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Coupling of protein antigens to erythrocytes through disulfide bond formation: Preparation of stable and sensitive target cells for immune hemolysis

[*N*-succinimidyl 3-(2-pyridyldithio)propionate/thiolation of erythrocytes]

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ABSTRACT **An efficient technique has been developed for coupling protein antigens to erythrocyte membranes. The procedure involves three steps. First, 3-(2-pyridyldithio)propionyl residues are introduced into the protein by reaction with a heterobifunctional reagent, *N*-succinimidyl 3-(2-pyridyldithio) propionate. Second, the addition of disulfide groups to sheep erythrocytes (SRBC) is achieved by coupling dithiodiglycolic acid to SRBC with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. The disulfide bonds of the dithiodiglycolyl-SRBC conjugate are then reduced with dithiothreitol. Finally, the 3-(2-pyridyldithio)propionyl-protein conjugate is covalently coupled to the thiolated SRBC through thiol/disulfide exchange to form the disulfide-linked antigen-SRBC conjugate. The procedure requires only 10–500 μg of protein antigen for the preparation of 50 μl of packed protein-coupled SRBC. Antibodies binding to antigen on the erythrocyte initiate a complement-dependent immune lysis of the target cells. Target cells prepared by this method are stable for at least 4 wk at 4°C in phosphate buffer (pH 7.2) and are capable of detecting as little as 40 pg of antibody in a hemolytic assay without noticeable nonspecific lysis.**

Erythrocytes to which protein, polysaccharide, or hapten antigens are attached have been used extensively as target cells to detect antibody in the serum and to quantitate antibody-forming cells and antigen-binding cells at the single-cell level (1). Such erythrocytes have been particularly useful as target cells in a rapid and sensitive technique for screening numerous samples of hybridoma cell culture fluids for anti-hapten antibody production (2). In the case of hybridoma-derived anti-protein antibodies, such a simple screening technique depends upon the availability of a simple and efficient way of attaching proteins to erythrocyte membranes. Ideally, only a very small quantity of protein antigen (Ag) should be required, and the resulting target cells should be stable and capable of reproducibly detecting very small amounts of anti-protein antibody in an immune hemolytic assay. Various methods have been reported for attaching protein to erythrocytes (3–10). However, these methods require substantial amounts of protein (which is often limited in supply) and may render the cells fragile and susceptible to spontaneous hemolysis.

This paper introduces a simple and efficient method for covalently coupling protein to erythrocytes for use in antibody/complement-mediated hemolytic assays. The procedure involves reagents that are all commercially available, and the conditions of the reaction that covalently links the protein to the erythrocytes are such that the protein-coupled cells remain stable (i.e., show no spontaneous lysis for weeks after the coupling reaction). We report here that this coupling technique has been used effectively to couple bovine serum albumin, human gamma globulin, bovine gamma globulin, monoclonal IgG (MIgG), sea urchin egg hyalin (Hy), and a prostatic tumor-associated antigen, human prostatic acid phosphatase (PAPase) to sheep erythrocytes (SRBC), resulting in highly stable target cells for immune hemolytic assays. The procedure requires only 10–500 μg of protein for the preparation of target cells that can detect as little as 40 pg of anti-protein antibody in an antibody/complement-mediated hemolytic assay.

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MATERIALS AND METHODS

Reagents. *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) was purchased from Pharmacia. Dithiodiglycolic acid (DTDG), dithiothreitol, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) were obtained from Aldrich. Guinea pig serum, used as a source of complement, was obtained from GIBCO.

Borate-Buffered Saline. The borate-buffered saline was prepared by diluting a borate buffer (11) (0.167 M H_3BO_3/0.134 M NaCl/0.022 M NaOH) 1:12 in 0.147 M NaCl, followed by adding 1 M HCl to pH 6 or 7.

Antigens. Bovine serum albumin (fraction V powder) and human gamma globulin (fraction II) were purchased from ICN. Bovine gamma globulin (fraction II) was obtained from Sigma. Hy was prepared by B. L. Hylander and R. G. Summers (12) as described (13). PAPase was prepared by C. Lee (14). MIgG with anti-phthalate specificity was prepared as described (15).

