Agglutination Kinetics of Enzymatically Treated Normal and Diabetic Rat Hepatocytes

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Abstract □ The effect of trypsin and neuraminidase treatments on concanavalin A-induced agglutination of viable hepatocytes from normal and diabetic rats are reported. Trypsin (1.0 μg/mL) treatment resulted in an increased rate of hepatocyte agglutination in both normal and diabetic cells in the presence of 100 μg/mL of concanavalin A. However, neuraminidase treatment resulted in a decrease in the rate of cytoagglutination in the normal cells and an increase in the rate in the diabetic counterpart. The results suggest that trypsin may have caused the removal of a surface protein and/or split a peptide bond on the agglutinin receptors resulting in identical receptor exposure and clustering in normal and diabetic cells. The neuraminidase data suggest that the arrangements of the neuraminic acid moieties on the receptors in normal cell membranes were different from those in the diabetic cells, eliminating the possible effect of changes in the surface charge density. In conclusion, normal cells carry numerous clustered (possibly some in the "cryptic" state) agglutinin receptors in the cell membrane as compared with cells from diabetic rats.

Keyphrases □ Agglutination—kinetics of enzymatically treated normal and diabetic rat hepatocytes, trypsin, neuraminidase □ Trypsin—agglutination kinetics of enzymatically treated normal and diabetic rat hepatocytes, neuraminidase □ Kinetics—agglutination of enzymatically treated normal and diabetic rat hepatocytes, trypsin, neuraminidase □ Neuraminidase—agglutination kinetics of enzymatically treated normal and diabetic rat hepatocytes, trypsin

The cell surface is considered to be an attractive target for the binding of proteins like lectins. The carbohydrate-binding specificity of the lectin concanavalin A has been useful in the detection, characterization, and study of membrane alterations and cytodifferentiations in cell surface membranes (1–5). Studies on the pretreatment of normal and transformed cells with proteolytic enzymes, neuraminidase, β-galactosidase, phospholipases, and other enzymes prior to agglutination have elucidated the mode and extent of cell membrane transformations (6–9).

Recently, it was reported (10) that rat hepatocytes dispersed by collagenase perfusion were highly agglutinable by concanavalin A. Furthermore, agglutination was twice as fast in normal cells as compared with cells obtained from streptozocin-treated diabetic rats. This manuscript reports cytoagglutination studies with concanavalin A after pretreating the normal and diabetic hepatocytes with trypsin and neuraminidase.

EXPERIMENTAL

Preparation of Rat Hepatocytes—Dispersed liver parenchymal cells were isolated and tested for viability by the procedure of Bikhazi *et al.* (10, 11). The trypan blue exclusion test for cellular viability was routinely performed before and after the experiments, and preparations showing ≥90% viable cells were used.

The metabolic state of the hepatocytes during the incubation period was examined by following the respiration rate of cell suspensions in several separate experiments. Oxygen tension was recorded continuously with the aid of a biological oxygen monitor. Rates were determined from the slopes of the oxygen tension curves and were calculated in terms of microliters of O_2 per milligram of cell protein. Protein was determined by the biuret reaction, with bovine serum albumin as a standard, using centrifuged cell pellets dissolved in 1 M NaOH to minimize possible interference from medium components.

Preparation of Diabetic Rats—Rats were rendered diabetic by injection with 65 mg/kg iv of streptozocin[1]. The procedure and blood glucose level tests were reported previously by Bikhazi *et al.* (10, 11).

Agglutination of Hepatocytes from Normal and Diabetic Rats—For the control experiments ~9×10^6 hepatocytes were suspended in 225 mL of Ca^{2+}- and Mg^{2+}-free phophate-buffered saline (pH 7.4). Cell counts were recorded kinetically by an automated counter[2] at 0, 5, 10, 20, and 30 min (10, 11).

Treatment with Concanavalin A—Two milliliters of Ca^{2+}- and Mg^{2+}-free phosphate solution containing concanavalin A was added to 225 mL of buffer to obtain a 100-μg/mL solution of the lectin. The hepatocytes were then dispersed in this solution, and cell counts were obtained as described above.

Treatment with Trypsin and/or Neuraminidase—The experiments included treatment of normal or diabetic cells with trypsin or neuraminidase and measuring agglutination in the absence of the lectin. The cells were dispersed in Ca^{2+}- and Mg^{2+}-free buffer solutions containing 0.22, 0.74, or 1.0 μg/mL of trypsin[3]. Treatment was allowed to proceed for 5 min, then cell counts were recorded at 0, 5, 10, 20, and 30 min. For neuraminidase[3] (88 ng/mL), the hepatocytes were treated with the enzyme for 15 min before cell counts were recorded. In another experiment, concanavalin A (100 μg/mL in the suspension) was added after the above treatments to induce agglutination.

