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Molecular Immunology 42 (2005) 31–38
Molecular Immunology
www.elsevier.com/locate/molimm

Investigation of the mechanisms of anti-complement activity in *Ixodes ricinus* ticks

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Received 18 January 2004; accepted 12 July 2004
Available online 21 August 2004

Abstract

The feeding success of a tick upon a host depends on its ability to suppress host anti-tick responses which include activation of the complement system. We investigated the mechanism of inhibition of the alternative pathway of complement by salivary gland extract (SGE) of the ixodid tick species, *Ixodes ricinus*. SGE treatment strongly inhibited C3a generation and factor B cleavage in serum when rabbit erythrocytes were used as complement activator, but not when cobra venom factor (CVF) was used as an activator. SGE treatment strongly inhibited C3b deposition on rabbit erythrocytes, and the turnover of C3 (to C3b/iC3b) in serum. However, there was no significant effect upon the formation, stability or activity of C3 convertase (C3bBb) when formed from purified C3b, factor B and factor D. SGE treatment of isolated C3 resulted in a shift in mobility of the α-chain (by about 5 kDa). N-terminal sequencing of this species suggests that cleavage occurs at the C-terminus of the α-chain of C3. Consistent with this hypothesis, the modified α-chain was still a substrate for pre-formed convertase. The activity was specific for the α-chain of C3 but not of C3(H_2O) nor the α′-chain of C3b. It is proposed that SGE-modified C3 does not participate in convertase formation, probably having a reduced affinity for factor B.

Keywords: Complement; Ticks; *Ixodes ricinus*; Alternative pathway of complement; Salivary glands

1. Introduction

Parasitism of hosts by ticks, and infection by tick-borne pathogens, are significant medical and veterinary problems and the associated economic losses are considerable (Service, 2001). In contrast to other hematophagous arthropods, the adults of many ixodid tick species feed on a host for extended periods of up to 14 days in duration, allowing ample time for activation of host responses including innate and adaptive immunity.

Proteins of the complement system are key effector molecules in blood and become activated in response to tick infestation. The mean C3 level of serum was shown to increase in rabbits in response to *Ixodes ricinus* feeding and to increase with subsequent re-infestations (Papatheodorou and Brossard, 1987). Deposition of complement was detected at the dermo-epidermal junction close to attachment sites of *Dermacentor andersoni* ticks feeding upon repeatedly infested guinea-pigs (Allen et al., 1979). Depletion of complement activity by cobra venom factor (CVF) treatment of tick-infested guinea-pigs resulted in an increase in mean engorgement weight of feeding *D. andersoni* larvae (Wikel and Allen, 1977). Intact C3 was detected in the gut of *I. scapularis* nymphs partially fed on C57BL/6 mice (Rathinavelu et al., 2003).

Abbreviations: SGE, salivary gland extract; CVF, cobra venom factor; GVB, gelatin-veronal buffered saline; EGTA, ethyleneglycoltetra-acetic acid; REAP, rabbit erythrocyte alternative pathway activation assay

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doi:10.1016/j.molimm.2004.07.001

The feeding success of a tick relies upon its ability to suppress host responses by utilizing bioactive molecules contained in the salivary glands of the tick and secreted into the feeding site in saliva (Ribeiro, 1987). Tick saliva and salivary gland extracts (SGE) contain many molecules capable of suppressing host immune activation such as histamine and IgG binding proteins (Paesen et al., 1999; Wang and Nuttall, 1995).

We previously reported the presence of anti-complement activity in the SGE of *I. ricinus*, *I. hexagonus* and *I. uriae* ticks, that was directed against the alternative pathway but not the classical pathway of complement (Lawrie et al., 1999). This activity was also observed in the saliva of *I. ricinus* ticks (Mejri et al., 2002). Anti-complement activity has also been detected in the salivary glands of *I. scapularis* (Ribeiro, 1987) and *Ornithodoros moubata* ticks (Astigarraga et al., 1997). Two novel proteins have subsequently been identified from the saliva of *I. scapularis* that inhibit host complement (Das et al., 2001; Valenzuela et al., 2000). Valenzuela and colleagues recombinantly expressed a protein in COS cells that the authors proposed accelerates the dissociation of Bb from the active convertase (C3bBb) (Valenzuela et al., 2000). Our data however suggested that *I. ricinus* anti-complement activity probably did not function by this mechanism. Consequently, we undertook a detailed investigation of the mechanisms of anti-complement activity in *I. ricinus* ticks, the European Lyme disease vector.

