Biochimica et Biophysica Acta, 641 (1981) 410—415

BBA Report

BBA 71496

ENHANCED CONCANAVALIN A AGGLUTINATION OF TRYPSINISED ERYTHROCYTES IS DUE TO A SPECIFIC CLASS OF AGGREGATION

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(Received May 6th, 1980)
(Revised manuscript received November 11th, 1980)

Key words Concanavalin A, Agglutination, Shear force; Viscosity, (Erythrocyte membrane)

Summary

Using the output of a rotational viscometer as a continuous index of aggregation, we have shown previously that the concanavalin A agglutination of native human erythrocytes can be resolved into three distinct classes of aggregation, static, type I and type II. Static aggregation occurs in the absence of shear forces while both type I and II aggregations are shear-induced. We now report that the increased concanavalin A agglutination of trypsinised erythrocytes is attributable to a specific enhancement in the development of type II aggregation. While type II formation in native cell suspensions requires high concanavalin A concentrations and continual shearing, an indistinguishable type of aggregation develops in suspensions of trypsinised red cells at considerably lower lectin concentrations and in the absence of applied shear forces.

Several groups have reported that the concanavalin A agglutination of human erythrocytes and other animal cells in enhanced by prior trypsinisation of the cell surface [1—4]. No adequate mechanism has been offered to explain this observation [5]. Previously, we reported that in the absence of applied shear forces concanavalin A caused only weak clumping of native erythrocytes (static aggregation). However, if suspensions in static aggregation were exposed to moderate shear stresses, or if concanavalin A was added to erythrocyte suspensions undergoing shear, a more extensive type of aggregation was rapidly

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formed (type I). Further shearing of type I led, after a lag period of the order of minutes, to the development of even stronger aggregation termed type II [6,7]. In the present investigation we have compared the development of the various classes of aggregation in suspensions of native and trypsin-treated erythrocytes.

The viscometric assay employed to measure the rate and degree of cell aggregation has been described previously [6,8]. It is based on the principle that if an aggregated cell suspension is to flow, intercellular bonds must be continuously broken down. The energy dissipated in this process appears as an increase in suspension viscosity, relative to the viscosity of an equivalent but non-aggregated sample. Since viscosity is directly proportional to the shear stress generated at the measuring element of a rotational viscometer (viscosity = shear stress/shear rate), the output of such an apparatus operated at a constant shear rate provides a continuous monitor of the degree of aggregation present in the cell suspension undergoing shear, provided no changes in suspending medium viscosity or cell deformability occur. For convenience, we use as an index of aggregation the ratio, R, of shear stress generated by aggregated and non-aggregated suspensions, measured at the same cell concentration and shear rate.

Fresh human red cells were washed three times in glucose-free Hepes-buffered Hanks' balanced salt solution, pH 7.4, and suspended at a hematocrit of 47%. Surface modification was performed by incubating washed erythrocytes with trypsin (Sigma; Type IX from porcine pancreas) at a final concentration of approx. 2000 BAEE units per 10^9 cells for 60 min at 37°C. Following one wash the suspension was incubated with excess trypsin inhibitor (Sigma, Type 1-S) for 5 min at 37°C. Cells were washed a further three times and suspended at a final hematocrit of 47%. The cell suspension (0.9 ml) was placed in the cup of a Contraves LS-2 couette viscometer fitted with a guard ring. A constant shear rate of 49.0 s^{-1} (37 rev./min with geometry used) was used throughout and the sample temperature was maintained at 37 ± 0.1°C by a water circulator. The equilibrium concanavalin A concentration and the number of lectin molecules bound per cell were estimated as described [6]. The coefficient of variation for replicate binding assays was less than 10%. Replicate agglutination assays on cells from the same donor were indistinguishable; donor variation was confined to different rates of type II aggregation (a factor of 2—3). However, the degree of enhancement in type II caused by trypsin treatment was consistent and independent of the donor. In the absence of concanavalin A the viscosities of trypsinised and native cell suspensions were identical. Including 9.8 mM α-methyl-D-mannoside in the medium completely inhibited type I and II aggregation for both native and trypsinised cells. Because the concanavalin A adsorption isotherm for native and trypsinised red cells differed, suitable lectin concentrations were selected in order that aggregation reactions could be compared under conditions where normal and surface modified cells bound similar amounts of concanavalin A.

