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FACTORS AFFECTING HEMAGGLUTINATION BY CONCANAVALIN A AND SOYBEAN AGGLUTININ

J. A. GORDON and M. D. MARQUARDT

Department of Pathology, University of Colorado Medical Center, Denver, Colo. 80220 (U.S.A.)

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SUMMARY

While investigating the effect of temperature on hemagglutination by concanavalin A, we noted three factors that seriously interfere with the usual microscopic agglutination assay and produce misleading or ambiguous results. (1) Adherence of concanavalin A-treated erythrocytes to surfaces of plastic Petri dishes, especially at (2) commonly used cell densities, effectively prevents determination of agglutination. (3) In addition, incubation times usually used may be insufficient to demonstrate agglutination. Failure to account for these factors may explain the previously reported temperature-sensitive, concanavalin A-mediated agglutination of trypsinized erythrocytes and transformed cells (Vlodavsky, I., Inbar, M. and Sachs, L., (1972) Biochim. Biophys. Acta 274, 364–369). By controlling these factors, we demonstrated that concanavalin A does agglutinate trypsinized, human erythrocytes equally well at 24 and 4 °C.

Investigation of the kinetics of erythrocyte agglutination by lectins revealed that the rate of agglutination by concanavalin A is markedly slower at lower temperatures while soybean agglutinin-mediated agglutination is faster at lower temperatures. Ultracentrifugation data indicate that at low temperature concanavalin A exists partially as a dimer (mol. wt 50 000) and at warmer temperatures exists mainly as a tetramer (mol. wt 100 000). The correlation of the effect of temperature on molecular weight with the agglutinating activity of concanavalin A suggests that temperature-dependent forms of concanavalin A may determine the rate of cell agglutination by this lectin. No temperature-dependent change in molecular form was observed with soybean agglutinin.

INTRODUCTION

Concanavalin A, the phytohemagglutinin from jack bean, exhibits a wide variety of interesting biological properties [1]. Aspects of the interaction between cells and concanavalin A under current investigation include (1) the chemical nature of the concanavalin A binding site on the surface of the cell membrane [2], (2) the mobility of these membrane sites, and (3) the surface alterations of cells following transformation [3–5]. Recently, temperature-dependent agglutinability by concanavalin A has been reported for virally and chemically transformed fibroblasts [6] and

trypsinized human erythrocytes [7]. For example, concanavalin A (at 100 or 250 μg/ml) was shown by Vlodavsky et al. [7] to cause some agglutination of trypsinized Group O erythrocytes within 30 min at 24 °C, but no agglutination was seen when the incubation was carried out at 4 °C. In marked contrast, agglutination by soybean agglutinin was shown to be independent of temperature at 4 and 24 °C. As the binding of concanavalin A to non-erythrocytic cell surfaces was found to be temperature-independent [6], these observations led to the suggestion that cell agglutination by concanavalin A, but not by soybean agglutinin, is associated with a temperature-sensitive activity of the cell membrane that may be of a metabolic nature [8].

This communication presents data both from equilibrium and kinetic studies on the agglutination of human erythrocytes at two temperatures, 4 and 24 °C, by concanavalin A and soybean agglutinin. We conclude that the previously reported temperature dependence of erythrocyte agglutination by concanavalin A at apparent equilibrium need not relate to changes on the cell surface. Our experiments do demonstrate a slower rate of hemagglutination by concanavalin A in a cold environment than in a warm environment. Additional evidence suggests that the slower rate of agglutination by concanavalin A in the cold is due to an alteration of the form and activity of concanavalin A.

MATERIALS AND METHODS

Concanavalin A, twice crystallized and stored in saturated NaCl, was obtained from Miles-Yeda, Ltd. Soybean agglutinin was prepared from saline-extracted soybean flour (Central Soya, Chicago) by affinity chromatography as previously described [9]. Human types A and O erythrocytes were obtained from 3-week-old whole blood. Lectin solutions and washed erythrocyte suspensions were prepared in phosphate-buffered saline, pH 7.2, of the following composition: 140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, and 1 mM KH_2PO_4. Washed erythrocytes were "trypsinized" by pretreatment with 1 mg of pancreatic extract (Difco, Detroit) per ml of an erythrocyte suspension of $2.4 \cdot 10^8$ cells/ml in phosphate-buffered saline for 1 h at 37–39 °C [10], resuspended and washed four times with phosphate-buffered saline. All cell centrifugations were done at about $100 \times g$.

