Enhancement of Immune Cellular Agglutination by Use of An Avidin–Biotin System

Shella M. Costello, Robert T. Felix, and Roger W. Giese

Classically, one can increase the titer of an agglutinating or first antibody with an antoglobulin or second antibody. We have used an avidin–biotin system in place of antoglobulin to similarly extend the agglutination by an initial anticellular antibody. Erythrocytes were agglutinated by adding in succession, caproylamidobiotin–antibody, avidin, and extender (caproylamidobiotin–macromolecule). The macromolecules evaluated as extenders, in order of increasing potency, were fibrinogen, albumin, succinylated polylysine, and ribonuclease A. From comparative testing, we found that antoglobulin raised the titer of antibody from 2560 to 20 480, and the avidin–biotin tool raised the titer of caproylamidobiotin–antibody from 2560 to 10 240 without extender and to 81 820 with an extender of caproylamidobiotin–ribonuclease. Thus noncovalent extenders add to the capability of the avidin–biotin system to facilitate and substitute for an antibody.

Additional Keyphrases: antibodies • immunoassay

Avidin is a protein found in egg white that binds four molecules of the vitamin biotin specifically and very strongly. The dissociation constant per site has been reported by Green to be 10⁻¹² mol/L (1). This binding interaction also is stable at extreme pH values and in the presence of several denaturing agents, proteolytic enzymes, and organic solvents (2). For these reasons, but even more so because at least some of this binding persists when biotin is attached to another molecule, the avidin–biotin system has attracted interest as a general molecular binding tool. Probably the earliest applications were the inhibitions of certain enzymes by avidin, showing that biotin was necessary for their activity (3). Several researchers more recently have used the avidin–biotin system to develop useful binding interactions, mostly in a variety of cell-labeling studies and affinity-isolation techniques, as have been recently reviewed or summarized (4–6).

Among the several studies of the avidin–biotin tool already reported by other groups, in two instances there was evaluation in regard to antibodies. Bayer et al. attached biotin to an anti-erythrocyte antibody, as well as antiserum, and showed that sequential treatment of erythrocytes with either of these reagents, followed by addition of a covalent conjugate of avidin and ferritin, gave specific labeling of the cell surface (7). Similarly, Heggness and Ash used biotin-labeled antimyosin antibody in conjunction with a fluorescein-labeled avidin to stain intracellular myosin (8). They compared the staining characteristics with those of a conventional approach involving a fluorescein-labeled antoglobulin (indirect immunofluorescence) and reported them to be the same. What clearly emerged from both of these qualitative studies is that avidin and biotin can facilitate and partly substitute for some of the routine applications of antibodies.

In this paper, we report a different application of the avidin–biotin tool in conjunction with antibodies, namely, the immune agglutination of erythrocytes. We demonstrate that the use of molecules we call "extenders" can render the avidin–biotin system more potent than a conventional antibody. For comparison and control purposes, we also agglutinate biotin-modified cells with avidin, a capability reported previously (9, 10). Overall, we use this agglutination system as a model to characterize some of the useful features as well as shortcomings of avidin and biotin as a tool.

Materials and Methods

We obtained sheep erythrocytes from Microbiological Associates, Bethesda, MD 20014, and human erythrocytes from an apparently normal volunteer. Before use, these cells were washed three times with phosphate-buffered saline (0.01 mol of sodium phosphate and 0.15 mol of sodium chloride per liter, pH 7.4) and made up to 15 mL of cells per liter (11). Avidin (A5759), d-biotin, fibrinogen (bovine fraction 1, F4000), albumin (bovine, A4378), and 2-(4'-hydroxyazobenzene)benzoic acid were from Sigma Chemical Co., St. Louis, MO 63178; poly-L-lysine HBr (71-120; M, 56 600) was from Miles Laboratories, Elkhart, IN 46514; 2,4,6-trinitrobenzenesulfonic acid was from Eastman, Rochester, NY 14650; ribonuclease A (RAF 1FC) was from Worthington Biochemical Corp., Freehold, NJ 07728. Rabbit anti-sheep erythrocyte antibody (64-406; cited as an IgG fraction purified by ion-exchange chromatography by the supplier) and goat anti-rabbit antibody (63-211; cited as a 2.3 g/L antibody protein by the supplier) were both from Cappel Laboratories, Inc., Thud Ridge Farm, Cochraville, PA 19330. The dimethylformamide (certified ACS grade) was from Fisher Chemical Co., Milwaukee, WI 53233, and was dried over molecular sieves (type 3A beads, Fisher Chemical Co.). All washing steps for cells were carried out with intermittent centrifugation. All protein concentration determinations were based on absorbance values at 280 nm and known absorptivities (12). The proteins were used as received.

Agglutination Procedures

The general sequence (with exceptions as noted) for the agglutination reactions was as follows: (a) Add 0.2 mL of a 15 mL/L suspension of cells or caproylamido-biotin-modified
cells to a series of 12 × 75 mm disposable glass tubes (60825-913; VWR Scientific, Boston, MA 02115). (b) Add the first agglutinating agent (e.g., Ab\(^B\)) at various dilutions to all of the appropriate tubes; mix the contents of all the tubes at once by manually shaking the entire rack several times, and incubate at 37 °C for 30 min. (c) Add and mix the other reagents similarly, but at room temperature and without incubation delays. (d) Centrifuge 12 tubes at a time for 45 s (3400 rpm; 1000 × g) in a Sorotuge (Clay Adams, Fisher Scientific Co., Medford, MA 02155). (e) Resuspend all samples together by manually shaking the entire rack several times; and score visually, based on degree of agglutination, from 1+ (complete agglutination) to 1+ (about 20% agglutination) and w (weak, about 5% agglutination).

