BACTERICIDAL PROTEINS GROUP IIA PHOSPHOLIPASE A_2 AND BACTERICIDAL/PERMEABLILITY-INCREASING PROTEIN IN HUMAN LACRIMAL GLAND AND TEARS

by

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ABSTRACT

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Bactericidal proteins group IIA phospholipase A_2 and bactericidal/permeability-increasing protein in human lacrimal gland and tears

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The open eye is constantly exposed to environmental pathogens and therefore, it is vulnerable to infections. Host defence requirements are supplied by the tear film. Tears contain various bactericidal proteins, derived mainly from the lacrimal glands. Recently discovered antimicrobial elements in tears have expanded the armamentarium of protection.

This study focused on the antimicrobial proteins group IIA phospholipase A_2 (PLA_2GIIA) and bactericidal/permeability-increasing protein (BPI) in the human eye, and on the development of fluoroimmunoassays for PLA_2GIIA and BPI. These assays were optimized for venous blood samples, and their applicability was evaluated with tear fluid samples from normal and diseased eyes.

In the first part of the study, immunohistochemical analysis showed that the lacrimal gland of a healthy eye contains PLA_2GIIA. Higher concentration levels of this protein were found in tear fluid than in venous samples of healthy individuals.

In the second and third parts of the study, the PLA_2GIIA concentration was measured in the tears of healthy and diseased eyes. Contact lenses may absorb PLA_2GIIA, but their use did not permanently affect the secretion of the protein. A diurnal concentration variation of PLA_2GIIA was detected, which is important for sampling. Comparison to normal tears showed that PLA_2GIIA levels were decreased in the eyes of atopic patients.

In the fourth part of the study, an antibody was generated and a fluoroimmunoassay was developed for BPI. After an evaluation, measurements in blood samples showed that plasma should be used. In the last part of the study, this fluoroimmunoassay was used to examine the BPI concentration in tears of healthy subjects. This protein was also identified by immunohistochemical and Western blot analyses in the lacrimal gland of human eye.

The present results show that PLA_2GIIA and BPI are noteworthy bactericidal agents in protection of human eyes against microbes. The fluoroimmunoassays which were developed will facilitate future clinical studies.

Key words: tears, lacrimal gland, fluoroimmunoassay, phospholipase A_2, bactericidal/permeability-increasing protein, bactericidal protein

TIIVISTELMÄ

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Bakteriosidiset proteiinit ryhmän IIA fosfolipaasi A_2 ja bactericidal/permeability-increasing proteiini ihmisen silmässä

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Silmä on auki ollessaan alttiina ympäristön patogeenien aiheuttamille infektioille. Koska sarveiskalvolla ei ole verisuonistoa, sen puolustus tapahtuu kyynelkalvon välityksellä. Kyynelnesteessä on useita bakteriosidisia proteiineja, jotka erittyvät pääosin kyynel- ja apukyynelrauhasista. Viime aikoina on löydetty uusia kyynelnesteen puolustusmekanismiin vaikuttavia yhdisteitä.

Tämän tutkimuksen tarkoitus oli selvittää kahden antimikrobisen proteiinin, ryhmän IIA fosfolipaasi A_2:n (PLA_2GIIA) ja bactericidal/permeability-increasing proteiinin (BPI) esiintymistä ihmisen silmässä sekä kehittää näille proteiineille luotettavampia immunomääritysmenetelmiä. Määritysmenetelmien avulla on tutkittu kyynelnesteestä PLA_2GIIA:n ja BPI:n pitoisuuksia sekä pyritty arvioimaan tulosten merkitystä silmäsairauksissa verrattuna terveeseen silmään.

Tutkimuksen ensimmäisessä osassa osoitettiin immunohistokemiallisin värjäyksin PLA_2GIIA:ta esiintyvän kyynel- ja apukyynelrauhasissa. Samalla mitattiin myös ensimmäistä kertaa hyvin korkeita tämän proteiinin pitoisuuksia verrattuna verinäytteistä saatuihin arvoihin.

Tutkimuksen toisessa ja kolmannessa osatyössä tutkittiin PLA_2GIIA:n pitoisuuksia terveessä ja sairaassa silmässä. Proteiinilla todettiin merkittävä vuorokautinen vaihtelu kyynelnesteessä, joka tulee huomioida määrityksiä suoritettaessa. Piilolasit voivat absorboida PLA_2GIIA:ta ja vähentää siten sen pitoisuutta kyynelnesteessä, mutta tämä vaikutus ei ole pysyvää käytön päättyessä. Atooppinen silmäluomen tulehdus vähentää PLA_2GIIA:n määrää kyynelnesteessä, mikä saattaa lisätä herkkyyttä bakteeritulehduksille.

Neljännessä osatyössä tuotettiin vasta-aine BPI:lle sekä kehitettiin fluoroimmunomenetelmä BPI:n määrittämiseksi verinäytteistä. Tutkimuksissa todettiin, että määrityksen luotettavuus edellyttää, että se suoritetaan plasmanäytteistä eikä seerumista. Viimeisessä osatyössä BPI vasta-aineella osoitettiin immunohistokemiallisin ja Western blot analyysein BPI:n esiintyvän ihmisen kyynelrauhasessa. Fluoroimmunomenetelmällä tutkittiin BPI:n pitoisuutta ihmisen normaalissa kyynelnesteessä.

Yhteenvetona voidaan todeta, että PLA_2GIIA ja BPI ovat tärkeä osa ihmisen silmän luonnollista puolustusjärjestelmää ja kehitetyt määritysmenetelmät helpottavat jatkotutkimuksia.

Avaintermit: kyynelneste, kyynelrauhanen, fluoroimmunomenetelmä, fosfolipaasi A_2, bactericidal/permeability-increasing proteiini, bakteriosidinen proteiini

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ABBREVIATIONS

AA	arachidonic acid
ABC	atopic blepharoconjunctivitis
ANCA	anti-neutrophil cytoplasmic antibodies
AP	alkaline phosphatase
BPI	bactericidal/permeability-increasing protein
BSA	bovine serum albumin
BUT	break up time
^{14}C	isotope 14 of carbon
CAP 57	57 kDa cationic antimicrobial protein (= BPI)
CD14	cluster of differentiation antigen 14
CL	contact lenses
CoA	coenzyme A
CONS	coagulase-negative staphylococci
$cPLA_2$	cytosolic phospholipase A_2
CRP	C-reactive protein
DTPA	diethylenetriaminepentaacetic acid
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EIA	enzymeimmunoassay
ELISA	enzyme-linked immunosorbent assay
FIA	fluoroimmunoassay
GAL	ß-galactosidase
GPI	glycophosphatidylinositol
^{3}H	isotope 3 of hydrogen (= tritium)
HEPC	2-hexadecanoylthio-1-ethylphosphorylcholine
HP	high performance
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidise
^{125}I	isotope 125 of iodine
IBS	interfacial binding surface
IEMA	immunoenzymometric
IFCC	International Federation of Clinical Chemistry
IFMA	immunofluorometric assay
IgA	immunoglobulin A
IgE	immunoglobulin E
IgG	immunoglobulin G
IL	interleukin
$iPLA_2$	calcium-independent phospholipase A_2
IQR	interquartile range
IRMA	immunoradiometric assay
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
MAPS	monoclonal antibody purification system

mRNA	messenger ribonucleic acid
OA	osteoarthritis
PAF	platelet-activating factor
PBS	phosphate buffered saline
PLA_2	phospholipase A_2
PLA_2GIIA	group IIA phospholipase A_2
PMN	polymorphonuclear leucocytes (neutrophils)
PsA	psoriatic arthritis
RA	rheumatoid arthritis
RIA	radioimmunoassay
sCD14	soluble form of cluster of differentiation antigen 14, CD14
SBC	staphylococcal blepharoconjunctivitis
SD	standard deviation
SDS	sodium dodecyl sulfate (lauryl sulfate)
SEM	standard error of mean
sIgA	secretory immunoglobulin A
SLPI	secretory leukocyte protease inhibitor (antileukoprotease)
$sPLA_2$	secreted phospholipase A_2
SPT	skin prick test
TLC	thin-layer chromatography
TNF	tumor necrosis factor
TR-FIA	time-resolved fluoroimmunoassay
TSA	tris saline azide (buffer solution)
TSL	tear specific lipocalin
VEGF	vascular endothelial growth factor

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, referred to in the text by Roman numerals. In addition, some unpublished results are presented.

I Nevalainen TJ, Aho HJ, Peuravuori H. Secretion of group 2 phospholipase A_2 by lacrimal glands.
Invest Ophthalmol Vis Sci 1994; 35: 417-421

II Aho VV, Paavilainen V, Nevalainen TJ, Peuravuori H, Saari KM.
Diurnal variation in group IIa phospholipase A_2 content in tears of contact lens wearers and normal controls.
Graefes Arch Clin Exp Ophthalmol 2003; 241: 85-88

III Peuravuori H, Kari O, Peltonen S, Aho VV, Saari JM, Collan Y, Määttä M, Saari KM. Group IIA phospholipase A_2 content of tears in patients with atopic blepharoconjunctivitis.
Graefes Arch Clin Exp Ophthalmol 2004; 242: 986-989

IV Häggblom JO, Jokilammi-Siltanen AB, Peuravuori H, Nevalainen TJ. Time-resolved fluoroimmunoassay for bactericidal/permeability-increasing protein.
Mediat Inflamm 1996; 5: 47-50

V Peuravuori H, Aho VV, Aho HJ, Collan Y, Saari KM.
Bactericidal/permeability-increasing protein in lacrimal gland and in tears of healthy subjects.
Graefes Arch Clin Exp Ophthalmol 2006; 244: 143-148

1. INTRODUCTION

The ocular surface, being constantly exposed to environmental pathogens, is particularly vulnerable to infection. Evolution has provided the ocular surface with multiple defence mechanisms, many of which operate through the tear fluid. Delivery of aqueous tears from the lacrimal gland is continuous during waking hours, and the tear film serves to lubricate the ocular surface preventing its dehydration. The transparent cornea of the eye lacks blood vessels and therefore, many of its defence requirements are supplied by the film of tears. This aqueous, isotonic layer is a complex mixture of electrolytes, glycoproteins and proteins derived mainly from the lacrimal and accessory lacrimal glands (Bron et al., 2004). Traditionally, lysozyme (muramidase) has been thought to be responsible for antimicrobial activity, especially in tears. However, tear fluid also contains other bactericidal compounds. In this context, two recently recognized host defence proteins are of interest, group IIA phospholipase A_2 (PLA_2GIIA) and bactericidal/permeability-increasing protein (BPI).

Phospholipase A_2 (PLA_2) forms a large family of phospholipid-hydrolyzing enzymes. These enzymes catalyse the hydrolysis of phospholipids into free fatty acids and lysophosholipids. Snake venom and mammalian secretory PLA_2s ($sPLA_2$) are calcium-dependent enzymes that play an important role in phospholipid metabolism, inflammation, host defence and digestion (Six and Dennis, 2000). PLA_2s are classified on the basis of their nucleotide and amino acid sequence into twelve distinct groups of PLA_2s, most having several subgroups. To date, 10 catalytically active $sPLA_2$s have been identified in mammals and all these enzymes have been sequenced, cloned and expressed in bacteria. In this diverse superfamily of PLA_2s, PLA_2GIIA has so far been shown to have the most important role in human diseases. This enzyme is involved in inflammation (Nevalainen et al., 2000) and is expressed at mRNA and protein levels in Paneth cells of the small intestinal mucosa, prostatic epithelial cells and cartilage (Kallajoki and Nevalainen, 1997, Leistad et al., 2004). PLA_2GIIA has antibacterial activity against gram-positive bacteria (e.g., in human serum; Grönroos et al., 2002), but as for the other $sPLA_2$s, the physiological and pathological functions of PLA_2GIIA are largely unknown.

High concentrations of PLA_2GIIA have been measured in tears of the normal human eye (Nevalainen et al., 1994), and this high level of PLA_2GIIA in tears has been shown to be capable of killing a wide range of gram-positive bacteria *in vitro* (Qu and Lehrer, 1998). The bactericidal action of PLA_2GIIA against gram-negative bacteria is more complex than its action against gram-positive bacteria. The enzyme is bactericidal against *Escherichia coli* (*E. coli*) only when acting simultaneously and synergistically with BPI, an antimicrobial protein produced by polymorphonuclear leucocytes (PMN) (Weiss et al., 1994). BPI partly destroys the lipopolysaccharide structure of gram-negative bacteria, and so enables phospholipid hydrolysis of the bacteria by PLA_2GIIA (Madsen et al., 1996). PLA_2GIIA binds to the bacterial cell surface of gram-positive bacteria and penetrates through the thick peptidoglycan layer which bears a highly anionic charge due to the presence of phosphate diester units of

lipoteichoic acid. PLA_2GIIA then degrades phospholipids in the cell membrane and activates bacterial autolysis (Foreman-Wykert et al., 1999).

The major inducers of host response against gram-negative bacteria are lipopolysaccharides (LPS), a group of high-molecular weight complexes which constitute the major cell wall component. BPI shows high affinity to the lipid A moiety, common to all LPSs (Gazzano-Santoro et al., 1992). BPI was identified in the azurophilic granules of human PMN and shown to be antibacterial towards *E. coli* and several other gram-negative bacteria (Weiss et al., 1978). Nanomolar concentrations of BPI in biological fluids may be adequate to kill gram-negative bacteria (Weiss et al., 1992). BPI is an attractive target for biopharmaceutical development, and recombinant protein fragments have been evaluated in pharmaceutical trials in humans (Levy 2002).

2. REVIEW OF THE LITERATURE

2.1. Tear film structure and function

The outer surface of the cornea is covered by the pre-corneal tear film (Figure 1). People normally blink their eye every sixth second to replenish the tear film. Tears act as both a delivery mechanism and an excretory route for nutrients and metabolic products of the corneal epithelium and stroma. Delivery of tears from the lacrimal glands is continuous during waking hours, and fresh fluid remains in the upper and lower marginal meniscus, and possibly under the upper lid, until after the next blink, when fluid is drawn from the menisci to form the tear film. The tear film improves the quality of the retinal image by smoothing out irregularities of the cellular surface. Tears wet the corneal epithelium. In addition, blinking spreads mucus over the epithelial surface and keeps the film hydrated allowing it to act as a lubricant and a protective barrier (Tutt et al., 2000). The nasolacrimal system serves as the drain for waste products (Figure 1).

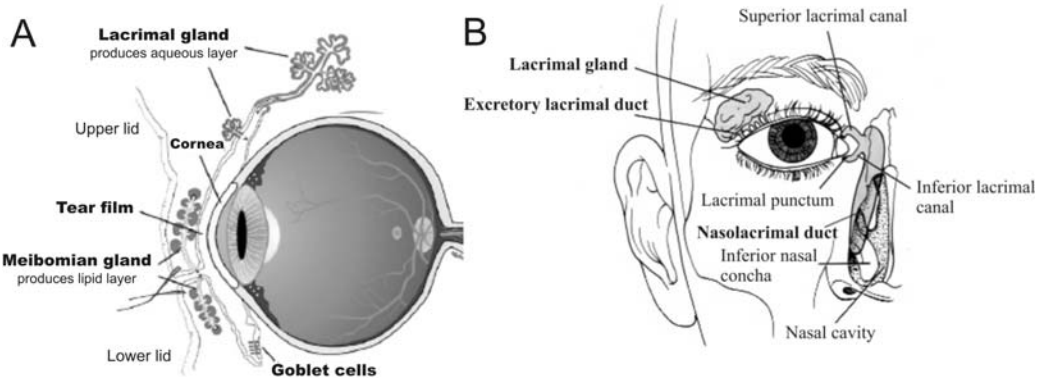

Figure 1. Schematic representation of the tear-producing tissues. Figure A shows a cross-section of the eye and Figure B the lacrimal gland localized in the orbit with drainage of tears. Figure A reprinted from Prog Retin Eye Res, 21, Dartt DA, Regulation of mucin and fluid secretion by conjunctival epithelial cells, 555-576, © 2002, with permission from Elsevier. Figure B modified from a picture of http://www.health-pictures.com.

Tear film is considered to have a three-layer structure: lipid layer, aqueous layer and deep mucin layer, which are in a dynamic equilibrium (Figure 2A). This classical description was provided first by Wolf (Wolf, 1946). The estimated thickness of the total film varies between 6 and 12 μm (Creech et al., 1998). The outer lipid or oil layer protects its inner lacrimal layer from evaporation. It also prevents the tears from flowing over the edge of the lower eyelid. The lipid component of the tear film is produced by sebaceous glands, known as Meibomian glands, and the glands of Zeis. The action of blinking squeezes or massages the Meibomian glands within the tarsal plates delivering fresh oil to the lid margin (Bron et al., 2004; Chew et al., 1993). The

lacrimal or aqueous middle is the thickest of the three tear layers. It is formed primarily by the glands of Krause and Wolfring and secondarily by the lacrimal gland, all of which are located in the eyelids (Figure 1). The lacrimal gland is the major producer of tears when one is crying or in the case of irritation of eyes by foreign bodies. Secretion of tears is regulated by neural and humoral stimuli (Dartt, 2002). The lacrimal fluid takes the main nutrients and oxygen to the cornea, delivers waste products away from the cornea, helps to prevent corneal infection, and maintains the tonicity of the tear film. The epithelial surface of the cornea is hydrophobic in nature and therefore, the third mucin layer is needed to keep the lacrimal layer from rolling off. This layer is spread onto the cornea by the goblet cells situated in the bulbar conjunctiva (Figure 1). Mucin layer is mainly composed of mucins and inorganic salts suspended in water (Johnson and Murphy, 2004). The mucins are a family of exceptionally large glycoproteins with at least half of their mass *O*-linked carbohydrates and are classified as transmembrane or secretory proteins. They are produced by conjunctival and corneal (ocular surface) epithelial cells. Depending upon the type, these mucins either form the glycocalyx, a filamentous coating on the apical surface of epithelial cells, or are secreted and become incorporated into the mucous layer in the tear film that covers the ocular surface. The mucous layer provides a physical and chemical barrier that protects the ocular surface from viral and bacterial pathogens, desiccation, chemical accidents, mechanical trauma, and thermal burns (Lamberts, 1994). The carbohydrate rich glycocalyx therefore separates the cellular corneal epithelial sheet and the overlying aqueous phase of the tear film (See Figure 2A).

In addition to the above-described and largely accepted three-layer model (Figure 2A), a model consisting of only two layers in the tear film has been presented (the bi-phasic model; McCulley and Shine, 1997: See Figure 2B). This model is supported by a large proportion of heterogenous hydrophobic lipids in the tear film (Bron and Tiffany, 1998) and the presence of active phospholipase A_2 (Qu and Lehrer, 1998). The model suggests that the polar glycocalyx directly anchors a continuous aqueous-mucin layer. Tear specific lipocalin (TSL), formerly known as tear specific pre-albumin (Redl 2000), enhances the spreading and stability of the lipid layer by forming complexes with polar lipids, thereby decreasing the surface tension of aqueous phase.

Because the cornea lacks blood vessels, host defense requirements are supplied by the tear film. The aqueous isotonic layer is a complex mixture of electrolytes, glycoproteins and proteins, derived mainly from the lacrimal and accessory lacrimal glands. The protein profile of lacrimal secretions consists of three major entities: lysozyme, lactoferrin, and TSL, as well as several minor entities (Sack et al., 1992; Molloy et al., 1997) augmented by increasing quantities of secretory immunoglobulin A (sIgA) as the rate of secretion decreases (Sack et al., 1992; Fullard and Snyder, 1990; Fullard and Tucker, 1991; See Table 1). TSL is the major lipid binding protein in tears, but it also scavenges contaminating lipids from the human corneal surface and delivers them into the aqueous phase of tears (Gasymov et al., 2005).

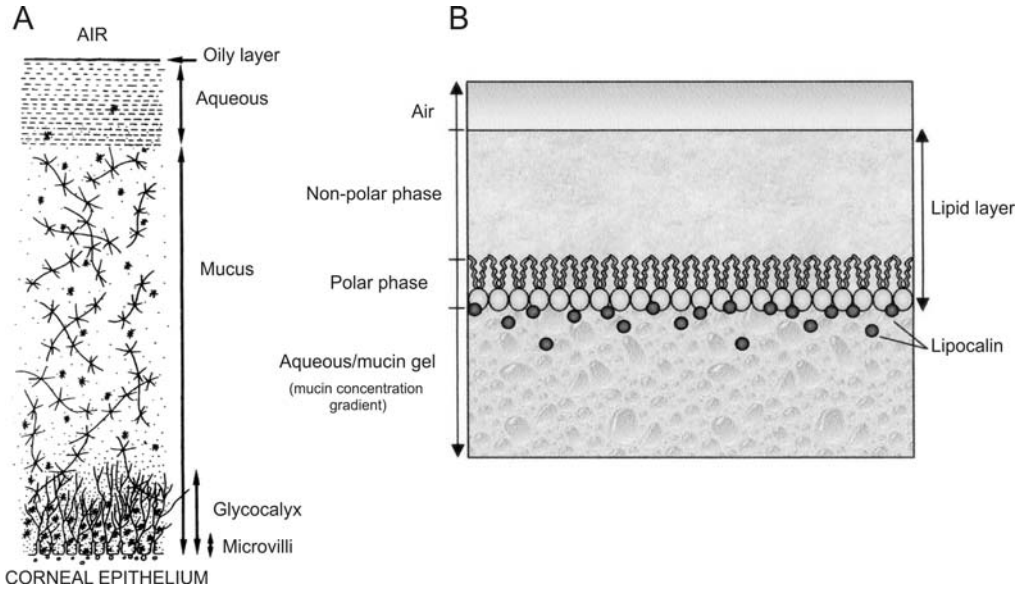

Figure 2. In Firure 2A is shown the three layer model of the tear film and in Figure 2B is shown the model where the lipid layer of the tear film has a biphasic structure. In Figure 2A is presented the aquous and mucin layers and the epithelium with its glycocalyx. In this model a large part of the thickness is occupied by the mucous layer. In Figure 2B of the biphasic model the relatively thick outer layer consists of nonpolar lipids and the inner layer contains polar phospholipids. The nonpolar lipids are generally thought to retard aqueous evaporation, whilst the underlying phospholipids facilitate interaction with the aqueous layer. Figure 2A is reprinted from Exp Eye Res, 78, Bron et al., Functional aspects of the tear film lipid layer, 347-360, © 2004 and Figure 2B from Prog Retin Eye Res, 23, Johnson ME, Murphy PJ, Changes in the tear film and ocular surface from dry eye syndrome, 449-474, © 2005, with permission from Elsevier.

