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THE MOLECULAR BASIS FOR COLD AGGLUTINATION: EFFECT OF RECEPTOR DENSITY UPON THERMAL AMPLITUDE OF A COLD AGGLUTININ

Chao-Ming Tsai[1], David A. Zopf[2], and Victor Ginsburg
National Institute of Arthritis, Metabolism, and Digestive Diseases
National Institutes of Health, Bethesda, Maryland 20014

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SUMMARY: Cold agglutinin MKV is a Waldenström macroglobulin that agglutinates human erythrocytes in the cold by binding N-acetylneuraminosyl-containing carbohydrate chains on their surfaces. Neuraminidase-treated cells are not agglutinated but their reactivity can be restored by allowing them to adsorb hematoside (NeuNAcα2-3Galβ1-4Glcβ1-ceramide). When between 7 x 10^4 and 10^6 molecules are adsorbed per cell, the cells are agglutinated at 0° but not at 37°. When over 10^6 molecules of hematoside are adsorbed, they are agglutinated at both 0° and 37°. The density of receptors on the erythrocyte surface can thus influence the thermal amplitude of cold agglutinins.

We have recently described a monoclonal IgM (agglutinin MKV) isolated from the serum of a patient with Waldenström's macroglobulinemia that agglutinates erythrocytes by binding to N-acetylneuraminosyl-containing carbohydrate chains on their surfaces (1). Human erythrocytes are agglutinated only in the cold while dog erythrocytes are also agglutinated at 37°, though less strongly than at 0°. The immunoglobulin has anti-Pr_2 specificity according to the classification of Roelcke (2) because treatment of the erythrocytes with ficin decreases the reactivity of human cells but increases the reactivity of dog cells and treatment of erythrocytes with neuraminidase abolishes the reactivity of both human and dog cells. The agglutinability of neuraminidase-treated erythrocytes is restored by allowing them to absorb exogenous ganglioside; when large amounts of ganglioside are absorbed the cells are agglutinated not only in the cold but also at 37° (1). The present paper presents the quantitative relationship between receptor density and agglutinability of erythrocytes by cold agglutinin MKV.

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[1]Present address: Bureau of Biologics, Food and Drug Administration, Bethesda, Maryland, 20014

[2]Present address: National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014

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MATERIALS AND METHODS

Cold agglutinin MKV is a cryoglobulin obtained from the serum of a patient with Waldenström's macroglobulinemia by repeated precipitation at 0° followed by solubilization in phosphate-buffered saline (0.15 M NaCl/0.05 M sodium phosphate, pH 7.2) at 37° (1). In order to render it soluble in the cold for the experiments reported here, the purified immunoglobulin was treated with neuraminidase as previously described (1) and then separated from the enzyme by passage through a column containing Sephadex G-200. The asialoprotein was iodinated with carrier-free ^{125}I (New England Nuclear Corporation, Boston, MA) by the chloramine T method (3) and the labeled product separated from free ^{125}I and other impurities by affinity chromatography, using a column containing 3'-sialyllactose-Sepharose-4B and thermal elution (4). The affinity-purified ^{125}I-labeled protein retained full hemagglutinating activity as compared to native cold agglutinin MKV. Bovine serum albumin was iodinated with carrier-free ^{131}I (New England Nuclear Corporation, Boston, MA) by the chloramine T method (3) and separated from free ^{131}I by gel filtration through a column containing Bio-Gel P2 (Bio-Rad Laboratories, Richmond, CA).

Human erythrocytes (donor D.Z.) were treated with Vibrio cholera neuraminidase (Calbiochem, La Jolla, CA) as follows: a 10% suspension of washed cells in phosphate-buffered saline, pH 6.0, containing 1 mM $CaCl_2$ and enzyme, 40 units/ml, was incubated for 40 min. at 37°C. The cells were then washed five times with 15 volumes of phosphate-buffered saline. The neuraminidase-treated cells lost over 90% of their original sialic acid residues [1.9 x 10^6 residues/cells after enzyme treatment as compared to 24 x 10^6 residues per cell before as determined with thiobarbituric acid reagent after hydrolysis (5)] and are no longer agglutinated by cold agglutinin MKV.