We observed agglutination as cell clumping (aggregates involving many cells) in all cases. All titr e end points were required to be 1+ or higher. Because each experiment typically comprised 150–250 tubes, and all additions of a given reagent were made before mixing, mixing was carried out anywhere from 1 to 20 min after reagent addition. All of these steps, except (b), were carried out at room temperature. The blanks routinely included in each procedure were as follows: no Ab, Ab, Ab + Ab', Ab + Av, Ab + Av + ExtB, AbB + Ext (no B), and AbB + Ab'.

Synthesis of Caprylomamidobiotin—N-hydroxysuccinimide Ester

Suspend biotin-N-hydroxysuccinimide [340 mg, 1 mmol, synthesized as described (13)] in 3 mL of dry dimethylformamide. To this add 4 mL of aqueous sodium bicarbonate (0.1 mol/L, pH 8.0) containing ε-aminocaproic acid (131 mg, 1 mmol). Stir the suspension magnetically for 4 h at room temperature. Most of the solvent can be removed on a rotary evaporator at reduced pressure (with use of a water aspirator). Suspend the moist, oily, white residue in approximately 10 mL of aqueous citric acid (100 g/L). Collect the suspension on Whatman no. 1 filter paper and wash with cold water five times. Dry the washed precipitate at reduced pressure at 45 °C over P\(_2\)O\(_5\) for three days. (We made no attempt to clean or characterize this intermediate further; the average yield, assuming pure product, was 87% at this stage.)

Dissolve this intermediate (310 mg, 0.87 mmol) in dry dimethylformamide (20 mL) at 95°C in a round-bottom flask. While magnetically stirring this solution, add all at once 5 mL of dimethylformamide containing carbonyldiimidazole (162 mg, 1 mmol). Maintain the temperature at 95 °C for 30 min, then allow the flask to cool at room temperature. Two hours later, add N-hydroxysuccinimide (100 mg, 0.87 mmol). Seal the flask with Parafilm and stir overnight.

Place the flask on a rotary evaporator, and remove the dimethylformamide at reduced pressure (water aspirator), using a bath temperature of approximately 55 °C. Transfer the resulting pale yellow oil to an Erlenmeyer flask with 15 mL of dry 2-propanol. After several hours the flask will contain an off-white solid precipitate. Aspirate the supernate and dissolve the solid with gentle heating in 25 mL of dry 2-propanol. Reduce the volume to approximately 10 mL by gentle boiling, and allow the flask to cool. Very fine white crystals will develop. After removing the supernate by decantation, dry the solid at reduced pressure over P\(_2\)O\(_5\) at 60 °C overnight. Our yield was 240 mg (61%) and the uncorrected melting point was 149–152 °C (d); the product gave a single spot when checked by thin-layer chromatography.

Avidin Dilutions and Saturation with Biotin

The stock solution of avidin was 5 g/L of phosphate-buffered saline. All dilutions were made with solutions of 20 mg of bovine serum albumin per liter of phosphate-buffered saline. To saturate avidin with biotin, 1.5 mL of a 0.1 g/L solution of avidin was treated with 0.025 mL of a 2 mmol/L solution of biotin (this was approximately a 5.7-molar excess over avidin binding sites).

Preparation of Caprylomamidobiotin-Conjugated Sheep Erythrocytes

Treat 10.0-mL aliquots of washed sheep erythrocytes (15 mL/L) each with either 2, 5, 10, 20, 40, 100, 200, 300, or 400 μL of a fresh solution of caprylomamidobiotin—N-hydroxysuccinimide ester (40 mmol/L) in N,N-dimethylformamide. Use 100 μL of dimethylformamide to make a control. After gentle mixing, incubate these reactions at 37 °C for 30 min. Similarly treat equivalent aliquots with biotin—N-hydroxysuccinimide ester. Then wash the cells three times with phosphate-buffered saline and make up to 2.5 mL with the phosphate-buffered saline solution of bovine serum albumin.

Preparation of Caprylomamidobiotin-Conjugated Macromolecules

Dissolve rabbit anti-sheep erythrocyte antibody, fibrinogen, bovine serum albumin, or RNase (14, 5, 6.9, and 10 g/L, respectively) in phosphate-buffered saline and treat 1-mL aliquots at room temperature with a fresh solution of caprylomamidobiotin—N-hydroxysuccinimide ester (approximately a 100-fold molar excess in each case) in dimethylformamide. For rabbit raised-antibody, dissolve 4.58 mg of the ester in 0.02 mL of dimethylformamide; for the fibrinogen sample, use 0.654 mg of ester in 0.10 mL of dimethylformamide; for bovine albumin, use 4.45 mg in 0.4 mL; and for RNase, 33 mg in 0.4 mL. In each case, use a control that involves treatment with an equivalent volume of dimethylformamide without the ester.

After immediate manual shaking, rotate each solution for 1 h at room temperature. Dialyze against three changes of phosphate-buffered saline (1 L each) at 4 °C for a total of 48 h. Measure the percentage of amino groups modified with caprylomamidobiotin with 2,4,6-trinitrobenzenesulfonic acid (14), and assay the avidin binding with 2-(4′-hydroxyazo-benzene)benzoic acid (15). Similarly, modify each protein with a 10-fold lower amount of caprylomamidobiotin—N-hydroxysuccinimide ester in the same volume of dimethylformamide, and modify the antibody and fibrinogen with the corresponding molar excesses of biotin—N-hydroxysuccinimide ester.

Treat with 8.0 mg of biotin—N-hydroxysuccinimide dissolved in 0.1 mL of dimethylformamide 1 mL of a 10 g/L solution of polysine in phosphate-buffered saline that has been dialyzed against phosphate-buffered saline. Treat a control with 0.1 mL of dimethylformamide. After rotation for 1 h at room temperature, dialyze the solutions against three changes of phosphate-buffered saline (1 L each) for 24 h at 4 °C, and then dilute to 5 g/L. Add sufficient sodium succinate to the solutions to make them 0.1 mol/L. Add succinic anhydride (7 mg; 100-fold molar excess over amino groups) in three aliquots over a period of 1.5 h. Keep the pH at approximately 7.5 by the addition of NaOH (5 mol/L). Then dialyze the solutions against phosphate-buffered saline for 36 h.

