INTERCELLULAR ADHESION OF NEURORETINA CHICK EMBRYO CELLS: ENHANCEMENT BY BOVINE SERUM ALBUMIN AND DERIVATES

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1. Introduction

Dissociated cells from various embryonic tissues are able to form multicellular aggregates spontaneously [1,2]. In particular, neuroretinas from early chick embryos can be dissociated mechanically without enzyme treatment and without chelating agent [3]. A quantitative method for measuring intercellular adhesion of neuroretinal cells has been reported in previous papers [3,4]. With that method, we have shown that isotypic adhesion of neuroretinal cells was enhanced by proteins or sera from various species [5]. Since bovine serum albumin (BSA), which is a well-defined holoprotein, displayed an activity similar to that of the other proteins which have been assayed [5], we decided to study the mechanism of intercellular adhesion enhancement by this protein.

In this paper, we report that monomeric and defatted BSA and more anionic derivatives display an activity similar to that of the native protein.

2. Materials and methods

Bovine serum albumins were purchased from Miles-Yeda Laboratory, Armour, Pentex. Basal medium of Eagle (BME) from Gibco; Rabbit antisera against bovine serum from Eurobio; [^{3}H]leucine from C.E.A.; Hepes from Calbiochem; Sephadex G-25 and Sephadex G-200 from Pharmacia; charcoal from Merck, Coomassie blue from Sigma. Chemicals were the purest available.

Liquid scintillation counter was Intertechnique SL 40; gyratory shaker was New Brunswick G 76. Analytical ultracentrifuge was Spinco Beckman model E, equipped with a Philpot-Svensson optical system; equilibrium sedimentation was performed in a 0.10 M NaCl, 0.005 M phosphate buffer, pH 7.5 at 13 000 rev/min.

Analytical disc polyacrylamide gel electrophoresis was carried out as described by Davis [6]. Immunoelectrophoresis [7] was performed in a 1% gel plate of Agar Noble (Difco) in veronal buffer pH 8.2 (I = 0.05). Gels were stained with Coomassie blue. Free amino groups were determined by the method of Fields [8] with trinitrobenzene-sulfonate.

2.1. *Measurements of intercellular adhesion*

The assay for intercellular adhesion has been described previously [3,5]. In brief, neuroretinas (N.R.) dissected from 9 day-old chick embryos were prepared for the modified collecting aggregate assay in which the number of labelled single cells adhering to the surface of unlabelled N.R. cells aggregates is measured. For labelling, 2 neuroretinas were incubated in 3 ml BME containing 0.2 mCi [^{3}H]leucine (38°C, 18 h) on a gyrotory shaker (70 rev/min). After extensive washings, the neuroretinas were dissociated into a single cell suspension by pipetting in BME. For preparation of aggregates, neuroretinas were first dissociated and equivalents of one neuroretina were dispended into 25 ml Erlenmeyer flasks containing 3 ml BME buffered with 10 mM Hepes, pH 7.2. The flasks were placed on the platform of a gyratory shaker (65 rev/min, 38°C, 18 h). Round aggregates with an average diameter of 0.5 mm were selected. The adhesion assay was initiated by adding 10^5 ^{3}H-labelled N.R. cells to each 25 ml Erlenmeyer flask

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containing 3 aggregates in 3 ml BME (control) or in 3 ml BME supplemented with the indicated amounts of native or modified BSA. The flasks were rotated at 38°C for one hour on a gyratory shaker (70 rev/min). The aggregates were then removed, washed and processed for liquid scintillation counting.

2.2. *Defatted BSA*

BSA was defatted according to Chen [9] and to Goodman [10].

Chen's defatted BSA: BSA (800 mg) was dissolved in distilled water (8 ml). Charcoal (0.4 g) was mixed into the solution, and the pH was lowered to 3 by addition of 0.2 N HCl. The solution was placed in an ice-bath and mixed for one hour. Charcoal was removed by centrifugation at 20 000 *g* for 20 min and the supernatant was brought to pH 7 by addition of 2 N NaOH. Further purification was performed by column chromatography on Sephadex G-25, eluted by distilled water, the solution was freeze-dried.

Goodman's method: The protein (75 mg) was suspended in a 5% glacial acetic acid–heptane mixture dried over Na_2SO_4. After 4 h, the solvent was removed and the extraction was carried out twice more. Then 4 extractions with heptane (dried on Na_2SO_4) were carried out. All these steps were conducted at 4°C. The protein was successively dried under vacuum for 6–10 h to remove the remaining heptane, dissolved in water, dialysed for 12–16 h at room temperature against distilled water, and freeze-dried.

2.3. *Monomeric BSA*

Preparation of monomeric albumin was performed by column chromatography on Sephadex G 200 (3 × 120 cm) in 0.1 M NaCl, 5 mM phosphate buffer, pH 7.5.

2.4. *Acetylated BSA [11]*

BSA (80 mg) was dissolved in 20 ml of 0.15 M NaCl. Acetic anhydride (80 μl) was added in small increments to the solution which was maintained at pH 7.5 with 1 N NaOH, under stirring for 30 min.

Under these conditions, amino groups were converted to acetamides and tyrosyls groups to acyl esters (*O,N*-acetylated BSA). The *O*-acyl tyrosyl residues were readily hydrolysed to regenerate the phenolic groups by 2 M hydroxylamine at pH 7.5 for one hour [12]. (*N*-acetylated BSA).

The *O,N*-acetylated BSA and the *N*-acetylated BSA solutions were dialysed against distilled water and then freeze-dried.

