 ± 0.9	71.5 ± 1.1
albumin	49.2 ± 1.0	44.7 ± 0.7	44.5 ± 0.6	43.2 ± 0.7	45.5 ± 1.0
α ₁ -globulin	2.5 ± 0.1	2.8 ± 0.2	2.5 ± 0.1	2.4 ± 0.1	2.3 ± 0.1
α ₂ -globulin	5.5 ± 0.2	7.4 ± 0.3	7.0 ± 0.3	6.8 ± 0.3	6.8 ± 0.2
β-globulin	5.7 ± 0.2	6.5 ± 0.3	7.4 ± 0.4	6.0 ± 0.2	6.5 ± 0.2
γ-globulin	10.3 ± 0.5	11.0 ± 0.5	10.5 ± 0.5	10.0 ± 0.3	10.4 ± 0.4

Erythrocyte aggregation was determined by syllectometry (Brinkman *et al.*, 1963). Syllectometry is a method based on measurement of the decrease in reflection of red light which occurs within 60 seconds after abruptly stopping the stirring of an erythrocyte suspension. The

Fibrinogen was determined by applying the biuret reaction to the fibrin clot according to Ware's method (1947).

Serum protein fractions were determined by electrophoresis on cellulose acetate (Cellogel). Strips were stained

with ponceau-S and the fractions were cut off and determined colorimetrically after dissolving in an appropriate volume of acetic acid 80 percent.

Haemoglobin was determined by the haemiglobin-cyanide method (Van Kampen and Zijlstra, 1961).

Results

Syllectometry values and fibrinogen concentration

In both groups of diabetics the mean values of the rate rouleaux formation, the corrected rate, the defibrinated rate, the fibrinogen rate and the fibrinogen concentration were higher than in the controls (Table 1). The significance varied from $p < 0.001$ to $p < 0.05$, except for the value of the defibrinated rate in group A after 6–12 months, when $p < 0.40$.

The correlation in each group between fibrinogen concentration and fibrinogen rate is shown in the

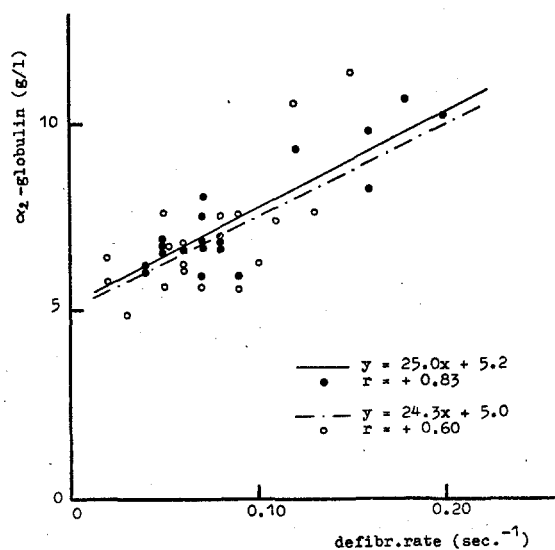


Fig. 2a. Scattergram of the relation between serum α_2 -globulin concentration and defibrinated rate in group A. Circles and broken line indicate values after 6–12 mo.

scattergrams in Fig. 1. A satisfactory regression was computed.

Within one group of diabetics only small, if any, differences in the average values of fibrinogen rate and fibrinogen concentration were found after 6–12 months, in spite of a sometimes considerable individual difference in fibrinogen rate and fibrinogen concentration in one subject after 6–12 months.

Syllectometry values and serum protein fractions

In defibrinated blood of both groups of diabetics a positive correlation exists between the defibrinated rate and the serum α_2 -globulin concentration (Fig. 2).

In group A there is also a correlation between the defibrinated rate and the β -globulin concentration. In group B, however, no such correlation could be de-

monstrated, because of the rather narrow range of β -globulin levels observed.

For the same reason no correlation could be demonstrated between the defibrinated rate and the serum globulin concentrations in the controls.

In both groups of diabetics the serum α_2 -globulin concentration was significantly higher ($p < 0.001$) and the albumin concentration significantly lower than in the controls ($p < 0.001$).