**Results**

Because avidin will combine with four unconjugated or

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2 Nonstandard abbreviations used: B, caprylomamidobiotin; Av, avidin; Ab, rabbit anti-sheep erythrocyte antibody; Ab'; goat anti-rabbit antibody; AdsAb, Ab adsorbed with sheep erythrocytes; ExtB or "extender," macromolecule conjugated with caprylomamidobiotin; Fib, fibrinogen; Alb, albumin; RNase, ribonuclease A; and SuPolys, succinylated polylysine. The extenders (each covalently modified with caprylomamidobiotin) are FibB, AlbB, RNaseB, and SuPolysB.
appropriately conjugated molecules of biotin, the reaction of avidin with biotin-conjugated antibody should be analogous to the reaction of antitibolin with its corresponding unmodified antibody. For the agglutination model we have selected, avidin should extend the agglutination titer of a biotin-modified anticalcellar antibody similarly to the extension of titer with an antitigulin.

The agglutination schemes we tested and compared are shown in Figure 1. The biotinyl residues involved were nearly always caproylamidobiotin groups covalently introduced onto the erythrocytes and macromolecules with the reagent shown in Figure 2. In the “direct” schemes the caproylamidobiotin residues were attached directly to the surface of the cells, and the cells were then washed with buffer. The “indirect” approach was characterized by initial reaction of the cells with caproylamidobiotin-modified antibody. All subsequent additions in both cases then were performed without intermediate washing steps.

The absence of further washing potentially created a more complex system when successive components were added, because reactions could take place both on the cell surface and in the bulk solution. However, this approach was analogous to the usual utilization of antitibolin, was convenient, and minimized damage to the cells from repeated washing steps.

**Fig. 1. Agglutination schemes**
The large circles represent cells; B, caproylamidobiotin; Av, avidin; Ab, antibody; EXT, extender macromolecule.

**Fig. 2. Structure of caproylamidobiotin-N-hydroxysuccinimide ester**

Further, we monitored the process with controls in each experiment, and when results were obtained where bulk reactions might have been important, analogous experiments were carried out with washing after every addition step.

The schemes in Figure 1 merely indicate in simplest terms the intended binding interactions. In this paper we show that the final structural details of the intercellular binding that actually developed in the agglutination mixtures depend critically on avidin–biotin interaction, but do not characterize it further.

One of the potential advantages of the avidin–biotin system is the opportunity to involve a variety of molecules in a binding process merely by attaching biotin to these molecules. In this application, we choose to call such molecules “extenders” and utilize the agglutination model to assess several structures of extenders. The alternate approach of involving ancillary molecules by covalently attaching them to avidin has been used by others but was not investigated here.

The caproylamidobiotin-conjugated molecules studied in this noncovalent context (extenders and antibody), and the extent and avidin-reactivity of the attached caproylamidobiotin are summarized in Table 1. Fibrinogen was chosen for its large size. Albumin and ribonuclease were chosen mostly for their incrementally smaller sizes. Polylsine provided a molecular weight comparable to that of albumin, but presumably was more flexible.

Except in the case of antibody, caproylamidobiotin was incorporated by reaction of each of these macromolecules with approximately a 100-fold molar excess of the N-hydroxysuccinimide ester of caproylamidobiotin (BNHS), which gave better results than a 10-fold excess. For antibody, some loss in agglutination titer was observed with this reagent excess, but a 10-fold lower amount of BNHS gave no change in agglutination titer and, therefore, we used the lesser amount. No trinitrobenzenesulfonic acid data are cited for fibrinogen.

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**Table 1. Characterization of Caproylamidobiotin-Modified Macromolecules**

<table>
<thead>
<tr>
<th>Macromolecule and no. of NH₂ groups</th>
<th>Extent of reaction with NH₂ (TNBS), %</th>
<th>Total B/mol (calc. from TNBS)</th>
<th>Avidin-reactive B/mol (calc. from HABA)</th>
<th>Avidin-reactive B, % of total B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen (Mₐ 340 000; 220 NH₂)</td>
<td>62.6</td>
<td>36.9</td>
<td>7.4</td>
<td>20.1</td>
</tr>
<tr>
<td>Albumin (Mₐ 68 000; 59 NH₂)</td>
<td>65.9</td>
<td>7.2</td>
<td>1.6</td>
<td>22.2</td>
</tr>
<tr>
<td>Ribonuclease (Mₐ 13 700; 11 NH₂)</td>
<td>15b</td>
<td>1.2d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antibody (Mₐ 160 000)</td>
<td>11.3c</td>
<td>49.5</td>
<td>7.8</td>
<td>15.8</td>
</tr>
<tr>
<td>Polylsine (Mₐ 58 600; 443 NH₂)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B, caproylamidobiotin; TNBS, 2,4,6-trinitrobenzenesulfonic acid; HABA, 2,4(4')-hydroxyazobenzenebenzoic acid.

*Approximately a 100-fold molar excess of B-N-hydroxysuccinimide was reacted with each molecule except in the case of antibody, where a 10-fold molar excess was used.

*This value was for modification with biotin–N-hydroxysuccinimide rather than caproylamidobiotin–N-hydroxysuccinimide.

*This value was based on a comparison of the starting material and product with a ninhydrin (39) rather than TNBS reaction because we encountered solubility problems with the latter.

*This value was for modification with BN-hydroxy succinimide, in contrast to b. 