Treatment with Neuraminidase, Trypsin, and Concanavalin A—After treating the hepatocytes for 15 min with 88 ng/mL of neuraminidase, trypsin (1.0 μg/mL) was added and the suspension was allowed to mix for 5 min. Concanavalin A was then added and the cytoagglutination was recorded as above.

Treatment with Trypsin, Trypsin Inhibitor, Neuraminidase, and Concanavalin A—Trypsin (1.0 μg/mL) was added to the cell suspension after cell counts were recorded. After 5 min, trypsin inhibitor (1.3 μg/mL) was added to quench the trypsin activity. Neuraminidase (88 ng/mL) was then added, the suspension was stirred for 15 min, and a second cell count recording was obtained. Afterward, concanavalin A (100 μg/mL) was added, and cell counts were recorded at 5, 10, and 20 min.

Data Analysis—The physical model on irreversible agglutination (10, 12) was used in the data analysis and interpretation. The general second-order rate relationship derived by Higuchi *et al.* (13) and further supported by Bikhazi *et al.* (10, 12) was used to describe the agglutination rate. The following equations were used:

$$\frac{1}{H_S} = \frac{1}{H_S^0} + Kt \qquad \text{(Eq. 1)}$$

where:

$$K = \frac{16\gamma K_0 r_S T}{3\eta} \qquad \text{(Eq. 2)}$$

and H_S is the number of hepatocyte singlets at any time t, H_S^0 is the number of singlets at $t = 0$, γ is the sticking probability constant (which measures the stickiness of the cells), K_0 is the Boltzmann constant, r_S is the hepatocyte radius, T is the temperature in degrees Kelvin, and η is the viscosity of the medium (10, 12).

RESULTS

Average Number, Mean Diameter, and Viability of Rat Hepatocytes—The average number of isolated cells per liver was ~1.2×10^8 hepatocytes. The mean hepatocyte diameter was taken as 20 μm (10), and cellular viability was routinely tested using the trypan blue exclusion test. The rate of oxygen production was estimated to be 1.0 μL/mg dry weight/h, which is within the normal range for mammalian cells (11). The protein content of hepatocytes was ~16% of the wet weight, near the value reported for whole liver (11).

[1] U-9889, lot number 60,273-9; courtesy of The Upjohn Co., Kalamazoo, Mich.
[2] Coulter Counter model A; Coulter Electronics, Hialeah, Fla.
[3] Sigma Chemical Co., St. Louis, Mo.

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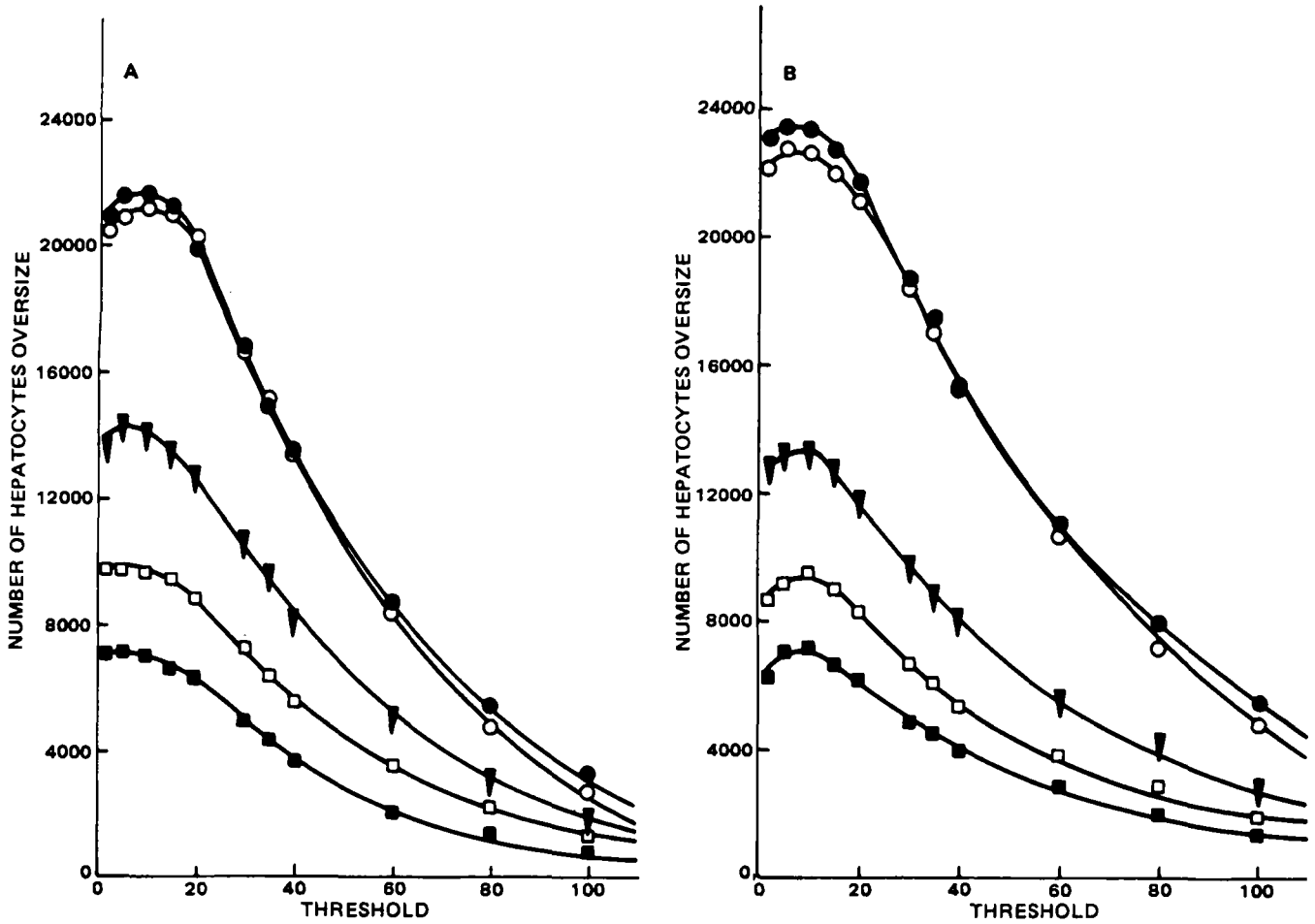