The agglutination assays were performed at 4 and 24 °C. Two parallel 20-tube series of decreasing lectin concentration in phosphate buffered saline were prepared by 2-fold serial dilution in glass test tubes. All lectin solutions and trypsinized erythrocyte suspensions were initially maintained at 4 °C. To each tube of a lectin series was added an equal volume of a dilute (10^7 cells/ml) or a concentrated (10^8 cells/ml) erythrocyte suspension. Then 0.2-ml of the dilute ($5 \cdot 10^6$ cells/ml) and 0.1 ml of the concentrated ($5 \cdot 10^7$ cells/ml) mixtures were transferred from each tube to corresponding wells of two Microtiter plates (Cooke Engineering Co.). The remaining solution (1.6–1.8 ml) of selected tubes was divided between two 35-mm plastic Petri dishes (Falcon Plastics, No. 3001) as used by Vlodavsky et al. [7]. After these operations were completed at 4 °C, one of the duplicate Microtiter plates and one set of Petri dishes were transferred to the 24 °C environment.

The Microtiter plates were first scored macroscopically 3 h after mixing with lectin. A pattern of uniform effacement of the bottom of the well by the erythrocytes indicated the presence of agglutination and was read as positive; a negative pattern

indicating nonagglutination consisted of a central button of erythrocytes surrounded by a concentric, clear zone devoid of erythrocytes [11]. The contents of the wells were also examined microscopically at 3 and 20 h for agglutination by resuspending the contents of each well with a Pasteur pipette and transferring a drop to a glass microscope slide and covering with a glass coverslip. The plastic Petri dishes were examined microscopically for agglutination after 30 and 90 min of incubation. The formation of erythrocyte aggregates of at least four or five cells that were not disrupted by gentle movement was considered as evidence of agglutination.

Kinetic studies on the agglutination reaction were carried out as follows: Mixtures of lectin and erythrocytes were prepared in phosphate-buffered saline as described above and kept in constant suspension by gentle magnetic stirring or gyro-rotation in glass beakers. The cell suspensions and lectin solutions were maintained at constant temperatures prior to mixing and for the duration of the experiments, except where otherwise indicated. At selected time intervals, aliquots were removed from the mixtures and diluted in 10-ml volumes of Isoton (Coulter Electronics, Hialeah, Fla) or phosphate-buffered saline. The samples were counted on a model A Coulter Counter (Coulter Electronics, Hialeah, Fla) with the lower threshold set so that the remaining single cells and aggregates were counted as a function of time. Samples were counted at initial levels of about 25 000 particles per 0.5 ml. Triple readings of duplicate samples were performed for each reaction mixture at selected time intervals. The averages were corrected for machine coincidence using the standard coincidence correction graph for the 100-μm aperture.

RESULTS

Determination of the lowest lectin concentration necessary for agglutination by visual examination of the Microtiter wells presented no ambiguities with the concentrated ($5 \cdot 10^7$ cells/ml) cell series. With the dilute ($5 \cdot 10^6$ cells/ml) cell series, macroscopic determination of agglutination was very ambiguous as the lower cell concentration gave indistinct patterns. Determination of agglutination in the dilute series was only possible by microscopic examination of the cells after resuspension of the contents of individual wells. The macroscopic and microscopic results from the Microtiter plate assays are summarized in Table I. At equilibrium there is no significant difference in lectin-mediated agglutinability of erythrocytes between the warm (24 °C) and cold (4 °C) experiments with either lectin, blood type, or cell concentration. Unequivocal agglutination by concanavalin A was present in the cold and in the warm at concentrations down to 2 μg/ml of concanavalin A, in marked contrast to previous reports [7]. Soybean agglutinin was found to be active under all conditions yielding agglutination down to at least 0.030 μg/ml of soybean agglutinin.