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because of the irreproducibility of the numbers and the unacceptably high content of apparent caproylamidobiotins (more than the number added). Although we noted no change in solubility, perhaps the fibrinogen molecules nevertheless were aggregating after modification with B-N-hydroxysuccinimide, preventing some of the amino groups from reacting with the acid. For the other macromolecules, the values were reproducible.

To evaluate the extent to which the caproylamidobiotins in these macromolecules could bind to avidin, we assayed with 2-(4'-hydroxyazobenzene)benzoic acid. This yellow dye forms an orange complex with avidin, which is disrupted by the addition of free or conjugated biotin. As seen in Table 1, the percentage of avidin-reactive caproylamidobiotins was comparable (15.8 to 22.2%) in the three cases fully evaluated. Presumably, steric factors play a role in limiting the extent of reaction of the conjugated caproylamidobiotin with avidin; e.g., the reaction of some of the conjugated caproylamidobiotins with avidin may block other molecules of caproylamidobiotin from reacting.

The tendency of avidin to adsorb to sheep erythrocytes and test tubes is summarized in Table 2. The buffer was phosphate-buffered saline–bovine serum albumin in all cases (and throughout the project) except that adsorption to sheep erythrocytes was also evaluated in the absence of bovine serum albumin. The nonspecific adsorption of avidin to human erythrocytes was only 9.4%, even when the amount of cells used (200 mL/L) was 13-fold greater, and the concentration of avidin (2 g/L) was 20- to 4000-fold greater, than the corresponding amounts used in the agglutination assays. This is in spite of the net negative charge of the erythrocyte surface, due mostly to sialic acid, and the highly basic nature of avidin (pI = 10.5). Nevertheless, the charge on these sialyl residues is contributed by carboxyl groups, which have only a weak ion-exchange capacity, and certainly some competition is provided by the saline content of the buffer.

Glass commonly adsorbs basic solutes strongly, and avidin is no exception. The limited ability of silanization to overcome adsorption (Table 2) might arise from the limited coverage of surface silanols that silanization achieves (16). Polyethylene shows a comparable ability to adsorb avidin. Because the final amounts of avidin in our agglutination mixtures were in the low-microgram range, where adsorption losses were as high as 10%, the cited concentrations of avidin in these solutions represent upper limits only.

The results from the direct and indirect agglutination schemes are summarized in Table 3. These data, for the most part, are the consequence of extensive optimization studies in which the concentrations of the agglutinating components were independently varied to give a maximum titer. The exceptions to this are the standard amount of cells, and the somewhat arbitrary dose of B-N-hydroxysuccinimide in the direct scheme at the bottom of Table 3.

No difference in agglutination titer was seen, whether the antibody, extender, or cells were modified with the N-hydroxysuccinimide ester of caproylamidobiotin or of biotin. For the sake of consistency, however, we used caproylamidobiotin for nearly all of the results reported in this paper. The polyanine–biotin conjugate was found to give nonspecific agglutination; to prevent this, the conjugate was further reacted with succinic anhydride to yield “SuPolysB.” The agglutination end points were scored visually, the reliability of which was confirmed in some of the early experiments by correlation with microscopic evaluation. The data were collected over a period of several months, involving new lots and new preparations of all biological reagents (except Ab and AbB), and the results were remarkably reproducible. The ability of agglutination systems to perform in this manner has been noted by others (17).

### Table 2. Adsorption of Avidin to Sheep Erythrocytes and Test Tubes

<table>
<thead>
<tr>
<th>Adsorbent</th>
<th>Avidin concn, g/L</th>
<th>Avidin loss, %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sheep erythrocytes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphate-buffered saline</td>
<td>5.0</td>
<td>6.3</td>
</tr>
<tr>
<td>Phosphate-buffered saline</td>
<td>2.0</td>
<td>9.3</td>
</tr>
<tr>
<td>Phosphate-buffered saline–bovine</td>
<td>2.0</td>
<td>9.4</td>
</tr>
<tr>
<td>Test tubes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glass (before/after silanization)</td>
<td>2.0</td>
<td>1.1/0.4</td>
</tr>
<tr>
<td>Polyethylene</td>
<td>0.02</td>
<td>12.1/10.2</td>
</tr>
</tbody>
</table>

- Based on $A_{280}$ values of the avidin solution before and after exposure to the adsorbent for 30 min at 37 °C.
- Packed cells (0.2 mL) plus 1.0 mL of avidin solution. A control involving sheep erythrocytes in phosphate-buffered saline allowed correction for any increases in $A_{280}$ from the cells (especially from hemolysis).
- The percentage loss of avidin in this case was determined by subjecting the avidin solution, before and after exposure to the sheep erythrocytes, to chromatography on a Lichrosorb DIOL column, 4.6 mm i.d. X 25 cm (Rainin Instruments, Brighton, MA 02135), with a mobile phase of 0.1 mol/L acetic acid/0.1 mol/L sodium sulfate (adjusted to pH 5.0 with sodium hydroxide) at 0.5 mL/min. The retention times of bovine serum albumin, avidin, and hemoglobin were 4.8, 4.9, and 5.6 min, respectively. This methodology will be described further elsewhere (17).

In all cases in which avidin was present, some thin smearing of the cells was observed on the sides of the glass tubes, whether agglutination was occurring or not. This smearing did not constitute agglutination when it was dialomed from the surface. An analogous observation is the report by Taylor that saline accelerates cell adhesion to glass (18).

The amplification factor in Table 3 is derived from the cited dilutions of primary reagent with and without helper. Thus, the amplification is considered to be the ability of the helper (as defined in Table 3) to extend the titer of the primary reagent. An extension by 8-fold is typical for the indirect agglutination of unfixed antibody-treated erythrocytes with an antiglobulin. As shown, the agglutination titer of the primary antibody was unchanged (2560) after conjugation with caproylamidobiotin at an effective concentration. Avidin alone extends the titer of AbB from 2560 to 10 240, which is less amplification than the titer of 20 480 with antiglobulin. However, the further addition of an extender provides higher titers up to a value of 81 820 when this reagent is RNaseB. Agglutination and qualitatively similar extension with ExtB was also possible in a direct scheme (Table 3). Incidentally, antiglobulin extended the titer of AbB and Ab to the same extent.