Hematoside (NeuNAcα2-3Galβ1-4Glcβ1-Ceramide) was isolated from dog erythrocytes (6). After purification by thin-layer chromatography (7) about 10 μmoles of hematoside were obtained from 200 ml of packed dog erythrocytes. The hematoside was ^3H-labeled by catalytic reduction with NaB^3H_4 in the presence of PtO_2 by the method of Hakomori(Sen-Itiroh Hakomori, personal communication) as follows: To 2 μmoles of hematoside in 0.9 ml of ethanol in a 14 x 100 mm screw cap tube was added 100 mCi of NaB^3H_4 (specific activity, 11 Ci/mmole; New England Nuclear Corporation, Boston, MA) and 5 mg of PtO_2. The tube was capped and the reaction mixture sonicated for 4 hr and then stirred overnight at room temperature. The catalyst was removed by centrifugation and after 25 μl of glacial acetic acid was added to destroy excess NaB^3H_4, the reaction mixture was evaporated to dryness under vacuum. Borate was removed by the repeated addition and evaporation of 2 ml aliquots of methanol (five times) and ^3H-labeled hematoside (specific activity, 370 mCi/mmole) was isolated and purified by thin-layer chromatography (7).

Neuraminidase-treated erythrocytes were allowed to adsorb ^3H-labeled hematoside as follows: a 50% suspension of cells, 0.25 ml, was added to 0.25 ml of phosphate-buffered saline, pH 7.2, containing from 1 to 400 μg of hematoside per ml. After incubation at 37° for 1 hour, the cells were collected by centrifugation and washed with 15-ml portions of phosphate-buffered saline until the ^3H-activity in the wash fell below 500 CPM/ml (generally from 5 to 20 washes). Aliquots of the cells were counted in a hemocytometer and their radioactivity determined in a scintillation counter with correction for quenching by hemoglobin. The number of hematoside molecules adsorbed per erythrocyte was then calculated. Under these conditions, adsorption of hematoside is roughly proportional to its concentration in the incubation media. For example, about 6 x 10^4 molecules are adsorbed per cell at 10 μg/ml and 4 x 10^5 molecules at 100 μg/ml.

Hemagglutination tests were performed in microtiter plates (IS-MRC-96, Linbro Chemical Co., New Haven, CT.) (8). A 2% suspension of erythrocytes in

phosphate-buffered saline, 25 µl, was added to serial dilutions of cold agglutinin MKV in 25 µl of phosphate-buffered saline. After incubation for 2 hr at 0° or 37°, agglutination was observed macroscopically.

The number of molecules of cold agglutinin MKV bound per erythrocyte was determined with ^{125}I-labeled cold agglutinin MKV (9) as follows: hemagglutination tests with the labeled agglutinin were performed as described above except the phosphate-buffered saline used for serial dilutions contained 0.1% ^{131}I-labeled bovine serum albumin in order to correct for the amount of cold agglutinin MKV unassociated with the cells and to minimize nonspecific adsorption to the cells and to the walls of the microtiter plates (10). After incubation for 2 hr at 0°, 25 µl of supernatant solution was collected. The remaining fluid containing the sedimented erythrocytes was removed and the wells washed twice with 100 µl of 0.1% unlabeled bovine serum albumin and the washings combined with the erythrocyte fraction. From the ^{125}I and ^{131}I in the supernatant solution compared to the combined erythrocyte fraction, the amount of cold agglutinin MKV bound to the erythrocytes can be estimated (9). As the specific activity of the cold agglutinin and the number of erythrocytes per well is known, the number of molecules bound per cell can be calculated.

RESULTS AND DISCUSSION

The agglutinability of neuraminidase-treated erythrocytes with cold agglutinin MKV can be restored by allowing them to adsorb hematoside(Fig. 1). Agglutinablility at 0° is first evident when the number of molecules of hematoside adsorbed per cell reaches 7×10^4 and increases to the level of untreated erythrocytes as the amount of hematoside adsorbed approaches 10^6 molecules per cell (untreated cells are agglutinated by 0.03 µg/ml of cold agglutinin MKV at 0° under the same conditions). At 10^6 or more molecules of hematoside adsorbed per cell, the erythrocytes are also agglutinated at 37°. Agglutination measurements at 37° with high levels of adsorbed hematoside are inaccurate as enough hematoside is shed by the cells into the medium during incubation so as to partially inhibit agglutination.