The levels of the major tear proteins decrease with age, as do the volume of tears (van Haeringen 1997). During sleep, secretion of all major proteins and water is inhibited, but sIgA release continues, producing a highly concentrated solution in a limited amount of fluid. Polymorphonuclear leucocytes (PMN) migrate into the conjunctival sac and secrete a variety of destructive and remodelling enzymes thus effectively creating a subclinical inflammatory state (Tan et al., 1993). The open eye tear film contains a resident pool of inactive PMN cells associated with the mucosal layer (Sakata et al., 1997). Sack et al. (1992) reported that the concentration of total tear proteins was 9 - 16 mg/ml between the states of reflex, open eye, and closed eye. Huth et al. (1981) found no diurnal variation in total protein concentration and this has been confirmed in a more resent study (Ng et al., 2001). Diurnal variation affects the levels of lysozyme in non-stimulated conditions, increasing from 9 a.m. to 12 noon and being lowest at midnight to 3 a.m., while there is no change in sIgA levels (Horwitz et al., 1978). Sen and Sarin (1986) used stimulated tears and also found significant daytime variation of lysozyme. However, in their study, the lysozyme level was lowest at 6 a.m. and increased to the highest value at 10 p.m. These different results may be

due the divergence in sampling methods, type of tears in study and analysis methodologies.

Table 1. Composition of human tears

Main components
Water
Electrolytes (Na^+, K^+, Cl^-, HCO^- and lesser levels of Ca^{2+}, Mg^{2+})
Proteins (lysozyme, lactoferrin, lipocalin, secretory IgA, phospholipase A_2, albumin and IgG, the latter two through leakage from conjunctiva)
Lipids (from Meibomian gland products, lipocalin-associated)
Mucins (epithelial membrane-anchored type, soluble goblet-cell type)
Defensins, collectins, related small molecules
Additional components
Lipid inflammatory mediators (leukotriene B_4, PAF)
Cytokines
Growth factors
Polymorphonuclear leukocytes
Antigens (e.g. cytokeratins, carcinoembryonic antigen, epithelial membrane antigen)
Signaling molecules
Complement components
Remodeling enzymes (e.g. matrix metalloproteases)

2.2. Bactericidal substances in tear fluid

The first-known antimicrobial substance in the human body, lysozyme, was discovered more than 80 years ago. Lysozyme was found to kill *Micrococcus lysodeikticus* (Fleming 1922; Fleming and Allison, 1922). Since then, the antimicrobial properties of tear fluid have been extensively studied and it has been shown that tear fluid is highly enriched with lysozyme and lactoferrin (Berta, 1991; Kijlstra, 1990). Lysozyme kills gram-positive bacteria by hydrolyzing linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine which stabilize the peptidoglycan constituting the cell wall (Van Haeringen, 1981). Tear fluid is highly enriched in lactoferrin, (Berta, 1991) inhibiting microbial metabolism by its strong iron-binding properties (Broekhuyse, 1974), and it may alter the membrane of gram-negative bacteria allowing subsequent lysis by lysozyme and other enzymes (Ellison and Giehl, 1991). Tear film immunoglobulins (e.g., sIgA) can bind the surface of microbial pathogens and block their interaction with specific epithelial surface receptors, or they may cause aggregation of bacteria thus preventing microbial access to surface receptors. Bacterial-bound sIgA may induce a respiratory burst and phagocytosis by PMN (Willcox and Lan, 1999; Said et al., 2004).

Recently discovered antimicrobial substances in tears have expanded the armamentarium of protection of eyes. These elements include secretory group IIA

phospholipase A_2 (Nevalainen et al., 1994; Qu and Lehrer, 1998), several members of the defensin family (Haynes et al., 1999; McNamara et al., 1999), the bi-functional antimicrobials/antiproteases, a specific secretory leukocyte protease inhibitor (SLPI, also known as antileukoprotease; Hiemstra et al., 1996), and pro-elafin (Sathe et al., 1998). In upper respiratory tract secretions, SLPI has been shown to be ionically associated with mucin (Nadziejko and Finkelstein, 1994). It can be assumed that other negatively charged small antimicrobial agents in tears are also associated with mucin. SLPI's broad anti-microbial activity and ability to rapidly inactivate serine proteases secreted by PMN, positions SLPI in co-operation with scavenging PMN cells to restrict microbial proliferation, while simultaneously protecting the underlying epithelium against damage. This is consistent with the finding that open-eye tear film has a resident pool of inactive PMN cells associated with the mucosal layer (Sakata et al., 1997).

2.3. Phospholipase A_2

Phospholipases A_2 (PLA_2s, phosphatidylcholine-2-acylhydrolases, EC 3.1.1.4) are enzymes that hydrolyze the sn-2 ester bond in membrane phospholipids to release free fatty acids and lysophospholipids (Figure 3).

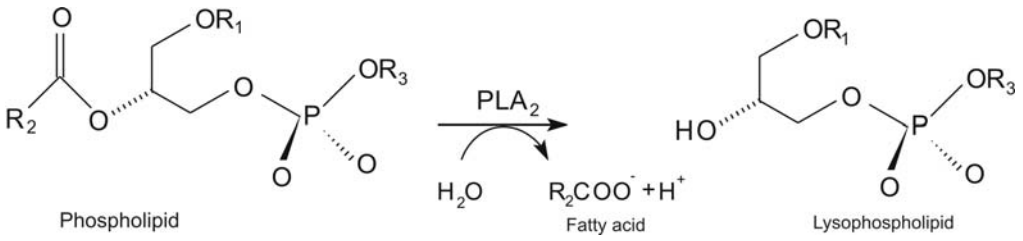

Figure 3. The hydrolysis of phospholipid by phospholipase A_2. R, alkyl group; H_2O, water molecule.

PLA_2s function in the breakdown of dietary lipids, in acyl-chain remodeling of phospholipids, in production of lipid-mediators and in host defense against microorganisms. The corresponding hydrolytic products are important precursors of bioactive substances. Arachidonic acid (AA) generated by PLA_2s is metabolized by cyclo-oxygenase and lipoxygenase to prostanoids and leukotrienes, which act as proinflammatory lipid mediators. Lysophospholipids function as biological mediators to trigger cellular signaling or as the precursors of the platelet-activating factor (PAF).

There are numerous genes in mammals encoding PLA_2 enzymes that comprise three main types: the secreted PLA_2s ($sPLA_2$) which require millimolar concentrations of Ca^{2+} (with exception of group XIIA $sPLA_2$ which requires micromolar levels of Ca^{2+} and group XIIB $sPLA_2$ which lacks enzymatic activity), the group IV cytosolic PLA_2s ($cPLA_2$) which require micromolar concentrations of Ca^{2+}, and the group VI calcium-independent PLA_2s ($iPLA_2$) which do not require Ca^{2+} for the activity (Six and Dennis, 2000; Valentin and Lambeau, 2000; Kudo and Murakami, 2002). Cells contain various

enzyme types of PLA_2s, suggesting that they play distinct functional roles. $sPLA_2$s are stored in cytosolic granules or synthesized upon stimulation and then secreted extracellularly. $cPLA_2$s and $iPLA_2$s are intracellular enzymes, but $cPLA_2$s are located in the cytosol and translocated into the membrane, while $iPLA_2$s are located both in the cytosol and in membrane fractions. None of the $sPLA_2$s have sequence homology with the intracellular PLA_2s, but they do share a number of common properties including a relatively low molecular mass of 14 - 19 kDa (except for group III $sPLA_2$), a large number of disulfides, and a similar Ca^{2+}-dependent catalytic mechanism (Berg et al., 2001). Currently ten distinct groups of $sPLA_2$s have been identified with several subgroups (Six and Dennis, 2000; Balsinde et al., 2002: See Table 2).

Table 2. Secreted phospholipases A_2 (modified from Balsinde et al., 2002)

Extracellular enzymes utilizing a catalytic histidine (exception group XIIB witch has no enzymatic activity, histidine mutated to leucine in the active site)				
Group		Initial/common sources	Size (kDa)	Disulfides
I	A	Cobra and krait venom	13-15	7
	B	Mammalian pancreas	13-15	7
II	A	Human synovial fluid, platelets and tears; rattlesnake and viper venom	13-15	7
	B	Gaboon viper venom	13-15	6
	C	Rat and mouse testis	15	8
	D	Human and mouse pancreas and spleen	14-15	7
	E	Human and mouse brain, heart and uterus	14-15	7
	F	Human and mouse testis and skin	16-17	7
III		Bee, lizard, and scorpion venom Human kidney, heart and skeletal muscle	15-18 55	5
V		Mammalian heart, lung and macrophages	14	6
IX		Marine snail venom (Conodipine-M)	14	6
X		Human spleen, thymus and leukocytes	14	8
XI	A	Green rice shoots	12.4	6
	B	Green rice shoots	12.9	6
XII	A	Mammalian heart, kidney, skin, pancreas and muscle	18.7	7
	B	Human and mouse liver, small intestine and kidney	19.7	7
XIII		Parvovirus	< l0	0
XIV		Symbiotic fungus and *Streptomyces*	13-19	2

PLA_2 was first identified in snake venom and mammalian pancreas (van den Bosch, 1980); PLA_2IA in cobra venom, and PLA_2IB in pancreatic acinar cell secretions. In the duodenal lumen PLA_2IB acts as a digestive enzyme for dietary phospholipids (Borgström, 1980). The secreted PLA_2s do not have specificity for arachidonate-

containing phospholipids. $cPLA_2$s are larger molecules (>60 kDa) and preferentially hydrolyze arachidonate-containing phospholipids. $iPLA_2$s (about 85 kDa) are not selective for arachidonate-containing phospholipids (Six and Dennis, 2000: See Table 3).

Table 3. Cytosolic and calcium-independent phospholipases A_2 (modified from Balsinde et al., 2002)

Intracellular enzymes utilizing a catalytic serine					
Group		Initial/common sources	Alternate name	Size (kDa)	Calcium requirement
IV	A	Human U937 cells and platelets	$cPLA_2\alpha$	85	<mM; membrane translocation
	B	Human pancreas, liver, heart and brain	$cPLA_2\beta$	114	<mM; membrane translocation
	C	Human heart and skeletal muscle	$cPLA_2\gamma$	61	None
VI	A-1	P388D, macrophages, CHO	$iPLA_2$ or $iPLA_2$-A	84-85	None
	A-2	Human B-lymphocytes and testis	$iPLA_2$-B	88-90	None
	B	Human heart and skeletal muscle	$iPLA_2\gamma$ or $iPLA_2$-2	88	None
VII	A	Human/mouse/porcine/bovine plasma	PAF-AH	45	None
	B	Human/bovine liver and kidney	PAF-AH (II)	40	None
VIII	A	Human and bovine brain	PAF-AH Ib α_1	26	None
	B	Human and bovine brain	PAF-AH Ib α_2	26	None

To date, 10 catalytically active $sPLA_2$s have been identified in mammals (PLA_2GIB, -GIIA, -GIIC, -GIID, -GIIE, -GIIF, -GIII, -GV, -GX and GXIIA; See Table 2) and all these enzymes have been sequenced, cloned and expressed in bacteria. The group I, II, V and X $sPLA_2$s are closely related, with a highly conserved Ca^{2+}-binding loop (Xxx, Cys, Gly, Xxx, Gly, Gly) and a catalytic site (Asp, Xxx, Cys, Cys, Xxx, Xxx, His, Asp). There are six conserved disulfide bonds and up to two additional unique disulfide bonds, which contribute to the high degree of stability of these enzymes (Kudo and Murakami, 2002; See Figure 4). Substrate hydrolysis proceeds through the activation and orientation of a water molecule by hydrogen bonding to the active site histidine. This dictates the pH dependence of 7-9 for all $sPLA_2$s. Adjacent to the histidine, there is a conserved aspartate residue, which, together with the Ca^{2+}-binding loop, acts as a ligand cage for Ca^{2+}. $sPLA_2$s hydrolyze the ester bond at the sn-

2 position of glycerophospholipids in the presence of micromolar to millimolar concentrations of Ca^{2+} without showing strict fatty acid selectivity. Most group II $sPLA_2$s act on anionic phospholipids in marked preference to charge-neutral phosphatidylcholine, PLA_2GV and -GX hydrolyze more efficiently zwitterionic phospholipids such as phosphatidylcholine and phosphatidylethanolamine, and PLA_2GIB is intermediate. PLA_2GIII and PLA_2GXIIA share homology with the group I, II, V and X $sPLA_2$s only in the Ca^{2+}-binding loop and catalytic site, thereby representing distinct group relatioship for group III and XII collections (Kudo and Murakami, 2002; Singer et al., 2002). The genes for PLA_2GIIA, -GIIC, -GIID, -GIIE, -GIIF and -GV are clustered in the same region of human chromosome 1 (Valentin et al., 2000), whereas structurally more distant PLA_2GIB, -GIII, -GX and -GXIIA lie on chromosomes 12, 22, 16 and 4, respectively (Gelb et al., 2000). All $sPLA_2$s have a signal sequence which is cleaved in the process of secretion of the mature protein. The only exceptions to this are PLA_2GIB and PLA_2GX, which are secreted with a propeptide that must be cleaved by trypsin to produce the mature active enzyme (See Figure 4).

Bactericidal properties of PLA_2s have been widely investigated. The best characterized bactericidal enzyme is PLA_2GIIA. Human PLA_2GV also possesses antibacterial potential against gram-positive bacteria, but the effect is weaker than PLA_2GIIA. PLA_2GV is bactericidal against *Listeria monocytogenes* (*L. monocytogenes*), methicillin-sensitive *Staphylococcus aureus* (*S. aureus*), *Enterococcus faecalis*, and *Enterococcus faecium* (Grönroos et al., 2001). The human PLA_2IB, -IIE, -IIF, and -X do not appear to have any antibacterial role against *Micrococcus lutenia* (Beers et al., 2002). Order of potency against *L. monocytogenes* and *S. aureus* is as follows: human PLA_2GIIA > -GX > -GV > -GXII > -GIIE > -GIB, -GIIF (devoid of bactericidal activity). Only human PLA_2GXII showed weak activity against gram-negative *E. coli* (Koduri et al., 2002). However, poorly bactericidal porcine pancreatic PLA_2IB was converted by site-directed mutagenesis to a stronger bactericidal agent against *E. coli* treated with bactericidal/permeability-increasing protein (BPI; Weiss et al., 1991). PLA_2GIIA is also bactericidal by synergistically acting against *E. coli* with BPI. Recent studies show that human PMN contain PLA_2GV and -X. It is postulated that PLA_2GV binds to *E. coli* and enhances hydrolysis of bacterial phospholipids after phagocytosis of the bacteria by PMN in line with PLA_2GIIA. PLA_2GV is present both in azurophilic (primary) and specific (secondary) granules, while PLA_2GX is contained solely in the former (Degousee et al., 2002). PLA_2IVA has been shown to be involved in neutrophil-mediated gram-negative bacterial killing and in the PAF biosynthesis of PMNs (Rubin et al., 2005). Many PLA_2s, including PLA_2IVA, are considered to be part of the innate immunity system. In fact, it seems that PLA_2s form a group of defensive proteins whose activity varies against different bacteria.

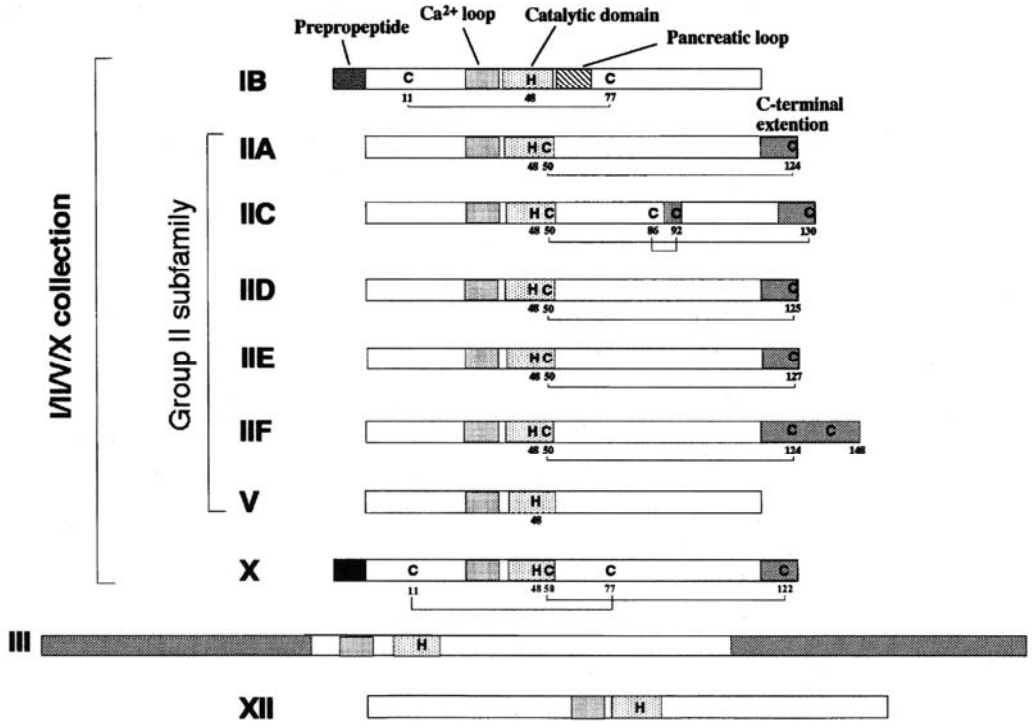

Figure 4. Structures of mammalian sPLA$_2$s with enzymatic activity. Positions of the catalytic domain and Ca^{2+}-loop, which are well conserved in all enzymes, and the group I-specific (Cys^{11}-Cys^{77}) and group II-spesific (Cys^{50}-C-terminal Cys) disulfides, are shown. The prepropeptide, which is removed by proteolytic cleavage, is shown present in PLA$_2$GIB and PLA$_2$GX. PLA$_2$GIB has a unique insertion called the 'pancreatic loop'. The group II subfamily of sPLA$_2$s, the genes for which are clustered in the same chromosome locus, have a C-terminal extension, PLA$_2$GIIC has an additional disulfide, and PLA$_2$GIIF has a uniquely long C-terminal extension containing a free Cys. Although PLA$_2$GV belongs to the group II subfamily, it lacks the group II-spesific disulfide and C-terminal extension. PLA$_2$GX possesses structural features of both groups I and II enzymes. PLA$_2$GIB, -GIIA, -GIIC, -GIID, -GIIE, -GIIF, - GV, and -GX are similar to one another in overall sturctures and thus, form the I/II/V/X collection. PLA$_2$GIII and PLA$_2$GXIIA are each distant from the group I, II, V and X sPLA$_2$s, and belong to disctinct relationship collections. PLA$_2$GIII has unique and large N- and C-terminal domains, and the central sPLA$_2$ domain shows the highest homology with bee venom PLA$_2$GIII. PLA$_2$GXIIA has a close structural resemblance with a mammalian PLA$_2$GXIIB. However, PLA$_2$GXIIB has a mutation in the active site, where a histidine has been replaced by a leucine. It is catalytically inactive because of this single point mutation and altered phospholipid-binding properties. Reprinted from Prostaglandins Other Lipid Mediat, 68-69, Kudo I and Murakami M, Phospholipase A$_2$ enzymes, 3-58, © 2002, with permission from Elsevier. Legend modified.

In human corneal epithelial cells, PLA$_2$GIII, -GX, -GXIIA and PLA$_2$IVA and -IVC have been detected by RT-PCR and Western blotting, but the expression of secreted PLA$_2$GIIA has not been confirmed. PLA$_2$s expressed by corneal epithelium could be part of the normal antibacterial activity of tears and in wound healing (Landreville et al., 2004). The presence of mRNA coding for PLA$_2$GIB, -GIIA, -GIIE, -GIIF, -GV and -GX was documented in rat retina (Kolko et al., 2004). Hypoxia-induced cPLA$_2$

activation results in expression of matrix metalloproteinases and the pro-angiogenic cytokine VEGF and an increased arachidonic acid release in endothelial cells of the monkey choroid and retina (Ottino et al., 2004).