Table 4 shows the dependence of agglutination on the concentration of avidin. For both the direct and indirect schemes, the lowest effective avidin concentration was in the range of 0.5 to 5 mg/L. In most cases, the ability to inhibit the agglutination reaction with an excess of avidin also was established. This result certainly is consistent with a specific agglutination mechanism. The range of avidin concentrations that gave agglutination was narrower at the lower concentration of B-N-hydroxysuccinimide or AbB.

The general behavior with FibB contrasts with that of the other extenders in the direct scheme. (This aspect was not evaluated significantly in the indirect approach.) Whether this is because of the much larger size of fibrinogen compared with...
Table 3. Recovery of Agglutination with a Helper

<table>
<thead>
<tr>
<th>Sequence a</th>
<th>Primary reagent</th>
<th>Helper</th>
<th>Lowest dilution of primary reagent giving agglutination b</th>
<th>Amplification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells + Ab ± Ab'</td>
<td>Ab</td>
<td>Ab'</td>
<td>2560</td>
<td>20 480 c</td>
</tr>
<tr>
<td>Cells + AbB ± Av</td>
<td>AbB</td>
<td>Av</td>
<td>2560</td>
<td>10 240 d</td>
</tr>
<tr>
<td>Cells + AbB ± (Av + ExtB)</td>
<td>AbB</td>
<td>(Av + FIBb)</td>
<td>2560</td>
<td>20 480 e</td>
</tr>
<tr>
<td>Cells + BNHS ± Av ± ExtB</td>
<td>BNHS</td>
<td>SuPolysB</td>
<td>4</td>
<td>16 f</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RNaseB</td>
<td>4</td>
<td>16 f</td>
</tr>
</tbody>
</table>

BNHS, caproylamidobiotin-N-hydroxysuccinimide.

The total incubation volume ranged from 0.32 to 0.52 mL from sheep erythrocytes (0.2 mL), Ab (0.2), Ab' (0.1) or Av (0.1), and extender (0.02).

b The reciprocal values are cited for the dilutions; twofold dilutions were made from the lowest to highest reciprocal values. In each case, except as noted in footnotes, the concentrations of the components other than primary reagent and cells were optimized for maximum agglutination titers.

c This agglutination titer was achieved by adding 0.2 mL of a 1/20 480 dilution of stock Ab to 0.2 mL of sheep erythrocytes (15 mL/L), incubating at 37 °C for 30 min, adding 0.1 mL of a 1/100 dilution in phosphate-buffered saline-bovine serum albumin of the stock solution of Ab' (stated by the supplier to be 2.3 g of antibody protein per liter), mixing, centrifuging, resuspending, and scoring. The same protocol, but with omission of helper (Ab' in this case) gave a titer of 2560, as cited, as is the case for all the subsequent titer results without helper.

d This agglutination titer was achieved by adding 0.2 mL of a 1/10 240 dilution of stock AbB (same concentration with respect to Ab as stock Ab-P in ) to 0.2 mL of sheep erythrocytes (15 mL/L), incubating at 37 °C for 30 min, adding 0.1 mL of avidin (50 mg/mL in phosphate-buffered saline-bovine serum albumin), mixing, centrifuging, resuspending, and scoring.

e These agglutination titers were achieved by proceeding similarly as in d except Ab was either 1/20 480, 1/40 960, or 1/81 820 as cited, and, immediately after the avidin addition, 0.02 mL of ExtB (30, 25, 30, or 10 mL/L, concentrations of FIBBHL, Ab, SuPolysB, or RNaseB, respectively) was added, followed by mixing, centrifuging, resuspending and scoring. A week agglutination at 1/81 820 was obtained with SuPolysB; thus, its titer is probably close to 60 000.

f This agglutination "titer" was obtained by adding 2 mL of a freshly prepared 18 g/L solution of BN-hydroxysuccinimide in dimethyldiformamide to 10 mL of a 15 mL/L suspension of sheep erythrocytes, incubating for 30 min at 37 °C, washing the cells three times with phosphate-buffered saline-bovine serum albumin, diluting to 10 mL, dispensing 0.2 mL into a tube, adding 0.1 mL of avidin solution (10 mg/mL in phosphate-buffered saline-bovine serum albumin), mixing, adding 0.02 mL of ExtB (10 mg of RNaseB or 30 mg of SuPolysB per liter, in phosphate-buffered saline-bovine serum albumin), mixing, centrifuging, resuspending, and scoring.

