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Research paper

Monoclonal anti-neutrophil elastase antibody characterisation: Ability to block function, detect free versus serpin-complexed enzyme and stain intracellular granules

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ABSTRACT

Four commercially available monoclonal antibodies (clones NP57, 256-3K1, 39A and 203) were characterised for their ability to block human neutrophil elastase (HNE) activity; capture free purified HNE or neutralised HNE in complex with alpha-1-antitrypsin (AAT); detect HNE and HNE–AAT by Western blot analysis; and detect intracellular HNE by flow cytometry. The ability to block small substrate cleavage by HNE ranged from 0% (265-3K1) to 15–18% (39A and 203) to 100% (NP57). All antibodies had the ability to capture free HNE with varying degrees of sensitivity, but HNE neutralisation by AAT resulted in complete loss of detection (NP57) to 2–4-fold decreased detection (39A and 203) to a 8-fold increase in detection (265-3K1). None of the monoclonal antibodies could detect 200 ng of free HNE, or HNE in complex with AAT, by Western blot analysis, which was easily detected by polyclonal antibodies. NP57 and 265-3K1 gave 10-fold higher fluorescence when detecting intracellular HNE than 39A and 203, and intracellular fluorescence decreased by 10–28% following maximal stimulation of purified neutrophils with fMLP and cytochalasin B (compared to 40% release determined by functional assay). However, for sub-maximal stimulation of neutrophils intracellular anti-HNE antibody binding increased, likely due to increased accessibility following redistribution of enzyme, indicating that measuring residual intracellular HNE as an index of release is a less reliable method than directly measuring extracellular HNE.

1. Introduction

Human neutrophil elastase (HNE) is an anti-microbial enzyme contained within azurophilic (or primary) granules in granulocytes (Lominadze et al., 2005), which also plays a role in tissue remodelling and possesses secretagogue actions that are now recognised as important to local inflammatory responses (Chua and Laurent, 2006). The primary role of this proteinase is degradation and disposal of pathogens following merging of azurophilic granules with endocytic vesicles containing engulfed microbes to form the phagolysosome; however, following graded levels of neutrophil stimulation increasing HNE (as well as other azurophilic granule proteinases) is released extracellularly. It is likely that this is a defensive mechanism whereby maximum stimulation results in mass disgorgement of proteinases into the extracellular space as the balance of phagocytosis and disposal of microbes is overwhelmed and non-specific measures are required to fend off invading organisms. However, there are emerging reports that support a role for neutrophil serine proteinases in regulating non-infectious inflammatory processes by activating specific receptors and modulating levels of cytokines (reviewed in Pham, 2006). Varying degrees of HNE release are also observed in many disease states, irrespective of microbial involvement and are thought to contribute to their pathogenesis; these include emphysema (Campbell et al., 1999), cystic fibrosis (reviewed in Taggart et al., 2005), chronic lung

Abbreviations: HNE, human neutrophil elastase; AAT, alpha-1-antitrypsin (SERPINA1); fMLP, formyl–methionine–leucine–phenylalanine; rPE, phycoerythrin; APC, allophycocyanin.

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inflammation in premature infants and adults (reviewed in Moraes et al., 2003; Speer, 2003) and asthma (reviewed in Guay et al., 2006).

HNE is rapidly neutralised through irreversible complex formation with serine proteinase inhibitors (serpins) in a 1:1 stoichiometric ratio. The serpin found in highest concentration in plasma is alpha-1-antitrypsin (AAT or SERPINA1), which is produced by the liver and normally found in plasma at a concentration of 18–48 μmol/l (83–220 mg/dl) (ATS/ERS workgroup, 2003; Stoller and Aboussouan, 2005). Its importance is emphasised by the development of emphysema in adults with AAT deficiency, as a consequence of uncontrolled HNE activity in the lungs.

The ability to measure HNE is most important to assessing acute and chronic lung disease, especially when sampling the environment in the lower reaches of the lung through bronchoalveolar lavage (BAL). A few commercially available methods for measuring released HNE are available, these include measurement of functional activity through conversion of colourless or quenched substrates to coloured or fluorescent cleavage products, respectively, and measurement of protein levels with ELISA-based methods. Functional assays best assess overwhelming, unregulated proteinase activity, but do not give an indication of HNE levels that have had transient degradative ability and were subsequently neutralised by serpins. The available ELISA methods routinely utilise polyclonal capture antibodies and often rely on complex formation with AAT to detect HNE (i.e. capture with polyclonal anti-HNE antibody and detect with conjugated polyclonal anti-AAT antibody); however, this method could underestimate HNE where neutrophil infiltration and degranulation has overwhelmed the endogenous inhibitory serpin capacity or in patients with low AAT, which may be a more common deficiency than expected (de Serres, 2003). Other ELISA methods utilise the same polyclonal antibody to capture and detect (the detection antibody is directly conjugated to peroxidase or alkaline phosphatase), which could suffer from a narrower detection range than using a high affinity monoclonal capture antibody and a polyclonal detection antibody. Furthermore, the conformation (and likely the antigenicity) of HNE is dramatically altered following structural distortion mediated by the covalent mechanism by which AAT and other serpins neutralise HNE (Silverman et al., 2001). This could complicate measuring a sample containing free and neutralized neutralised HNE and also raises questions about the form of HNE provided as a standard in these methods.

