AFFINITY CYTOCHEMISTRY: THE LOCALIZATION OF LECTIN AND ANTIBODY RECEPTORS ON ERYTHROCYTES VIA THE AVIDIN–BIOTIN COMPLEX

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1. Introduction

Ferritin-conjugated proteins, such as lectins and antibodies, are widely used for localization of cell surface receptors in the electron microscope [1,2]. Procedures designed to effect direct covalent coupling of ferritin to the binding-protein are, for the most part, cumbersome and inefficient. The resultant complex is of high molecular weight – often a multimer – thus affecting both the physical and chemical binding characteristics and the biological activity of the conjugate.

Biotin, derivatized to bacteriophages via the terminal carboxy moiety, has previously been shown to be available for subsequence interaction with avidin, resulting in phage inactivation [3]. A later investigation demonstrated that ferritin–avidin conjugates can be used for the specific labeling of biotin-tagged proteins and membrane preparations [4]. Recently, we have described an improved technique for the preparation of ferritin–avidin conjugates for use as an ultrastructural marker in the localization of surface receptor sites on biotin-derivatized whole cells [5]. The visualization of tRNA hybridized to DNA has also been reported [6].

In the present communication, we describe the further use of ferritin–avidin conjugates for the visualization of Con A**, PNA and antibody receptor sites on erythrocytes. The study is based on the interaction between ferritin–avidin and biotinylated binding proteins and is comprised of the following steps: (a) Biotin is covalently attached to the desired binding protein. (b) The characterized conjugate is incubated with appropriate cells. (c) Subsequent incubation with FAv enables the visualization of the given cell surface receptor. It is hoped that this approach may unify affinity cytochemical techniques and circumvent some of the problems related to ferritin–protein conjugation.

2. Materials and methods

2.1. *Chemicals and synthesis*

BNHS was prepared from biotin and *N*-hydroxysuccinimide (both purchased from Sigma Chem. Corp.) in the presence of dicyclohexylcarbodiimide (Fluka) as described previously [3,7]. Other reagents were of the highest commercial grade available.

2.2. *Proteins*

The preparation of ferritin–avidin conjugates (FAv) was described previously [5]. Concanavalin A (Con A) was purchased from Miles Yeda Ltd. Peanut agglutinin (PNA) was kindly provided by N. Sharon [8]. Rabbit antimouse RBC antiserum and goat antirabbit RBC antiserum were prepared as previously described [9]. Goat Ig was separated by ammonium sulfate fractionation.

** *Abbreviations:* Con A, concanavalin A; PNA, peanut agglutinin; rAb, goat gamma globulin against rabbit red blood cell membranes; mAs, antiserum against mouse erythrocyte membranes; B-Con A, B-PNA B-rAb, B-mAs – biotinylated-Con A, etc.; FAv, ferritin–avidin conjugates; rRBC, mRBC, hRBC – rabbit, mouse and human erythrocytes, respectively; BNHS, biotinyl-*N*-hydroxysuccinimide ester; PBS, phosphate buffered saline, pH 7.2; VBS, Veronal-acetate buffered saline, pH 7.4.

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2.3. *Preparation of biotin derivatized proteins*

Con A was treated with various molar ratios of BNHS in a solution of 0.1 M sodium bicarbonate containing 1 M sodium chloride (see table 1). The reaction was allowed to proceed for 4 h at room temperature, and the biotinylated samples were dialyzed overnight against PBS with several buffer changes.

The amount of biotin per conjugate was determined by deamination and subsequent amino acid analysis of biotin-protected lysines [10]. Quantification of the extent of exposed biotin moieties/conjugates was accomplished by retention on avidin-Sepharose columns [11]. Affinity chromatography on avidin columns was performed in the presence of 0.2 M glucose. The effect of the attached biotinyl residues on the activity of Con A was analyzed by an agglutination assay of rabbit erythrocytes using a Fragiligraph [12].

2.4. *Procedure for labeling cell surface receptors*

Rabbit, mouse or human erythrocytes were isolated from fresh heparinized blood by sedimentation, rinsed twice in VBS and fixed briefly for 2 min at room temperature with 2% glutaraldehyde in VBS. Aliquots of 10^7 cells were washed three times in VBS and then incubated for 30 min at room temperature with an appropriate solution of B-lectins or B-rAb at a concentration of 1 mg protein/ml, or in 5% B-mAs. Neuraminidase treatment preceding B-PNA incubation was carried out as previously described [8]. Controls were performed in the presence of 0.2 M of the competitive sugar or with underivatized lectins and antibodies. After the previous step, cells were washed three times with VBS and treated with FAv (1 mg/ml in ferritin) for 15 min at room temperature. Next, cells were rinsed twice with VBS and additionally fixed with 2% glutaraldehyde for 30 min. Processing for electron microscopy was carried out as previously reported [5].