Figure 1—*Representative cumulative size distribution curves for normal (A) and diabetic (B) rat hepatocytes with respect to time in the presence of 1 μg/mL of trypsin and 100 μg/mL of concanavalin A. Key: (●) 0 min, trypsin-treated cells without concanavalin A; (○) 0 min, concanavalin A added after trypsin treatment; (▼) 5 min; (□) 10 min; (■) 20 min.*

Second-Order Rate Plots for Agglutination—The value of H_S (Eq. 1) was estimated from the cumulative size distribution curves as exemplified in Fig. 1. The cell counts (H_S) at threshold 30 (volume 4.2×10^3 μm^3) and at 0, 5, 10, and 20 min were used to draw the linear curves of $1/H_S$ *versus* time (t). All data were treated similarly to Fig. 2, which represents the least-squares line fitted to the experimental data. The slopes of the least-squares lines were estimated, and γ values (Eq. 2) were calculated (10, 12) (Table I).

Hepatocyte Agglutination—Bikhazi *et al.* (10) observed that the agglutination rate of normal cells was double that of diabetic cells in the presence of 100 μg/mL of the lectin. Hepatocytes were not agglutinable after trypsin treatment. Figure 1 represents typical time-dependent cumulative size distribution plots of normal and diabetic cells after treatment with trypsin followed by concanavalin A. The effect of trypsin treatment was obvious when compared with the non-trypsin-treated cells (Table I).

Curves similar to those of Fig. 1 were observed after treatment with trypsin and trypsin inhibitor followed by concanavalin A, except that the rate of agglutination was less than when the inhibitor was absent. These findings suggest that trypsin has no proteolytic action on concanavalin A.

Table I—Sticking Probability Constants for Hepatocyte Agglutination with Concanavalin A after Various Treatments

Treatment[a]	Sticking Probability Constants[b], $\gamma \times 10^7$ cm^{-1}/hepatocyte	
	Normal Cells	Diabetic Cells
Concanavalin A	8.78 ± 0.92 (A)	3.23 ± 0.46 (G)
Trypsin	3.23 ± 0.02 (B)	4.16 ± 0.92 (H)
Neuraminidase	0	0
Trypsin plus concanavalin A	36.05 ± 3.23 (C)	34.20 ± 4.16 (I)
Neuraminidase plus concanavalin A	4.53 ± 0.69 (D)	8.78 ± 0.92 (J)
Trypsin plus trypsin inhibitor plus concanavalin A	13.86 ± 3.70 (E)	9.70 ± 1.84 (K)
Trypsin plus trypsin inhibitor plus neuraminidase plus concanavalin A	7.85 ± 1.85 (F)	13.86 ± 0.46 (L)

[a] Concentrations: concanavalin A, 100 μg/mL; trypsin, 1.0 μg/mL; neuraminidase, 88 ng/mL; trypsin inhibitor, 1.3 μg/mL. [b] Mean ± *SEM*; $n = 6$. Letters in parentheses are codes for statistical analysis. Significant differences: $p < 0.001$ (A,C), (A,G), (B,C), (B,E), (C,E), (G,I), (G,K), (H,I), (K,L), (G,J); $0.001 < p < 0.01$ (E,F), (J,L); $0.01 < p < 0.02$ (F,L), (H,K); $0.02 < p < 0.05$ (A,E), (E,K); $0.05 < p < 0.1$ (B,H); $0.1 < p < 0.2$ (A,D), (D,J). No other combinations showed statistical differences.