2.3.1. Measurements of PLA_2 enzyme activity and concentration

In the diverse superfamily of PLA_2s, PLA_2GIIA has so far been shown to have the most important role in human diseases. Catalytic activity assays are substrate specific for PLA_2s, but it is often impossible to distinguish separate sub-types of this enzyme family on the basis of substrate preference. This is especially the case with samples like tissue homogenates. However, Yang et al. (1999) have published separate assays for catalytic activity of PLA_2GVI, PLA_2GIV, PLA_2GIIA and PLA_2GV, but their use has been insignificant. With specific antibodies it has been possible to localize separate PLA_2s. Human sPLA_2s have been found in many organs, but only PLA_2GIB and PLA_2GIIA have so far detected in venous samples (Nevalainen et al., 2005). PLA_2GIIA has also been measured in other body fluids, for example, in tears (Nevalainen et al., 1994, Qu and Lehrer, 1998). Therefore this presentation focuses only on the most commonly used methods for PLA_2GIB and PLA_2GIIA determination in mammalian samples.

2.3.1.1. Catalytic activity methods

The presence of PLA_2 in biological fluids or tissue homogenates can be analyzed with assays based on catalytic enzyme activity. Titrimetric, radiometric, fluorometric and spectrophotometric methods have been described. The titrimetric method (de Haas et al., 1971) is rather insensitive and cannot usually be applied to intracellular phospholipases. In a radiometric analysis, *E. coli* lipids are labelled with ^{3}H- or ^{14}C-oleic acid during bacterial growth. Autoclaved bacterial membranes are then used as a substrate for PLA_2 (Patriarca et al., 1972). The three main phospholipids of *E.* coli are phosphatidylethanolamine, phosphatidylglycerol and cardiolipin, of which phosphatidylethanolamine carries most of the radioactivity. Shakir (1981) developed a method, modified by Schädlich and co-workers (1987), based on an ^{3}H- or ^{14}C-label at the sn-2 position fatty acid, phosphatidylcholine, in mixed micelle substrates. The cleaved and separated fatty acids carrying the label indicate the proportional activity of PLA_2 in the sample. In addition to these two methods, several techniques have been used for detecting the split fatty acids. These include high-performance liquid chromatography (HPLC; Reynolds et al., 1991), thin-layer chromatography (TLC; Lister et al., 1988) and a commercial scintillation proximity assay using radioactive probes (PerkinElmer Life Sciences, Boston MA, USA).

Fluorometric assays can be classified into continuous and discontinuous methods. In continuous assays the substrate is labeled with a fluorophore such as pyrene (Hendrickson and Rauk, 1981; Radvanyi et al., 1989). The fluorescence of pyrene can be quenched intramolecularly by the trinitrophenyl group of the label. Upon hydrolysis, the monomer emission increases as pyrenehexanoic acid is released into the aqueous phase and the quenching is relieved (Thuren et al., 1988). The cleaved fluorescent fatty acids can be caught by proteins like albumin or fatty acid binding

protein from rat liver (Wilton, 1990). During the binding process the fluorescence properties of the fluorochrome change and this can be measured continuously as a kinetic measurement of the PLA_2 activity without any separation steps. In discontinuous assays, phospholipids like naphtyl- or dansyl-phosphatidylcholine are used. The cleaved fluorescent fatty acids are separated by HPLC or TLC and then measured (Hendrickson et al., 1987; Hendrickson et al., 1990). Thuren et al. (1985) has used pyrene-labelled phospholipid analogs as substrates and separated the fluorescent product from unreacted substrate by liquid-liquid phase partition.

Photometric methods for measuring PLA_2 catalytic enzyme activity are quite rapid to carry out. The method developed by Hoffmann et al. (1986) is based on the use of phosphatidylcholine vesicles as a substrate. After cleavage of free fatty acids from the substrate by PLA_2, the amount of released free fatty acid is measured by a coenzyme A (CoA) coupled assay. The enzyme acyl CoA oxidase oxidizes acyl CoA to enoyl CoA which results in generation of hydrogen peroxide. Coupling this oxidase with acyl CoA synthetase enables the quantification of free fatty acids by another enzymatic process in which the activation of free fatty acids by CoA synthetase is followed by production of hydrogen peroxide. The hydrogen peroxide generated by the oxidase is measured by oxidative coupling of 4-antipyrine and phenol (Shimizu et al., 1980). The assay can be performed using gelatine blocked microtitre plates and commercially available reagents (Santos et al., 1994). Other continuous photometric methods developed into microtitre formats use substrates like 2-hexadecanoylthio-1-ethylphosphorylcholine (HEPC; Aarsman and van den Bosch, 1977; Bhat et al., 1993) and the 1,2-dithio analog of diheptanoyl phosphatidylcholine (Reynolds et al., 1992). Upon hydrolysis of the ester bond at the sn-2 position by PLA_2, free thiols are detected using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB a.k.a. Ellman's reagent; Ellman, 1959). Reagent kits using both substrates are commercially available.

2.3.1.2. Immunoassay techniques

An immunoassay can be defined as a technique that exploits antibodies or reagents with biospecific affinities for the analysis of sample components. Immunoassays can be generally divided into those which use labels and those which do not. The specific nature of antibody binding allows development of assays that are highly selective and that can be used within complex matrices like blood or urine. The combined use of antibodies along with labels such as radioisotopes or enzymes also provides immunoassays with extremely low detection limits. Although less sensitive, non-labeled methods based on detection by nephelometry, turbidimetry, particle counting, and latex agglutination are still used in some clinical laboratories. Traditional immunoassays remain the primary method of analysis in clinical diagnostics in spite of novel developments like antibody microarray and immunosensor methods (Ekins, 1998; Seydack, 2005).

In the majority of immunoassays, either antibody or antigen is coupled to a solid phase. The solid phase enables easy separation of non-specific components from the sample. Drawbacks to this method, especially with microtiter wells, are relatively long diffusion and reaction times. An important parameter in an immunoassay is the choice

of label. The early assays used radioisotopes as labels. However, because of apparent health risks to workers and the stability of the label with long half-life time, fluorescent, luminescent, light-scattering, and enzyme labels have widely displaced radioactive labels. Homogenous assays have the benefit of short reaction times. In these methods the reagents have been designed in such a way that binding of labeled antibody to antigen modulates the measurable properties of the label (Wild and Davies, 1994).

Briefly, immunoassays can be divided on the basis of reagent ratio (Ekins, 1989). In a reagent limited assay, the amount of antibody is limited compared to the amount of analyte (antigen) used. For instance, in a radioimmunoassay (RIA), labeled antigen competes with the analyte in the sample. Therefore the term "competitive immunoassay" is used for this type of heterogeneous method. After the incubation, antibody-bound fraction is separated and its radioactivity is measured. The signal is inversely proportional to the amount of analyte in the sample. This kind of assay was first developed for insulin (Yalow and Berson, 1959). Immunometric, non-competitive assays (reagent in excess) utilize mainly the solid phase in the method design. Depending on the label used, the terms immunoradiometric (IRMA), immunofluorometric (IFMA), immunoentzymometric (IEMA), etc. are used to describe these assays (Gosling, 1990). In this type of assay, capture antibody is immobilized on the surface of the solid phase and sample is introduced. After incubation and washing, excess amount of labeled antibody (tracer) is added and incubated. Thereafter, further washing removes the unbound tracer and, depending on the label in question, the assay is finalized by detection steps. The measured signal is proportional to the amount of analyte in the sample. In the literature, the terms "two-site-" or "sandwich" immunoassays are synonymous with immunometric assays.

The sensitivity of an assay can be defined as the minimal detectable concentration or the slope of the dose-response standard curve (Ekins, 1998). Sensitivity in a non-competetive assay is theoretically maximal when the amount of antibody approaches infinity whereas, in a competive assay, it is attained when antibody concentration is close to zero. Both assay types are dependent on the affinity of the antibody. Generally the non-competetive assays may be considered even two orders of magnityde more sensitive than competetive assays. However in RIA, the counting efficiency is high for the radioactive isotope 125 of iodine (^{125}I); about 80 % (Wild and Davies, 1994). Specificity is less in a non-competitive assay with an excess of antibody due to cross-reactions, but this can be limited by the selection of an appropriate combination of monoclonal antibodies. In a competitive assay, specificity is dependent on the affinity of cross reactants and purity of labeled antigen (Davies, 1994; Ekins and Edwards, 1997).

2.3.1.3. Immunoassays for PLA_2

PLA_2s have been isolated from a variety of cells and tissues. Since catalytic methods cannot separate these proteins into different classes of PLA_2s, antibodies against different PLA_2 groups have been used in class-specific assays.

The first human PLA_2 antibodies were raised against PLA_2IB, and soon followed by the development of immunoassays by Nishijima et al. (1983) and Eskola et al. (1983). The former was a RIA method, whereas the latter was an IFMA method, using a new fluoroimmunotechnique (fluoroimmunoassay, FIA) which together with enzymeimmunotechniques (enzymeimmunoassay, EIA), have now largely replaced assays with radioactive labels. These studies provided a basis for further assays since they measured specific PLA_2IB concentrations in fluids of healthy human individuals for the first time. According to these and other studies, normal PLA_2IB values in serum and plasma are below 10 μg/l (Kaiser, 1999).

Assays utilizing microtitre plates are based on FIA and EIA techniques. Drawbacks of the former are more expensive instrumentation and reagents. Eskola et al. (1983) used a time-resolved fluoroimmunoassay (TR-FIA), a method that takes advantage of a short time lapse to minimize the background interference before measuring the fluorescence of rare-earth metal chelate probes with long fluorescence lifetimes. Various enzymatic systems can be used in EIA techniques; those methods based on horseradish peroxidase (HRP), alkaline phosphatase (AP), or ß-galactosidase (GAL) are the most common. This group of analytical methods includes several types, but an IEMA method, enzyme-linked immunosorbent assay (ELISA) is most often used in determinations of PLA_2s. These assays are based on absorbance measurements by microtiter plate readers. A rare enzyme label, inorganic pyrophosphatase, has also been used for a rat PLA_2IB assay (Kortesuo et al., 1993), and it has been used for some human clinical determinations (Peuravuori and Korpela, 1993). Commercial ELISA kits are available for human PLA_2GIIA as well as antibodies for some other PLA_2s.

All secreted PLA_2 proteins have been produced by recombinant technology in sufficient quantities to produce antibodies for immunochemical analyses. Nevalainen et al. (2005) developed an IFMA method (TR-FIA) for all hitherto known human $sPLA_2$s using polyclonal antibodies raised against recombinant proteins and studied these assays using serum samples.

2.3.2. Group IIA phospholipase A_2 (PLA_2GIIA)

PLA_2GIIA was originally purified and cloned from human platelets and synovial fluid (Kramer et al., 1989; Hara et al., 1989; Seilhamer et al., 1989). It is present in a variety of inflammatory processes and it is included in the group of acute phase proteins. Increased serum levels of PLA_2GIIA appear in acute pancreatitis, septic shock, chronic inflammatory diseases such as Crohn's disease and patients with burns (Nevalainen et al., 2000; Talvinen et al., 2001; Haapamäki et al., 1998; Yamada et al., 1998). Upon stimulation platelets, mast cells, fibroblasts, and macrophages synthesize and secrete PLA_2GIIA (Horigome et al., 1987; Murakami et al., 1992; Shinohara et al., 1992; Hidi et al., 1993). The enzyme and its mRNA have also been identified in Paneth cells of the gastrointestinal tract (Nevalainen et al., 1995). PLA_2GIIA is expressed in columnar epithelial cells of inflamed colonic mucosa and in metaplastic Paneth cells (Haapamäki et al., 1999). The mRNA and protein are also expressed in the lacrimal gland and in cartilage (Kallajoki et al., 1997). Northern blot analysis and *in situ* hybridization have shown that PLA_2GIIA can be synthetized in the main and accessory

lacrimal glands of the human eye. The cells synthesizing PLA_2GIIA are distinct from those synthesizing lysozyme (Aho et al., 1996). PLA_2GIIA in nasal fluid originates mainly from the tear fluid, although minor amounts are detectable in the mucosa of nasolacrimal ducts (Aho et al., 1997; Paulsen et al., 2001).

2.3.2.1. Reference values for PLA_2GIIA concentration

Several studies have analysed the concentration of PLA_2GIIA in normal human venous samples. Matsuda et al. (1991) published a RIA method using a monoclonal antibody and found a PLA_2GIIA serum concentration of 2.2 ± 0.7 µg/l (mean ± SD) for 47 control individuals (range 1.4 - 4.2 µg/l). Soon after, values of 7.3 ± 2.6 µg/l (mean ± SD) were found in serum samples for 22 controls measured with an ELISA method (Vadas et al., 1992). Nevalainen et al. (1992) developed a TR-FIA and measured a mean PLA_2GIIA serum concentration of 3.7 µg/l with a 95 % reference interval of 1.3 - 10.8 µg/l (IFMA, 59 control individuals). Specificity of the antibodies used in these early studies was tested against human and porcine PLA_2GIB and bee or cobra venom PLA_2s. Kugiyama et al. (2000) had a control group of 94 blood donors and measured PLA_2GIIA concentrations with a RIA method (Matsuda et al., 1991). The calculated 95 % reference interval for these EDTA plasma samples was 0.9 - 4.9 µg/l (mean 2.2 µg/l, SD 1.0, median 1.9 µg/l).

Recently Nevalainen et al. (2005) published a modified TR-FIA method (IFMA) for PLA_2GIIA and found a median value of 1.8 µg/l for a group of 28 healthy blood donors. The 95 % reference interval was 0.2 - 5.8 µg/l for these serum samples (mean 1.8 µg/l, SD 1.3, unpublished data). In this study they showed that PLA_2GIB and PLA_2GIIA were present in serum samples of control individuals at low levels, and that elevated levels were found in sera of patients with acute pancreatitis (PLA_2GIB) and septic infections (PLA_2GIIA). However, in the same serum samples of septic patients and healthy blood donors, the concentrations of the other secreted PLA_2s could not be detected in their separate TR-FIA assays. Specificity of each polyclonal antibody in this study was tested by Western blotting (Degousee et al., 2002). In a large research by Boekholdt et al. (2005), 2209 control individuals were matched by sex. The measurements were done in serum samples with an ELISA method with two different monoclonal antibodies raised against recombinant PLA_2GIIA. In this study PLA_2GIIA median values were 10.4 µg/l (IQR 7.3 - 16.3 µg/l) for 813 women and 7.3 µg/l (IQR 5.3 - 10.6 µg/l) for 1396 men. For the whole reference group of 2209 individuals, the median PLA_2GIIA value was 8.3 µg/l (IQR 5.8 - 12.6 µg/l).

Depending on the research group and the methodology used in determinations, there are still variations in the reference values for PLA_2GIIA (See Table 4). However, we may consider that the normal serum and plasma concentration of PLA_2GIIA in man is below 10 µg/l.

Table 4. Reference values for group IIA phospholipase A_2 concentration

Mean ± SD, number of controls	95 % reference interval	Published
2.2 ± 0.7 µg/l, n = 47 (RIA)		Matsuda et al., 1991
7.3 ± 2.6 µg/l, n = 22 (ELISA)		Vadas et al., 1992
3.7 µg/l, n = 59 (IFMA)	1.3 - 10.8 µg/l	Nevalainen et al., 1992
2.2 ± 1.0 µg/l, n = 94 (RIA)	0.9 - 4.9 µg/l	Kugiyama et al., 2000
1.8 ± 1.3µg/l, n = 28 (IFMA)	0.2 - 5.8 µg/l	Nevalainen et al., 2005

Abbreviations: RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay; IFMA, immunofluorometric assay. The 95% reference interval is with the 2.5^{th} and 97.5^{th} centiles.

2.3.2.2. Antibacterial properties of PLA_2GIIA

Bactericidal properties of PLA_2GIIA were originally found together with BPI in rabbit PMN (Weiss et al., 1979). The enzyme was bactericidal against *E. coli* only when acting synergistically with BPI (Weiss et al., 1994). BPI perturbs the lipopolysaccharide capsule of gram-negative bacteria through its interaction and enables phospholipid hydrolysis of the bacteria by PLA_2GIIA (Madsen et al., 1996). Recent studies have shown that human PMN do not express (at mRNA level) and release PLA_2GIIA during inflammation (Degousee et al., 2002), even though PLA_2GIIA plays an important role in coping with bacteria. It is now postulated that the antisera used in earlier studies (Rosenthal et al., 1995) cross-reacted with other sPLA_2s.

PLA_2GIIA is a readily water-soluble protein as are all other secreted PLA_2s, but it must bind to the membrane-water interface to access its substrate. The efficiency of the enzyme in attacking gram-positive bacteria depends on whether it can bind to and penetrate the cell wall to gain access to phospholipids in the membrane. This process includes binding of the enzyme to the bacterial cell surface and penetration through a thick peptidoglycan layer with a highly anionic charge due to the presence of the phosphate diester units of lipoteichoic acid. This results in degradation of the cell membrane phospholipid, and activation of bacterial autolysis (Foreman-Wykert et al., 1999). Studies on the crystal structure and analysis of the surface electrostatic potentials show that PLA_2GIIA is highly cationic. It contains cationic patches on the putative interfacial, nearly planar, binding/recognition surface that surrounds the deep active site (Snitko et al., 1997). Human PLA_2GIIA is the most basic (pI = 9.4) and the most efficacious against gram-positive bacteria of all sPLA_2s. It contains 13 lysines and 10 arginines scattered over its surface, but lacks interfacial tryptophan (Bezzine et al., 2002; Beers et al., 2003). The enzyme has extremely low activity on condensed zwitterionic plasma membranes, such as phosphatidylcholine and sphingomyelin rich mammalian cells. On the other hand it has high activity on anionic phospholipid interfaces including phosphatidylglycerol-rich, gram-positive bacterial membranes which are not normally characteristic of the outer monolayer of the eukaryotic cell

membrane. Substrate specificity is dictated by the membrane interface to which the enzyme prefers to bind (interfacial specificity) and by the type of phospholipids which are accommodated in the catalytic site (catalytic site specificity) (Baker et al., 1998; Bezzine et al., 2002; Berg et al., 2001). PLA_2GIIA does not act on corneal endothelial cells because it cannot bind to the phosphatidylcholine rich outer plasma membrane of these cells (Koduri et al., 1998).

PLA_2GIIA purified from human tears kill *Listeria monocytogenes* at a concentration of 1.1 µg/l, and two *S. aureus* strains at concentrations of 15 µg/l and 80 µg/l, respectively (Qu and Lehrer 1998). PLA_2GIIA concentrations of 250 µg/l for *Enterococcus faecium* and 300 µg/l for *Micrococcus luteus* are required to kill these bacteria. Permeabilization of the highly cross-linked cell wall of *S. aureus* by protease lysostaphin effectively enhances PLA_2GIIA-mediated hydrolysis of cell membrane phospholipids (Buckland et al., 2000). Bactericidal properties of PLA_2GIIA have also been studied in rabbit tears. These studies demonstrated that the enzyme is able to kill *S. aureus* (Moreau et al., 2001; Girgis et al., 2003).

2.4. Bactericidal/permeability-increasing protein (BPI)

The PMNs are the central cellular effectors of the primary defence system, developing in the bone marrow wherein the cytoplasmic granules are synthesized. Diversity of PMN granule subpopulations has been defined (Borregaard and Cowland, 1997), and the antimicrobial proteins and peptides appear to be confined to both azurophilic (early) and specific granules. The former are characterized by their content of hydrolytic antibacterial proteins such as elastase, lysozyme, defensins and myeloperoxidase. BPI was also identified in human azurophilic granules and shown to be potentially antibacterial towards *E. coli* and several other gram-negative bacteria (Weiss et al., 1978). BPI is an abundant constituent of PMNs and plays an important role in the oxygen-independent mechanism for bacterial killing (Weiss and Olsson, 1987). It is also detected in granules of human eosinophils (Calafat et al., 1998). BPI has been detected in human mucosal epithelial cells of the esophagus and colon (Canny et al., 2002), and in nasolacrimal ducts (Paulsen et al., 2001). Human BPI is considered to belong to the family of lipid-interactive/binding proteins, with a structural similarity to lipopolysaccharide (LPS) binding protein (LBP; Bingle and Craven 2004), a liver-derived plasma component that delivers LPS to its receptor host cells. LBP was first isolated from rabbit acute-phase serum by Tobias et al. (1986) and it is included in the group of acute phase proteins (Schumann and Zweigner, 1999).

2.4.1. Antibacterial properties of BPI

Lipopolysaccharides (LPSs, endotoxins) are major inducers of host responses of gram-negative bacteria. LPSs are high-molecular-weight complexes that constitute the major cell wall component in all gram-negative bacterial families. LPSs play a vital function for bacterial viability and when released free, induce potent pathophysiological effects in mammalians primarily via induction of high quantities of tumor necrosis factor α (TNF-α) and interleukin 1 (IL-1) cytokines. Gram-negative bacterial sepsis is a disease with a mortality rate of ~30 %. It is estimated that there are

750,000 sepsis cases annually in the United States alone (Angus et al., 2001). In 2004 *E. coli* was the most common cause of bacterial infections in humans and caused 21.2 % of the total bacterial infections among working population in Finland. For these reasons, there is wide interest in the investigation of adjuvant therapies, including reagents that bind to and neutralize LPS.