3. Results and discussion

3.1. *Characterization of derivatized Con A*

The successful application of this method requires that the biotinylated protein be subject to unhindered interaction both with its receptor as well as the ferritin-avidin conjugate. In other words, the amount of biotin derivatized to the protein should be sufficient to react with avidin and, at the same time, should not be too much so as to disturb the binding of the protein to its receptor. The example of biotin-conjugated Con A will demonstrate this principle.

The derivatization of biotin, by means of its *N*-hydroxysuccinimide ester, to Con A was nearly quantitative as determined by amino acid analysis (table 1). In order to check the avidin-binding capacity of these conjugates, samples were adsorbed to a column of avidin-Sepharose. In a conjugate containing an average of about 5 biotin residues per tetramer, 60% was retained on avidin-Sepharose. An average of 15 biotin molecules/tetramer resulted in 80% binding to such columns. Full binding could not be achieved until attachment of 30 biotins/tetramer. It is apparent that nearly total derivatization is necessary in order to ensure complete interaction between the biotinylated protein and avidin-Sepharose. This phenomenon is surprising, considering the extraordinary affinity between biotin and avidin [13]. Accordingly, a portion of the derivatized biotin residues are apparently unexposed and not available for combination with avidin-Sepharose. It should be noted, however, that this does not exclude the

Table 1
Analysis of biotin-derivatized Con A samples

B-Con A Sample	Amount (mg) Con A	BNHS	Biotin/dimer Molar ratio	Retained on avidin column (%)	Protected lysines (per dimer)
A	100	2.8	4.4	59	2-3
B	100	5.6	8.8	79	7-9
C	100	11.2	17.6	92	n.d.
D	10	5.0	77.8	n.d.	total

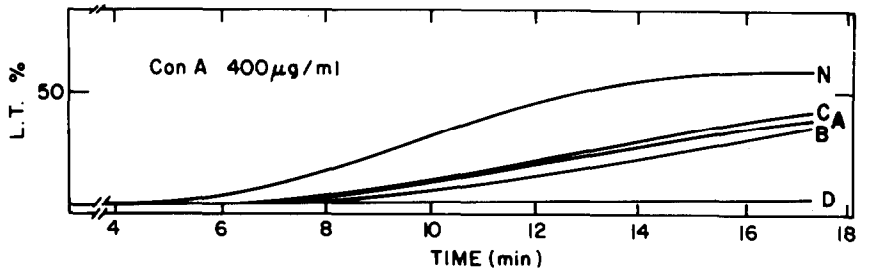

Fig.1. Rate of rRBC agglutination by native (N) and biotinyl-Con A preparations (Samples A-D, table 1). L.T.: Light transmission.

possibility of complete interaction between FAv and B-proteins in solution. Binding of the latter to membrane receptors may cause conformational changes, resulting in the exposure of more biotinyl residues for complex formation with avidin.

In order to determine the effect of biotinylation on biological activity, an agglutination assay using rRBC was performed. As can be seen from fig.1, most of the derivatives were still capable of agglutinating the cells. However, supersaturation of Con A with biotin (sample D) had several undesirable attributes. First, rRBC agglutination was completely inhibited (fig.1), indicating that the biological activity of this sample was significantly altered - but not destroyed, since total retention by affinity chromatography on Sephadex G-75 was observed. The combined lack of rRBC agglutination versus binding to Sephadex, implies that this sample may have been converted to a monovalent form of Con A by heavy biotinylation – similar to that obtained by chemical [14] or by enzymatic means [15,16]. The final inadequacy of this sample was observed under the electron microscope in the form of extensive aggregates.

In view of these results, it is therefore advisable to derivatize proteins under near-saturating conditions, but to avoid excessive treatment with BNHS. A sample, at least 80% of which binds to avidin-Sepharose, is probably appropriate for the studies described in this report. Similar results were also obtained with biotinylated PNA and antibodies.

3.2. *Specific labeling of the cell surface*

rRBC, incubated with B-PNA, B-Con A or B-rAb were specifically labeled with FAv (figs.2 and 3). When

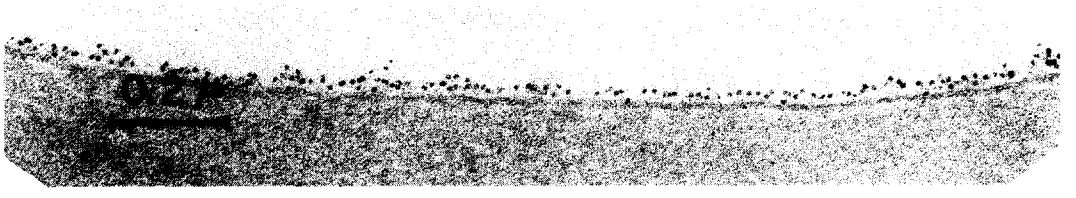

Fig.2. rRBC treated with B-Con A and labeled with FAv.