Table 4. Dependence of Agglutination on the Concentration of Avidin

<table>
<thead>
<tr>
<th>Sequence a</th>
<th>Amounts of avidin tested (and amounts of avidin giving agglutination) b, mg/L</th>
<th>At an intermediate</th>
<th>At an end point</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct</td>
<td></td>
<td>[BNHS] or [Ab]</td>
<td>[BNHS] or [AbB]</td>
</tr>
<tr>
<td>sheep erythrocytes + Av</td>
<td>500, 100, (50, 10, 5, 1), 0.5 c</td>
<td>500, 100, 50, 10, 5, (1, 0.5), 0.1 d</td>
<td></td>
</tr>
<tr>
<td>sheep erythrocytes + Av + FIBb</td>
<td>500, 100, 50 (10, 5), 1, 0.5 c</td>
<td>500, 100, 50, 10, (5), 1, 0.5 e</td>
<td></td>
</tr>
<tr>
<td>+ AlB</td>
<td>500, (100, 50, 10, 5), 1, 0.5</td>
<td>500, 100, 50, 10, (5), 1, 0.5</td>
<td></td>
</tr>
<tr>
<td>+ SuPolysB</td>
<td>500, (100, 50, 10, 5), 1, 0.5</td>
<td>500, 100, 50, 10, (5), 1, 0.5</td>
<td></td>
</tr>
<tr>
<td>+ RNaseB</td>
<td>500, (100, 50, 10, 5), 1, 0.5</td>
<td>500, 100, 50, 10, (5), 1, 0.5</td>
<td></td>
</tr>
<tr>
<td>Indirect</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sheep erythrocytes + Ab + Av</td>
<td>200, 100, 50, 5, 1, 0.1 f</td>
<td>50, 10, (5), 1, 0.5, 0.1 g</td>
<td></td>
</tr>
<tr>
<td>sheep erythrocytes + Ab + Av + FIBb</td>
<td>(50, 10, 5, 1)</td>
<td>100, 50, 10, (5), 1</td>
<td></td>
</tr>
<tr>
<td>+ AlB</td>
<td>(10, 5, 1)</td>
<td>10, (5), 1</td>
<td></td>
</tr>
<tr>
<td>+ SuPolysB</td>
<td>(10, 5, 1)</td>
<td>10, (5), 1</td>
<td></td>
</tr>
<tr>
<td>+ RNaseB</td>
<td>(10, 5, 1)</td>
<td>10, (5), 1</td>
<td></td>
</tr>
</tbody>
</table>

BNHS, caproylamidobiotin-N-hydroxysuccinimide.

The volumes are as defined in Table 3.

The cited values are the amounts tested. The values in parentheses gave agglutination. The amount of ExtB throughout was 0.6 μg/tube for FIBBHL, 0.5 for AlB, and 0.2 for RNaseB. These values were especially optimum for the indirect agglutination at an end-point amount of AlB, and roughly optimum for the direct cases.

For these series, sheep erythrocytesB were aliquoted from a suspension of 10 mL of sheep erythrocytes (15 mL/L) treated with 40 μL of a fresh 0.04 μmol/L solution of BNHS in dimethyldiformamide.

c In d except 10 μL of BN-hydroxysuccinimide solution was used. Essentially the same results were also obtained at 0 °C.

d As in c except 2 μL of BN-hydroxysuccinimide solution was used. Essentially the same results were also obtained at 0 °C.

e Ab dilution was 1/5120, which was one dilution past the titer of 1/2560 where Ab alone agglutinated the cells.

f The end point [AbB] in each case is the titer cited in Table 3.

The other extenders, some size heterogeneity due to an inherent tendency to form polymers, or some other aspect of this preparation is not defined. The contrasting behavior for FIBb is documented further in Figure 3. Here, the concentration range of extender that provides extension of agglutination is broader for FIBb than RNaseB, whereas the latter provides a greater degree of amplification at its optimum concentration. Indeed, the extended end point with RNaseB is very sharp. If the concentration of RNaseB was increased or decreased twofold away from its critical concentration, no agglutination...
Fig. 3. Extension of indirect agglutination as a function of ExtB concentration for ExtB = Fib (O - - - O) or RNaseB (V - - - V) was seen for antibody dilutions of either 40 960 or 81 820. The concentrations for avidin that give agglutination for an end point amount of BN-hydroxy succinimide or AbB, shown in the right column in Table 4, are similarly sharp.

A final point in regard to Table 4 is the behavior in agglutination at an intermediate amount of BN-hydroxy succinimide for the avidin concentrations of 50 and 100 mg/L. Aside from the contrary behavior of the FibB, the extenders all provide agglutination at a dose of avidin that is inhibiting in their absence. Here, it is likely that the caproylamidobiotin on the cells is fully reacted with avidin, and the extender then bridges the cells together. Such a mechanism would be expected to persist at higher avidin concentrations, once excess avidin is washed away, because such washing would prevent any consumption of added extender by residual avidin in the bulk-solution phase. This was found to be the case. When bovine conjugated to sheep erythrocytes was reagent with 500 mg of avidin per liter, washed, and treated with extender, we observed 4+ agglutination.

Several types of control experiments were carried out to assure both the specificity of the agglutination enhancement by avidin and biotin and the validity of the general characteristics of this system developed with this model. These experiments and their results are summarized in Table 5. The dependency on avidin–biotin binding was shown in controls A, C, D, and F, where the absence of conjugated caproylamidobiotin, or blocking of the avidin–caproylamidobiotin linkage by saturation of the avidin with biotin either before or after addition of avidin to the system, reduced agglutination to the background level for the types of sequences being evaluated. Controls B and E further demonstrated the absence of non-specificity in the AbB reagent. In B, human cells were used in place of sheep cells; adsorbed AbB was tested in E. Taken together with the results from Table 4, which show inhibition of agglutination at higher avidin concentrations, and the several blanks routinely included in all of the assays, these results constitute strong evidence for the critical dependence of agglutination enhancement on the intercellular development of bonds among avidin–biotin–conjugated molecules.

Discussion

It is interesting to consider why the term "tool" has arisen for the avidin–biotin system. This term also has been adopted for lectins, another class of binding proteins (19). Perhaps the intention is twofold: first, these types of systems can be used to probe and explore chemical and biological systems; secondly (with some overlap), they will be useful for the physical manipulation of other molecules and of microscopic materials such as organelles and cells. The work reported in this paper is intended to be relevant to both viewpoints. A discussion of some characteristics of the avidin–biotin system should help its development into a well-defined probe and demonstrate its ability to manipulate cells in conjunction with antibodies.