Antisera. Rabbit antisera to bovine serum albumin and bovine and human gamma globulin were prepared by injecting each of three rabbits intravenously three times at successive 1-wk intervals with 10-mg portions of antigen in 1-ml of borate-buffered saline. The animals were bled 2 wk after the final injection, and the sera were processed and stored at −20°C. Rabbit anti-Hy was prepared by B. L. Hylander and R. G. Summers (12). Rabbit anti-PAPase was prepared by C. Lee of this institute (14). For the preparation of anti-mouse gamma globulin antibodies, a rabbit was immunized intramuscularly in multiple sites with 1 mg of MIgG in 1 ml of 50% complete Freund's adjuvant and was given a booster injection 1 mo later. The animal

Abbreviations: PDTP, 3-(2-pyridyldithio)propionyl group; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; DTDG, dithiodiglycolic acid; SRBC, sheep erythrocytes; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; TG, thioglycolyl group; Ag, protein antigen; MIgG, monoclonal IgG; Hy, sea urchin egg hyalin; PAPase, human prostatic acid phosphatase; AG-TG-SRBC, conjugates of protein antigen and sheep erythrocytes prepared by reaction of PDTP-Ag with TG-SRBC; Ag-SRBC (EDCI), conjugates of protein antigen and sheep erythrocytes prepared with carbodiimide.

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was bled 1 wk after the last injection. The antiserum was affinity-purified by adsorption on and elution from an immunoadsorbent prepared by coupling normal mouse gamma globulin to CNBr-activated Sepharose-4B (16).

SRBC. SRBC were obtained weekly by venous puncture of a sheep held at the Springville Laboratories of Roswell Park Memorial Institute. The blood was defibrinated as described (17). The erythrocytes were washed three times and made up to a 50% (vol/vol) suspension in borate-buffered saline (pH 7) for use in coupling experiments.

Introduction of 3-(2-pyridyldithio)propionyl (PDTP) Groups into Proteins by SPDP. The PDTP groups were introduced into the proteins by reaction with SPDP as described (18), and the excess of SPDP reagent was removed by extensive dialysis against phosphate-buffered saline. The concentrations and volumes used of the different protein solutions and of the SPDP reagent are shown in Table 1. The content of PDTP groups in the modified proteins (PDTP-Ag) was determined by the method of Carlsson *et al.* (18). Briefly, one ml of a properly diluted PDTP-Ag solution (0.5–1.0 mg/ml) in phosphate-buffered saline was treated with 0.1 ml of 1 M dithiothreitol at room temperature for 40 min. This treatment effects the release of pyridine-2-thione, which has a molar absorptivity of 8.08×10^3 at 343 nm (19). The amount of pyridine-2-thione released is equivalent to the content of PDTP groups in PDTP-Ag.

Coupling of DTDG to SRBC by EDCI. DTDG (182 mg) was dissolved in one ml of 2 M NaOH. The DTDG solution was then diluted to 25 ml by adding borate-buffered saline (pH 6). One ml of a 50% (vol/vol) suspension of SRBC in borate-buffered saline (pH 7) was added. After thorough mixing, a freshly prepared solution containing 250 mg of EDCI in 2.5 ml of water was added with mixing. The preparation was left at room temperature for 30 min with occasional swirling to keep the erythrocytes suspended. During the course of mixing the reagents, the pH of the reaction mixture rose from 5.3 to 8.4. The cells (DTDG-SRBC) were then washed four times with 45-ml portions of phosphate buffered saline (0.075 M KH_2PO_4/0.075 M NaCl, pH 7.2) and stored in the same buffer at 4°C until used.

Reduction of DTDG-SRBC to Thioglycolyl-SRBC (TG-SRBC) by Dithiothreitol. One-half ml of freshly prepared 1 M dithiothreitol and 12.5 ml of a 2% (vol/vol) suspension of DTDG-SRBC in phosphate-buffered saline were mixed in a 15-ml centrifuge tube and stoppered. The reaction was continued at room temperature for 1 hr on a rotating mixer. The resulting TG-SRBC were washed four times with 15-ml portions of saline and made to a 50% (vol/vol) suspension in preparation for coupling with the PDTP-Ag as described below. Because the thiol group is very reactive and can take part in unwanted reactions, we recommend that the TG-SRBC be prepared immediately prior to the coupling.

