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Coupling of C3b to Erythrocytes by Disulfide Bond Formation: Preparation of EC3b for Hemolytic and Complement Receptor Assays [1]

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We describe a new method of preparing C3-coated erythrocytes by coupling C3 to thiol-activated erythrocytes. The procedure involves three steps. Firstly, sheep erythrocytes were treated with N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) to introduce 3-(2-pyridyldithio) propionyl residues into membrane proteins. Secondly, C3 was cleaved with trypsin or CoVF, Bb enzyme to obtain C3b exposing the SH group (C3b-SH). Finally, the C3b-SH was coupled to the thiol-activated erythrocytes (TA-E) through thiol/disulfide exchange to form the TA-EC3b conjugate. E coated with C3d was prepared by treating TA-EC3b with KSCN inactivated serum and plasmin.

Studying the rosette formation between TA-EC3b or TA-EC3d and cells expressing C3b (CR_1) and C3d (CR_2) receptors and the inhibition thereof with anti-CR_1 and anti-CR_2 antibodies as well as with C3-sheep E membrane protein complexes, we found that TA-EC3b and TA-EC3d bound exclusively to CR_1 and CR_2, respectively. In addition, TA-EC3b like EAC1423b bound factors B and H as tested by hemolytic and direct binding assays. The advantage of TA-EC3 for complement receptor and hemolytic assays are the simplicity of the preparation method and the general applicability of the TA-EC3.

Key words: *thiolation of erythrocytes – third component of complement – complement receptors*

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Abbreviations: Bb, activated factor B; BDVEA, 1% bovine serum albumin (BSA), 3.2% dextrose, 35 mM veronal buffer, pH 7.2 with 20 mM EDTA, and 2 mM sodium azide; C, complement; C3b major fragment of C3 activation, 190,000 daltons; C3d, 30,000 daltons, fragment of C3b that retains to complexes following proteolytis of bound C3b; CR1, complement receptor type one, receptor specific for the C region of C3b and C4b; CR2 complement receptor type two, receptor specific for the d region of C3b; E, erythrocyte; EA, IgM-antibody sensitized E; EAC, EA coated with complement components; EC3, sheep E-C3 complexes prepared by C3 activation and fixation onto sheep E; GVB-Ni, gelatin veronal buffer; containing 0.15 mM nickelous chloride, PDP, 3-(2-pyridyldithio) propionyl groups TA-E, thiol-activated erythrocytes; TA-EC3, TA-E coated with C3; SPDP, N-succinimidyl 3-(2-pyridyldithio) propionate.

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Introduction

A number of methods exist for the detection of receptors for the various cleavage products of C3 and of complement activation as it is determined by hemolysis of erythrocytes. Most of these methods involve coating of erythrocytes with C3 by means of activation of the alternative or classical pathway. These methods require relatively large amounts of different complement components C1, C2, C4 and C3 or factors B, D, nephritic factor and C3 (Ross and Polley, 1976; Pangburn and Müller-Eberhard, 1978). This means that specific assays for complement receptors or the hemolytic assays are not feasible for laboratories which do not specialize in C purification. Recently, a very sensitive, specific and simple assay for the detection of complement receptors has been reported (Lambris and Ross, 1982; Ross and Lambris, 1982). Even though that method which involves coupling of C3b, iC3b or C3d to fluorescent microsphere is more sensitive than the other complement receptor assays, it does not have a wide application. For theoretical reasons one has to assume that the microspheres bind the C3 fragments in a non-specific way: some C3b molecules may be bound in such a way that they expose the site for binding to C3b receptors, other C3b molecules may be bound to the beads by *just this site*. Therefore, the fluorescent microsphere coated with C3b and C3d does not seem to be optimal for studies involving the binding of different ligands such as factors B and H and in fluid phase assays for the detection of complement receptors. Furthermore, hemolytic assays cannot be performed with the beads.

In this report a new method for preparing EC3b and EC3d is described. This method requires only partially purified C3, and this in smaller quantities than required for other coating techniques. This method is based on the fact that C3b expresses an SH-group (Tack et al., 1980), and it involves coupling of that C3b to thiol-activated erythrocytes.

Materials and Methods

C receptor cells

Human E, peripheral blood lymphocytes, neutrophils and tonsil cells were isolated as described previously (Ross et al., 1978; Dobson et al., 1981). Raji and Daudi cells (Burkitt lymphoma derived lymphoblastoid cell lines) were maintained in RPM I640 supplemented with 10% heat-inactivated fetal bovine serum and antibiotics.