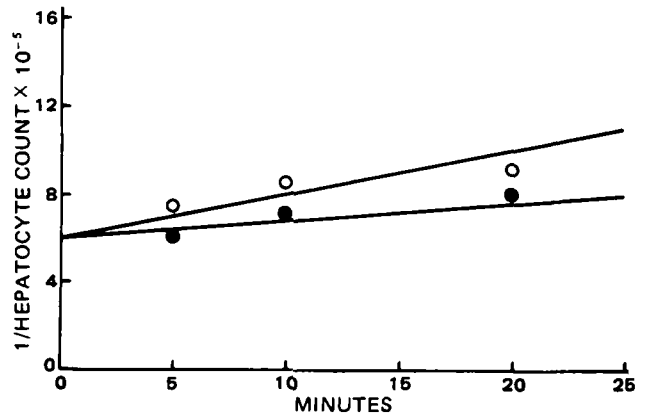

Figure 2—*Representative second-order plot of diabetic rat hepatocyte agglutination, 1/hepatocyte count* versus *time. Straight lines are least-squares fit to the experimental data in the presence of 88 ng/mL of neuraminidase and 100 μg/mL of concanavalin A. Key: (●) 100 μg/mL of concanavalin A; (○) 88 ng/mL of neuraminidase plus 100 μg/mL of concanavalin A.*

No agglutination was seen after neuraminidase treatment. Table I shows that there was no effect of neuraminidase treatment on concanavalin A-induced agglutination in normal cells, but a drastic increase in cytoagglutination was observed in diabetic cells.

Neuraminidase treatment after trypsin treatment caused a marked decrease of γ in normal cells, while the reverse was true for diabetic cells (Table I). After treatment with both enzymes, the agglutination rate of normal cells remained the same as those of the untreated cells. However, diabetic hepatocytes showed an increase in the agglutination rate after trypsin treatment followed by the neuraminidase effect. This was also observed for the neuraminidase *versus* the trypsin-neuraminidase results.

DISCUSSION

A number of postulations have been reported in the literature on the effect of proteolytic enzymes on normal and transformed cell agglutination induced by lectins. These include the exposure of cryptic receptors (14, 15), clustering of receptors (16–18), and changes in surface morphology characterized by a reduction (19) or increase in surface microvilli (20, 21).

In the absence of trypsin inhibitor, the proteolytic effect of trypsin continued throughout the course of the experiments, suggesting that the 5-min enzymatic treatment was not sufficient for complete receptor exposure and/or rearrangement. The agglutination rates of both cell types with concanavalin A after trypsin treatment (with and without inhibitor) were almost the same. Therefore, the proteolytic action of trypsin may have caused the removal of a surface protein and/or split a peptide bond on the agglutinin receptors, which could result in identical exposure and clustering (rearrangements) of the receptors in normal and diabetic cells.

Neuraminidase is an enzyme which normally hydrolyzes peripheral *n*-acetylneuraminic (sialic) acids. After hydrolysis, the removal of the surface sialic acid could cause a reduction in the net negative surface charge of the cells thus reducing the forces of repulsion between adjacent cells, favoring cytoagglutination (22). The results presented in Table I indicated cytodispersion and repulsion rather than agglutination after normal cells were treated with neuraminidase. It is, therefore, possible that the hydrolysis of the neuraminic acid moieties (the target moieties for concanavalin A-induced agglutination) on the glycoprotein receptors actually took place. However, in the case of diabetic cells, the opposite effect was observed. Therefore, in normal hepatocytes, the arrangements of the neuraminic acid moieties on the glycoprotein receptors seemed to be different from those in the diabetic cell membranes. The surface charge density may not be a factor since after neuraminidase treatment and in the absence of concanavalin A, agglutination was not observed (Table I).

The pretreatment of normal cells first with trypsin and then neuraminidase had no effect on the rate of concanavalin A-induced hepatocyte agglutination (Table I). This was contrary to diabetic cells, where the rate of agglutination drastically increased after consecutive treatment with both enzymes. The data suggest that normal cells (as compared with diabetic cells) carry clustered agglutinin receptors, some of which may be in the "cryptic" state. Trypsin normally causes increased receptor mobility and further exposure of the "cryptic" receptors. In the presence of both trypsin and neuraminidase, agglutination of the diabetic cells was maximal.

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