BPI is a basic (pI = 9.8) 55 kDa protein with elongated, "boomerang" shaped structure, consisting of two similar domains of nearly equal size (Figure 5). The protein molecule comprises two α-helical domains, a cationic lysine-rich N-terminus linked to a central highly twisted β-sheet segment which in turn is linked to an anionic, hydrophobic C-terminal domain (Beamer, 2003). The antibacterial and LPS-neutralizing activity of BPI is localized in the N-terminal half of the protein (Ooi et al., 1991), whereas the C-terminal half enhances the opsonic activity of the molecule, i.e., its capability to promote phagocytosis (Iovine et al., 2002).

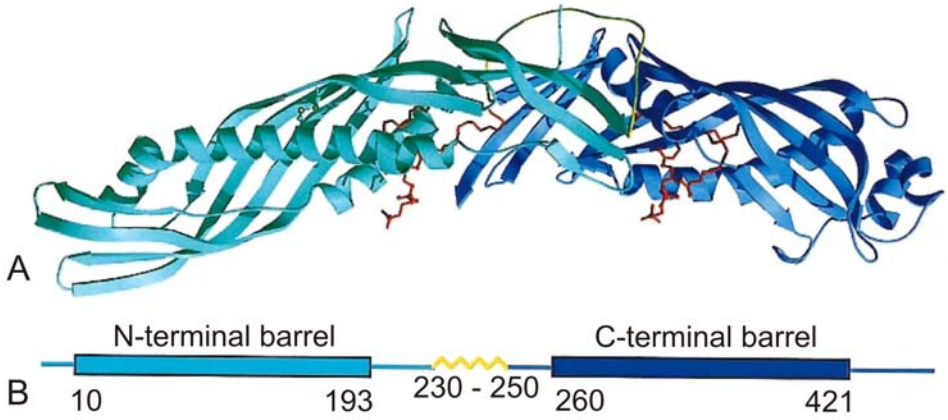

Figure 5. A ribbon diagram of human bactericidal/permeability-increasing protein (shown top, A). The N-terminal domain is on the left and C-terminal on the right. Proline-rich (amino acids 230-250, shown below, B) linker together with the ß-sheet structure connects these domains. Amino acid numbers for each structural unit are indicated below. Reprinted from Biochem Pharmacol, 57, Beamer LJ, Carroll SF, Eisenberg D, The three-dimensional structure of human bactericidal/permeability-increasing protein: implications for understanding protein-lipopolysaccharide interactions, 225-229, © 1999, with permission from Elsevier.

The broad-range, innate immune recognition of gram-negative bacteria by BPI results from its interactions with sites within the conserved inner core sugar and lipid A regions (Figure 6). The conserved hydrophobic lipid A region is β1→6-linked to disaccharide of *N*-acetylglucosamine. It is substituted with non-hydroxylated and 3-OH fatty acids by amide and ester linkages and attached to a carbohydrate chain of variable length and composition, including several acidic groups in close proximity (Rietschel et al., 1994). Direct binding of BPI to the bacterial envelope is critical for its antimicrobial action, and BPI shows high affinity to the lipid A moiety of LPS (Gazzano-Santoro et al., 1992). Since BPI binds the lipid A region that is common to all LPSs, it is able to neutralize endotoxin from a broad array of gram-negative

pathogens. It has been proposed that BPI binding to the negatively charged outer membrane followed by its insertion will cause rigidification of the the acyl chains of the LPS layer and destabilization of the layer, eventually provoking membrane rupture. The extension of long polysaccharide chains from outer membrane impedes binding of BPI. The binding and insertion of the protein leads to significant changes in membrane current influencing membrane permeability for hydrophobic molecules. Binding also changes transmembrane potential which influences channel gating and thereby causes membrane dysfunction (Wiese et al., 1997).

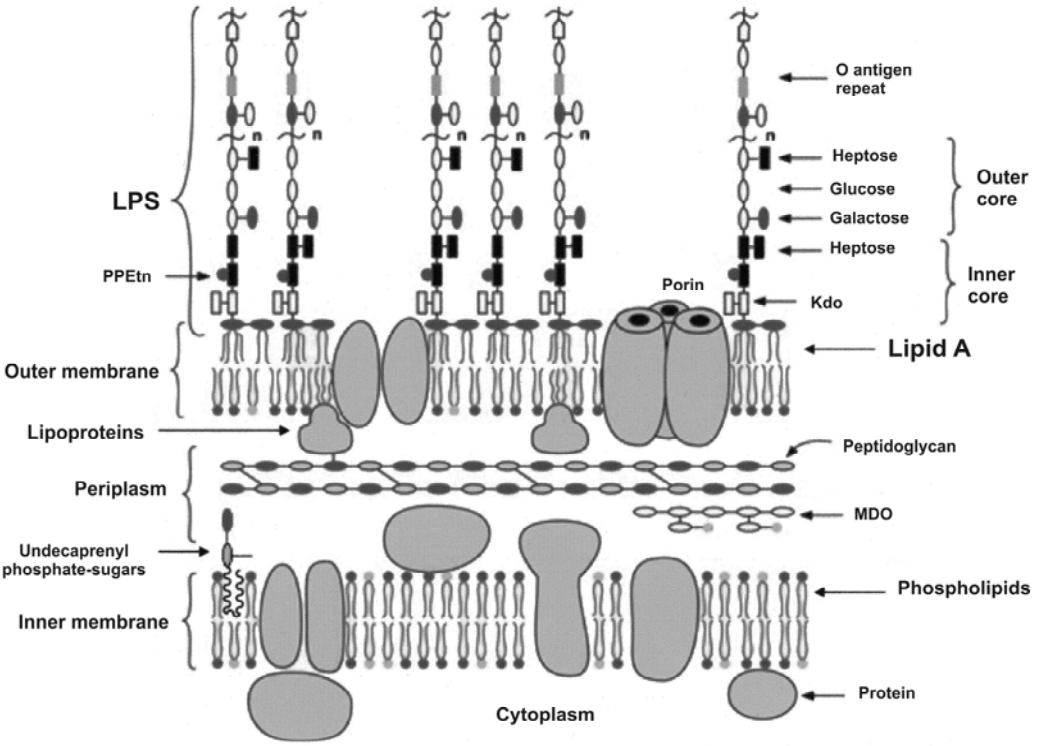

Figure 6. Molecular organization of the envelope of *Escherichia coli*. In the external chains ovals and rectangles represent sugar residues, whereas circles represent the polar head groups of glycerophospholipids. Abbreviations: PPEtn, pyrophosphoethanolamine; Kdo, 3-deoxy-D-manno-octulosonic acid; LPS, lipopolysaccharide; MDO, membrane-derived oligosaccharides. Reprinted, with permission, from the Annu Rev Biochem, Volume 71, © 2002 by Annual Review www.annualreviews.org

BPI has a close structural relationship with a 60 kDa LBP, sharing 45% amino acid identity (Beamer et al., 1999). The most significant functional difference between these proteins is their endotoxin binding. LBP greatly amplifies the endotoxin activity of LPS by sequestering monomers from LPS aggregates and delivering the monomers to cellular CD14 and Toll-líke receptor complex. Meanwhile, BPI blocks delivery of LPS and prevents LPS signalling, possibly by increasing the size of LPS aggregates (Tobias et al., 1997). BPI binding initiates first sublethal, then lethal, bacterial injury (Elsbach et al., 1999), whereas equivalent amounts of LBP binding have no cytotoxic effect on

bacteria (See Figure 7). BPI and LBP are functionally antagonistic and the affinity of LBP for lipopolysaccharide is even 70-fold lower than that of BPI (Elsbach 1998). Both proteins have opsonics effects enhancing the uptake of bacteria by phagocytic cells. LBP-coated gram-negative bacteria are taken up mainly by monocytes or macrophages through CD14 (Iovine et al., 2002), whereas BPI-coated bacteria are taken up mainly by PMNs, independent of CD14. CD14 is a 55 kDa glycophosphatidylinositol (GPI) linked membrane protein mainly expressed on monocytes (the monocyte differentiation antigen), but also on macrophages and neutrophils (Wright et al., 1990; Fujihara et al., 2003). Concentration of the soluble form (sCD14) is markedly lower in neonatal than in adult normal plasma (Levy et al., 2004). A similar difference was detected for BPI (Levy et al., 2002a; Nupponen et al., 2002). However, LBP concentrations were on the same level in newborn and adult plasmas (Bortolussi et al., 1997). Sepsis increases the concentration of sCD14, LBP and BPI in neonatal plasma (Blanco et al., 1996; Berner et al., 2002; Nupponen et al., 2002).

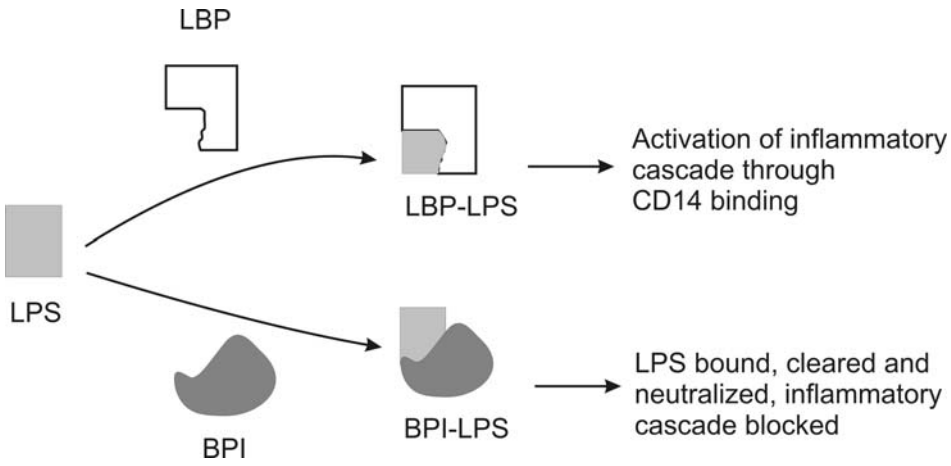

Figure 7. Different effects of BPI and LBP on the LPS-induced inflammatory cascade, modified from Beamer et al. (1999). Abbreviations: BPI, bactericidal/permeability-increasing protein; CD14, cluster of differentiation antigen 14; LBP, lipopolysaccharide binding protein; LPS, lipopolysaccharide.

Complement is a central part of the primary defense system. It mediates control against infectious agents and links primary and adaptive immunity. In humans the complement system is composed of nearly 40 molecules which are either distributed as soluble plasma proteins or are expressed as integral membrane proteins (Walport, 2001). Components of both the classical and alternative complement pathways are present in ocular tissues and ocular fluids (Wilcox et al., 1997). The complement system is also involved in the host defence against gram-negative bacteria. Nishimura and co-workers (2001) found that BPI could accelerate complement activation to opsonize gram-negative bacteria, followed by promotion of phagocytosis by PMNs via complement receptors.

Nanomolar concentrations of BPI in biological fluids, such as serum, plasma and whole blood, may be sufficient to kill gram-negative bacteria. Even the N-terminal fragment of BPI is effective in blocking bacterial proliferation as well as other LPS-mediated effects (Weiss et al., 1992). These properties have rendered BPI an attractive target for biopharmaceutical development and led to therapeutic trials of humans with antibacterially active, recombinant protein fragments in humans (Levy, 2002b). Gram-positive bacteria cause the majority of bacterial infections in newborns; nevertheless, gram-negative bacteria are involved in up to 40% of all infections. Levy and co-workers (2000) observed that adding $rBPI_{21}$, a recombinant 21-kDa modified N-terminal fragment of human BPI, enhances the antibacterial activity of newborn cord blood against *E. coli* and *Citrobacter koseri*. BPI and $rBPI_{21}$ are potentially bactericidal against *Neisseria meningitidis*. $rBPI_{21}$ has been in human clinical phase III trials for severe meningococcal sepsis (Levin et al., 2000), though so far these experiments have been unsuccessful. $rBPI_{21}$ has also been studied in children undergoing cardiopulmonary bypass, in the treatment of Crohn's disease, and in proliferative retinal diseases such as advanced diabetic retinopathy (Rauniyar et al., 2002). It has been shown that PMNs mediate fungistatic activity against *Histoplasma capsulatum* yeasts and surprisingly, BPI can inhibit their growth, although yeasts do not contain LPS (Newman et al., 2000). Khan and co-workers (1999) found *in vitro* and in mice that $rBPI_{21}$ has activity against a protozoan parasite, *Toxoplasma gondii*, and this activity is significantly increased when in combination with sulfadiazine.

2.4.2. Measurements of BPI concentration

Pereira et al. (1989) developed the first quantitative ELISA method for BPI, then termed 57 kDa cationic antimicrobial protein, CAP 57. Amino acid sequence analysis and monoclonal antibodies later showed that CAP 57 and BPI are the same protein (Pereira et al., 1990). Schindler et al. (1993) observed that BPI levels increased during four hour hemodialysis treatments from the pre-treatment value of 0.41 µg/l to 4.5 µg/l measured in heparinized plasma. They also found that blood samples should be processed rapidly, otherwise the BPI concentration increases depending on time and temperature. White and co-workers (1994) detected BPI concentrations ranging between < 0.2 - 2.1 µg/l in citrated plasma (mean 0.8 µg/l) and 4.9 - 72.1 µg/l (mean 27.1 µg/l) in the serum of the same healthy donors. They also concluded that BPI levels varied depending upon the elapsed time of collection and processing. Furthermore, they found that BPI should be measured in plasma instead of serum, thereby avoiding artefacts caused by the destruction of PMNs and release of BPI during the process of coagulation. Dentener et al. (1995) continued to study the effect of blood sampling. They found the concentration of BPI in 32 healthy individuals was below the detection level of their ELISA, i.e., <0.2 µg/l, when measured from ethylenediaminetetraacetic acid (EDTA) or heparinized plasma separated from blood cells immediately after collection. However, when plasma of heparinized blood stayed for 0.5 - 1 hour at room temperature before separation, BPI concentrations increased to 1 - 2 µg/l. Dentener and co-workers measured BPI values of up to 60 µg/l after prolonged incubation of 24 hours at room temperature before separation. In plasma of EDTA-treated blood incubated up to 4 hours at room temperature, BPI was not

detectable. Therefore they concluded that EDTA plasma is to be preferred in BPI determinations.

In a later study, Kimmings and co- workers (2000) found BPI values of < 0.5 µg/l in citrated plasma of healthy individuals using an ELISA method (White et al., 1994). Using a TR-FIA method (IFMA) Rintala et al. (2000) measured a BPI concentration ranging 0.9 - 24 µg/l (median 5.2 µg/l) in EDTA plasma of a group of 42 healthy donors. Nupponen et al. (2002) used the same method, and found a BPI concentration range of 0.7 - 18.4 µg/l (median 7.3 µg/l) in citrated plasma for a separate group of 15 healthy donors. However, Shäfer et al. (2002) used the ELISA method of White and co-workers (1994) and found higher plasma values of 18.7 ± 19.2 µg/l (mean ± SD) for 11 donors. Furthermore BPI values of 18.0 ± 0.9 µg/l (mean ± SD) were found in citrated plasma of 22 donors using a commercial ELISA method (Bokarewa et al., 2003; See Table 5).

Table 5. Bactericidal/permeability-increasing protein concentration in healthy individuals (controls)

Mean ± SD, number of controls	**Type of sample**	**Research group, published**
0.8 µg/l, n = 20	citrated plasma	White et al., 1994
< 0.2 µg/l, n = 32	EDTA and heparinized plasma	Dentener et al., 1995
0.48 ± 0.4 µg/l, n = 16	citrated plasma	von der Möhlen et al., 1996
18.7 ± 19.2 µg/l, n = 11	citrated (?) plasma	Shäfer et al., 2002
18.0 ± 0.9 µg/l, n = 22	citrated plasma	Bokarewa et al., 2003

Abbreviations: SD, standard deviation; EDTA, ethylenediaminetetraacetic acid

Elevated BPI concentrations have been found in patients undergoing coronary artery bypass grafting and operated on with cardiopulmonary bypass (Fransen et al., 1998), a method that is known to induce a generalized inflammatory response. The same research group found that two-day-old red blood cell units had a BPI concentration of 10 µg/l, but after 31 days the BPI level had increased to 50 µg/l (stored at 4°C). They stated that storage could account for the higher BPI levels found in patients who had received transfusions intraoperatively, >20 µg/l, compared with patients without transfusion, ~ 12 µg/l. Preoperative values were ~ 2 µg/l (Fransen et al., 1999).

Sepsis increases BPI values in plasma. The mean BPI value of 31 patients was 7.1 µg/l (range 0.5 - 58.0 µg/l, SD 11.4), and the mean value of 16 healthy control subjects was 0.48 µg/l (range 0.10 - 1.30 µg/l, SD 0.4), measured in citrated plasma samples (von der Möhlen et al., 1996). Rintala et al. (2000) confirmed these findings in EDTA plasma. Higher values were measured in gram-negative (median 16.8 µg/l, range 1.6 - 205 µg/l) than in gram-positive (range 1.6 - 60 µg/l) sepsis, while the values for

healthy donors were much lower (median 5.2 µg/l, range 0.9 - 24 µg/l). In acute cholangitis, the BPI concentration was 7.1 µg/l during acute phase, but did not change significantly when measured a week later (4.6 µg/l), although the overall inflammatory responses reduced after endoscopic treatment (Kimmings et al., 2000). The detection limit of BPI in healthy individuals was 0.5 µg/l. Juffermans and co-workers (1998) found elevated BPI values of 8.3 µg/l in patients with active tuberculosis (range 0.4 - 123.0 µg/l) and 3.9 µg/l in individuals, who have been in close contact with these patients. Schäfer et al. (2002), who found the normal BPI plasma level of 18.7 µg/l, reported elevated values for a group of 30 patients with alcoholic liver disease, mean 50.1 µg/l.

Rheumatoid arthritis (RA) increases BPI in citrated plasma, from a normal value of 18 µg/l to 23 µg/l (Bokarewa et al., 2003). The BPI concentration in synovial fluid of these patients with RA was 293 µg/l. Punzi et al. (2000) measured BPI in synoval fluid of RA, psoriatic arthritis (PsA) and osteoarthritis (OA) patients, and obtained values of 307.7 µg/l, 145.3 µg/l, and 151.1 µg/l, respectively. However, Inman and Chiu (1996) did not find detectable levels of BPI in the synovial fluid of patients with OA. They found a broad range of BPI values in RA (343 - 2570 µg/l, mean 1226 µg/l, n = 8) and in reactive arthritis (4.6 - 333 µg/l, mean 168 µg/l, n = 8).

Research groups have even found tenfold BPI concentration differences in control individuals using the same measuring method (See Table 5). This has delayed finding valid reference values for it. Patents around BPI have also hindered the research and limited the number of groups which have obtained the tools needed to work with the protein, especially in the United States. However, these determinations will yet be made, especially since pharmaceutical development has shown such promising results in clinical trials.

2.4.3. BPI autoantibodies

Zhao et al. (1995) identified the first autoantibodies against BPI. Autoimmunity can be defined as a failure of an organism to recognise its own tissue, and includes any immune response to the host's own tissue, whether it is humoral (e.g., circulating autoantibodies) or cellular (e.g., delayed hypersensitivity). An intact immune system has the ability to distinguish self antigens from non-self antigens. There is however, always a chance that this system might break down. If this happens autoantibodies, i.e., antibodies capable of reacting with "self" components, are produced. Anti-neutrophil cytoplasmic antibodies (ANCA) are autoantibodies which react with antigens in the cytoplasm of PMNs and monocytes (Wiik, 1989). They were first described in 1982 by Davies et al. in a few patients with necrotizing glomerulonephritis and symptoms of systemic vasculitis. BPI-ANCA, like other autoantibodies, is currently routinely measured by ELISA methods.

3. AIMS OF THE STUDY

The present study focused on two primary defense proteins in humans: group IIA phospholipase A_2 (PLA_2GIIA) and bactericidal/permeability-increasing protein (BPI). Most of the anti-microbial proteins in tears are secreted by the main and accessory lacrimal glands, but the localization of PLA_2GIIA and BPI in the eye is largely unknown.

The study had three specific aims:

1. To show the presence of PLA_2GIIA and BPI in the lacrimal gland of the human eye.

2. To optimize fluoroimmunoassays for PLA_2GIIA and BPI using venous blood samples and to study their applicability to tear fluid samples.

3. To study PLA_2GIIA and BPI in tears of healthy eye, and the levels of PLA_2GIIA effected by the wear of contact lenses and an ocular disease.

4. MATERIALS AND METHODS

4.1. Lacrimal gland specimens, studies I and V

Lacrimal gland specimens were obtained in all from twelve autopsied subjects. Specimens from eight subjects (2 men and 6 women, 56 - 84 years, mean 72 years) were used for PLA_2 and BPI assays. Portions of the main glands and the adjacent conjunctiva from four of these eight subjects, as well as from four additional autopsied subjects, were fixed in 10% buffered formalin for immunohistochemical analysis (3 men and 5 women, 44 - 87 years, mean 71 years). The tissue specimens were from cadavers with no known ocular disease. Specimens from the pectoralis major muscle served as negative controls both in the biochemical and immunohistochemical analysis. Because the samples came from routine clinical autopsies performed 2 to 4 days after death, tissue integrity was verified by light microscopy with hematoxylin and eosin staining to rule out excessive autolysis.