We have investigated four commercial monoclonal anti-HNE antibodies for their ability to block HNE functional activity and compared their ability to detect free HNE and HNE–AAT complexes. We have also investigated the ability of these antibodies to detect intracellular HNE in purified neutrophils and compared the intracellular staining in pre- and post-stimulated neutrophils as an alternative method of investigating HNE release (by measuring residual HNE in neutrophils).

2. Materials and methods

2.1. Antibodies

Monoclonal anti-HNE antibodies were purchased from the RDI division of Fitzgerald Industries Intl. (Concord, MA): clone 203, isotype IgG1; Dako UK Ltd. (Ely, UK): clone NP57, isotype IgG1; Biogenesis Ltd. (Poole, Dorset, but now part of AbD Serotec, Oxford, UK): clone 39A, IgG1. Monoclonal anti-HNE antibody clone 265-3K1 (IgG1) was also provided as generous gift from Hycult Biotechnology b.v. (Uden, Netherlands). Phycoerythrin-conjugated goat anti-mouse immunoglobulin antibody was purchased from Dako UK Ltd and peroxidase-conjugated donkey anti-rabbit and anti-mouse immunoglobulin antibodies (minimum cross-reactivity with other species) were purchased from Jackson Immunoresearch (via Stratech Scientific Ltd., Newmarket, UK). Rabbit polyclonal anti-HNE antibodies were purchased from Calbiochem (via Merck Chemicals Ltd, Beeston, Nottingham, UK) and Athens Research and Technology (Athens, GA). Antibodies were directly conjugated to either phycoerythrin (rPE) or cross-linked allophycocyanin (APC) using Innova Bioscience's (Oxford, UK) Lightning-Link technology: briefly, antibodies were buffer exchanged into PBS and concentrated to 1 mg/ml using Vivaspin 10 kDa molecular mass cut-off centrifugal concentrators (purchased from Fisher Scientific Ltd UK), if they were not already provided as such, prior to 1/10 volume addition of provided modifier and reconstitution of lyophilised conjugate. Following an overnight incubation in the dark at room temperature, unreacted conjugate was neutralised with addition of a 1/10 volume of provided quencher for 1 h at room temperature in the dark. Antibodies were then ready to use as primary conjugates without further purification or manipulation.

2.2. Functional assay

Purified HNE was purchased for standard curves from Athens Research and Technology. Lyophilised HNE was reconstituted in 0.1 M sodium acetate (pH 5.5), 150 mM NaCl to 0.5 mg/ml and diluted in assay buffer (0.1 M Tris, pH 7.4, 500 mM NaCl) prior to functional assay. Assay buffer was supplemented with different detergents including Tween20, Brij 35 and Triton X-100 at final concentrations from 1.0% to 0.001% and used to create doubling dilution standard curves of purified HNE from 40 nM (1.2 μg/ml) to 0.32 nM. An equal volume of assay buffer (lacking deterents) containing 3 mg/ml Suc-Ala-Ala-Pro-Val-pNA (Bachem AG, Bubendorf, Switzerland) as the chromogenic substrate was added to diluted HNE just prior to measurement. HNE conversion of the colourless substrate to a yellow cleaved product was measured kinetically using an MRX revelation plate reader (Dynex Technologies, Chantilly, VA). The plate reader was set to 37 °C and took measurements every 60 s for 1 h at 405 nm. The integral software then calculated HNE concentration against the known standard curve (which was diluted in 0.5% Triton X-100) using the rate of conversion (slope). Since the same aliquot of purified HNE was used to generate all dilution curves (which only varied in the type and concentration of detergent), the ratio of relative calculated activity and actual HNE concentration could be determined. The ability of monoclonal antibodies to block HNE function was determined by incubating a molar ratio of antibody to HNE at 1:1 or 4:1 (not taking into consideration the bivalency of Fab recognition sites) in detergent-free activity buffer for 20 min at room temperature prior to addition of chromogenic substrate and comparison against activity