The increase in aggregation index in suspensions of native and trypsinised erythrocytes under shear is compared at different concanavalin A concentrations in Fig. 1. We have no unequivocal evidence that cell deformability is unaltered under these conditions, but have demonstrated that its contribution

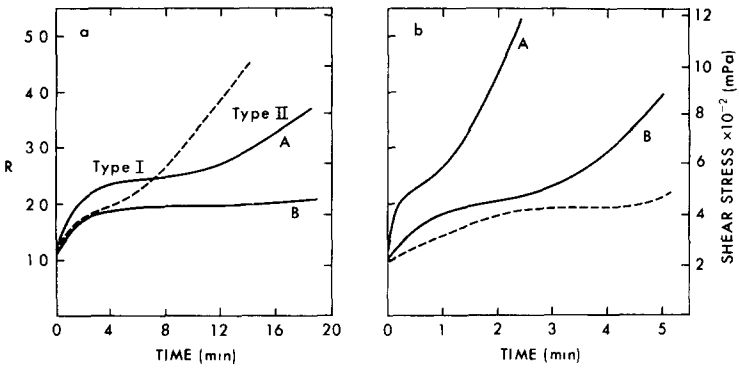

Fig. 1. The development of concanavalin A-induced aggregation in suspensions of native and trypsinised erythrocytes under shear. Concanavalin A (Miles) was added in a constant volume (40 μl) at zero time with no detectable increase in suspending medium viscosity and the progression of the aggregation reaction, as measured by the subsequent increase in shear stress (right-hand axis), was monitored over 20 min. The index of aggregation, R (left-hand axis), is the ratio of the shear stress measured for aggregated and non-aggregated (pre-concanavalin A) suspensions. (a) Native cells; (b) trypsinised cells (note expanded time scale). (A) 170 000 molecules bound per cell, (B) 75 000 molecules bound per cell. Mixed cell population containing equal volumes of native and trypsinised erythrocytes (- - - - -), 75 000 molecules bound per cell.

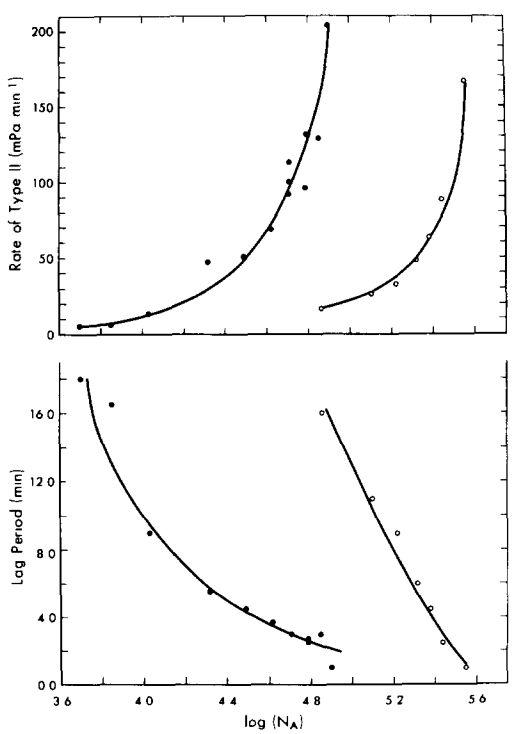

Fig. 2. The dependence on lectin concentration of concanavalin A-induced agglutination of native and trypsinised erythrocytes under shear. Experimental conditions as for Fig. 1. (a) The rate of type II aggregation and (b) the lag period, defined as the time elapsed between concanavalin A addition and the onset of type II aggregation, preceding type II formation as a function of the total number of molecules bound per cell (N_A). Native erythrocytes (○), trypsinised erythrocytes (●).