Syllectometry values in Haemacel

There was no significant difference in the mean values of the Haemacel rate between the two groups of diabetics or between the diabetics and controls.

Although in group B the rate, the corrected rate, the defibrinated rate and the fibrinogen rate are higher no significant difference could be demonstrated in these values between groups A and B.

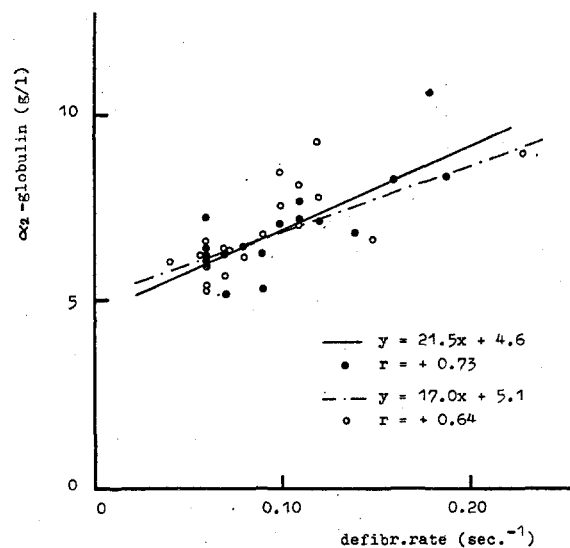


Fig. 2b. Scattergram of the relation between serum α_2 -globulin concentration and defibrinated rate in group B. Circles and broken line indicate values after 6–12 mo.

Discussion

The relatively fast rate of rouleaux formation, as deduced from syllectometry, represents the first step in the rather slowly developing series of events in erythrocyte aggregation *in vitro* occurring during blood sedimentation (Thorsen and Hint, 1950).

Plasma fibrinogen is the main component governing the rouleaux formation of erythrocytes in the controls and in the diabetics, as is shown in Table 1 and Fig. 1. Within the age limits of our groups there is no increase of plasma fibrinogen with age, as was found by Ogston and Ogston (1966). Defibrination results in a rate of rouleaux formation that is but a third to a quarter of the rate in whole blood. Sometimes no rouleaux formation takes place in defibrinated blood (van Haeringen and Glasius, 1970). This is in accord-

ance with the greatly reduced viscosity values in defibrinated blood found by Merrill *et al.* (1963). The effect of fibrinogen upon erythrocyte aggregation also can be studied with pure fibrinogen, thus excluding the effects of other proteins (Wells *et al.*, 1964 and Chien *et al.*, 1967). Cell aggregation was observed through a microscope and semiquantitative estimates were made from photomicrographs.

The existence of an erythrocyte factor (Hirschboeck, 1947 and Jansonius and Zijlstra, 1965) could not be demonstrated in Haemacel as standard fluid. Haemacel was preferred over a standard plasma pool or fibrinogen solution, since it is commercially available as a sterile solution of constant composition and is stable at room temperature. Our findings that syllectometry values are significantly higher in diabetic patients than in controls are in agreement with the results of Ditzel and Moinat (1955), who found that intravascular erythrocyte aggregation in the conjunctival vascular bed occurs more frequently in diabetic patients than in non-diabetic controls. They found a relationship between the intensity of erythrocyte aggregation and the degree of retinopathy and nephropathy. We also found higher syllectometry values in diabetic patients with retinopathy, but the difference, as compared with diabetic patients without retinopathy, was not statistically significant. This may be because there is not an essential difference between the two groups of diabetic patients, but only a gradual difference between those not yet having developed retinopathy and those with retinopathy, the latter group gradually increasing at the expense of the former, until almost all patients have developed retinopathy after two decades of manifest diabetes (Vink, 1969).

Repeated investigation after the relatively short period of 6–12 months gave mean values that again showed no significant difference between the two groups of diabetic patients.

If the abnormal erythrocyte aggregation in blood of diabetics, as shown in our syllectometry results, should take place also *in vivo*, then vascular occlusion at the level of the microcirculation might occur, thus promoting the development of diabetic retinopathy. This fits in well with the clinical observation that in high viscosity syndromes a retinopathy may develop which in some respects resembles diabetic retinopathy. This may, for instance, be seen in retinal vein thrombosis, where fluorescein angiography of the fundus may show the development of many microaneurysms, areas of capillary closure and neovascularisation (Oosterhuis and Vink, 1968).