The tissue samples for PLA_2 and BPI assays, as well as for Western blotting were homogenized with an Ultra-Turrax (IKA Laboratechnik, Staufen, Germany) device in 0.01 M sodium phosphate buffer, pH 7.6 (1 g wet tissue per 5 ml of the buffer) containing protease inhibitors (Boehringen Mannheim, Mannheim, Germany) and then centrifuged at 10,000 g for 1 h at 4°C. The supernatants were used for analysis.

4.2. Subjects, studies I – V and unpublished experiments

The principles of the Declaration of Helsinki (World Medical Association) were followed and the study protocols were approved by the Ethics Committee of the University of Turku and the Turku University Central Hospital, Finland. Written informed consent was obtained from each healthy subject providing tear samples. The 83 blood samples in the previously unpublished experiments were taken during blood donations at the Finnish Red Cross Transfusion Service in Turku, Finland. Accordingly, 37 patients with rheumatoid arthritis (RA; 13 men and 24 women, median age 58 years, age range 27 – 83, treated at either Turku University Central Hospital, Turku or Central Finland Central Hospital, Jyväskylä, Finland) and 48 patients with Crohn's disease (18 men and 30 women, median age 32 years, age range 18 – 69, treated at Turku University Central Hospital, Turku) gave informed consent to use of their blood samples. In study I, an oral informed consent was obtained from four healthy volunteers giving the tear specimens.

In study I, tear specimens were obtained from 4 healthy female volunteers (aged 5 to 42 years, mean 29 years; See Table 6). In study II, tear samples of 22 subjects who did not use CLs were used as normal controls (6 male and 16 female; age 24.8 ± 2.0 years, mean ± SD). Accordinly, in study III as normal age-matched controls, we studied 29 non-atopic subjects, 11 male and 18 female, with ages ranging from 20 to 63 years (37.0 ± 12.0, mean ± SD). None of these control subjects wore CLs, and all of them passed a routine ophthalmological examination.

In study II, the PLA_2GIIA content of tears was measured in eyes of 20 healthy contact lens (CL) wearers (4 men and 16 women), whose ages ranged from 19 to 34 (22.6 ± 3.2, mean ± SD) years. The subjects had used soft CLs for 5.2 ± 3.1 years.

In study III, 29 patients (11 men and 18 women) with atopic blepharoconjunctivitis (ABC), with ages ranging from 10 to 61 years (36.3 ± 12.7, mean ± SD) were studied. All patients had typical symptoms of ABC, characterized by type I hypersensitivity reactions, high immunoglobulin E (IgE) levels, and multiple positive skin prick tests (SPT). The diagnosis of ABC was confirmed by a positive SPT; the presence of chronic atopic dermatitis in lids, face and elsewhere in the body; and by using conjunctival brush cytology. All patients were treated by intermittent preservative-free sodium chromoglycate (eye drops for lubrication). The treatment was stopped in all patients 3 days before the examination. All patients had bacterial cultures positive for *Staphylococcus aureus* within the range of 1+ to 3+.

In study IV, serum and heparinized plasma samples were collected from unselected hospitalized patients with or without infection (42 women and 48 men). The mean age was 61 years (range 14 - 93 years). Samples were stored frozen until assayed.

In study V the concentration of BPI in tears was studied in 42 healthy volunteers (13 men and 29 women) with ages ranging from 22 to 30 (24.7 ± 2.1, mean ± SD) years. None of the subjects had any general disease or eye disease. None of the subjects wore CLs. All of the subjects had normal visual acuity (at least 20/20) without or with spectacle correction. In all of them, routine ophthalmological examinations revealed normal findings.

Table 6. Subjects in studies I - V

	Subjects	**Sex**	**Mean age (range)**
Study I	4 healthy donors	female	29 years (5 - 42)
Study II	22 healthy donors	6 male, 16 female	24.8 years
	20 healthy donors with CLs	4 male, 16 female	22.6 years (19 -34)
Study III	29 healthy donors	11 male, 18 female	37 years (20 - 63)
	29 patients with ABC	11 male, 18 female	36.3 years (10 - 61)
Study IV	90 patients with/without infection	48 male, 42 female	61years (14 - 93)
Study V	42 healthy donors	13 male, 29 female	24.7 years (22 - 30)

Abbreviations: CLs, contact lenses; ABC, atopic blepharoconjunctivitis

4.3. Collection of tears, studies I - III and V

In study I, spontaneous emotional tears were collected into microcentrifuge tubes (Eppendorf AG, Hamburg, Germany).

In sudies II, III and V, nonstimulated tears were collected using disposable microcapillaries (Microcaps 5 µl, Drummond Scientific, Broomall, PA, USA or 20 µl Duran Ringcaps, Hirschmann Laborgeräte, Eberstadt, Germany) under a Haag-Streit 900 biomicroscope (Haag-Streit AG, Bern, Switzerland). The samples were collected sporadically during the day from the marginal tear strip of the lower lid near the lateral canthus, with care being taken not to irritate the conjunctiva, cornea, or lid margin. In all cases, the collection time was limited to 5 min. Tear samples were immediately placed on dry ice and kept at -70 °C until analyzed. Tear secretion rate was measured using Schirmer tear test strips (Clement Clarke, Essex, UK).

In study II, the tear samples were collected from the CL wearers at 4 p.m. after a break of at least 16 h without CLs. After 1 or 2 days, the same subjects applied the CLs to their eyes at 8 a.m. and they were studied after 4, 8 and 12 h use of CLs (at noon, 4 p.m. and 8 p.m.). To determine the normal diurnal variation in the PLA_2GIIA content of tears, tear samples were collected from normal controls at 8 a.m., noon, 4 p.m. and 8 p.m. Between 0.15 µl and 5 µl of tear fluid was collected, and the volume was measured using a calibrating scale beside the microcapillary. The tear samples were diluted from 1:300 to 1:1100 with physiological saline in study II, while in study V the samples were diluted just before measurements. All samples were kept frozen at −70 °C until assayed.

4.4. Conjunctival cytology, study III

Conjunctival brush (Accellon Multi Biosampler, Medscand Medical AB, Malmö, Sweden) cytology was taken from 21 patients with ABC from the lower and upper lid tarsal conjunctiva of the eye after the collection of tear fluid. The sample was spread on a glass slide and stained using the Papanicolau method. The presence of eosinophils, PMNs, lymphocytes, squamous and columnar epithelial cells, metaplastic cells and goblet cells were examined for by light microscope (Kari, 1988).

4.5. Phospholipase A_2 assays, study I

The catalytic activity of PLA_2 was expressed as units per liter or units per gram of protein, where unit was defined as millimoles of substrate hydrolyzed per minute at 40 °C. A mixture of 2-[^{3}H]palmitoyl phosphatidylcholine and unlabeled dipalmitoyl phosphatidylcholine served as the substrate (Schädlich et al., 1987).

The concentration of PLA_2GIB was measured by a TR-FIA (IFMA) using a monoclonal antibody to human pancreatic PLA_2 immobilized on the solid phase and a europium labelled polyclonal antibody for detection (Santavuori et al., 1992). The results were expressed as micrograms per liter.

4.6. Determination of PLA_2GIIA and BPI concentration, studies I - V

Time-resolved fluorescence was measured with a 1230 Arcus fluorometer (Perkin Elmer Wallac, Turku, Finland). The plate washer (Delfia Platewash) and plate shaker (Delfia Plateshake) used in the fluoroimmunoassay were both from Perkin Elmer Wallac. Data were handled with MultiCalc data management software (Perkin Elmer

Wallac), including the quality control follow-up with Shewhart/Levey-Jennings control chart (Westgard et al., 1977).

4.6.1. Production of antibodies and purification of IgG, studies I - V

Antisera to recombinant PLA_2GIIA and recombinant BPI were raised in rabbits. The rabbits were immunized subcutaneously four times at 2- to 3-week intervals with 0.05 - 0.2 mg of human recombinant PLA_2GIIA (Ciba-Geigy Ltd, Basel, Switzerland) or human recombinant BPI (Incyte, Palo Alto, CA, USA) in Freund's complete adjuvant at the first immunization, and in Freund's incomplete adjuvant on subsequent injections. Serum was collected 2 weeks after the last booster injection. Ig fractions were purified with a 5 ml HiTrap Protein A HP (high performance) column (Amersham Biosciences, Uppsala, Sweden) or Protein A MAPS (monoclonal antibody purification system) II kit (Bio-Rad Laboratories, Richmond, CA, USA) according to the manufacturer's instructions. Phosphate buffered saline (PBS, 20 mM sodium phosphate buffer, pH 7 containing 0.9% NaCl), was used as the binding buffer, and 0.1 M glycine, pH 2.5 as the elution buffer. After immediate overnight dialysis against PBS, the purified IgG was frozen in 1.0 mg aliquots. The specificity of the antibodies was ascertained by immunohistochemistry and Western blot analysis (data not shown).

4.6.2. Labelling of IgG with europium chelate, studies I - V

Delfia Eu-labelling reagent, europium chelate of N^1-(p-isothiocyanatobenzyl)diethylenetriamine-N^1,N^2,N^3,N^3-tetraacetatic acid (Perkin Elmer Wallac, Turku, Finland), was dissolved into the IgG-PBS solution in the appropriate ratio, pH adjusted to 9.5, and incubated overnight at 4 °C according to the manufacturer's instructions. Eu-labelled IgG was isolated from the unreacted Eu-labelling reagent by size exclusion chromatography with Superdex 75 (Pharmacia, Uppsala, Sweden) and subsequent 45 cm Trisacryl GF 2000 (LKB, Bromma, Sweden) gels. The column was equilibrated and eluted with 50 mM Tris-HCl buffer, pH 7.75, 0.9 % NaCl and 0.05 % NaN_3 (TSA). High molecular weight fractions of Eu-chelate/IgG were pooled and stored at 4 °C.

4.6.3. Coating of microtitre plates with antibody, studies I - V

To coat microtitre wells (96-well Delfia microtitre plates, Perkin Elmer Wallac, Turku, Finland), the plates were incubated overnight at room temperature with protein A-purified anti-PLA_2GIIA antibody (25 µg/ml in 50 mM Tris-HCl buffer, pH 7.75, 0.9 % NaCl, 0.05 % NaN_3 (TSA); 200 µl/well) or anti-BPI antibody (25 µg/ml in TSA, 200 µl/well in study IV or 10 µg/ml in TSA, 100 µl/well in study V). The antibodies were pretreated with three volumes of HCl/water (125 µl of 11.6 M HC1 in 50 ml of water) for 5 min before coating. The plates were washed with TSA-based wash solution. For blocking, 300 µl of TSA containing 0.1 % BSA, 6 % D-sorbitol, 3.9 % diethylenetriaminepentaacetic acid (DTPA, Titriplex V, Merck, Darmstadt, Germany) and 1 mM $CaCl_2$ were added and, after an overnight incubation at room temperature, the fluid was aspirated. The plates were stored at 4 °C in a moist atmosphere.

4.6.4. Fluoroimmunoassays for PLA_2GIIA and BPI, studies I - V

PLA_2GIIA standards were prepared by serial dilution of human recombinant PLA_2GIIA (Ciba-Geigy Ltd, Basel, Switzerland) from a stock solution of 10 µg/ml in human serum with Delfia assay (TSA buffer, pH 7.75, containing BSA, bovine gammaglobulins, Tween 40, DTPA and an inert red dye, Perkin Elmer Wallac) to five concentrations (0.25, 1.5, 9, 54 and 324 µg/l, study I; 1.5, 9, 18, 54 and 324 µg/l, studies II and III). BPI standards were prepared from recombinant human BPI (Incyte, Palo Alto, CA, USA) stock solution into assay buffer (final concentrations 4.07, 9.76, 48.8, 122 and 305 µg/l in study IV and 1.22, 4.07, 9.76, 48.8 and 122 µg/l in study V) and stored in frozen aliquots.

Coated plates were washed two times, and 25 µl of either PLA_2GIIA standards or BPI standards or analyte were pipetted into the wells followed by 175 µ1 of assay buffer. After 1 h incubation with shaking at room temperature and six washes, 25 µl of Eu-labelled IgG (2.5 µg/ml diluted with assay buffer) and 175 µ1 assay buffer were added. After 1 h incubation with shaking at room temperature and six washes, 200 µl of Delfia enhancement solution (Perkin Elmer Wallac, Turku, Finland) was added. Fluorescence was measured after a further 5 min shaking and 10 min incubation at room temperature.

In study V, 10 µ1 of tear sample or standard solution, and 90 µ1 of Delfia assay buffer were added to the IgG-coated (anti-BPI antibody) microtitre wells and incubated at room temperature for 30 min with shaking at 240 cycles/min. After four washings with TSA-based washing solution, 10 µ1 of secondary antibody (Eu-labelled IgG solution diluted to 1 µg/ml with assay buffer) and 90 µ1 of assay buffer were added and the plate was incubated for 30 min with shaking. After washing, 100 µ1 of Delfia enhancement solution was incubated in the wells for 5 min with shaking as above, and after 10 min standing at room temperature, fluorescence was measured with a 1230 Arcus or a Victor fluorometer (Perkin Elmer Wallac) at excitation and emission wavelengths of 340 nm and 615 nm, respectively.

4.6.4.1. Evaluation of fluoroimmunoassays for PLA_2GIIA and BPI, and PLA_2 catalytic activity determination, unpublished

The original TR-FIA (IFMA) for PLA_2GIIA was published by Nevalainen et al. (1992). This assay was used in Study I. In studies II and III there were some modifications made to the assay design. A different polyclonal antibody against recocombinant PLA_2GIIA was raised in a rabbit. The standard concentrations in the assay protocol were also changed (0.25, 1.5, 9, 54 and 324 µg/l, study I; 1.5, 9, 18, 54 and 324 µg/l, studies II and III). The catalytic activity determination (Schädlich et al., 1987) was also modified by changing the original labelled portion of the substrate to *L*-α_1-palmitoyl phosphatidyl ethanolamine containing the ^{14}C-labelled archidonic acid in the sn-2 position (NEC-783, PerkinElmer Life Sciences, Boston MA, USA). Moreover, the volume of this portion of the substrate was decreased from 10 µl to 5 µl per assay tube. However, the catalytic determinations of PLA_2 were done in study I using the original method by Schädlich et al. (1987).

In order to determine normal reference values for serum and EDTA plasma, concentrations of PLA_2GIIA were measured in control specimens, first comprising 42 persons (28 men and 14 women, median age 45 years, age range 21 - 64 years) and then expanded to include 83 voluntary adult blood donors (41 men and 42 women, mean age 44.4 years, median age 45 years, age range 21 - 64 years, Finnish Red Cross Blood Transfusion Service in Turku, Finland)

The serum and EDTA plasma samples of 42 healthy blood donors of PLA_2GIIA studies were also used for BPI determination.

Estimations of the reference values for serum and EDTA plasma were done by using the 95% reference interval with the 2.5th and 97.5th centiles (Wright and Royston 1999) according to the IFCC recommendations (IFCC 1987). Therefore, it is postulated that 5% of 'normal', randomly selected reference individuals, have values outside the levels of the group of majority population.

Three internal serum-based controls (2.7, 26.3 and 82.3 μg/l) were included in every analysis for PLA_2GIIA. These controls were prepared from pooled control sera and stored as frozen aliquots for daily use. For PLA_2 activity measurements, two internal controls were prepared from *Crotalus atrox* venom PLA_2 (Sigma Chemical, St. Louis, MO, USA). These controls were divided into aliquots, stored frozen and used in every analysis. Tests were done with different sample buffers for tissue homogenates and protein mixtures without PLA_2 activity for blank solutions.

To test the analytical recovery of the BPI assay, 49.3 μg/l of BPI in human citrated plasma was added to 34 plasma samples. As reference material, BPI was measured in citrated plasma of 15 healthy adult donors.

To evaluate PLA_2GIIA and BPI TR-FIA assays in elevated concentrations, we studied serum and EDTA plasma samples of 48 patients with Crohn's disease and synovial fluid of 37 patients with RA. As reference material, synovial fluid of 35 subjects autopsied at the Department of Pathology, University of Turku, with no known arthropathy was used. PLA_2GIIA was also measured in sera of these 37 RA patients.

4.7. Immunohistochemistry, studies I, IV and V

For immunohistochemistry, sections of formalin-fixed paraffin-embedded tissue specimens were reacted with a monoclonal anti-human PLA_2GIB antibody (Santavuori et al. 1992), a polyclonal rabbit anti-human recombinant GIIA PLA_2 antiserum or with an IgG fraction of polyclonal rabbit anti-human recombinant BPI antiserum (see 4.6.1.). These primary antibodies were affinity purified with protein A (Amersham Biosciences, Uppsala, Sweden). Alternatively, formalin-fixed paraffin-embedded tissues containing the lacrimal gland and the adjacent conjunctiva were digested with pepsin, 4 mg/ml in 10 nM HCl, and reacted with an IgG-fraction (2.84 mg/ml, dilution 1:5000) of a rabbit anti-human recombinant BPI antiserum. To exclude possible cross-reaction, other antibodies used were monoclonal antibody HHF 35 for muscle actin

(Enzo, Farmingdale, NY, USA; dilution 1:50), monoclonal antibody for vimentin (clone V9, catalog no. 08-00520, Zymed Laboratories, San Francisco, CA, USA; dilution 1:50), monoclonal antibody 34 beta E 12 for high molecular weight cytokeratins 1, 5, 10 and 14 (Enzo, Farmingdale, NY, USA; dilution 1:5) and polyclonal antiserum for S-100 (Dako, Glostrup, Denmark; dilution 1:1000). The primary immunoreaction was localized according the avidin-biotin complex method (Hsu et al., 1981) by using Vectastain avidin-biotin-peroxidase complex kits (Vector Laboratories, Burlingame, CA, USA). For controls, the primary antibodies were replaced by nonimmune rabbit serum or bovine serum albumin. The sections were counterstained lightly with hematoxylin.

4.8. Western blotting, study V

All reagents and equipment used in Western blotting were purchased from Amersham Pharmacia, Uppsala, Sweden. Samples from tissue homogenate supernatant and tear specimen were diluted (1:10) in non-reducing Laemmli electrophoresis buffer and heated to 95°C for 5 min. Electrophoresis was performed on a MultiPhor system with homogeneous 15% SDS gels. Separated proteins were transferred to Hybond ECL (enhanced chemiluminescence) nitrocellulose membranes by using a MultiPhor II Nova Blot system according to the manufacturer's instructions. For analysis, the same recombinant BPI polyclonal antibody as in immunohistochemistry was used at dilution of 1:1000, followed by enhanced chemiluminescence with ECL Western blotting reagents.

4.9. Statistical analyses, studies I - V

The descriptive statistics are given as mean ± SD. All p values lower than 0.05 were considered statistically significant.

The difference in PLA_2-values in homogized tissue and tear samples was analyzed using the Mann-Whitney *U-test*. (I)

The baseline comparisons between healthy controls and CL wearers without CLs at 4 p.m. were conducted using the Wilcoxon rank sum test. The diurnal variation in the PLA_2GIIA content of tears was compared between the healthy controls and the subjects wearing CLs by using analysis of variance for repeated measurements. The mean change of the PLA_2GIIA content studied within the control group was also analyzed by means of repeated-measures analysis of variance. (II)

Statistical analyses were performed with program SAS for Windows version 8.2 (SAS Institute, Cary, NC, USA) After a test for normality (Shapiro-Wilk's *W-test*), the difference in the PLA_2GIIA content between the patients with ABC and the healthy controls, and the effect of age on the PLA_2GIIA concentration of tears was analyzed using the Student's *t-test*. Difference in the PLA_2GIIA content of tears between ABC patients with dry eyes and ABC patients with normal tear secretion rate was tested with a two-way *t-test*. Linear regression analysis was used in comparison of the PLA_2GIIA concentration of tears with each off the different conjunctival cells (eosinophils,

PMNs, lymphocytes, squamous epithelial cells, columnar epithelial cells and goblet cells) separately and with the inflammatory cells (eosinophils, PMNs and lymphocytes) together. Pearson's correlation was determined between the PLA_2GIIA concentration of tears and the quantity of eosinophils. (III)

Student's *t-test* and Pearson's linear regression were used in the analysis of BPI-values in serum samples. (IV)

The difference in BPI-values in tears between the genders was analyzed using the Mann-Whitney *U-test*. In addition, Spearman rank order correlation was determined between the tear flow rate and the concentration of BPI in tears of healthy subjects. (V)

The Bland and Altman procedure (Bland and Altman 1986; Bland and Altman 1999) was used in comparison of the unpublished PLA_2 and BPI determination data. This involves graphical techniques and calculations of the differences between observations made by using the two methods on the same subjects. The 95% limits of agreement, estimated by mean difference of ± 1.96 standard deviation of the differences, provide an interval within which 95% of differences between measurements by the two methods are expected to lie.

5. RESULTS

5.1. Presence of PLA_2GIIA in tissues and in serum/plasma samples, studies I - III

5.1.1. Evaluation of PLA_2GIIA fluoroimmunoassay and PLA_2 catalytic activity determination in fluids of healthy and diseased individuals, unpublished

The specificity of the polyclonal antibody used in studies II and III was confirmed in the laboratory of professor Edward Dennis (University of California, San Diego, USA) by Western blot (personal communication). This antibody did not cross-react with human PLA_2GV, a structurally close relative of PLA_2GIIA.

5.1.1.1. Measurements in samples of control individuals

In an analysis of 83 control sera from healthy blood donors the mean PLA_2GIIA concentration was 6.0 µg/1 (SD 2.7, range 1.6 - 12.3 µg/1, median 5.7 µg/l; See Figure 8).