Table 1. Introduction of PDTP groups into proteins by SPDP

Protein type	Protein, mg/ml	Protein, ml	SPDP,* ml	Substitution degree, [PDTP]/[Protein]†
Gamma globulin				
Human	40	2	0.25	1.9
Bovine	40	2	0.3	4.0
BSA	30	2	0.25	3.2
Hy	1	2	0.3	8.2
PAPase	12	0.5	0.05	5.6
MIgG	12	0.5	0.05	11

* The 20 mM SPDP solution was freshly prepared in absolute ethanol.
† The molecular weights used for the calculation of molar concentration of proteins are as follows: human gamma globulin, 160,000; bovine gamma globulin, 160,000; bovine serum albumin (BSA), 67,000; Hy, 300,000; PAPase, 100,000; MIgG, 160,000.

Coupling of PDTP-Ag to TG-SRBC. PDTP-Ag (0.01–2 mg) in phosphate-buffered saline (25–200 μl) was mixed with 0.1 ml of a 50% (vol/vol) freshly prepared TG-SRBC suspension on a rotating mixer at room temperature overnight. The resulting protein-coupled cells (Ag-TG-SRBC) were washed three times with 15-ml portions of saline and a 6% (vol/vol) suspension prepared for hemolytic assays or for storage at 4°C until used.

Antibody/Complement-Mediated Hemolysis. The antibody/complement-mediated hemolytic spot test conducted on slides layered with the target cells in agarose (20) was modified as described by Bankert *et al.* (10).

Direct Coupling of Ag to SRBC with EDCI. The direct coupling of human gamma globulin and bovine serum albumin to SRBC with EDCI was as described by Golub *et al.* (9).

RESULTS AND DISCUSSION

A general method for the coupling of proteins to SRBC through disulfide bond formation is summarized in Fig. 1 *a–c*. The procedure involves three steps. First, the PDTP residues are introduced into the protein antigen by the reaction of a portion of the amino groups of the protein with a heterobifunctional reagent, SPDP, as described by Carlsson *et al.* (18) (Fig. 1*a*). Second, the addition of disulfide groups to SRBC is achieved by coupling DTDG to the amino groups on the erythrocyte membrane proteins by using EDCI as the coupling reagent. The disulfide bonds of the DTDG-coupled SRBC are then reduced by using dithiothreitol (Fig. 1*b*). Finally, the PDTP-protein conjugate is covalently coupled to the TG-SRBC through thiol/disulfide exchange to form the disulfide-linked protein-SRBC conjugate (Ag-TG-SRBC) (Fig. 1*c*).

In the first reaction (Fig. 1*a*), the degree of substitution (mol of PDTP groups/mol of protein) can be varied by using different amounts of reagent. Under the experimental conditions used, only a small fraction of the protein amino groups were coupled to PDTP groups in order to preserve the structural integrity and antigenicity of the protein (Table 1).

Utilizing this approach, we attempted to couple a variety of unrelated proteins to SRBC and tested these protein-coupled cells for their ability to detect antibodies of the appropriate specificity. Six different proteins were successfully coupled to SRBC resulting in target cells capable of detecting anti-protein antibodies in an immune hemolytic reaction (Table 2). In addition to testing the immune serum with the appropriate protein-coupled target SRBC, each undiluted serum was tested for lysis of control SRBC (i.e., TG-SRBC were treated the same as the experimental target SRBC, except that the specific protein was omitted from the coupling procedure). No lysis of the control TG-SRBC was observed with any of the immune sera.

Table 2 also contains data comparing the sensitivity of AG-TG-SRBC prepared with different amounts of PDTP-Ag in detecting anti-protein antibodies. The optimal quantity of PDTP-Ag required for sensitization of TG-SRBC ranged from 10–500 μg in 0.025–0.04 ml of solution.

To determine the sensitivity of Ag-TG-SRBC in terms of the amount of antibody required to produce visible hemolysis, a rabbit anti-mouse gamma globulin was purified by means of affinity chromatography. We observed that as little as 40 pg of anti-mouse gamma globulin could be detected by immune hemolysis with MIgG-coupled target SRBC (Table 2). Less than 500 μg of MIgG was utilized in preparing these target cells.