Dintenfass *et al.* (1966) found a high viscosity syndrome due to an excessive aggregation of the erythrocytes in various thrombotic states. Van Haeringen (to be published) found abnormal syllectometry values in elderly patients with various thrombotic disorders of the retina.

The results presented here show striking parallels with the findings of Heath *et al.* (1971) that platelet aggregation *in vitro* is increased in diabetics with deteriorating retinopathy. However, it has to be emphasized that the pathogenesis of diabetic angiopathy is a problem of great complexity, in which increased erythrocyte aggregation tendency is only one of the aggravating factors.

References

1. Brinkman, R., Zijlstra, W.G. and Jansonius, N.J.: Quantitative evaluation of the rate of rouleaux formation of erythrocytes by measuring light reflection ("syllectometry"). *Proc. kon. ned. Akad. Wet.* **66**, 412 (1963).
2. Chien, S., Usami, S., Dellenback, R.J., Gregersen, M.I., Nanninga, L.B. and Guest, M.M.: Blood viscosity: influence of erythrocyte aggregation. *Science* **157**, 829 (1967).
3. Dintenfass, L., Julian, D. and Miller, G.: Viscosity of blood in normal subjects and in patients suffering from coronary occlusion and arterial thrombosis. *Amer. Heart. J.* **7**, 587 (1966).
4. Ditzel, J. and Moinat, P.: Lipoproteins and glycoproteins in diabetic subjects. *J. Lab. clin. Med.* **57**, 873 (1959).
5. Haeringen, N.J. van and Glasius, E.: The relation between rouleaux formation in blood and fibrinogen content in plasma. *Clin. chim. Acta* **29**, 55 (1970).
6. Heath, H., Brigden, W.D., Canever, J.V., Pollock, J., Hunter, P.R., Kelsey, J. and Bloom, A.: Platelet adhesiveness and aggregation in relation to diabetic retinopathy. *Diabetologia* **7**, 308 (1971).
7. Hirschboeck, S.: Lecithin and the erythrocyte factor in the blood sedimentation phenomenon. *Blood* **2**, 578 (1947).
8. Jansonius, N.J. and Zijlstra, W.G.: Various factors influencing rouleaux formation of erythrocytes, studied with the aid of syllectometry. *Proc. kon. ned. Akad. Wet.* **68**, 121 (1965).
9. Kampen, E.J. van and Zijlstra, W.G.: Standardisation of haemoglobinometry II. The haemoglobin-cyanide method. *Clin. chim. Acta* **6**, 538 (1961).
10. Merrill, E.W., Cokelet, G.C., Britten, A. and Wells, R.E.: Non-Newtonian rheology of human blood — Effect of fibrinogen deduced by "subtraction". *Circulat. Res.* **13**, 48 (1963).
11. Ogston, C.M. and Ogston, D.: Plasma fibrinogen and plasminogen levels in health and in ischaemic heart disease. *J. clin. Path.* **19**, 352 (1966).
12. Oosterhuis, J.A.: Fluorescein fundus photography in retinal vein occlusion. *Perspectives in Ophthalmology*, p. 29. Rotterdam: (Excerpta Medica Foundation), (1967).
13. Skovborg, F., Nielsen, Aa.V., Schlichtkrull, J. and Ditzel, J.: Blood-viscosity in diabetic patients. *Lancet* **1966 I**, 129.
14. Thorsen, G. and Hint, H.: Aggregation, sedimentation and intravascular sludging of erythrocytes. *Acta chir. scand.* **154** (1950).

15. Vink, R.: Fluorescein angiography in diabetic retinopathy. Leiden: Thesis (1969).
16. Ware, A.G., Guest, M.M. and Seegers, W.H.: Determination of fibrinogen in plasma by applying the biuret reaction to the fibrin clot. *Arch. Biochem.* **13**, 231 (1947).
17. Wells, R.E., Gawronski, T.H., Cox, P.J. and Pepera, R.D.: Influence of fibrinogen on flow properties of erythrocyte suspensions. *Amer. J. Physiol.* **207**, 1035 (1964).

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