Considerable difficulty was encountered in attempting to ascertain the presence of agglutination by the technique involving microscopic examination of the contents of plastic Petri dishes even after ninety minutes incubation (Table II). Within 30 min we observed extensive adherence of the settled erythrocytes to the bottom surfaces of the plastic dishes. Probably because of this adherence to the plastic of most of the erythrocytes in the dilute cell suspension, no aggregates could be seen at 4 °C and at 24 °C even with concanavalin A concentrations of 1000 μg/ml. However, with the more concentrated ($5 \cdot 10^7$ cells/ml) cell suspension, aggregates were observed with

TABLE I

AVERAGE LOWEST LECTIN CONCENTRATION CAUSING HUMAN ERYTHROCYTE AGGLUTINATION IN MICROTITER PLATES AT TWO TEMPERATURES

Washed and trypsinized erythrocytes in phosphate-buffered saline were used as described in text. The data are expressed as the average of 3 independent experiments.

ABO type	Erythrocyte concentration (cells/ml)	Temperature (°C)	Concanavalin A (μg/ml)		Soybean agglutinin (μg/ml)	
			Macro*	Micro**	Macro*	Micro**
O	$5 \cdot 10^6$	4	i***	~1	i***	~0.002
		24	i***	~1	i***	~0.004
O	$5 \cdot 10^7$	4	~1	~1	~0.010	~0.010
		24	~1	~1	~0.020	~0.030
A	$5 \cdot 10^6$	4	i***	~2	i***	~0.003
		24	i***	~1	i***	~0.002
A	$5 \cdot 10^7$	4	~2	—	~0.002	~0.002
		24	~1	—	~0.003	~0.002

* Lowest concentration of lectin giving agglutination by macroscopic (visual) observation of well contents after 3 h of incubation. Maximum error of one doubling dilution in either direction.

** Microscopic observation of well contents transferred to a glass microscope slide and scored for minimum agglutination taken as clumps of 4–5 cells stable to gentle shaking. Cells without lectin were negative. Recorded values were taken from the observations at 3 h except for the dilute cell series with concanavalin A where the values at 20 h indicated that 3 h was insufficient for equilibrium. Maximum error is one doubling dilution in either direction.

*** Indeterminant for technical reasons, see Results.

TABLE II

LOWEST LECTIN CONCENTRATION CAUSING HUMAN ERYTHROCYTE AGGLUTINATION IN PLASTIC PETRI DISHES AT TWO TEMPERATURES

Plates incubated for 30 min (24 °C) and 90 min (4 °C) before scoring the lowest lectin concentration giving agglutination.

ABO type	Erythrocyte concentration (cells/ml)	Temperature (°C)	Concanavalin A (μg/ml)	Soybean agglutinin (μg/ml)
O	$5 \cdot 10^6$	4	i*	~0.002
		24	i*	~0.002
O	$5 \cdot 10^7$	4	~2	~0.002
		24	~2	~0.002
A	$5 \cdot 10^6$	4	i*	~0.002
		24	i*	~0.002
A	$5 \cdot 10^7$	4	~1	~0.002
		24	~1	~0.002

* Ambiguous and difficult to score as the cells adhered to the bottom of the plate following settling (see Results). With the more concentrated cells, aggregation apparently occurred before the single cells became attached to the surfaces of the plates, see Figs 1–2.

concentrations of concanavalin A greater than 2 μg/ml at both 4 and 24 °C with A and O erythrocytes. These aggregates of cells probably formed before cell settling was complete (kinetics of agglutination described later). With soybean agglutinin, agglutination could be detected with both cell concentrations down to soybean agglutinin concentrations of 0.002 μg/ml; aggregation probably proceeded faster than erythrocyte settling. The results from the Petri dish assays (Table II) with the concentrated cell suspensions are in agreement with the results of the Microtiter plate experiments, i.e. concanavalin A agglutinates types A and O, trypsinized, human erythrocytes in the concentrated suspensions equally well in the warm and cold down to concentrations of 2.0 μg/ml of concanavalin A.