The efficiency of coupling proteins to SRBC by our method and by the EDCI method (9) was compared by testing the resulting target cells in an immune hemolytic assay. (Table 3). To achieve the same sensitivity (hemolytic titer), the quantity of

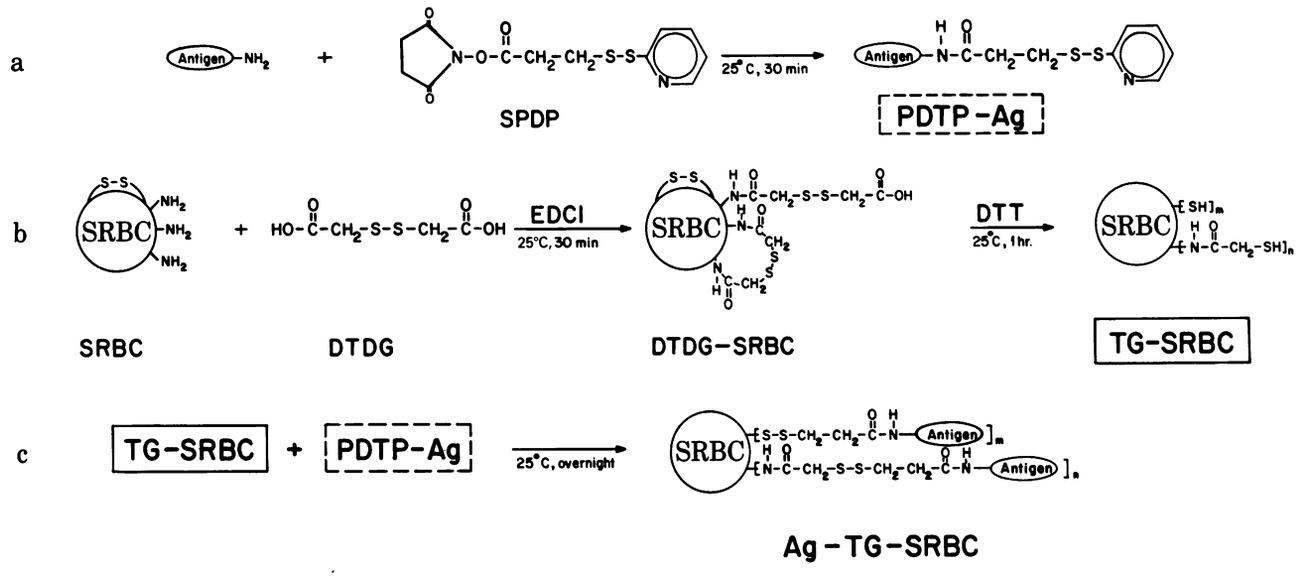

FIG. 1. Coupling of protein antigen to SRBC. (*a*) Introduction of PDTP groups into a protein antigen by aminolysis. (*b*) Thiolation of SRBC. The coupling of DTDG to cell membrane protein with EDCI as the condensing agent is followed by the conversion of the disulfide bonds on the DTDG-coupled SRBC into aliphatic thiols by reaction with dithiothreitol. (*c*) Reaction between the modified protein antigen containing PDTP groups and the thiolated-SRBC through thiol/disulfide exchange to form the disulfide-linked protein-SRBC conjugate.

antigen required for our Ag-TG-SRBC preparations was smaller by a factor of 120–600 than that required for EDCI coupling.

Intermolecular linking of protein antigen to the proteins of the SRBC membranes by means of coupling reagents such as EDCI or homobifunctional reagents (e.g., bis-diazotized benzidine or 1,3-difluoro-4,6-dinitrobenzene, in which the two reactive groups are identical) may be complicated by intramolecular reactions on individual protein molecules. Such side reactions obviously decrease the efficiency of coupling the protein to the SRBC membranes. Accordingly, such coupling procedures characteristically require larger quantities of protein for the production of satisfactory target erythrocytes. With the heterobifunctional reagent SPDP, the two reactive groups are directed towards different functional groups (i.e., amino and thiol groups), and one can modify the protein to PDTP-Ag, which is reactive toward thiol groups, and then separately couple the PDTP-Ag to TG-SRBC (Fig. 1a–c). Because the thiol/disulfide exchange involved in the coupling of PDTP-Ag and TG-SRBC is selective, a highly efficient conjugation of protein and SRBC is achieved.

Another advantage of using SPDP as the key reagent is that the modification of the protein and the subsequent coupling of the modified protein to TG-SRBC proceed in aqueous media under very mild conditions. Thus, destruction of the antigenic structure of the protein is avoided and the integrity of the erythrocytes is maintained.