C components, C-coated sheep erythrocytes and C3-coated microspheres

C components and nephritic factor (NF) were isolated and used to prepare EAC1423b and EC3b as described (Lambris et al., 1980). EC3b and EAC1423b were converted into EC3d with KSCN-inactivated serum and plasmin (Lambris et al., 1980). Fixation of C3 onto E was quantitated by measuring the uptake of ^{125}I-labeled monoclonal anti-C3 (Bethesda Research Laboratories, Gaithersburg, MD) onto a small sample of the EC3b or EAC143b. Microspheres (Covalent Technology Corp., CA) coated with C3b (C3bms) and C3d (C3dms) were prepared as previously described (Lambris and Ross, 1982).

Preparation of antibodies specific for CR1 and CR2

Antibodies to CR_1 and CR_2 were prepared in rabbits by immunization with purified protein antigens (Dobson et al., 1981; Lambris et al., 1981). The $F(ab')_2$ fragments of these antibodies were prepared by digestion with pepsin (Ross and Winchester, 1980).

Preparation of C3 fragments

C3b was generated from purified C3 (1 mg/ml) either by incubation with trypsin (10 μg/ml/TPCK treated; Serva Heidelberg, F.R.G.) for 1 min followed by the addition of soybean trypsin inhibitor (40 μg/ml; Serva, Heidelberg, F.R.G.) or by incubation with preformed CoVF, Bb for 90 min at 37°C (Burger et al., 1982). C3 fragments bound to sheep erythrocyte proteins (C3b0R and C3d0R) were prepared as described by Fearon et al. (1981). Briefly, the membranes of 10^{10} EC3 bearing 7.1×10^5 C3d per E or 8×10^5 C3b per E were produced by lysis of the EC3 in 5 mM phosphate buffer. After solubilization with 0.25% Nonidet P40 and removal of the detergent with Bio-Beads SM-2 (Bio-Rad Laboratories, Richmond, CA) the C3b0R and C3d0R were concentrated to 2 ml, dialyzed against BDVA and stored at 4°C.

Coupling of C3b to sheep erythrocytes

SPDP (3.9 mg) was dissolved in 25 μl DMSO. The SPDP solution was then added to 2.5 ml of a suspension of sheep erythrocytes (2×10^9/ml) in PBS and the mixture was shaken vigorously. The preparation was allowed to react at room temperature for 60 min with continuous rotation to keep the erythrocytes in suspension. The thiol-activated erythrocytes (TA-E) were then washed four times with phosphate buffered saline (PBS) and resuspended to a concentration of 5×10^9/ml PBS. Then C3b made either with trypsin or COVF, Bb enzyme was mixed with the thiol-activated erythrocytes on a rotating mixer at room temperature for 60 min (see Results). The resulting C3b-coated cells (TA-EC3b) were washed three times with BDVEA, resuspended to a concentration of 2×10^8/ml and stored at 4°C until use. TA-EC3b were converted into TA-EC3d with KSCN-inactivated serum and plasmin (Lambris et al., 1980).

C receptor assays

C receptors were detected by a rosette assay (Lambris et al., 1980) and by immunofluorescence (Lambris and Ross, 1982). The inhibition of rosette formation by anti-CR_1 (200 μg/ml) and anti-CR_2 (250 μg/ml) was performed as previously described (Lambris et al., 1980; Lambris and Ross, 1982). The inhibitory effect of C3-sheep E membrane complexes (C3b0R and C3d0R) was tested by treatment of C-receptor cells with 100 μl of either C3b0R or C3d0R before the addition of indicator cells.

Binding of factor H and B to TA-EC3b

Purified factors H and B were labeled with ^{125}I by the Iodogen method (Fraker and Speck, 1978) resulting in a specific activity of 4.2×10^6 and 2.9×10^6 cpm/μg

protein, respectively. The specific binding of ^{125}I-H and ^{125}I-B to TA-EC3b or EC3b was measured as previously described (Kazatchkine et al., 1979). The ability of factor B to bind to TA-EC3b and to form the C3 convertase was determined by hemolytic assay for lysis of TA-EC3b (1×10^8/ml) by incubation first with different amounts of B and a constant amount of D (1 μg/ml) in GVB-Ni (3 min at 37°C) (Fishelson and Müller-Eberhard, 1982) followed by a second incubation with guinea pig serum (1 : 15 with 0.04 M EDTA-GVB) for 60 min at 37°C. The ability of factor H to serve as a cofactor for the cleavage of C3b to iC3b by factor I was determined by inhibition of lysis of TA-EC3b by B, D and EDTA-guinea pig serum.