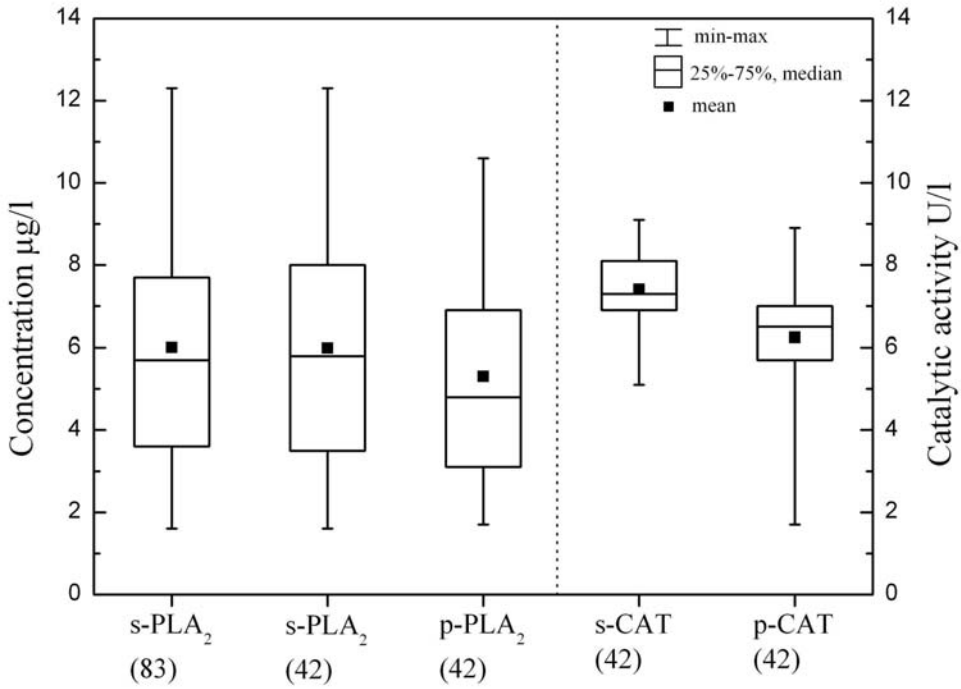

Figure 8. Concentration (µg/l) and catalytic activity (U/l) of PLA_2GIIA in control serum and EDTA plasma samples (mean, median, 25th and 75th percentile and range). s-PLA_2 (83) shows the values of PLA_2GIIA in 83 control sera, s-PLA_2 (42) and p-PLA_2 (42) shows the PLA_2GIIA values in sera and EDTA plasma of 42 control individuals, and s-CAT (42) and p-CAT (42) shows the values of PLA_2 catalytic activity in sera and EDTA plasma of 42 control individuals.

The mean concentration of PLA_2GIIA in 42 individuals was 6.0 µg/1 (SD 2.6, range 1.6 - 12.3 µg/1, median 5.8 µg/1) in serum and 5.3 µg/1 (SD 2.5, range 1.7 - 10.6 µg/1, median 5.2 µg/1) in EDTA plasma samples (Figure 8). Figure 9A shows the result of the Bland and Altman procedure for the same 42 reference individuals. The mean difference was 0.7 µg/1 with 95 % confidence interval 0.2 to 1.2 µg/1 (limits of agreement). Thus TR-FIA measurement of PLA_2GIIA tends to give higher values for serum between 0.2 and 1.2 µg/1.

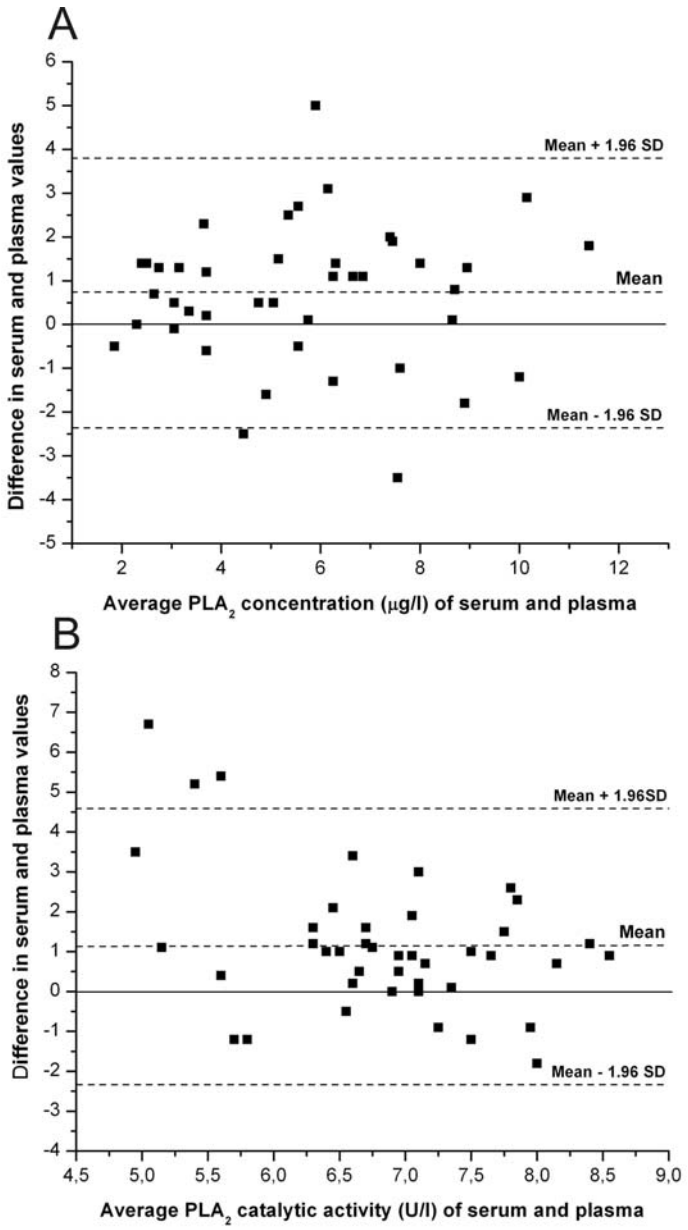

Figure 9. Bland-Altman plot of TR-FIA for PLA_2GIIA (A) and PLA_2 catalytic activity (B), when 42 serum and EDTA plasma samples were compared.

The mean catalytic activity of PLA_2 was 7.4 U/l (SD 1.0, range 5.1 - 9.1 U/l, median 7.4 µg/1) in serum and 6.3 U/l (SD 1.5, range 1.7 - 8.9, median 6.5 µg/1) in EDTA plasma samples of 42 healthy donors, respectively (See Figure 8). Figure 9B shows the Bland-Altman plot for these measurements. The mean difference in catalytic activity measurement of PLA_2 was 1.2 U/l with 95 % confidence interval 0.6 to 1.7 U/l. This method tends to give higher catalytic activity values of PLA_2 for serum between 0.6 and 1.7 U/l.

Estimations of PLA_2GIIA reference values for serum and EDTA plasma were made using the 95% reference interval with the 2.5th and 97.5th centiles. In Figure 10, the reference interval of 1.8 - 11.6 µg/l in serum for 83 randomly selected blood donors is shown. Accordingly, reference intervals for a smaller group of 42 individuals were 2.3 - 11.6 µg/l and 1.8 - 10.5 µg/l in serum and EDTA plasma, respectively. Reference values of PLA_2 catalytic activity were 5.2 - 9.0 U/l in serum and 2.8 - 8.4 U/l in EDTA plasma for the same group of 42 blood donors. These results are in line with earlier published reference values of PLA_2GIIA, i.e., the normal serum and plasma concentration of PLA_2GIIA in man is below 10 µg/l. Also, it can be concluded that the reference catalytic activity of PLA_2 for human normal serum and EDTA plasma is below 10 U/l (Kaiser 1999).

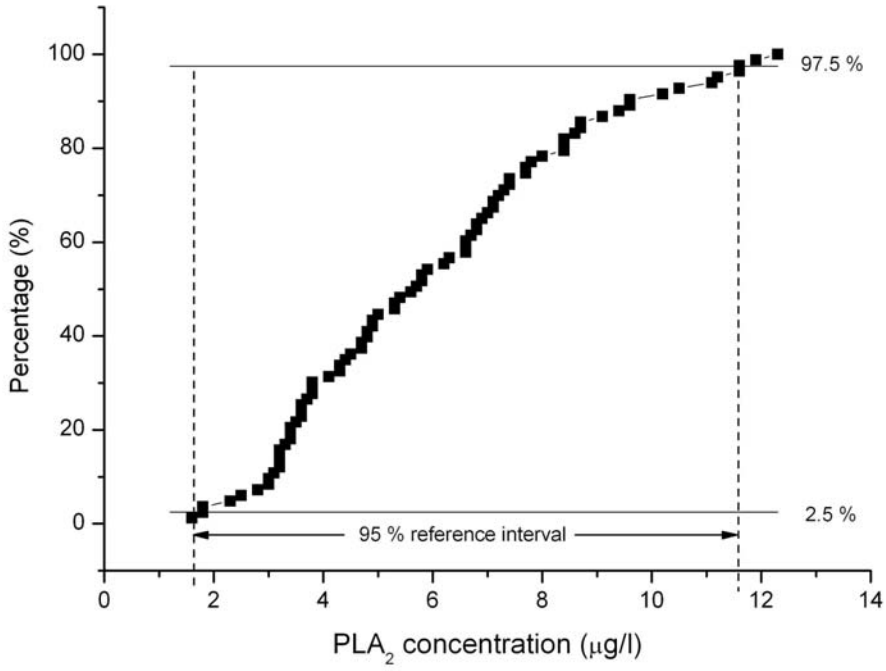

Figure 10. Cumulative frequency plot of PLA_2GIIA TR-FIA measurement with 95 % reference interval for 83 serum samples. The PLA_2GIIA concentration is at the 2.5th centile 1.8 µg/l and at the 97.5th centile 11.6 µg/l.

5.1.1.2. Measurements in patient samples

The PLA_2GIIA concentration of 48 patients with Crohn's disease was 19.4 ± 36.8 µg/l in serum (mean ± SD, range 1.5 - 245.0 µg/l) and 21.8 ± 45.2 µg/l (mean ± SD, range 2.5 - 300.0 µg/l) in EDTA plasma samples. Figure 11 shows the result of the Bland and Altman procedure for these samples. When the amount of random error increases as the measured values increase, the data are said to be heteroscedastic (Atkinson and Nevill, 1998). In this situation, Bland and Altman (1986) recommend the natural logarithmic transformation of the data before the calculation of limits of agreement. The mean difference was – 0.05 µg/l on the log scale and back-transform (antilog) the data gave for the mean difference 1.0 µg/l with 95 % confidence interval 0.9 to 1.1 µg/l (limits of agreement). TR-FIA measurement of PLA_2GIIA tends to give lower values for serum between 0.9 and 1.1 µg/l. Immunoassays of PLA_2GIIA in serum and EDTA plasma of healthy individuals and patients with Crohn's disease showed that both methods for sample collection are equally valid for TR-FIA determination.

The PLA_2GIIA concentration in serum of 37 patients with RA was 66.0 ± 80.5 µg/l (mean ± SD, range 10.4 - 417.0 µg/l) and in synovial fluid 894.5 ± 474.5 µg/l (mean ± SD, range 114.0 - 1770.0 µg/l). PLA_2GIIA concentration in synovial fluid of 35 autopsied subjects was 629.6 ± 487.4 µg/l (mean ± SD, range 42.0 - 2068.0 µg/l). These results are in line with the published data showing significantly higher levels of PLA_2GIIA in patients with RA than in healthy controls (Kaiser 1999, Pruzanski 2005).

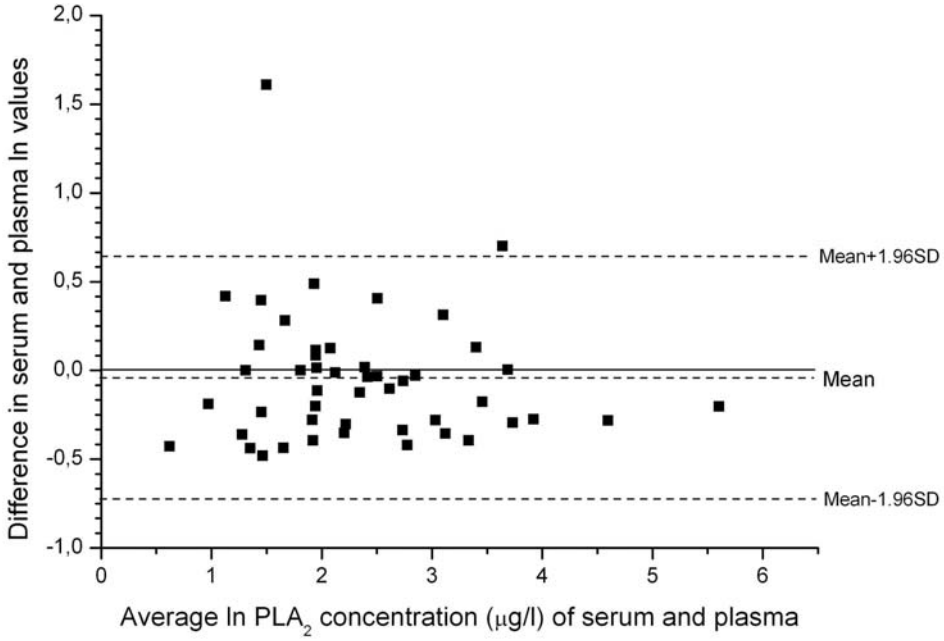

Figure 11. Bland-Altman plot of TR-FIA for PLA_2GIIA, when the measurements in serum and EDTA plasma of 48 patients with Crohn's disease were compared. Natural logarithmic (ln) transformation of the data has been performed.

5.1.2. Detection of PLA_2 in the lacrimal gland, study I

The concentration of PLA_2GIIA was 2458.8 ± 1762.1 µg/l (332.3 ± 142.6 µg/g protein) in the 8 lacrimal glands and 38.3 ± 43.3 µg/1 (10.1 ± 12.7 µg/g protein) in the 8 muscle homogenates ($p < 0.001$, Mann-Whitney *U-test*). The concentration of PLA_2GIB was below the detection limit of the assay (0.28 µg/1) in the lacrimal gland, muscle, and tear specimens. The mean (± SD) catalytic activity of PLA_2 was 40.2 ± 12.4 U/1 (6.2 ± 6.2 U/g protein) in the 8 lacrimal gland homogenates and 0.25 ± 0.51 U/1 (0.07 ± 0.14 U/g protein) in the 8 skeletal muscle homogenates ($p < 0.001$, Mann-Whitney *U-test*). Therefore, the catalytic activity present in lacrimal homogenates and tears is due to the presence of PLA_2GIIA.

Immunohistochemical analysis was used to identify cells expressing PLA_2GIIA. Immunoreactivity was seen in the apical cytoplasm of the acinar cells of lacrimal gland. The apical granules in the acinar cells were most intensely labelled. In addition, some reactivity was present in the apical parts of ductal epithelial cells. Diffuse weak staining was seen in some cells of the conjunctival epithelium. No staining was found in the ductal myoepithelial cells. Interacinar plasma cells and lymphocytes were also negative. Elastic fibers in the connective tissue and arterial walls showed some labelling. Lacrimal glands did not contain immunoreactive PLA_2GIB. Neither PLA_2GIB nor PLA_2GIIA was detected in skeletal muscle by immunohistochemistry.

5.1.3. Concentration of PLA_2GIIA in tears of normal and diseased eye, studies I - III

5.1.3.1. Measurements in samples of healthy individuals

In study I, the mean concentration of PLA_2GIIA in emotional tear samples was 1451.3 ± 156.0 µg/1 (mean ± SD) for 4 healthy donors, which is markedly higher than values measured in venous samples (<10 µg/1). The catalytic activity of PLA_2 was 595.5 ± 305.2 U/1 in these 4 samples (mean ± SD), as compared to below 10 U/l in normal plasma/serum (Kaiser 1999).

5.1.3.2. Measurements in patient samples

In study II, the mean PLA_2GIIA concentration in non-stimulated tears of healthy controls collected with microcapillaries was 80.6 ± 47.8 mg/l (mean ± SD). The concentration increased between 8 a.m. (69.9 ± 37.5 mg/l) and noon (95.2 ± 48.2 mg/l; $p = 0.006$), and decreased between 4 p.m. (92.3 ± 48.2 mg/l) and 8 p.m. (64.9 ± 41.2 mg/l; $p = 0.003$). Multiple comparisons were not adjusted for in the statistics cited.

The PLA_2GIIA content of tears of CL wearers was 56.3 ± 30.3 mg/l at noon, 54.1 ± 24.3 mg/l at 4 p.m. and 49.7 ± 30.5 mg/l at 8 p.m. The PLA_2GIIA values in CL wearers at noon ($p=0.0001$) and 4 p.m. ($p = 0.0002$) were statistically significantly lower than the values in the healthy controls at the same times of day. There was no statistically significant difference in the PLA_2GIIA content of tears between the CL wearers and control individuals at 8 p.m. ($p = 0.11$). Multiple comparisons were not adjusted for in the statistics cited.

In study III, the PLA_2GIIA content of tears was 43.8 ± 33.0 mg/l (mean ± SD) in patients with atopic blepharoconjunctivitis (ABC) and 67.1 ± 23.3 mg/l (mean ± SD) in control individuals. The difference between these two groups was statistically significant ($p = 0.0018$). A significant decrease in the concentration of PLA_2GIIA occurred with increasing age ($p = 0.025$). The mean concentration of PLA_2GIIA in tears was 25.8 ± 23.6 mg/l (mean ± SD) in patients with ABC and dry eye, and 56.6 ± 33.3 mg/l (mean ± SD) in ABC patients with normal tear secretion rate. The difference between these groups was statistically significant ($p = 0.011$). Linear regression analysis did not reveal any statistically significant difference between the PLA_2GIIA content of tears and the presence of different cell types gathered from conjunctiva by the brush cytology. Conjunctival eosinophilis were present in 15 patients of 21 (71%). A trend was found between the PLA_2GIIA concentration of tears and the quantity of conjunctival eosinophils (linear regression analysis $p = 0.07$; Pearson's $r = 0.41$, $p = 0.06$).

5.2. Presence of BPI in healthy and diseased individuals, studies IV and V

5.2.1. Evaluation of BPI by fluoroimmunoassay, unpublished

Many research groups have studied how BPI should be measured (White et al., 1994; Dentener et al., 1995), and they have concluded that BPI should be measured in plasma, thereby avoiding artefacts caused by the PMN destruction and release of BPI during the process of coagulation. However, the original TR-FIA for BPI (study IV) was developed using serum samples. Thus, the behavior of TR-FIA was evaluated with different sample materials in order to determine normal reference values.

The BPI concentration of 42 control individuals was 21.4 ± 16.5 µg/1 (mean ± SD, range 2.3 - 77.2 µg/1, median 17.1 µg/1) and 7.1 ± 5.7 µg/1 (mean ± SD, range 0.9 - 24.1 µg/1, median 5.2 µg/1) in serum and EDTA plasma samples, respectively. In the 42 EDTA plasma samples the variation was large and some samples gave unexpectedly high values. Therefore, the reference interval for these samples was 1.35 - 23.0 µg/l (See Figure 12A). The BPI concentration of 48 patients with Crohn's disease was 18.2 ± 16.2 µg/1 (mean ± SD, range 0.3 - 71.0 µg/1, median 14.0 µg/1) and 3.6 ± 2.3 µg/l (mean ± SD, range 0.3 - 12.3 µg/1, median 3.1 µg/1) in serum and EDTA plasma samples, respectively. BPI concentration is not known to rise in patients with Crohn's disease. On the contrary autoantibodies against BPI may be found. The reference interval for EDTA plasma samples of 48 patients with Crohn's disease was 0.9 - 8.5 µg/1, see Figure 12B.

The analytical recovery of the BPI assay was 97.2 ± 11.2 % (mean ± SD, n = 34), measured in citrated plasma samples. The mean value for 15 healthy adult donors was 7.2 µg/l in citrated plasma (SD 4.3, range 0.7 - 18.4 µg/l, median 7.3 µg/l).

The BPI concentration in synovial fluid of 37 patients with rheumatoid arthritis was 533.3 ± 1120.6 µg/l (mean ± SD, range 1.9 - 6018.1 µg/l) as compared to 8.1 ± 5.2 µg/l (mean ± SD, range 0.5 - 23.6 µg/l, median 7.2 µg/1) in synovial fluid of 35 autopsied subjects.