It is readily apparent that the rate of agglutination of erythrocytes by concanavalin A is slower in a cold (1 °C) than a warm (24 °C) environment with concanavalin

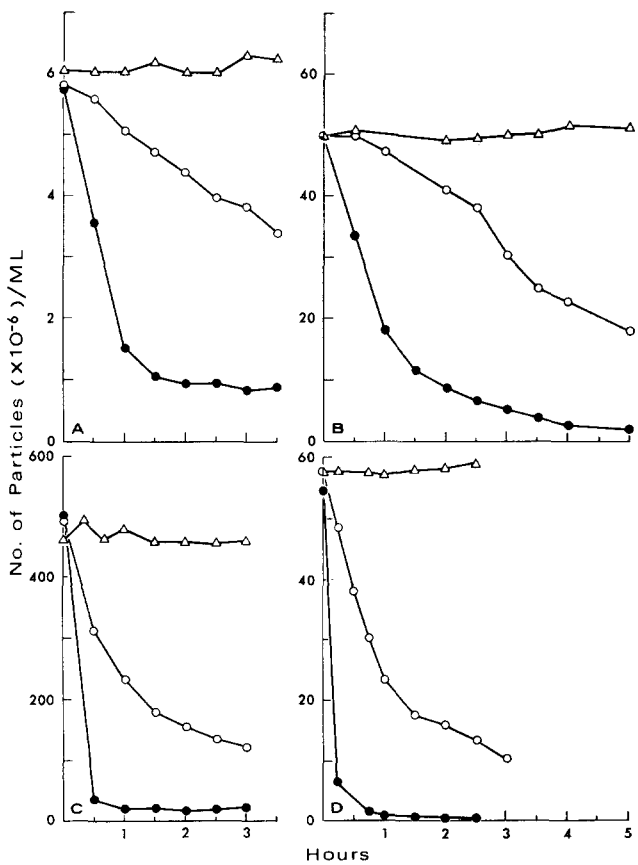

Fig. 1. The effect of temperature, erythrocyte density, and lectin concentration on the kinetics of agglutination of trypsinized human erythrocytes (○+) by concanavalin A. The experiments were performed at 24 (●—●) and 1 °C (○—○) with controls within lectin (△--△). (A) About $6 \cdot 10^6$ cells/ml, 100 μg/ml concanavalin A. (B) About $50 \cdot 10^6$ cells/ml, 100 μg/ml concanavalin A. (C) About $500 \cdot 10^6$ cells/ml, 100 μg/ml concanavalin A. (D) About $60 \cdot 10^6$ cells/ml, 1000 μg/ml concanavalin A.

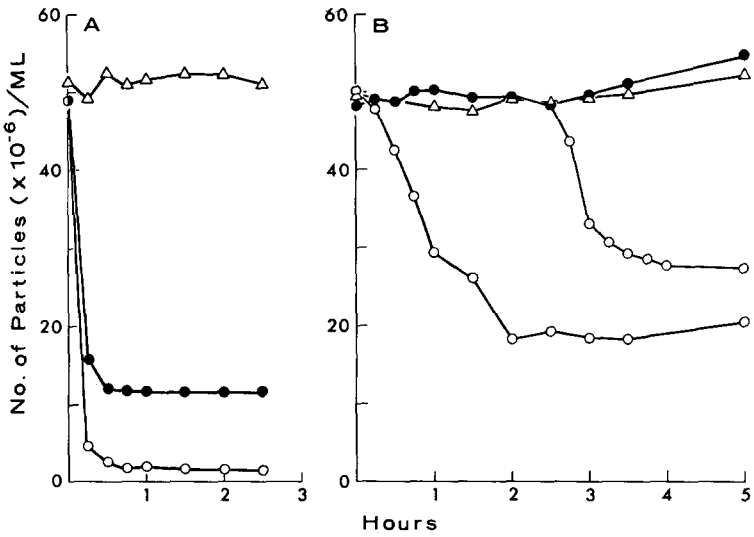

Fig. 2. The effect of temperature and lectin concentration on the kinetics of agglutination of trypsinized, human erythrocytes (○+) by soybean agglutinin. Erythrocyte concentration was $5 \cdot 10^7$ cells/ml. ●—●, warm (about 24 °C); ○—○, cold (about 1 °C); △—△, control (about 24 °C). (A) 1 μg/ml soybean agglutinin. (B) 0.1 μg/ml soybean agglutinin. In (B) at 2.5 h of incubation an aliquot of the 24 °C reaction mixture was quickly brought to approx. 1 °C.