Results and Discussion

A simple method for preparing erythrocytes coated with C3b or C3d through disulfide bond formation is summarized in Fig. 1a–c. The procedure involved three steps. Firstly, pyridyldithiopropionyl (PDP) groups were introduced into sheep erythrocytes by the reaction of amino groups on erythrocyte membrane proteins with the heterobifunctional reagent SPDP (Fig. 1a). Secondly, C3 was cleaved to C3b and C3a with either trypsin or the CoVF, Bb enzyme to expose the SH group on C3b (C3b-SH) (Fig. 1b). Finally, the C3b-SH was covalently coupled to the TA-E through thiol-disulfide exchange to form the disulfide linked TA-EC3b conjugate (Fig. 1c).

In the first reaction (Fig. 1a), the degree of substitution (molecules of PDP per E) can be varied by using different concentrations of reagent. The maximum concentration not causing lysis of erythrocytes and yet at the same time effecting optimal substitution was 5 mM SPDP. Using this concentration, 7×10^{11} pyridyldithiopropionyl groups could be introduced per erythrocyte. This was determined by

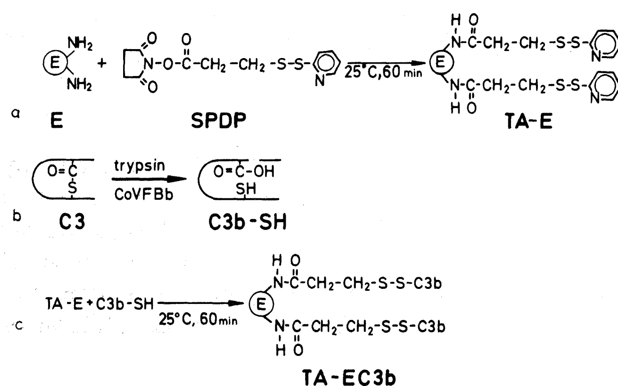

Fig. 1. Coupling of C3b to E. a: Introduction of 3-(2-pyridyl-dithio) propionyl groups to erythrocyte membrane proteins. b: Cleavage of C3 to C3b with trypsin or CoVF, Bb enzyme to expose the SH group. c: Reaction between modified erythrocytes and C3b through thioldisulfide exchange to form the disulfide linked TA-EC3b conjugate.

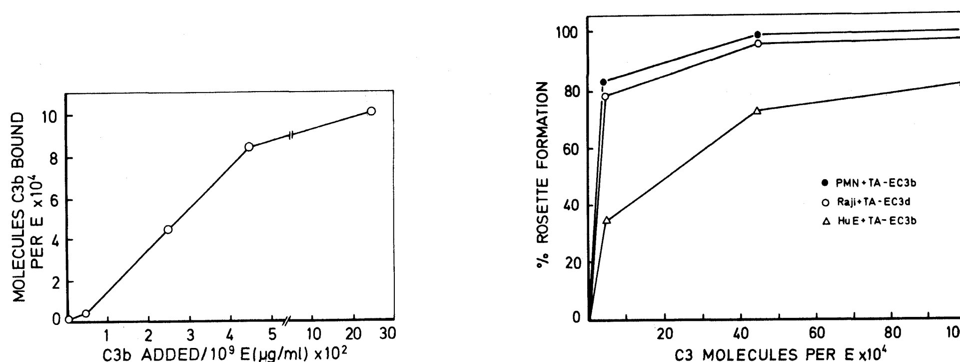

Fig. 2. Dose-dependence of C3b coupling to thiol-activated erythrocytes. 1×10^9 TA-E were incubated with increasing concentrations of C3b in PBS for 60 min at room temperature. Then, the TA-EC3b were washed three times with BDVA and the number of C3b molecules coupled to TA-E was measured by using ^{125}I-labeled monoclonal anti-C3c.

Fig. 3. C3 dose-dependent effect on the rosette formation of TA-EC3b and TA-EC3d with PMN, HuE and Raji cells, respectively. 100 μl of a suspension of TA-EC3b or TA-EC3d (2×10^8/ml) carrying different amounts of C3 were mixed with 100 μl of a suspension of C receptor cells (4×10^6/ml), incubated for 15 min at 37°C and rosette formation was assessed.

elution of the pyridyl groups with dithiothreitol and measuring the absorbance at 343 nm (Carlsson et al., 1978). Even though a lot of PDP groups are introduced per erythrocytes, only some of these are accessible on the outer surface because the SPDP can cross the cell membrane and bind inside the cell.