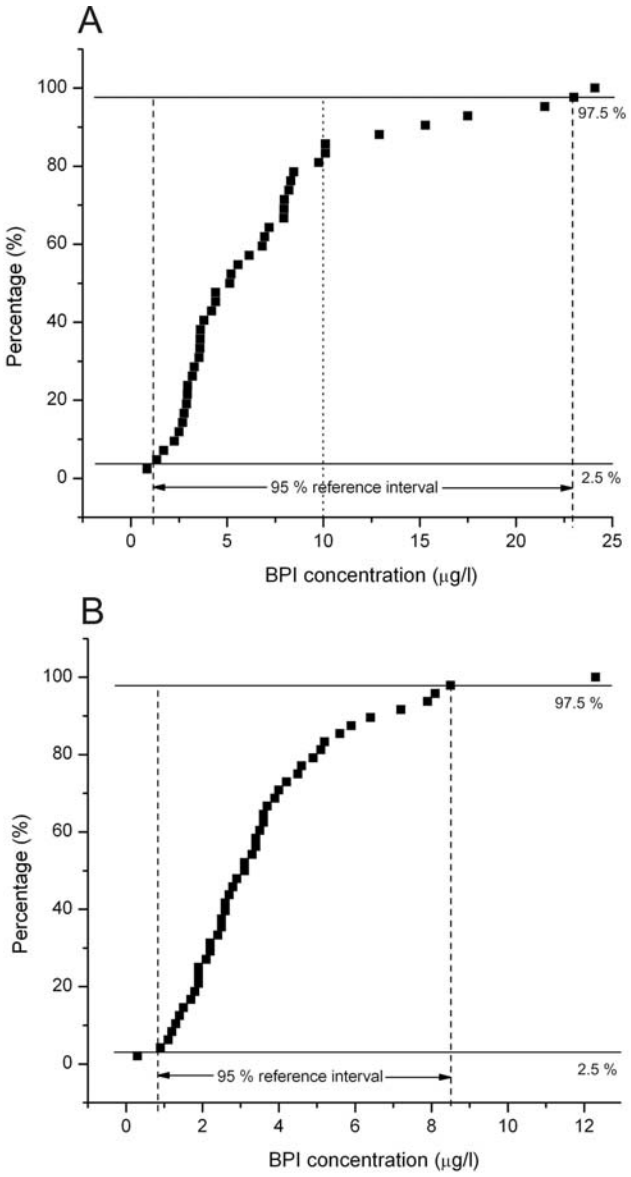

Figure 12. Cumulative frequency plot of BPI TR-FIA measurement with 95 % reference interval for EDTA plasma samples of 42 control individuals (A) and 48 patients with Crohn's disease (B). The BPI concentration is 1.35 µg/l at the 2.5th centile and 23.0 µg/l at the 97.5th centile in (A), and 0.9 µg/l at the 2.5th centile and 8.5 µg/l at the 97.5th centile in (B).

5.2.2. Fluoroimmunoassay, serum and heparinized plasma samples, study IV

The linear range for the BPI standard curve was 5 - 500 µg/1. The detection limit of the assay was 1.6 µg/1 corresponding to the mean plus 3 SD of the zero standard (blank) fluorescence counts. The mean BPI concentration was 28.3 µg/1 (range 1.64 - 132, SD 26.8, n = 83) in serum samples and in heparinized plasma 52.3 µg/1 (range 0.9 - 403, SD 60.6, n = 90), respectively.

The difference in the serum concentration of BPI between unselected hospitalized patients with and without infection was statistically significant ($p < 0.0001$). There was a positive correlation between the concentration of BPI in serum and the white blood cell count ($r = 0.589$, $p < 0.0001$, $n = 83$). There was also a positive correlation between serum BPI and CRP (C-reactive protein) levels ($r = 0.39$, $p < 0.05$, $n = 59$). However, in heparinized plasma samples there was no correlation between BPI and white blood cell count or CRP.

At the time of these tests, the plasma samples were not collected with the necessary care. Therefore, the samples were stored in room temperature for too long period of time and the plasma was not separated rapidly enough. These erroneous procedures in sampling explain why for all individuals tested, the BPI values were consistently higher in heparinized plasma samples compared to the matched serum samples. There was no difference between unselected hospitalized patients with and without infection in heparinized plasma samples.

5.2.3. Fluoroimmunoassay, tear samples of healthy eye, study V

The mean concentration (mean ± SD) of BPI in tears of healthy subjects was 26.8 ± 19.7 µg/l in 13 men, 28.2 ± 33.3 µg/l in 29 women, and 27.8 ± 29.5 µg/l in the combined groups ($n = 42$). There was no statistically significant difference in the BPI content of tears between the genders ($p = 0.45$). The tear flow rate was 4.5 ± 2.6 µl/min in men, 11.9 ± 16.2 µl/min in women, and 9.1 ± 13.2 µl/min in the overall analysis. The concentration of BPI decreased significantly with an increase in the tear flow rate (Spearman $r = -0.84$, $p < 0.0001$).

The concentration of BPI was 23.1 µg/l in the lacrimal gland and 3.6 µg/1 in the pectoralis major muscle homogenate of the same autopsied subject (our unpublished data).

5.2.4. Detection of BPI in tissues, studies IV and V

The presence of BPI in various tissue compartments was studied immunohistochemically.

In study IV, intense immunoreaction for BPI was seen in polymorphonuclear leukocytes at numerous locations (e.g., in the vascular compartment of kidney glomeruli and colonic mucosa). Control sections reacted with preimmune serum were devoid of immunoreaction.

In study V, BPI immunoreactivity was detected in the cytoplasm of outer basal epithelial cell layers of all intercalated, intralobular, interlobular and excretory ducts and also very faintly and focally in myoepithelial cells around acini.The staining pattern was identical in the eight subjects tested. Ductal basal cells and myoepithelium were negative when stained with normal control serum. Periacinar myoepithelial cells of spindle type were positive for muscle actin, vimentin and S-100. Staining of variable intensity for cytokeratin 34 beta E 12 were present in acini, ducts and conjunctiva and

also in some myoepithelial cells. Occasional dendritic melanocytic S-100 and vimentin positive cells were found in excretory ducts.

5.2.5. Western blot analysis of lacrimal gland and tear fluid, study V

Western blot analysis with a BPI antibody of the homogenized tissue sample of the lacrimal gland showed a single protein band with an approximate molecular weight of 55 kDa. BPI in the tear fluid specimen was also detected in Western blot analysis. However, the protein band visualized with the antibody BPI in the tear fluid migrated at a slightly elevated molecular weight, approximately 60 - 65 kDa, probably due to glycosides attached the protein.

6. DISCUSSION

The aqueous isotonic layer of the tear film is a complex mixture of electrolytes, glycoproteins and proteins, derived mainly from the lacrimal and accessory lacrimal glands (Bron et al., 2004). Since the cornea lacks blood vessels, the host defence requirements are supplied by the tear film. Human tears have been shown to contain high levels of the antimicrobial enzyme lysozyme (Saari et al., 1983) and other recently found proteins. In the current study we showed that human lacrimal gland is enriched with PLA_2GIIA and BPI, and it secretes these proteins into tear fluid where, in concert with other bactericidal agents, their concentrations are high enough to protect the eye against bacterial invasions.

6.1. Group IIA phospholipase A_2, studies I - III

6.1.1. Measurement of PLA_2GIIA

The catalytic determination of PLA_2 originates back to the early 1980's (Shakir 1981) based on the use of radioactive substrates and an extraction step. The more sensitive and convenient time-resolved fluoroimmunoassay (TR-FIA), specific for PLA_2GIIA, was developed a decade later and published in 1992 by Nevalainen and co-workers. These assays have made it possible to comprehend the role of PLA_2GIIA in protection against microbes. In theory, these methods could measure different parameters (activity and amount), but investigations in our and other laboratories have shown that in practice, both assay principles give comparable results. Laboratories have usually developed their assay methods in-house or used commercially available kits. These analyses provide a good understanding of PLA_2GIIA concentrations in normal human serum and plasma compared with the values measured in patients with many different diseases. Unfortunately, PLA_2 is not yet routinely determined in clinical laboratories for diagnostic purposes. The early immunoassays (Matsuda et al., 1991; Nevalainen et al., 1992; Vadas et al., 1992) are, undoubtedly, relevant for clinical laboratories. However, when the measurements are made from tissue homogenates or other biological fluids than serum or plasma, the immunoassays may not result in correct conclusions. The majority of errors occur in sampling, especially when factors like the diurnal variation and sample homogeneity is not taken into account. Comparison of the available data from this thesis against other published literature shows that common practices are required for establishing diagnostic values for PLA_2 in body fluids like tears.

6.1.2. PLA_2GIIA in lacrimal gland and tear fluid, study I

The present work (study I) showed for the first time that PLA_2GIIA is located in, and secreted from lacrimal glands in human eye. This finding was subsequently verified by Aho and co-workers (1996). They also showed that PLA_2GIIA is secreted apart from lysozyme. More recently, PLA_2GIIA has been purified and detected in human tears by Western blotting (Qu and Lehrer 1998; Song et al., 1999).

PLA_2GIIA values measured in the non-stimulated tears of healthy subjects were three orders of magnitude higher (37.7 mg/l; microfiltration and enhanced chemilunimescence detection, Qu and Lehrer 1998) than in serum. However, lower PLA_2GIIA values (27.4 mg/l) were measured in onion vapour-stimulated tears.

We measured PLA_2GIIA from emotional tears collected from four voluntary donors. Comparison with the results of later studies by Qu and Lehrer (1998) showed that our samples were diluted. It can be concluded, therefore, that the samples must be taken directly from the eye.

Many anti-microbial proteins in tears are synthesized in the main and accessory lacrimal glands and their concentrations follow a diurnal rhythm and decrease with an increase in age. This has also been observed for PLA_2GIIA with our TR-FIA (Saari et al., 2001). Similar findings were demonstrated with tears collected from rabbit eyes, where the catalytic activity of PLA_2 decreases with increasing age (Girgis et al., 2003). Drainage of tears into the nasolacrimal system serves to remove waste products. PLA_2GIIA in nasal fluid originates mainly from the tear fluid, although minor amounts are detectable in the mucosa of nasolacrimal ducts (Aho et al., 1997; Paulsen et al., 2001).

6.1.3. Concentration of PLA_2GIIA in tears of normal and diseased eye, studies II and III

Qu and Lehrer (1998) measured PLA_2GIIA concentrations which are comparable with our studies (II and III), although they used a different method. They used a Western blot analysis with densitometer detection. This method is accurate but unsuitable for larger amount of samples. The drawback of the studies by Qu and Lehrer (1998) were that they did not take into account the diurnal variation which had already been demonstrated to affect lysozyme concentration (Horwitz et al., 1978) and that the main tear proteins may decrease with age (van Haeringen 1997). Their control individuals were between 25 and 36 years old. The mean normal PLA_2GIIA concentration in this study of six healthy donors (36.7 ± 7.3 mg/l) was lower than published by Saari et al. (2001, 54.5 ± 33.9 mg/l) for 122 healthy volunteers. The original TR-FIA for PLA_2GIIA of our studies was optimized for the serum measurements. However, efforts to improve this assay were made. Most of the variations in results in our studies were found to result from sampling due to difficulties in obtaining them homogeneously as well as possible factors affecting sample dilution. Despite certain variations in the values due to sampling and other factors, the relatively high and constant concentrations of the membrane-attacking enzyme PLA_2GIIA in tears implied that PLA_2GIIA is one of the principal bactericides in human tears. Recent studies have shown that PLA_2GIIA in serum samples of patients with bacterial infections, although present at much lower concentration than in normal tear fluid, is cabable of killing 90% of *Staphylococcus aureus* and 99% of *Listeria monocytogenes in vitro*, and even at the lower concentrations found in normal serum PLA_2GIIA killed 90% of *Listeria monocytogenes* (Grönroos et al., 2002).

It was thought that contact lens use may induce distortion of secretion of antimicrobial enzymes. I studied the PLA_2GIIA concentration in tears of persons who had used CLs for many years. I found that after a break of at least 16 h without CLs the enzyme concentration returned to a normal range. This is an important practical result showing that even the long-term use of CLs does not cause permanent changes in the secretion of antimicrobial proteins in tears. However, we found a statistically significant decrease in PLA_2GIIA values in tears of the CL wearers using CLs at noon and at 4 p.m. when compared to the healthy controls. This may be due the absorption of the protein into the CLs. Hume and co-workers (2004) found that one type of CL material can absorb more PLA_2GIIA than another type, but showed that there might be a saturation point for each type of CL. Thus, the protective effect of PLA_2GIIA may depend on the type of CL and the usage time.

Diurnal variation affects the normal values of PLA_2GIIA in human tears considerably. The concentration increased between 8 a.m. (69.9 ± 37.5 mg/l) and noon (95.2 ± 48.2 mg/l), and decreased between 4 p.m. (92.3 ± 48.2 mg/l) and 8 p.m. (64.9 ± 41.2 mg/l) in a group of 22 individuals. This is consistent with the diurnal variation shown for lysozyme (Horwitz et al., 1978). The work by Saari and co-workers (2001) showed that PLA_2GIIA concentration is highest in the age group of 20 - 29 years (81.6 ± 32.0 mg/l, mean ± SD), decreasing thereafter with increasing age. They found with our TR-FIA a PLA_2GIIA concentration of 54.5 ± 33.9 mg/l (mean ± SD) for 122 age- and gender-matched healthy individuals which represented the adult population well. They showed that there was no difference in PLA_2GIIA concentration between genders, as showed also for lysozyme (Saari et al., 1983). Our findings for the reference individuals support this result.

Our further studies showed that the PLA_2GIIA concentration in tears decreases in patients with atopic blepharoconjunctivitis (ABC). Non-stimulated tear samples were taken from middle-aged ABC patients who did not wear contact lenses. Almost one-half (48%) of the patients in the present study had dry eyes. PLA_2GIIA values decrease in tears of older subjects (Saari et al., 2001) and also in reflex tears of normal individuals (Aho et al. 2002b). Similarly, the PLA_2GIIA concentration decreases two days after photorefractive keratectomy surgery, although 1 week postoperatively, it was higher than preoperatively (Aho et al., 2003). On the other hand, PLA_2GIIA values increase markedly in patients with keratoconjunctivitis sicca (Aho et al., 2002a). It is not clear why ABC lowers PLA_2GIIA in tears. The lower amounts may be associated with atopy since the lysozyme concentration of tears decreased in patients with atopy (Saari and Klockars, 1983). Several studies have shown that the occurrence of *Staphylococcus aureus* in the conjunctiva and lid margin is a consistent finding in patients with ABC (Laouini et al., 2003), as also observed in our patients. Continuous infection in the conjunctiva may stimulate the secretion of antimicrobial proteins of tears which, with longstanding hyper-secretion, may cause an increased consumption of antimicrobial proteins of tears and, in this way, a decrease in the PLA_2GIIA content of tears. While PLA_2GIIA in tears of patients with ABC were below the normal values, they were still considerably above the level required for effective killing of gram-positive bacteria (Qu and Lehrer, 1998). However, in the patients with both ABC and

dry eye, the PLA_2GIIA concentration of tears may occasionally be insufficient for effective killing of gram positive bacteria, since the levels of lysozyme are also decreased in patients with dry eye (Seal et al., 1986). Interestingly, when I measured PLA_2GIIA in tears of patients with ocular rosacea, I found significantly lower concentrations compared to normal subjects, especially in patients with dry eye symptons. These results were published elwewhere (Kari et al., 2005). Furhermore, PLA_2GIIA concentration of tears was normal in 22 patients with chronic staphylococcal blepharoconjunctivitis (SBC, unpublished finding). PLA_2GIIA activity was increased in tears from infected eyes (Girgis et al., 2003), but our unpublished study showed that the concentration of PLA_2GIIA in tears of patients with SBC was normal obviously due to an equally increased secretion and depletion of PLA_2GIIA.

6.2. Bactericidal/permeability-increasing protein, studies IV and V

6.2.1. Measurement of BPI, study IV

When the first BPI determinations were published, researchers did not agree on whether BPI should be measured from serum or plasma. Through evaluation of the methods, and especially through better knowledge of BPI, it is shown that BPI should be measured from plasma, thereby avoiding artefacts caused by the destruction of neutrophils and release of BPI during the process of coagulation. It has been emphasized that BPI levels may vary depending upon the elapsed time for collection and processing of the sample (Dentener et al., 1995). We developed a TR-FIA for BPI and showed that both EDTA-treated and citrated plasma, synovial fluid, and with slight modifications, tears can be used. EDTA chelates with lanthanides such as europium, but the assay has a two stage protocol with a washing step. Therefore, EDTA in the sample does not come in contact with the label and does not interfere with the assay. The assay still needs further evaluation, though the main focus for venous samples is timely processing of samples in which plasma has separated. Experience shows that BPI levels in separated EDTA plasma are quite stable during a few freeze-thaw cycles, similarly to PLA_2GIIA in serum and plasma samples. In conclusion, the methods for BPI measurement need further examination as evidenced by variable results for control levels of the protein in the literature.

6.2.2. BPI in lacrimal gland and in tears of healthy subjects, study V

Our study showed for the first time that human lacrimal gland and tears contain BPI in concentrations which are effective against a broad spectrum of gram-negative bacteria. Tears also contain defensins and lactoferrin which are active against gram-negative bacteria (Haynes et al., 1999; Ellison and Giehl 1991).

Immunohistochemical analysis has shown that the cytoplasm of basal ductal epithelial cells of lacrimals glands contains BPI. Basal epithelial cells as the origin of a bactericidal protein secreted into tears may appear unexpected. However, cultured squamous epithelial cells as well as hair bulb keratinocytes and dermal fibroblasts have also been shown to express BPI (Reichel et al., 2003; Takahashi et al., 2004). Western blot analysis of the homogenized tissue of the lacrimal gland showed one protein band with a molecular weight of 55 kDa, consistent with the size of BPI. These

immunochemical analyses, along with the unpublished data, support the existence of BPI in human lacrimal gland.

The results of the present study have shown that the mean concentration of BPI in tears of healthy subjects was 27.8 ± 29.5 μg/l (mean ± SD, n = 42). This concentration markedly exceeds the level found in normal plasma. The mean concentration of BPI of the tear fluid exhibited a very high SD, in line with observations on PLA_2GIIA. This may be due to variations in reflex and basal tears under normal conditions. Part of the variation probably originates from sampling, factors affecting the dilution of the sample, and difficulties in taking the sample homogeneously. Related problems are well known from analysis of saliva, but despite the potential variability of its composition, a number of analyses mainly for hormones and drugs have been succesfully developed (Wilde, 1994; Streckfus and Bigler, 2002). However, the results obtained indicate that BPI belongs to the defense protection system in tears and its concentration is normally sufficiently high for killing of gram-negative bacteria. The bactericidal activity of BPI against common gram-negative bacteria, including *E. coli*, is seen at nanomolar concentrations in biologic fluids such as serum, plasma, and whole blood (Weiss et al., 1992) as well as in inflammatory peritoneal exudates generated *in vivo* (Weinrauch et al., 1995).

Our study suggests that BPI present in human tears is secreted from the lacrimal gland. However, even non-stimulated tear samples may contain small amounts of PMN cells which are rich in BPI. Thus, a fraction of the BPI measured in tears with TR-FIA may actually be derived from the PMN cells lysed during freezing or thawing and dilution for the measurement. Tear fluid from closed eye is known to be rich of PMN cells (Sakata et al., 1997), but our samples were taken during the day time using microcapillaries. The open eye does not contain significant numbers of PMN cells. However, Sakata and co-workers suggest that there is a pool of inactive PMNs under the lower lid capable of adhering to the mucosal layer. Therefore, a critical factor in all types of tear analysis is to obtain a representative and reproducible tear sample.

6.3. Future directions

Studies with phopholipase A_2 have shown that it is an expanding family group. Of note, BPI has recently also formed a "superfamily" with the identification of homology to a murine epithelial expressed gene named plunc (palate, lung and nasal epithelium clone), expressed in upper airways and nasopharynx (Bingle and Craven, 2002). Earlier found related proteins with BPI are LBP, with its antagonistic functions, and cholesteryl ester transfer protein and phospholipid trasfer protein (Hailman et al., 1996). BPI's actions are amplified by extracellular factors including the complement system and secretory phopholipase A_2 (Elsbach et al., 1994), and members of defensin and cathelicidin anbimicrobial peptide families synergistically enhance BPI's antimicrobial activity (Levy et al., 1994). PLA_2GIIA and BPI, together with their family group proteins, will be focused not only as host defence antimicrobial proteins, but also as a future source in development of pharmaceutical compounds.

7. CONCLUSIONS

The presence of two novel proteins in human eye, PLA_2GIIA and BPI, was shown by immunohistochemistry.

Methods were developed for measuring PLA_2GIIA and BPI in human body fluids, including tears, for the purpose of assessing the diagnostic value of the proteins. The diagnostic value of measuring the two proteins is hampered by unpredictable variation of the daily and individual concentrations. Both the tear fluid sampling and the assay methodology need improvements.

The normal concentrations for the proteins in tears were determined, taking into account diurnal variation. Tear fluid was found to be enriched with PLA_2GIIA and BPI.

It was shown that contact lenses do not permanently affect the secretion of the proteins from lacrimal glands. In atopic eye disease the values of PLA_2GIIA decreased.

The results of the present thesis indicate that PLA_2GIIA and BPI may have a crucial role in the defence of the ocular surface against microbial invasions, together with other known bactericides. Improvement of the sampling procedure will enable studies of different eye diseases and thereby provide better understanding of the biological functions of PLA_2GIIA and BPI. Since BPI is under biopharmaceutical development, it is possible that in future, patients with eye diseases will also benefit from BPI-based pharmaceutical compounds.