A concentrations of 100 μg/ml and 1000 μg/ml (Fig. 1), and the reaction rate is slower at the lower concentration of concanavalin A. With soybean agglutinin concentrations of 0.1 μg/ml the reaction rate is much more rapid in the cold than in the warm environment (Fig. 2B). If the soybean agglutinin concentration is raised to 1 μg/ml the reaction rates are too fast to detect kinetic differences in the warm and cold environments, but the reaction proceeds further (more cells agglutinated and/or fewer and larger aggregates) at 1 °C than at 24 °C (Fig. 2A). Note that the soybean agglutinin–erythrocyte agglutination reaction very rapidly reaches equilibrium. This is not true for the concanavalin A–erythrocyte reaction where, in the cold, equilibrium was not attained at 4 h.

The kinetics of the concanavalin A–erythrocyte agglutination reaction are also affected by the cell concentration (Fig. 1A–C). If the erythrocyte concentration ($5 \cdot 10^7$ cells/ml) is increased ten times (to approx. $5 \cdot 10^8$ cells/ml), holding concanavalin A at 100 μg/ml, the rate of the agglutination reaction is dramatically increased (Fig. 1B–C).

DISCUSSION

A previous report by Vlodavsky et al. [7] has indicated that concanavalin A, but not soybean agglutinin, agglutinates human erythrocytes at 24 °C but not at 4 °C [7]. Since binding of concanavalin A to cells (nonerythrocytes) was found to be temperature-independent [6], the existence of a specific temperature-sensitive activity on the erythrocyte membrane was suggested as an explanation for hemagglutinability at 24 °C but not at 4 °C [7]. The temperature-sensitive phenomenon has also been

explained by an hypothesis involving a redistribution of concanavalin A receptors by temperature-dependent lateral diffusion of receptor sites in the membrane [12]. Both of these explanations involve membrane changes as the primary events.

The reported experiments by Vlodavsky et al. [7] had been performed in plastic Petri dishes with suspensions of trypsinized cells ($2.5 \cdot 10^6$–$4 \cdot 10^6$ cells/ml). With concanavalin A (100–250 μg/ml) and O erythrocytes, they found no agglutination at 4 °C and weak agglutination at 24 °C after 30 min. With soybean agglutinin they found strong agglutination at both 4 and 24 °C. We have confirmed the latter result. However, with a cell suspension of $5 \cdot 10^6$ cells/ml, we found no apparent agglutination of erythrocytes by concanavalin A concentrations up to 1000 μg/ml assayed in plastic Petri dishes at 4 or 24 °C even with 90 min incubation. Simply raising the cell concentration 10-fold gave agglutination at both temperatures down to concanavalin A concentrations of 2 μg/ml in Petri dishes (Table II) and in Microtiter plates (Table I). We conclude that critical cell densities facilitate the assessment of concanavalin A mediated agglutination by these visual assay techniques as has been noted with isohemagglutinins [13].

Another reason for the inadequacy of the Petri dish assay with concanavalin A and a dilute cell system that probably contributed to the previously reported negative results with concanavalin A [7], is the settling and adherence of most of the erythrocytes to the plastic surface of the bottoms of the dishes before agglutination can take place. Indeed, enhancement of cellular adherence to plastic surfaces by concanavalin A has now been noted by others [14, 15]. This problem can be prevented by continually agitating the cell suspensions during the incubation period [16], or by using a smaller ratio of settling surface area to sample volume, as in the wells of Microtiter plates.

Measuring kinetics of hemagglutination with the quantitative Coulter Counter assay, we have shown that the concanavalin A–erythrocyte agglutination reaction proceeds more slowly when the temperature is lowered (Fig. 1). The opposite is true for the soybean agglutinin–erythrocyte agglutination reaction (Fig. 2). Thus the time of observation can be an important variable in assessing the presence of erythrocyte agglutination by lectins.