to the increase in R could at worst represent a small fraction of the values observed [13]. With both native and trypsinised cells the aggregation reaction progresses through similar stages. The addition of concanavalin A initiates the rapid formation of type I aggregation followed by type II development. Comparison of Fig. 1a and b demonstrates that the most pronounced difference in the concanavalin A agglutination of native and trypsinised erythrocytes is the enhanced rate of type II formation in suspensions of trypsinised red cells. In contrast, no significant difference is observed in either the rate of development or the level of type I aggregation. As described previously, the type I R value (R^I) for native erythrocytes increases rapidly with lectin concentration and reaches a limiting value (2.5) at approx. $14 \cdot 10^4$ concanavalin A molecules bound per cell [6]. Trypsinised cells behave similarly (Greig, R.G. and Brooks, D.E., unpublished observations).

A detailed comparison of the various stages of the agglutination reaction for native and trypsinised cells is presented in Fig. 2. When equal numbers of concanavalin A molecules are bound at the erythrocyte surface the rate of type II formation is much greater in suspensions of trypsinised red cells (Fig. 2a) and the lag period preceding type II development is correspondingly shorter (Fig. 2b). At a surface concanavalin A concentration of 70 000 molecules per cell, for example, type II formation is 20-times faster and the lag period over 10 min shorter in suspensions of trypsinised cells. In addition, the minimum surface concanavalin A concentration required for type II development is 10-times lower for the modified erythrocytes. Thus, in suspensions of trypsinised cells, far fewer adsorbed concanavalin A molecules are required to promote the development of type II aggregation. When type II aggregation is complete, however, the final R value (approx. 10) is similar for both native and trypsinised cells (Greig, R.G. and Brooks, D.E., unpublished results). Thus, as manifest in our assay, the principal difference in the agglutinability of native and trypsin-modified erythrocytes is not the final level of agglutination, but the rate at which this level is achieved through the development of type II aggregation.

In this respect, our results are at variance with those of previous workers who reported that the rate of agglutination of trypsinised red cells was similar to that of suspensions containing equal volumes of native and trypsinised erythrocytes [4]. Repeating this experiment in the viscometer we found that the rate of type I aggregation, the type I R value, the lag period and the rate of type II development for the mixed cell suspension assumed values intermediate between those found for homogeneous suspensions of native and trypsinised red cells (Fig. 1).

To relate our observations to those in the literature, we have compared the development of concanavalin A-induced aggregation in suspensions of native and modified cells in the absence of applied shear forces (Fig. 3). Under such conditions, native cells agglutinate weakly and the formed aggregates are characterized by a distinct morphology and erythrocyte sedimentation rate (Fig. 3a). Upon exposure to shear at higher lectin concentrations, type II aggregation can be induced in these suspensions. The morphology and erythrocyte sedimentation rates of type II aggregates are distinctly different from those formed in the absence of applied shear (Fig. 3b). In contrast to