Table 2. Antibody/complement-mediated hemolysis of target cells prepared by the coupling of PDTP-Ag to TG-SRBC*

Target cell	PDTP-Ag, mg/ml	PDTP-Ag, ml	Antibody preparation†	Hemolytic titer‡
HγG-TG-SRBC	20	0.025	Rabbit anti-HγG	64
HγG-TG-SRBC	20	0.1	antiserum	128
BSA-TG-SRBC	16.5	0.025	Rabbit anti-BSA	64
BSA-TG-SRBC	16.5	0.1	antiserum	64
BγG-TG-SRBC	10	0.025	Rabbit anti-BγG	512
BγG-TG-SRBC	10	0.1	antiserum	512
Hy-TG-SRBC	0.4	0.025	Rabbit anti-Hy	64
Hy-TG-SRBC	0.4	0.1	antiserum	128
Hy-TG-SRBC	0.4	0.2		128
PAPase-TG-SRBC	12	0.025	Rabbit	64
PAPase-TG-SRBC	12	0.1	anti-PAPase antiserum	64
MIgG-TG-SRBC	11.6	0.04	Affinity-purified rabbit anti-MγG antibody	§

HγG, human gamma globulin; BγG, bovine gamma globulin; MγG, mouse gamma globulin; BSA, bovine serum albumin.

* All slides were incubated with a goat anti-rabbit immunoglobulin antiserum diluted 1:50 as a facilitator before incubating with guinea pig serum diluted 1:10. PDTP-Ag reaction was with 0.1 ml of 50% (vol/vol) TG-SRBC.

† The rabbit antisera used in this experiment were preadsorbed with an equal volume of packed SRBC at 37°C for 1 hr. There was no hemolysis of the control TG-SRBC.

‡ Values in this column indicate the reciprocal of the highest dilution of the antibody preparation that gave an unequivocal positive reaction.

§ The quantity of antibody present at the end point is 40 pg.

Table 3. Comparison of Ag-TG-SRBC and Ag-SRBC (EDCI)* in detecting antibody/complement mediated-hemolysis†

Target cells	Ag,‡ mg	Antiserum§	Hemolytic titer¶
HγG-TG-SRBC	2	Rabbit anti-HγG	128
HγG-TG-SRBC	0.5	Rabbit anti-HγG	64
HγG-SRBC (EDCI)	60	Rabbit anti-HγG	64
BSA-TG-SRBC	1.6	Rabbit anti-BSA	64
BSA-TG-SRBC	0.4	Rabbit anti-BSA	32
BSA-SRBC (EDCI)	240	Rabbit anti-BSA	32

HγG, human gamma globulin; BSA, bovine serum albumin.

* Ag-SRBC (EDCI) were prepared by direct coupling of proteins (HγG and BSA) to SRBC by EDCI as described (9).

† All slides were incubated with a goat anti-rabbit immunoglobulin antiserum diluted 1:50 as a facilitator before incubating with guinea pig serum diluted 1:10.

‡ Quantity of Ag used to prepare 0.05 ml of packed target cells.

§ The rabbit antisera were preadsorbed with an equal volume of packed SRBC at 37°C for 1 hr. There was no hemolysis of the control TG-SRBC.

¶ Values in this column indicate the reciprocal of the highest dilution of antiserum that gave an unequivocal positive reaction.

The Ag-TG-SRBC were found to be stable without significant lysis for at least 4 wk of storage at 4°C in phosphate-buffered saline. Because DTDG-SRBC were also stable in phosphate-buffered saline at 4°C for at least 4 wk, a large quantity of DTDG-SRBC can be prepared and stored, thereby facilitating the routine preparation of protein-coupled target cells as needed.

This method of preparing target cells will be very useful in screening a large number of microcultures of hybridomas for specific antibody, as in our earlier sampling technique (2), especially when the quantity of protein antigen involved is very limited.

Our recent experience with one particular protein—the PAPase associated with patients with prostatic cancer—indicates the value of our procedure. Here, with as little as 300 μg of PDTP-PAPase, we were able to prepare target cells (PAPase-TG-SRBC) that were used successfully to screen hybridoma microcultures for the production of anti-PAPase antibodies. In fact, the hemolytic assay with PAP-TG-SRBC permitted the identification of anti-PAPase antibody-forming cell hybrids which were not detected by an established radioimmunoassay (unpublished data).

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