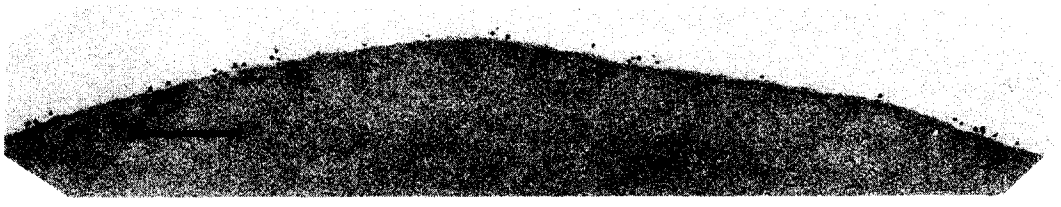

Fig.3. rRBC incubated with B-rAb and labeled with FAv.

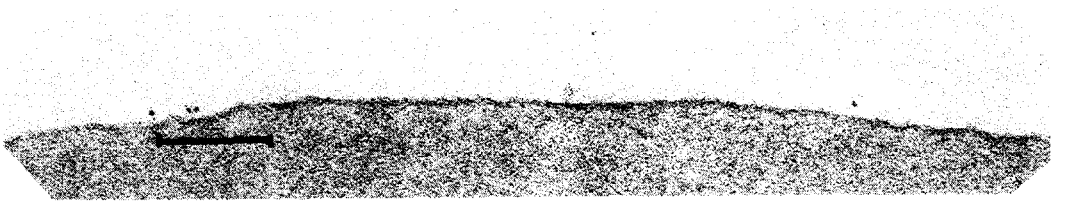

Fig.4. rRBC treated with B-Con A in 0.1 M glucose and labeled with FAv.

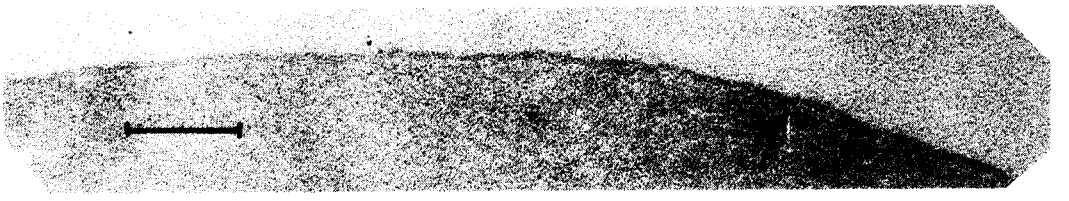

Fig.5. hRBC treated with B-PNA and FAv.

Fig.6. hRBC treated with 50 U/ml neuraminidase, incubated with B-PNA and labeled with FAv.

the cells were incubated with B-Con A or B-PNA in the presence of their competitive sugar, no labeling was observed (fig.4). hRBC also lacked labeling when incubated with B-PNA followed by FAv, since membrane receptors for this lectin are not exposed (fig.5). However, treatment of these cells with neuraminidase removes sialic acid residues and exposes the Gal-β-(1→3)GalNAc sequence [8]. These cells were highly labeled by sequential treatment with B-PNA and FAv (fig.6). Localization of cell receptors is not confined to purified protein preparations, since specific labeling was also obtained by biotin-derivatized unfractionated antiserum. Thus mRBC (but neither rRBC nor hRBC) were labeled with FAv upon prior incubation with B-mAs.

An interesting observation involved the presence of FAv labeling of rRBC after incubation with underivatized Con A. Since avidin is a mannose-bearing glycoprotein, the free binding sites of the membrane-bound, multi-subunit lectin are available for further interaction with FAv. This explanation is supported by the fact that neither underivatized PNA, Ab or As affected FAv labeling of the cell surface. Indeed, such 'nonspecific' Con A-induced labeling could be prevented by prior fixation of Con A treated cells with glutaraldehyde, followed by incubation with FAv in a glucose-containing medium. Under these conditions, the label was more evenly distributed.

The distribution of FAv on cells treated with biotinylated proteins was similar in every case to that of the respective protein–ferritin conjugate. These results indicate that chemical binding of biotin to lectins and antibodies does not interfere with their specificity and binding capacity to cell surface receptors and antigenic determinants.

In summation, the above-outlined procedure is appealing for a variety of reasons: (a) Only one ferritin–protein conjugate (FAv) need be prepared

and characterized for all affinity systems; (b) Biotin can be attached to small ligands and macromolecules efficiently and under very mild conditions; (c) The size, physical characteristics, and biological activity of the biotin-derivatized proteins examined, are only nominally affected; (d) The biotin–avidin complex is of exceptionally high affinity and stability; and (e) Avidin and biotin are both commercially available in large quantities.

Since fixation does not interfere with FAv labeling, cells may be fixed at various time intervals after interaction with biotinylated proteins. Subsequent treatment with FAv or fluorescent-labeled avidin may enable kinetics studies of the interaction between such proteins and cell membranes. This topic is currently under investigation.

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