We consider that the avidin–biotin system is advantageous as a tool not so much because of any special capability, but because of a variety of features whose relative importance will vary with the application. Sometimes, in fact, some of these properties are likely to be undesirable, and an alternative molecular-binding system will be more appropriate. Currently established properties of avidin and biotin that are relevant

<table>
<thead>
<tr>
<th>Incubation sequence</th>
<th>Avidin, µg/tube</th>
<th>Extender, µg/tube</th>
<th>Agglutination or agglutination liter of Ab or AbB</th>
</tr>
</thead>
<tbody>
<tr>
<td>A, biotin-saturated avidin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B – sheep erythrocytes + AvB</td>
<td>10</td>
<td>—</td>
<td>no</td>
</tr>
<tr>
<td>B – sheep erythrocytes + AvB + FibB</td>
<td>10</td>
<td>0.6</td>
<td>no</td>
</tr>
<tr>
<td>sheep erythrocytes + AbB + AvB</td>
<td>(10)</td>
<td>—</td>
<td>1280</td>
</tr>
<tr>
<td>sheep erythrocytes + AbB + AvB + FibB</td>
<td>(10)</td>
<td>0.6</td>
<td>1280</td>
</tr>
<tr>
<td>B, human cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human erythrocytes + AbB + Av + FibB</td>
<td>5, 1</td>
<td>0.6</td>
<td>none</td>
</tr>
<tr>
<td>C, unmodified Ab</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sheep erythrocytes + Ab + Av + FibB + AlbB</td>
<td>50, 10, 5, (1)</td>
<td>0.6</td>
<td>2560</td>
</tr>
<tr>
<td>D, unmodified extender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sheep erythrocytes + AbB + Av + Fib + Alb</td>
<td>50, 10, 5, (1)</td>
<td>0.6</td>
<td>10 240</td>
</tr>
<tr>
<td>E, adsorbed Ab</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sheep erythrocytes + AdsAbB + Av + FibB</td>
<td>50, 10, 5, (1)</td>
<td>0.6</td>
<td>1280</td>
</tr>
<tr>
<td>sheep erythrocytes + AdsAb + Av + FibB</td>
<td>(5)</td>
<td>0.6</td>
<td>40</td>
</tr>
<tr>
<td>F, biotin after avidin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sheep erythrocytes + AbB + Av + B + extB</td>
<td>(5)</td>
<td>0.5</td>
<td>640 or</td>
</tr>
</tbody>
</table>

* Only values in parentheses gave agglutination.

b ExtB was FibB, AlbB, SuPolysB, or RNaseB.
to their use as a tool include: high affinity and multivalent binding for free and conjugated biotin, excellent stability (except for the rapid inactivation of free avidin by oxidizing agents), relatively small size, homogeneity, ready availability, the polar nature of biotin, and ease of attachment of biotin to other molecules (2).

Aside from lectins, probably the two other binding systems that are currently most analogous to the avidin–biotin system are antibodies and protein A (S. aureus). Aside from their extensive utilization in the binding of antigens and free hapten, antibodies have also been used in conjunction with bound hapten in a manner that is especially analogous to the use of the avidin–biotin system, as has been reviewed (20). A recent study particularly analogous to the work reported here involves the agglutination by concanavalin A of bovine erythrocytes synthetically modified with α-D-mannosyl residues (21).

Let us first consider the high affinity of avidin for biotin. The other binding systems cited all have lower affinities for their ligands, and the order of decreasing affinities is approximately avidin > antibodies > protein A > lectins. Avidin binds biotin with an affinity of $10^{15}$ L/mol (1), antibodies show a $K_a$ typically ranging from $10^9$ to $10^{11}$ L/mol for their hapten (22), protein A binds $I_c$ with an affinity of about $10^6$ L/mol (23), and lectins bind simple sugars with a $K_a$ of approximately $10^{2}$–$10^{4}$ L/mol ($10^6$–$10^7$ L/mol for membrane-binding sites) (24). However, these differences probably tend to become blurred when multivalent interactions become available, which greatly increases apparent affinity constants (25); i.e., the binding becomes avidity rather than affinity (22). Another consideration that might reduce the effect of differences in these affinity constants in practice is the occurrence of a weaker link or a more critical parameter elsewhere in a given system. Finally, for the particular case of avidin, it is not fully clear how much of the affinity for biotin is retained when biotin is conjugated to a macromolecule. The general impression that the affinity is just as strong could relate in some cases to avidity effects. At least, it is reported that the carboxyl group of biotin can be modified with minimum (26) or apparently minimum (27) change in binding to avidin, and avidin-affinity columns can bind biotin enzymes very strongly (28). However, a dissociation rate constant approximately $10^4$-fold higher than the corresponding rate constant for biotin was reported for an analogous spin-label derivative of biotin (29). Although a complex of avidin with biotinyl-hexaglycyl-1-(1-naphthoxy)-3-N-ethylamino)-propan-2-ol was found to undergo no measurable dissociation at 4 °C during a 2-h incubation, at 25 °C and 37 °C the half-life was 15.5 and 2 h, respectively (30). A control experiment with a complex of biotin with avidin showed no measurable dissociation after 3 h at 37 °C. Thus, additional work may be needed on this general aspect of the affinity of avidin for biotin conjugates.

In this context, it is interesting that the lowest concentration of concanavalin A that agglutinates mixed erythrocytes is typically 1–10 mg/L (21, 31), which is in the same range as the lowest agglutinating dose of avidin (direct or indirect, with or without extender) in this study. It might be useful to know the number of receptors involved in each case (this number is not defined in our work) before drawing conclusions. However, the total number of concanavalin-A–binding sites in the work cited earlier (involving erythrocytes synthetically modified with mannosyl residues) was reported not to correlate with the extent of concanavalin-A-induced agglutination (21).

Thus it may be necessary to invoke one or more of the factors cited above for blurring affinity differences to account for this comparable potency in cellular agglutination of a "very weak" and "very strong" binding agent.