2. Materials and methods

2.1. Preparation of salivary gland extract (SGE)

I. ricinus and *I. hexagonus* ticks were reared and maintained at CEH Oxford according to standard methods (Jones et al., 1988). Adult female ticks (and an equal number of males) were fed on hamsters for 5 days. The engorged females were removed and their salivary glands dissected. The salivary glands were homogenized in tissue culture PBS (137 mM NaCl, 2.7 mM KCl, 7.4 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2) using a glass homogenizer. Particulate matter was removed by centrifugation at 13,000 × *g* for 10 min. The soluble fraction (i.e. SGE) was aliquoted and stored at −20 °C. Protein concentration was determined using a modified Bradford assay (Bio-Rad protein assay) (Bradford, 1976). Standard curves were prepared using bovine serum albumin.

2.2. Polyacrylamide gel electrophoresis (PAGE)

Gradient (4–12%) Bis-Tris NuPAGE gels obtained from Invitrogen (Paisley, UK) were used in accordance with the manufacturer's instructions using supplied MOPS (4-morpholinepropanesulfonic acid) buffer. MultiMark or See-Blue 2 pre-stained molecular weight markers from the same manufacturer were used throughout. Gels were run with or without SDS, as described in figure legends. Samples run under non-denaturing conditions were applied in the loading buffer (5×), 400 mM Tris, pH 6.8, 100 mM EDTA, 50% glycerol and bromophenol blue 0.13 mg ml^{-1}. Samples run under denaturing conditions were first boiled in loading buffer (5×), 400 mM Tris (pH 6.8), 0.5 M DTT, 10% (w/v) SDS, 100 mM EDTA, 50% glycerol and bromophenol blue 0.13 mg ml^{-1}, for 5 min before loading.

Gels were either stained for total protein content using Coomassie blue stain R-250 (Sigma, UK) in 10% acetic acid/50% methanol, or were electroblotted onto nitrocellulose membranes for western blotting analysis in accordance with standard protocols (Sambrook et al., 1989). Dilutions of anti-sera used are shown in text and figure legends. Anti-(rabbit IgG) horseradish peroxidase conjugate or anti-(mouse IgG) horseradish peroxidase conjugate (both at 1/3000 dilution) (Sigma, UK) were used as secondary antibodies. Blots were developed using a chemiluminescence substrate, ECL+ kit from Amersham (Bucks, UK) in accordance with the manufacturer's instructions. Polyacrylamide gels containing ^{125}I–C3 were dried onto filter paper (using BioRad thermal gel dryer) and exposed to Kodak X-ray film for varying times (see figure legends).

2.3. Activation of complement alternative pathway by rabbit erythrocytes (REAP assays)

Reactions based on standard alternative pathway hemolytic assays (AH_{50}) were used (Coligan, 1994). Human serum (10 μl) and approximately 10^7 unsensitized rabbit erythrocytes in GVB/EGTA/10 mM $MgCl_2$ buffer (150 μl total volume) were incubated at 37 °C for various times as indicated in text and figure legends. When comparing with activation by CVF (Fig. 3A), fewer erythrocytes (10^6 cells) were added to REAP assays (2 μl) in order to equalize the kinetics of the turnover of factor B in the absence of SGE.

To test the effect of SGE in REAP assays, 5 μl (20 μg total protein) was added to the diluted serum (10 μl) before the activator (erythrocytes). All timed reaction samples were stopped by the addition of loading buffer (containing EDTA), either denaturing or non-denaturing as indicated in the figures, and placed on ice until gel loading.

2.4. Activation of complement alternative pathway by cobra venom factor (CVF)

Reactions contained 10 μl of human serum and 15 μl (1 mg ml^{-1} in PBS) of CVF (Sigma, UK) in GVB/EGTA/10 mM $MgCl_2$ buffer (total volume 150 μl), in the presence or absence of SGE (20 μg total protein). The SGE was added to the diluted serum before the addition of the activator (CVF). Reactions were incubated at 37 °C. All timed reaction samples were stopped by the addition of non-denaturing loading buffer (containing EDTA) and placed on ice until gel loading.