2.5. *Succinylated BSA [13]*

Protein (100 mg) was dissolved in 40 ml of distilled water. Succinic anhydride (110 mg) was added and the solution was maintained under stirring at pH 8 with 1M NaOH, for one hour. The solution was dialysed and freeze-dried.

2.6. *Tryptophan-modified BSA [14]*

An aqueous solution of BSA (50 mg/20 ml) was allowed to react (pH 2.7, room temperature in the dark) with 2-hydroxy 5-nitrobenzyl bromide (4.4 mg) dissolved in dry dioxan (0.5 ml). The pH was maintained at pH 2.7 by addition of 1 N NaOH. The labelled protein was purified from excess reagent by gel filtration on a column (4 × 20 cm) of Sephadex G-25 in 0.18 M acetic acid. Pooled protein fractions were dialysed against distilled water and freeze-dried. By spectral absorption, it was found that 1.4 tryptophan residues per monomer had been substituted.

3. Results

Immunoelectrophoresis of bovine serum albumins from various sources showed that only BSA obtained from Miles-Yeda Laboratory gave one single precipitation band with a rabbit antiserum against bovine serum. Polyacrylamide gels electrophoresis showed 4 bands corresponding to the monomer and oligomers of BSA. The monomer isolated by gel filtration showed only one band in polyacrylamide gel electrophoresis, and had a mol. wt of 62 700 as determined by equilibrium sedimentation experiments. Table 1 shows that the enhancement of cell adhesion by the isolated monomer was similar to that displayed by the mixture of monomer and oligomers. Furthermore table 1 shows that the adhesion promoting effect of BSA defatted by two different methods was unchanged.

By acetylation, 80 p. 100 of the free amino groups were substituted; thus the *O,N*-acetylated BSA carried about 45 less positive charges than the native protein. By succinylation, 56 p. 100 of the free amino groups were substituted, so about 33 positive charges have been substituted by 33 negative charges; there-

Table 1
Enhancement of intercellular adhesion by native, monomeric and defatted bovine serum albumin (BSA) expressed as the ratio of:
number of cells per aggregate in BSA-supplemented BME / number of cells per aggregate in BME

	BME supplemented with BSA			
Experiments	Native	Monomeric	Chen's defatted	Goodman's defatted
1	6.0	6.0	6.5	6.5
2	8.5	–	8	9.5
3	3.0	–	3.5	3.0

Isotypic adhesion of chick embryo neuro-retinal cells was carried out at 38°C for 1 h, as described in Materials and methods, in BME (control) or in BME supplemented with BSA. In each assay, the bovine serum albumin concentration was 1 mg/ml (≃ 15 μM/liter).

fore the total net charges of the protein has been increased by 66 negative charges. Under the conditions used here, the phenolic groups were not succinylated since addition of 2 M hydroxylamine at pH 7.5 did not change the 280 nm absorbance.

Table 2 shows that the increase of negative charges did not alter the capacity of bovine serum albumin to enhance cell adhesion. Moreover, the *N*-acetylated BSA and the *O,N*-acetylated BSA had similar activities showing that the *O*-acetylation of tyrosine residues (60%) does not decrease the adhesion promoting effect of BSA. A similar result was obtained after specific modification of tryptophan (enhancement ratio: 6). So, neither tryptophan, nor the exposed tyrosines were required to enhance cell adhesion.

4. Discussion

Various extracellular macromolecules, not released by the cells on which they are assayed, are able to enhance intercellular adhesion: polylysine which is a strongly basic molecule promoted agglutination of HeLa cells [15]; conversely, acid mucopolysaccharides promote the aggregation of tissue culture cells [16]; also an Ig M-like protein from horse serum increases the disappearance of single neuroretinal cells [17]; and some lectins (Concanavalin A or Wheat Germ Agglutinin) agglutinate enzymatically or chemically dissociated neuroretina cells [18]. Bovine serum albumin appears to be a new type of adhesion promoting macromolecule. It is noteworthy that the monomer and the defatted bovine serum albumin display the same activity as the native serum albumin. The enhancing effect cannot be related to the oligomeric state of the protein or to the presence of some hydrophobic molecules bound to serum albumin.

Elimination of positive charges as in *N*-acetylated BSA, elimination of positive charges and adhesion of negative charges as in *N*-succinylated BSA lead to a strongly acidic macromolecule. These dramatic changes in net charges did not destroy the adhesion enhancing effect. This result could be expected either if the adhesion activity does not involve ionic interactions or if the adhesion activity of the native molecule cancelled by the chemical modifications, is overcome by a new ionic charge [19]. Moreover, these results show that neither exposed amino groups nor exposed phenol or indol groups are required to enhance the intercellular adhesion. In a preliminary experiment, it was found that partial substitution of carboxylic groups (8 blocked carboxyl groups) did not alter the adhesion enhancing effect.

Table 2
Enhancement of intercellular adhesion by chemically modified bovine serum albumin expressed as in table 1

	BME supplemented with BSA			
Experiments	Native	*N,O*-acetylated	*N*-acetylated	*N*-succinylated
1	2.5	3.0	1.5	3.0
2	4.5	5.5	2.0	–
3	3.5	6.5	6.0	–
4	3.0	–	–	6.5

Experimental conditions are identical to table 1. In each assay, the bovine serum albumin concentration was 0.2 mg/ml (≃ 3 μM/liter).

Since the native protein which is only slightly acidic, and since the more acidic or more basic derivatives did not loose their adhesion activity, it seems that the enhancement of cell adhesion by BSA cannot be explained by an unspecific ionic effect but more likely by a triggering of a specific interaction between some portion(s) of BSA and receptor(s) of the cell membrane.

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