Therefore, our results indicate that the previously reported temperature-sentive agglutination of erythrocytes by concanavalin A does not need to be ascribed to a specific metabolic activity on the membrane surface. At equilibrium, concanavalin A agglutinates erythrocytes equally well at 24 and 4 °C. The previously reported results can be explained on the basis of several interrelated parameters discussed above. These parameters need not involve changes in the cell membrane.

It may be noted that the minimal lectin concentrations producing agglutination differ between the results of the experiments performed with the more static conditions (Petri dish and microtiter plate assays) and those of the more dynamic conditions in the assays of agglutination kinetics. Such differences may be explained on the basis of varying sensitivities to agglutination by the different assay techniques. The degree of agitation of the reacting mixture during the assay procedure will effect the endpoint of the hemagglutination reaction. One would expect a hemagglutination reaction under the conditions of agitation in the experiments measuring kinetics to achieve an equilibrium state when the rate of agglutination by lectin was equal to the rate of disaggregation caused by the shear forces of centrifugal stirring. The level of the equili-

brium state and the extent of agglutination at any time point would be dependent upon the shear forces produced during stirring. Under the more static conditions of the Petri dish assay and the microtiter plate assay, such disruptive shear forces would be at a minimum as the cells settle under gravity. Thus, one might expect differences in agglutinability under different conditions of assay.

It might be argued that the slower kinetics of concanavalin A-mediated agglutination at lower temperatures reported here (Fig. 1) could be due to a slowed metabolic activity on the cell surface and/or a decreased mobility of the concanavalin A receptor site [12,17] (assuming such mobility is a necessary function for agglutination to occur). This argument would require that the receptor for concanavalin A and/or its mobility be relatively unusual or be unusually affected after concanavalin A attachment. Soybean agglutinin, other lectins [18,19], and naturally occurring ABO isoagglutinins [20–23] mediate hemagglutination reactions which are unaffected or stronger at lower temperatures (exothermic [21]).

It is therefore not unreasonable to suggest that the slowed rate of agglutination by concanavalin A at lower temperatures might reflect changes in the agglutinating ability of concanavalin A and not changes in the cell surface. Support for this suggestion is found in the known temperature dependence of the concanavalin A tetramer–dimer dissociation [24,25] which we have confirmed under our conditions and which was not observed with soybean agglutinin (Table III).

That concanavalin A apparently binds to some cell types (no data are available for erythrocytes) equally well at warm and cold temperatures [6] is compatible with an explanation involving the partial dissociation of concanavalin A to a less active agglutinating form (dimer) at lower temperatures. Our hypothesis that the temperature-dependent molecular form of concanavalin A is important in determining the rate of hemagglutination by concanavalin A is actually compatible with either equal or unequal binding of the two forms of concanavalin A at the two temperatures. However, there is evidence to suggest that dimers and tetramers may bind equally well.

TABLE III

DEPENDENCE OF APPARENT MOLECULAR WEIGHT OF CONCANAVALIN A AND SOYBEAN AGGLUTININ ON TEMPERATURE*

Lectin	Protein concentration (mg/ml)	Temperature (°C)	Percent distribution according to protomer association	
			Dimer	Tetramer
Concanavalin A	1.20**	22	< 5	approx. 95
		4	approx. 40	approx. 60
Soybean agglutinin	1.00	22		100
		4		100

* Sedimentation analysis carried out at 60000 rev./min in a Model E ultracentrifuge (Beckman Instruments, Palo Alto) equipped with Schlieren optics. Refrigerated temperature was monitored in the pre-cooled AnD rotor by the RITC unit. Bovine serum albumin was also run in phosphate-buffered saline in a second 12-mm Kel-F cell, in place of the counterbalance, for comparison purposes.

** Initial concentration.

For example, recent chemical derivatizaton of tetrameric concanavalin A has been shown to convert the protein to a dimeric molecule with a decreased ability to agglutinate erythrocytes unaccompanied by changes in binding ability [26]. The agglutination reaction is conveniently thought of in terms of a two-step mechanism [21]. The first step involves the association of the lectin with the cell surface without agglutination. The second step involves the agglutination of cells. Both the tetrameric and dimeric forms of concanavalin A could participate in the first step, but only the tetrameric form may be able to bridge cells and cause agglutination.

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