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Mouse Autoantibodies Bind to a Phospholipase-C-Sensitive Structure on Red Blood Cells

S.J.		Hardy
K.O.		Cox

School of Biological Sciences, Flinders University of South Australia, Bedford Park, Australia

Abstract

After culturing mouse peritoneal cells in vitro for 4 days, high numbers of cells can be detected that secrete autoantibodies against isologous red blood cells (RBC), modified with the proteolytic enzyme bromelain (Brom). **Plaque-forming** cell numbers against mouse Brom RBC were significantly reduced by **pre-treating** mouse Brom RBC prior to haemolytic assay with phospholipase C, an enzyme that hydrolyzes phospholipids, notably phosphatidylcholine. In contrast, further treatment of mouse Brom RBC with Brom, neuraminidase, β -chymotrypsin, trypsin, or papain had no effect on **plaque-forming** cell numbers. These results show that phosphatidylcholine is an integral part of the mouse RBC autoantigen exposed by Brom treatment.

Correspondence to: Dr. K.O. Cox, School of Biological Sciences, Flinders University of South Australia, Bedford Park, SA 5042 (Australia)

Cells taken from the lymphoid tissues of untreated, healthy mice (including germ-free mice) are continuously secreting autoantibodies that react with antigens buried within the membranes of their own red blood cells (RBC). These antigens, which are not detected on the surface of normal RBC, can be revealed by partial proteolysis with the enzyme bromelain (Brom) [1–3]. As well as binding to mouse Brom RBC, these autoantibodies also react with the constant region of mouse IgG [4] and the ubiquitous membrane phospholipid phosphatidylcholine [5]. The number of detectable autoreactive plaque-forming cells (PFC) can be increased by culturing cells from the peritoneal cavity for several days prior to assay [2,6].

Until relatively recently, little was known about the molecular nature of the mouse RBC autoantigen revealed by Brom treatment. In 1981 Serban et al. [7] postulated that the trimethylammonium moiety represented a significant portion of the mouse Brom RBC autoantigen. However, their system required very high concentrations of trimethylammonium-containing compounds to produce effective inhibition of autoantibody activity. Later work indicated that phosphatidylcholine, itself a trimethylammonium-containing compound, could specifically inhibit serum autoantibody activity and splenic PFC against mouse Brom RBC at concentrations approximately 2,000 times lower than those seen for other trimethylammonium-containing compounds [5]. Marcolini et al. [8] have also demonstrated that phosphatidylcholine can inhibit binding of autoreactive Ly-1 + B lymphomas to mouse Brom RBC. In addition, these authors report direct binding of fluorescently labelled phosphatidylcholine-containing liposomes to autoreactive B cells. All these studies have, however, been performed using either inhibition of autoantibody activity or direct binding as the crucial experimental evidence. Given the implications of an

autoantibody against such a common membrane phospholipid, it was decided to use an entirely different experimental design to test whether phosphatidylcholine was the RBC autoantigen. It was reasoned that as phosphatidylcholine could inhibit autoantibody activity when added to haemolytic assays [5] it should be possible to reduce the number of detectable PFC against mouse Brom RBC by treating the target RBC prior to assay with phospholipase C, an enzyme that hydrolyzes phosphatidylcholine. The results show that phospholipase C treatment of mouse Brom RBC specifically decreases the number of detectable PFC, indicating that a reduction in the number of intact phosphatidylcholine molecules in the membrane corresponds with a reduction in PFC activity.

Peritoneal cells from 10- to 11-week-old C3H mice

Phospholipase C Reduces PFC against Mouse Bromelain-Modified RBC

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were collected and cultured essentially as described [6]. After 4 days in culture, the PFC activity against mouse Brom RBC and enzyme-treated mouse Brom RBC was determined as reported [3].

All enzymes were freshly prepared before each experiment in Dulbecco's phosphate-buffered saline at twice the concentration listed in table 1. Brom (EC 3.4.22.4), from pineapple stems; phospholipase C (EC 3.1.4.3), from *Clostridium perfringens*, and β -chymo-trypsin (EC 3.4.21.1), from bovine pancreas, were all obtained from Sigma Chemical (St. Louis, Mo., USA), while neuraminidase (EC 3.2.1.18) from *C.per-fringens*, was obtained from Boehringer Mannheim (FRG), trypsin (EC 3.4.21.4), from bovine pancreas, from Worthington Diagnostic Systems (Freehold N.J., USA) and papain (EC 3.4.22.2), from African papaya, from Calbiochem-Behring (San Diego, Calif., USA).

RBC from 7- to 8-week-old male C3H mice were prepared as outlined [9]. After washing, the cells were pooled and packed at 1,100 g for 4 min. Routinely, 10 ml of blood was collected per experiment. All RBC were modified once with 11.5 U/ml (final) Brom by incubating the packed RBC with the enzyme as a 50% suspension at 37 °C for 30 min, mixing every 10 min. Brom-modified RBC were washed as described above and aliquoted prior to packing. Mouse Brom RBC were modified with the second enzyme in the same manner (table 1), washed four times, and made up to a 21% suspension prior to use. Control RBC (Brom treated once) were prepared as above, but incubated with Dulbecco's phosphate-buffered saline alone during the second modification procedure.