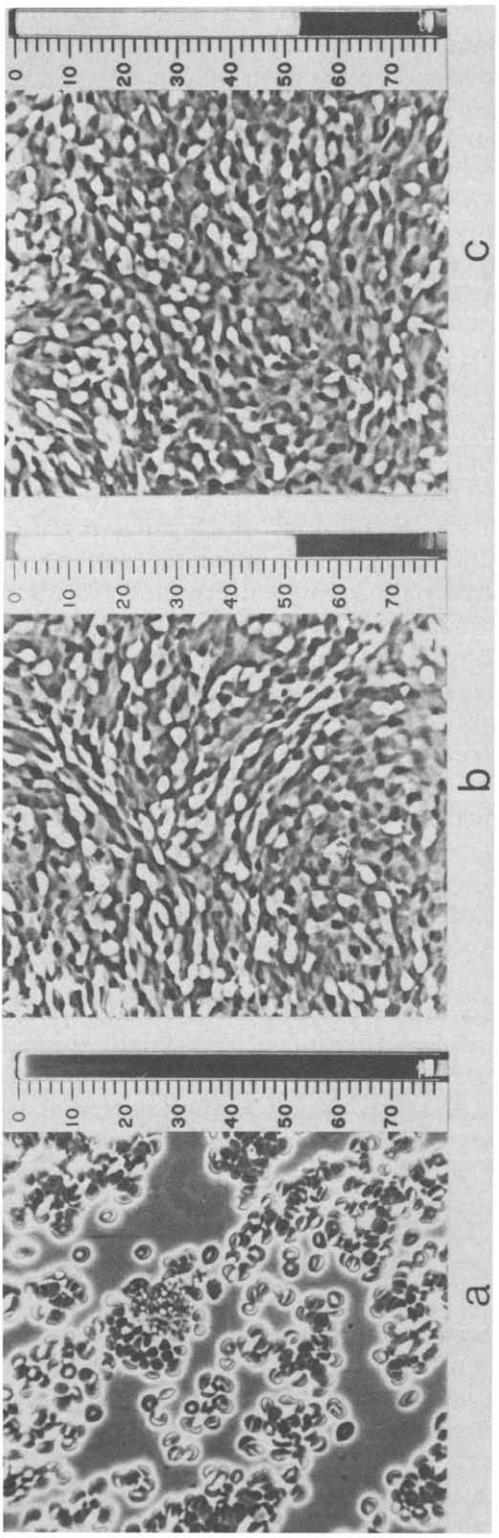

Fig. 3. The formation of type II aggregation in suspensions of native and trypsinised erythrocytes. Suitable concanavalin A concentrations were incubated with suspensions of native or trypsinised red cells for 60 min at 37°C. The extent of aggregation was assessed by microscopic examination and by measurement of the erythrocyte sedimentation rate. The erythrocyte sedimentation rates were estimated 60 min following the application of the diluted suspension (1 : 1 with Hanks' balanced salt solution, final hematocrit 23.5%) to 1.0 ml Seditubes (Becton-Dickinson). (a) Native cells incubated with concanavalin A (25 000 molecules bound per cell) in the absence of applied shear (static aggregation); (b) type II aggregation generated in suspensions of native cells carrying high surface concanavalin A concentrations (170 000 molecules bound per cell) and exposed to shear (49.0 s^{-1}) for 20 min; (c) Trypsinised cells incubated with concanavalin A (25 000 molecules bound per cell) in the absence of applied shear. For explanation see text.

native erythrocytes, when trypsinised red cells are incubated with concanavalin A in the absence of applied shear, the class of aggregation generated, as judged by the morphology and erythrocyte sedimentation rate of the formed aggregates, is type II (Fig. 3c). Thus, the widely reported observation that the concanavalin A agglutinability of human erythrocytes is enhanced by prior trypsinisation can be attributed to the formation of type II aggregation in these suspensions. Type II aggregations does not develop in native cell suspensions unless they are exposed to shear stresses greater than about 50 mPa.

The reason for the enhanced secondary shear-induced aggregation of trypsinised cells is not known. Under normal conditions, native red cells are only weakly agglutinated by concanavalin A. However, by perturbing the static structure of the cell membrane either by shearing or trypsinisation, a much stronger class of aggregation can be expressed. It would appear, therefore, that native red cells possess a kinetic barrier to the formation of type II aggregation. We speculate that this barrier prevents the formation of intercellular lectin bridges by a combination of electrostatic [9] and steric factors [10,11]. The effects of this barrier are reduced, and type II aggregation promoted, by tryptic digestion of cell surface structures. The kinetic barrier may prevent lectin molecules bound to one cell surface from interacting with sites on adjacent cells. Alternatively, it may inhibit the reorganization of bound lectin into a more stable configuration. The mechanism by which shearing in the presence of high concanavalin A concentrations mimics the effect of trypsin in promoting type II aggregation is similarly unclear, although in principal a combination of increased intercellular collision rates and shear-induced rearrangement of membrane components could produce the observed effects.

We thank Mandy Hoskins for meticulous technical assistance. Financial support from MRC Canada and the British Columbia Health Care Research Foundation is gratefully acknowledged.

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