Alternatively, it is conceivable that any lower affinity of the specific binding in the lectin–sugar system relative to the avidin–biotin system actually could be favorable for cellular agglutination. This might arise from the greater reversibility of the weaker lectin bonds. For example, concanavalin A might be comparable to avidin in achieving agglutination because this lectin is more able to switch from intracellular to intercellular binding when the cell surfaces are in contact.

A second feature of our agglutination work, which can be compared with lectin agglutination and which may similarly relate to the affinity differences between avidin and lectins, is the agglutination behavior at a high concentration of binding protein. The agglutination of mannos-modified erythrocytes cited here earlier (21) persisted even at concanavalin A concentrations near 500 mg/L (32). The agglutination of native erythrocytes with lectins persists as well at very high concentrations of lectin (33). In contrast, we observed sharp and total inhibition of agglutination with avidin at concentrations as low as 5 to 100 mg/L, depending upon the nature and amount of the other reagents (see Table 4). Perhaps a weaker lectin-receptor binding leads to significantly less saturation of receptors and (or) renders the saturation of the receptors more reversible than with avidin, so that a greater shift to intercellular binding occurs with the lectin when the cells are brought together. An alternative speculation is that "nonspecific" adhesion develops at the greater concentrations of lectin.

Avidin was only half as able as antiligubulin to extend the titer of its primary reagent, as defined and summarized in Table 3. Both the larger size and the hinge flexibility for the antiligubulin may have contributed to this difference. Presumably, antibodies inherently possess hinge flexibility to facilitate their ability to cross-link antigens. The lengthened version of biotin (caproylamidobiotin) was synthesized and tested with this partly in mind. However, we observed the equivalent titers in our agglutination system for caproylamidobiotin and biotin.

How do we rationalize the amplification provided by the extenders, and the potency order for the extenders of FibB < AlbB < SuPolysB < RNaseB? (As noted in footnote e of Table 3, the behavior of SuPolysB was consistently intermediate between that of AlbB and RNaseB. The amplification per se can be attributed in general terms to the formation of additional bonds and distances in the system analogous to the use of antiglobulin in agglutination. Although the order of increasing potency essentially is the same as the order of decreasing size (except for the unknown effective size of SuPolysB), the number of extenders tested thus far is small.

Even though the various extenders differed in their potency, nevertheless all of them, comprising a variety of size, shape, and charge characteristics, were able to function effectively in conjunction with avidin and biotin. Although FibB was the weakest extender of the titer of the antiligubulin antibody, it still provided the advantage of extending the agglutination with a lower dependence on its own concentration. This especially contrasts with the behavior of RNaseB, where a much more critical concentration dependence is seen, as shown in Figure 3. Each of these agents might therefore be preferred in a given system, depending on the experimental objective.

The behavior of the other two extenders tested, AlbB and SuPolysB, was intermediate in this regard. Overall, these data suggest that avidin–biotin tool can be both universal and particular concerning its interactions with biotin–conjugated proteins.

One of the limitations involved in attaching to a protein is the tendency of the resulting conjugate to be less soluble. Apparently this problem is most severe with hydrophobic ligands. This tends to limit the usefulness of antibodies as tools because some of the excellent haptons are quite nonpolar. Biotin, a water-soluble vitamin, should thus be an advanta-
This variety of tenders has been seen to some extent. Antibody response was also countered in these cases, and it was decided that this was supported by higher antibody titers in the group. In addition, antigen availability was considered important for the system.

The choice of erythrocyte agglutination as an initial model to pursue some of the characteristics of the avidin–biotin system in conjugation with antibodies was based on several considerations. The model is relevant to several potential applications of avidin and biotin in laboratory medicine, the components are readily available, the results are easily collected for semiquantitative evaluation and optimization studies, and the system is well defined in certain respects in regard to analogous facilitation with antiligand (Coombs's test), allowing comparison testing with antibodies. We further decided to use only native (unfixed) erythrocytes to emphasize and evaluate the usefulness of avidin and biotin under biologically nondisruptive conditions, a basic advantage for this system that has been cited previously (9).

This agglutination system also posed some limitations. Cellular agglutination is a complex phenomenon for which a variety of factors have been considered to play a role (19, 34). This placed some limits on the degree to which our model could be used to reveal and develop characteristics of the avidin–biotin system. Along these lines, it was important to maintain constant conditions and procedures and to use extensive controls to assure the primary dependence of the system on the specific interaction of avidin and biotin.

One factor that may have influenced some of the results, and which has received much attention in agglutination studies, is receptor movement, cited by various authors as both promoting and inhibiting cellular agglutination (34). We considered, for example, whether the reduced ability of avidin to achieve agglutination in the presence of extender rather than in its absence, for both the direct and indirect cases as seen in the right column of Table 4, might be due to the development of clustering upon addition of extender. For example, Bach and Schnebli observed by electron microscopy that anti-concanavalin A causes clustering of concanavalin A molecules specifically attached to an erythrocyte surface (35). Thus, we repeated the direct agglutination with and without all of the extenders at 0 °C, where receptor mobility is considered to be prevented. However, there was essentially no change in extent or enhancement of agglutination as a function of avidin concentration, so that this hypothesis was not supported. Consequently, the mechanism may involve simply an occupancy of some of the avidin sites, without intercellular cross-linking, by the extender. The amplification that extender provides then proceeds to develop optimally at a higher avidin concentration than in the absence of extender. Analogously to our observation that the extent of direct agglutination without extender is the same at 0 °C and at room temperature, Schnebli and Bachi observed no change in the extent of agglutination of erythrocytes by concanavalin A over the same temperature change (33).

Already established as a universal binding system, the avidin–biotin tool is seen here to gain in versatility in conjunction with antibodies by the involvement of noncovalent extenders. We expect that other applications of this tool also will benefit by the use of such extenders.

References


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