From studies on various "anti-I" cold agglutinins, Oleson estimates that binding of 50-500 molecules of agglutinin per cell are required for the agglutination of human erythrocytes at 5° (9). As shown in Table I, about 90 molecules of cold agglutinin MKV are bound per erythrocyte at the lowest level of immunoglobulin that causes agglutination at 0°. However, it is likely that only part of these bound molecules are actually cross-linking erythrocytes as there is comparatively little difference between the number of cold agglutinin MKV molecules bound to untreated cells and neuraminidase-treated cells, which are

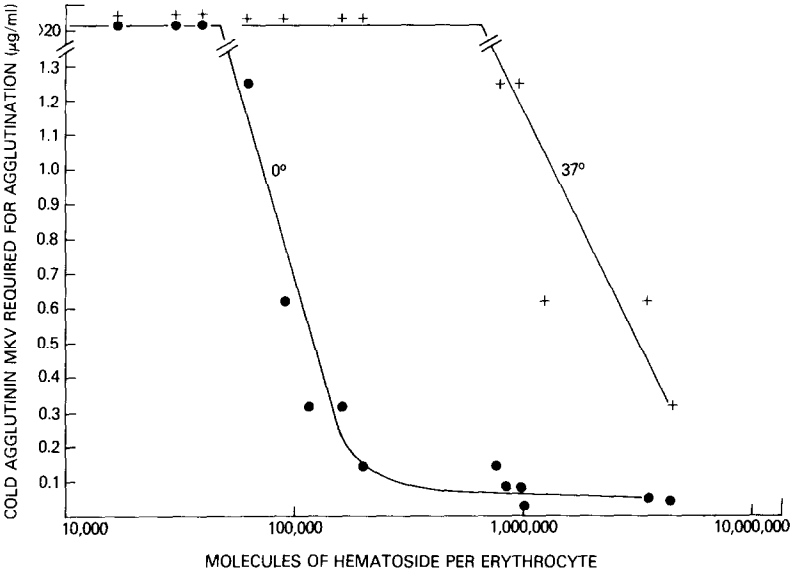

Fig. 1. Concentration of cold agglutinin MKV required to agglutinate neuraminidase-treated human erythrocytes at 0° and 37° after adsorption of varying amounts of ^{3}H-labeled hematoside. The number of hematoside molecules adsorbed per cell and the hemagglutination titer was determined as described in "Materials and Methods".

not agglutinated at any level of agglutinin. This suggests that most molecules of cold agglutinin MKV are bound "unproductively" (i.e., bound to only one cell as opposed to cross-linking two cells) and that treatment with neuraminidase preferentially destroys binding sites favorable for cross-linking, perhaps because they are more accessible to the enzyme. This interpretation is supported by the data in Table I showing that neuraminidase-treated erythrocytes that have adsorbed enough hematoside to render them agglutinable bind little more agglutinin than their parent cells and that the number of molecules of agglutinin bound per cell that results in agglutination decreases as the number of hematoside molecules adsorbed per cell increases. The chances for "productive" binding would increase with increasing density of receptors that are favorable for cross-linking on opposing membranes.

The finding that receptor density can influence the thermal amplitude of cold agglutinins explains the fact that cold agglutinin MKV agglutinates dog

TABLE I

Effect of concentration of cold agglutinin MKV on its binding to erythrocytes before and after treatment with neuraminidase and after treatment with neuraminidase and adsorption of hematoside

Treatment of erythrocytes	Concentration of cold agglutinin MKV (μg/ml)							
	0.02	0.05	0.14	0.37	0.90	2.4	6.3	14.3
	molecules of cold agglutinin MKV bound per cell [a] (stippling indicates dilutions that agglutinate erythrocytes)							
Untreated	40	90	290	700	1,700	4,600	10,500	27,900
Neuraminidase	30	80	180	420	1,100	2,400	6,000	12,400
Neuraminidase with 6×10^4 molecules of adsorbed hematoside per cell	30	80	200	520	1,390	3,100	7,000	16,700
Neuraminidase with 1.85×10^5 molecules of adsorbed hematoside per cell	50	120	240	650	1,800	3,800	7,000	16,900
Neuraminidase with 4.30×10^5 molecules of adsorbed hematoside per cell	50	120	335	790	1,990	4,400	8,800	16,800

[a] The number of molecules bound per cell was determined as described in "Materials and Methods".

erythrocytes at 37° almost as well as at 0° (1). Hematoside is the major ganglioside of dog erythrocytes (6) and from the yield of hematoside obtained from these cells (see "Materials and Methods"), we estimate there are at least 10^7 molecules per erythrocyte.

That receptor density can influence thermal amplitude also supports the view that cold agglutinins exhibit large thermal amplitudes simply because they are low affinity antibodies that require multivalent attachment for the formation of bonds strong enough to cross-link erythrocytes (11, 12). The requirement for multivalency explains their extraordinary thermal amplitude in that relatively small increases in the affinity of individual binding sites at lower temperatures (13) are magnified exponentially because of the cooperativity inherent in multivalent attachment (14). It is unnecessary to postulate a temperature-dependent change in conformation of either the cold agglutinin or its antigen [cf.(15)] in order to explain the thermal amplitude of their interaction.

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