After culturing unimmunized mouse peritoneal cells in vitro for 4 days, a high number of PFC against mouse Brom RBC were detected (routinely > 104 PFC/106 viable cells cultured on day 0). This number of PFC was taken to be 100% for each experiment.

When mouse Brom RBC were further modified with a second enzyme as listed in table 1, only phospholipase C treatment caused any significant reduction in PFC numbers relative to the mouse Brom RBC control. When analyzed by one-way analysis of variance, the PFC numbers for all groups, except the phospholipase-C-treated mouse Brom RBC, were found to be homogeneous. ($F_{5,15} = 2.43$, $p > 0.05$), whereas plaque numbers against phospholipase-C-treated cells were significantly lower ($F_{6,17} = 12.73$, $p < 0.01$). This suggests that phospholipase C treatment is modifying, masking, destroying, or reducing

Table 1. Reduction in the number of detectable PFC against mouse Brom RBC following treatment of the target RBC with phospholipase C

Second enzyme	Final enzyme n	Percentage of control
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treatment (30 min concentration at 37°C)	units/ml	(mean $\pm$ SD) ^a
None -	6	100 $\pm$ 5.3
Brom 11.55b	3	94.4 $\pm$ 11.9
Neuraminidase	1 3	86.5 $\pm$ 4.8
β -Chymotrypsin	80 3	90.7 $\pm$ 2.6
Trypsin	575 3	91.2 $\pm$ 5.3
Papain 1.925c	3	92.7 $\pm$ 1.8
Phospholipase C	0.02 3	63.5 $\pm$ 6.6

Peritoneal cells cultured in vitro for 4 days were assayed for PFC activity against mouse Brom RBC modified as described above. Assays were performed in triplicate and scaled as a percentage relative to the number of PFC obtained against untreated mouse brom RBC to accommodate any interexperimental variation. Unit definitions as outlined by the manufacturers. a PFC/106 cells cultured on day 0 between 27,000 and 45,000 for all experiments.

b Equivalent to 10 mg/ml, as previously reported [3]. c Milk-clotting units per milliliter. the number of autoantibody-binding sites on the treated mouse Brom RBC.

These findings support the hypothesis that phosphatidylcholine represents either the entire mouse brom RBC autoantigen or at least a major portion of it [5]. Another possibility is that the autoantibodies are recognizing a structure comprising both protein and phospholipid or a protein-phospholipid microdomain. Phospholipase treatment would interfere with the integrity of such a structure by altering or destroying the protein-phospholipid interaction. Such a change would also result in a reduction of plaque numbers. Against this possibility are two important observations. Firstly, the activity of autoantibodies against mouse Brom RBC can be inhibited by artificial phosphatidylcholine vesicles devoid of protein [5]. In addition, cells reactive to the mouse Brom RBC have been shown to bind phosphatidylcholine vesicles, again in the absence of protein [8]. As phosphatidylcholine is the highly preferred substrate for phospholipase C from *C. perfringens* [10] the most likely hypothesis to explain these results is that phospholipase C treatment is reducing the number of available

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surface autoantigens by hydrolyzing phosphatidylcholine molecules in the cell membrane of the mouse Brom RBC. As phosphatidylcholine constitutes the major membrane phospholipid in both mouse and mouse Brom RBC [Hardy, et al., in preparation], it follows that the experimental approach used here would not result in a total loss of PFC activity against mouse Brom RBC, because extensive hydrolysis of RBC membrane phosphatidylcholine would cause cell lysis [11]. These results also indicate that the entire phosphatidylcholine molecule (or a group of such molecules) is required if autoantigen integrity is to be maintained. Due to their lipophilic nature, the diglyceride and phosphatidic acid moieties produced following phospholipase C cleavage of phosphatidylcholine would remain within the RBC membrane. Thus, if these fragments were the autoantigen, no reduction in PFC numbers should have been observed. In addition, when diglycerides and phosphatidic acid were added to haemolytic assays using antimouse Brom RBC serum autoantibodies, no reduction in haemolytic activity was detected, suggesting neither molecule was recognized by the autoantibodies [5]. It is also highly unlikely that the phosphorylcholine liberated by phospholipase C treatment is inhibiting the antimouse Brom RBC PFC. This is because phosphorylcholine is soluble and, therefore, would be removed after

the second enzyme modification by the RBC washing procedures, making it unavailable to the autoantibodies produced during PFC enumeration.

As the various enzymes used in this study hydro-lyze quite different substrates, it is not possible to match the potency or severity of each enzyme treatment. The enzyme concentrations thus chosen were based either on those previously reported to interfere with biological responses, or those observed during preliminary experiments not to cause too much lysis of the RBC following the modification procedures (data not shown). These discrepancies do not alter the overall conclusion from this study, however.

Linder and Edgington [12] reported circulating analogues of the mouse Brom RBC autoantigens in the serum of New Zealand Black mice. These analogues may constitute phospholipid liposomes or membrane fragments containing phosphatidylcholine. Thus, the autoantibodies, in addition to the hypotheses already proposed [5] could provide a mechanism for removing or tagging circulating insoluble membrane fragments.

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