Fig. 2 shows the uptake of C3b molecules by thiol-erythrocytes when different amounts of C3b were offered. As an example, by using 250 μg C3b per 10^9 cells, 4.5×10^4 C3b molecules could be coupled per cell. That amount of molecules is sufficient for most complement receptor assays. In order to determine the number of C3 molecules required for optimum rosette formation with different cell types, erythrocytes were coated with different amounts of C3. TA-E carrying as few as 10^4 molecules C3b or C3d per cell were capable of forming over 80% rosettes with neutrophils and Raji cells, respectively (Fig. 3). In the case of HuE rosettes with TA-EC3b, 4×10^5 molecules per cell were required for optimum rosette formation

TABLE I

COMPARISON OF DIFFERENT METHODS FOR ASSAY OF CR_1 AND CR_2

C receptor cell type	CR_1 assays (%)			CR_2 assays (%)		
	EAC143b	TA-EC3b	C3bms	EAC143d	TA-EC3d	C3dms
HuE	76	78	98	0	0	0
Blood lymphocytes	17	17	20	7	7	9
Blood neutrophils	94	94	98	0	0	0
Tonsil lymphocytes	65	63	67	70	71	75

TABLE II

INHIBITION OF TA-EC3 ROSETTE FORMATION BY ANTIBODIES SPECIFIC FOR C RECEPTORS AND BY C3 FRAGMENTS BOUND TO SHEEP ERYTHROCYTE MEMBRANE PROTEINS

TA-EC3 type	C receptor cells	Inhibition by			
		anti-CR_1 (%)	anti-CR_2 (%)	C3b0R (%)	C3d0R (%)
TA-EC3b	Erythrocytes	100	0	100	0
	Neutrophils	95	0	100	0
TA-EC3d	Raji	0	98	0	98

(Fig. 3). These data together with the data shown in Table I, comparing the different complement receptor assays, indicate that the TA-EC3b indicator cells can be applied for complement receptor assays instead of indicator cells made with the classical or alternative pathway (EAC1423, EAC3).

The specificity of TA-EC3b and TA-EC3d for CR1 and CR2 was further confirmed by blocking experiments with anti-CR1 and anti-CR2 antibodies as well as with C3b0R and C3d0R complexes. The latter complexes possess high affinity for the C receptors (unpublished observations). In all cases anti-CR1 antibodies and C3b0R totally inhibited TA-EC3b binding to CR1 whereas anti-CR2 antibodies and C3d0R totally inhibited TA-EC3d binding to CR2 (Table II). To determine whether the conformation of C3b bound to TA-E is similar to that of C3b fixed to EAC143b binding of ^{125}I-labeled H and B to TA-EC3b and EAC143b carrying comparable numbers of C3b molecular cells was investigated. As can be seen from Table III the same amounts of labeled ^{125}I-H and ^{125}I-B bound to either TA-EC3b or EA143b while TA-E and EAC14 showed background binding (Table III). The binding of ^{125}I-labeled ligands could be inhibited by an excess of unlabeled ligands, H and B. In addition to the capability of TA-EC3b to bind factor B, it was found that TA-EC3b was capable of forming the C3 convertase after the addition of factor B and D as tested by lysis of erythrocytes when guinea pig serum containing 20 mM EDTA was added as a source of terminal components. Incubation of TA-EC3b with

TABLE III

BINDING OF ^{125}I-LABELED FACTOR H AND B TO TA-EC3b AND EAC143b

Cell type	^{125}I-H bound per 10^7 cells in presence of		^{125}I-B bound per 10^7 cells in presence of	
	Buffer (cpm)	Excess unlabeled H (cpm)	Buffer (cpm)	Excess unlabeled B (cpm)
TA-EC3b	17525	175	27151	887
TA-E	215	210	840	856
EAC143b	19065	190	23041	832
EAC14	165	180	299	815

factors B (17.3 μg/ml) and D (1 μg/ml) caused 60% lysis after the addition of guinea pig serum containing 20 mM EDTA. Factor H bound to TA-EC3b exhibited the ability to serve as a cofactor of factor I for the cleavage of C3b to iC3b as tested by inhibition of the formation of the C3 convertase with factors B and D.

Using the procedure described, only C3 is required for the preparation of sheep erythrocytes coated with C3 fragments. Because only proteins with a free sulfhydryl group can bind to TA-E, partially purified C3 may be used for preparation of TA-EC3b. The TA-EC3b were found to be stable without significant lysis and loss of activity for at least 3 weeks when stored at 4°C in BDVAE. Since TA-E were also stable in phosphate buffered saline (PBS) containing 1 mM EDTA at 4°C for at least 3 weeks, a large quantity of TA-E can be prepared, thereby facilitating the routine preparation of target cells coated with C3.

This method of preparing target cells may also be very useful for coupling other proteins with free sulfhydryl groups to erythrocytes. It may also be used for other tests such as hemolytic assays or for the detection of monoclonal antibodies against proteins bound to TA-E.

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