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9. REFERENCES

Aarsman AJ, van den Bosch H. A continuous spectrophotometric assay for membrane-bound lysophospholipases using a thioester substrate analog. FEBS Lett 1977; 79: 317-320

Aho HJ, Grenman R, Sipilä J, Peuravuori H, Hartikainen J, Nevalainen TJ. Group II phospholipase A_2 in nasal fluid, mucosa and paranasal sinuses. Acta Otolaryngol 1997; 117: 860-863

Aho HJ, Saari KM, Kallajoki M, Nevalainen TJ. Synthesis of group II phospholipase A_2 and lysozyme in lacrimal glands. Invest Ophthalmol Vis Sci 1996; 37: 1826-1832

Aho VV, Holopainen JM, Tervo T, Moilanen JA, Nevalainen TJ, Saari KM. Group IIA phospholipase A_2 content in tears of patients having photorefractive keratectomy. J Cataract Refract Surg 2003; 29: 2163-2167

Aho VV, Nevalainen TJ, Saari KM. Group IIA phospholipase A_2 content of tears in patients with keratoconjunctivitis
sicca. Graefes Arch Clin Exp Ophthalmol 2002a; 240: 521-523

Aho VV, Nevalainen TJ, Saari KM. Group IIA phospholipase A_2 content of basal, nonstimulated and reflex tears. Curr Eye Res 2002b; 24: 224-227

Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. Crit Care Med 2001; 29: 1303-1310

Atkinson G, Nevill AM. Statistical methods for assessing measurement error (reliability) in variables relevant to sports medicine. Sports Med. 1998; 26: 217-238

Baker SF, Othman R, Wilton DC. Tryptophan-containing mutant of human (group IIA) secreted phospholipase A_2 has a dramatically increased ability to hydrolyze phosphatidylcholine vesicles and cell membranes. Biochemistry 1998; 37: 13203-13211

Balsinde J, Winstead MV, Dennis EA. Phospholipase A_2 regulation of arachidonic acid mobilization. FEBS Lett 2002; 531: 2-6

Beamer LJ. Structure of human BPI (bactericidal/permeability-increasing protein) and implications for related proteins. Biochem Soc Trans 2003; 31: 791-794

Beamer LJ, Carroll SF, Eisenberg D. The three-dimensional structure of human bactericidal/permeability-increasing protein: implications for understanding protein-lipopolysaccharide interactions. Biochem Pharmacol 1999; 57: 225-229

Beers SA, Buckland AG, Giles N, Gelb MH, Wilton DC. Effect of tryptophan insertions on the properties of the human group IIA phospholipase A_2: mutagenesis produces an enzyme with characteristics similar to those of the human group V phospholipase A_2. Biochemistry 2003; 42: 7326-7338

Beers SA, Buckland AG, Koduri RS, Cho W, Gelb MH, Wilton DC. The antibacterial properties of secreted phospholipases A_2: a major physiological role for the group IIA enzyme that depends on the very high pI of the enzyme to allow penetration of the bacterial cell wall. J Biol Chem 2002; 277: 1788-1793

Berg OG, Gelb MH, Tsai MD, Jain MK. Interfacial enzymology: the secreted phospholipase A_2-paradigm. Chem Rev 2001; 101: 2613-2654

Berner R, Furll B, Stelter F, Drose J, Muller HP, Schutt C. Elevated levels of lipopolysaccharide-binding protein and soluble CD14 in plasma in neonatal early-onset sepsis. Clin Diagn Lab Immunol 2002; 9: 440-445

Berta A. Anatomy and Physiology of the Lacrimal System. In: Enzymology of the Tears. CRC Press, Florida, USA. 1992, pp. 19-28

Bezzine S, Bollinger JG, Singer AG, Veatch SL, Keller SL, Gelb MH. On the binding preference of human groups IIA and X phospholipases A_2 for membranes with anionic phospholipids. J Biol Chem 2002; 277: 48523-48534

Bhat MK, Mueller-Harvey I, Sumner IG, Goodenough PW. Simplified methods for the synthesis of 2-hexadecanoylthio-1-ethylphosphorylcholine and for the determination of phospholipase A_2 activity. Biochim Biophys Acta 1993; 1166: 244-250

Bingle CD, Craven CJ. PLUNC: a novel family of candidate host defence proteins expressed in the upper airways and nasopharynx. Hum Mol Genet 2002; 11: 937-943

Bingle CD, Craven CJ. Meet the relatives: a family of BPI- and LBP-related proteins. Trends Immunol 2004; 25: 53-55

Blanco A, Solis G, Arranz E, Coto GD, Ramos A, Telleria J. Serum levels of CD14 in neonatal sepsis by Gram-positive and Gram-negative bacteria. Acta Paediatr 1996; 85: 728-732

Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1986; 1: 307-310

Bland JM, Altman DG. Measuring agreement in method comparison studies. Stat Methods Med Res 1999; 8: 135-160

Boekholdt SM, Keller TT, Wareham NJ, Luben R, Bingham SA, Day NE, Sandhu MS, Jukema JW, Kastelein JJ, Hack CE, Khaw KT. Serum levels of type II secretory phospholipase A_2 and the risk of future coronary artery disease in apparently healthy men and women: the EPIC-Norfolk Prospective Population Study. Arterioscler Thromb Vasc Biol 2005; 25: 839-846

Bokarewa MI, Jin T, Tarkowski A. Intraarticular release and accumulation of defensins and bactericidal/permeability-increasing protein in patients with rheumatoid arthritis. J Rheumatol 2003; 30: 1719-1724

Borgström B. Importance of phospholipids, pancreatic phospholipase A_2, and fatty acid for the digestion of dietary fat: in vitro experiments with the porcine enzymes. Gastroenterology 1980; 78: 954-962

Borregaard N, Cowland JB. Granules of the human neutrophilic polymorphonuclear leukocyte. Blood 1997; 89: 3503-3521

Bortolussi R, Rajaraman K, Qing G, Rajaraman R. Fibronectin enhances in vitro lipopolysaccharide priming of polymorphonuclear leukocytes. Blood 1997; 89: 4182-4189

van den Bosch H. Intracellular phospholipases A. Biochim Biophys Acta 1980; 604: 191-246

Broekhuyse RM. Tear lactoferrin: a bacteriostatic and complexing protein. Invest Ophthalmol Vis Sci 1974; 13: 550-554

Bron AJ, Tiffany JM. The meibomian glands and tear film lipids. Structure, function, and control. Adv Exp Med Biol 1998; 438: 281-95

Bron AJ, Tiffany JM, Gouveia SM, Yokoi N, Voon LW. Functional aspects of the tear film lipid layer. Exp Eye Res 2004; 78: 347-360

Buckland AG, Heeley EL, Wilton DC. Bacterial cell membrane hydrolysis by secreted phospholipases A_2: a major physiological role of human group IIa $sPLA_2$ involving both bacterial cell wall penetration and interfacial catalysis. Biochim Biophys Acta 2000; 1484: 195-206

Calafat J, Janssen H, Tool A, Dentener MA, Knol EF, Rosenberg HF, Egesten A. The bactericidal/permeability-increasing protein (BPI) is present in specific granules of human eosinophils. Blood 1998; 91: 4770-4775

Canny G, Levy O, Furuta GT, Narravula-Alipati S, Sisson RB, Serhan CN, Colgan SP. Lipid mediator-induced expression of bactericidal/permeability-increasing protein (BPI) in human mucosal epithelia. Proc Natl Acad Sci USA 2002; 99: 3902-3907

Chen J, Engle SJ, Seilhamer JJ, Tischfield JA. Cloning and recombinant expression of a novel human low molecular weight Ca^{2+}-dependent phospholipase A_2. J Biol Chem 1994; 269: 2365-2368

Chew CKS, Hykin PG, Jansweijer C, Dikstein S, Tiffany JM, Bron AJ. The casual level of meibomian lipids in humans. Curr Eye Res 1993; 12: 255-259

Creech JL, Do LT, Fatt I, Radke CJ. *In vivo* tear-film thickness determination and implications for tear-film stability. Curr Eye Res 1998; 17: 1058-1066

Dartt DA. Regulation of mucin and fluid secretion by conjunctival epithelial cells. Prog Retin Eye Res 2002; 21: 555-576

Davies C. Concepts. Technical performance concents. In: Wild D (Ed.), The immunoassay handbook, MacMillan Press, London, UK, 1994, pp. 83-115

Davies DJ, Moran JE, Niall JF, Ryan GB. Segmental necrotizing glomerulonephritis with antineutrophil antibody: Possible arbovirus etiology? Br Med J 1982; 285:606

Degousee N, Ghomashchi F, Stefanski E, Singer A, Smart BP, Borregaard N, Reithmeier R, Lindsay TF, Lichtenberger C, Reinisch W, Lambeau G, Arm J, Tischfield J, Gelb MH, Rubin BB. Groups IV, V, and X phospholipases A_2s in human neutrophils: role in eicosanoid production and gram-negative bacterial phospholipid hydrolysis. J Biol Chem 2002; 277: 5061-5073

Dentener MA, Francot GJ, Smit FT, Froon AH, Pennings HJ, Wouters EF, Buurman WA. Presence of bactericidal/permeability-increasing protein in disease: detection by ELISA. J Infect Dis 1995; 171: 739-743

Ekins RP. Multi-analyte immunoassay. J Pharm Biomed Anal 1989; 7: 155-168

Ekins RP. Ligand assays: from electrophoresis to miniaturized microarrays. Clin Chem 1998; 44: 2015-2030

Ekins R, Edwards P. On the meaning of "sensitivity". Clin Chem 1997; 43: 1824-1831

Ellison RT, Giehl TJ. Killing of gram-negative bacteria by lactoferrin and lysozyme. J Clin Invest 1991; 88: 1080-1091

Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959; 82: 70-77

Elsbach P. The bactericidal/permeability-increasing protein (BPI) in antibacterial host defense. J Leukoc Biol 1998; 64: 14-18

Elsbach P, Weiss J, Doerfler M, Shu C, Kohn F, Ammons WS, Kung AH, Meszaros KK, Parent JB. The bactericidal/permeability increasing protein of neutrophils is a potent antibacterial and anti-endotoxin agent in vitro and in vivo. Prog Clin Biol Res 1994; 388: 41-51

Elsbach P, Weiss J, Levy O. Oxygen-independent Antimicrobial Systems of Phagocytes, in: Gallin, JI, Snyderman R, Nathan C (Eds.), Inflammation. Basic Principles and Clinical Correlates, 3rd Edition, Lippincott-Raven, Philadelphia, USA, 1999, pp. 801-817

Eskola JU, Nevalainen TJ, Lövgren TN. Time-resolved fluoroimmunoassay of human pancreatic phospholipase A_2. Clin Chem 1983; 29: 1777-1780

Fischer W. 1994. Lipoteichoic acid and lipids in the membrane of *Staphylococcus aureus*. Med Microbiol Immunol 1994; 183: 61-76

Fleming A.On a remarkable bacteriolytic ferment found in tissues and secretions. Proc R Soc Ser B 1922; 93: 306-317

Fleming A, Allison VD. Observations on a bacteriolytic substance ("lysozyme") found in secretions and tissues. Br J Exp Pathol 1922; 3: 252-260

Foreman-Wykert AK, Weinrauch Y, Elsbach P, Weiss J. Cell-wall determinants of the bactericidal action of group IIA phospholipase A_2 against Gram-positive bacteria. J Clin Invest 1999; 103: 715-721

Fransen E, Maessen J, Dentener M, Senden N, Buurman W. Impact of blood transfusions on inflammatory mediator release in patients undergoing cardiac surgery. Chest 1999; 116: 1233-1239

Fransen E, Maessen J, Dentener M, Senden N, Geskes G, Buurman W. Systemic inflammation present in patients undergoing CABG without extracorporeal circulation. Chest 1998; 113: 1290-1295

Fujihara M, Muroi M, Tanamoto K, Suzuki T, Azuma H, Ikeda H. Molecular mechanisms of macrophage activation and deactivation by lipopolysaccharide: roles of the receptor complex. Pharmacol Ther 2003; 100: 171-194

Fullard RJ, Snyder C. Protein levels in non-stimulated and stimulated tears. Invest Ophthalmol Vis Sci 1990; 31: 1119-1126

Fullard RJ, Tucker DL. Changes in human tear protein levels with progressively increasing stimulus. Invest. Ophthalmol Vis Sci 1991; 32: 2290-2301

Gachon AM, Lacazette E. Tear lipocalin and the eye's front line of defence. Br J Ophthalmol 1998; 82: 453-455

Gasymov OK, Abduragimov AR, Prasher P, Yusifov TN, Glasgow BJ. Tear lipocalin: evidence for a scavenging function to remove lipids from the human corneal surface. Invest Ophthalmol Vis Sci 2005; 46: 3589-3596

Gazzano-Santoro H, Parent JB, Grinna L, Horwitz A, Parsons T, Theofan G, Elsbach P, Weiss J, Conlon PJ. High-affinity binding of the bactericidal/permeability-increasing protein and a recombinant amino-terminal fragment to the lipid A region of lipopolysaccharide. Infect Immun 1992; 60: 4754-4761

Gelb MH, Valentin E, Ghomashchi F, Lazdunski M, Lambeau G. Cloning and recombinant expression of a structurally novel human secreted phospholipase A2. J Biol Chem 2000; 275: 39823-39826

Girgis DO, Dajcs JJ, O'Callaghan RJ. Phospholipase A_2 activity in normal and Staphylococcus aureus-infected rabbit eyes. Invest Ophthalmol Vis Sci 2003; 44: 197-202

Girgis DO, Reed JM, Monds KS, Dajcs JJ, Marquart ME, Thibodeaux BA, O'Callaghan RJ. Pathogenesis of Staphylococcus in the rabbit anterior chamber. Invest Ophthalmol Vis Sci 2005; 46: 1371-1378

Gosling JP. A decade of development in immunoassay methodology. Clin Chem 1990; 36: 1408-1427

Grönroos JO, Laine VJO, Janssen MJW, Egmond MR, Nevalainen TJ. Bactericidal properties of group IIA and group V phospholipases A_2. J Immunol 2001; 166: 4029-4034

Grönroos JO, Laine VJO, Nevalainen TJ. Bactericidal group IIA phospholipase A_2 in serum of patients with bacterial infections. J Infect Dis 2002; 185: 1767-1772

Haapamäki MM, Grönroos JM, Nurmi H, Alanen K, Nevalainen TJ. Gene expression of group II phospholipase A_2 in intestine in Crohn's disease. Am J Gastroenterol 1999; 94: 713-720

Haapamäki MM, Grönroos JM, Nurmi H, Söderlund K, Peuravuori H, Alanen K, Nevalainen TJ. Elevated group II phospholipase A_2 mass concentration in serum and colonic mucosa in Crohn's disease. Clin Chem Lab Med 1998; 36: 751-755

de Haas GH, Bonsen PP, Pieterson WA, van Deenen LL Studies on phospholipase A and its zymogen from porcine pancreas. 3. Action of the enzyme on short-chain lecithins. Biochim Biophys Acta 1971; 239: 252-266
van Haeringen NJ. Clinical biochemistry of tears. Surv Ophthalmol 1981; 26: 84-96

van Haeringen NJ. Aging and the lacrimal system. Br J Ophthalmol 1997; 81: 824-826

Hailman E, Albers JJ, Wolfbauer G, Tu AY, Wright SD. Neutralization and transfer of lipopolysaccharide by phospholipid transfer protein. J Biol Chem 1996; 271: 12172-12178

Hara S, Kudo I, Chang HW, Matsuta K, Miyamoto T, Inoue K. Purification and characterization of extracellular phospholipase A_2 from human synovial fluid in rheumatoid arthritis. J Biochem (Tokyo). 1989; 105: 395-399

Haynes RJ, Tighe PJ, Dua HS. Antimicrobial defensin peptides of the human ocular surface. Br J Ophthalmol 1999; 83: 737-741

Hendrickson HS, Hendrickson EK, Rustad TJ. Synthesis of a naphthylvinyl-labeled glycerol ether analog of phosphatidylcholine and its use in the assay of phospholipase A_2. J Lipid Res 1987; 28: 864-872

Hendrickson HS, Kotz KJ, Hendrickson EK. Evaluation of fluorescent and colored phosphatidylcholine analogs as substrates for the assay of phospholipase A_2. Anal Biochem 1990; 185: 80-83

Hendrickson HS, Rauk PN. Continuous fluorometric assay of phospholipase A_2 with pyrene-labeled lecithin as a substrate. Anal Biochem 1981; 116: 553-558

Hidi R, Vargaftig BB, Touqui L. Increased synthesis and secretion of a 14-kDa phospholipase A_2 by guinea pig alveolar macrophages. Dissociation from arachidonic acid liberation and modulation by dexamethasone. J Immunol 1993; 151: 5613-5623

Hiemstra PS, Maassen RJ, Stolk J, Heinzel-Weiland R, Steffens GJ, Dijkam JH. Antibacterial activity of antileukoprotease. Infect Immun 1996; 64: 4520-4524

Hoffmann GE, Schmidt D, Bastian B, Guder WG. Photometric determination of phospholipase A. J Clin Chem Clin Biochem 1986 24: 871-875

Horigome K, Hayakawa M, Inoue K, Nojima S. Purification and characterization of phospholipase A_2 released from rat platelets. J Biochem (Tokyo) 1987; 101: 625-631

Horwitz BL, Christensen GR, Ritzmann SR, Diurnal profiles of tear lysozymes and gamma A globulin. Ann Ophthalmol 1978; 10: 75-80

Hsu SM, Raine L, Fanger H. The use of avidin-biotinperoxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 1981; 29: 577-580

Hume EB, Cole N, Parmar A, Tan ME, Aliwarga Y, Schubert T, Holden BA, Willcox MD. Secretory phospholipase A_2 deposition on contact lenses and its effect on bacterial adhesion. Invest Ophthalmol Vis Sci 2004; 45: 3161-3164

Huth SW, Miller MJ, Leopold IH. Calcium and protein in tears: diurnal variation. Arch Ophthalmol 1981; 99: 1628-1633

IFCC (International Federation of Clinical Chemistry, Solberg HE). Approved recommendation (1987) on the theory of reference values. Part 5. Statistical treatment of collected reference values. Determination of reference limits. Clin Chim Acta 1987; 170, S13-S32

Inman RD, Chiu B. Comparative microbicidal activity of synovial fluid on arthritogenic organisms. Clin Exp Immunol 1996; 104: 80-85

Iovine N, Eastvold J, Elsbach P, Weiss JP, Gioannini TL. The carboxyl-terminal domain of closely related endotoxin-binding proteins determines the target of protein-lipopolysaccharide complexes. J Biol Chem 2002; 277: 7970-7978

Johnson ME, Murphy PJ. Changes in the tear film and ocular surface from dry eye syndrome. Prog Retin Eye Res 2004; 23: 449-474

Juffermans NP, Verbon A, van Deventer SJ, Buurman WA, van Deutekom H, Speelman P, van der Poll T. Serum concentrations of lipopolysaccharide activity-modulating proteins during tuberculosis. J Infect Dis 1998; 178: 1839-1842

Kaiser E. Phospholipase A_2: its usefulness in laboratory diagnostics. Crit Rev Clin Lab Sci 1999; 36: 65-163

Kallajoki M, Nevalainen TJ. Expression of Group II Phospholipase A_2 in Human Tissues. In: Uhl W, Nevalainen TJ, Büchler MW (Eds.), Phospholipases A_2: Basic and Clinical Aspects in Inflammatory Diseases, Progress in Surgery, vol. 24, Karger, Basel, Germany, 1997, pp. 8-16

Kari O. Atopic conjunctivitis. A cytologic examination. Acta Ophthalmol 1988; 66: 381-386

Kari O, Aho VV, Peltonen S, Saari JM, Kari M, Määttä M, Collan Y, Saari KM. Group IIA phospholipase A_2 concentration of tears in patients with ocular rosacea. Acta Ophthalmol Scand. 2005; 83: 483-486

Khan AA, Lambert LH Jr, Remington JS, Araujo FG. Recombinant bactericidal/permeability-increasing protein (rBPI21) in combination with sulfadiazine is active against Toxoplasma gondii. Antimicrob Agents Chemother 1999; 43: 758-762

Kijlstra A. The role of lactoferrin in the nonspecific immune response on the ocular surface. Reg Immunol 1990; 3: 193-197

Kimmings AN, van Deventer SJ, Rauws EAJ, Huibregtse K, Gouma DJ. Systemic inflammatory response in acute cholangitis and after subsequent treatment. Eur J Surg 2000; 166: 700-705

Koduri RS, Baker SF, Snitko Y, Han SK, Cho W, Wilton DC, Gelb MH. Action of human group IIa secreted phospholipase A_2 on cell membranes. Vesicle but not heparinoid binding determines rate of fatty acid release by exogenously added enzyme. J Biol Chem 1998; 273: 32142-32153

Koduri RS, Grönroos JO, Laine VJ, Le Calvez C, Lambeau G, Nevalainen TJ, Gelb MH. Bactericidal properties of human and murine groups I, II, V, X, and XII secreted phospholipases A_2. J Biol Chem 2002; 277: 5849-5857

Kolko M, Christoffersen NR, Barreiro SG, Bazan NG. Expression and location of mRNAs encoding multiple forms of secretory phospholipase A_2 in the rat retina. J Neurosci Res 2004; 77: 517-524

Kortesuo PT, Hietaranta AJ, Jamia M, Hirsimäki P, Nevalainen TJ. Rat pancreatic phospholipase A_2. Purification, localization, and development of an enzyme immunoassay. Int J Pancreatol 1993; 13: 111-118

Kramer RM, Hession C, Johansen B, Hayes G, McGray P, Chow EP, Tizard R, Pepinsky RB. Structure and properties of a human non-pancreatic phospholipase A_2. J Biol Chem 1989; 264: 5768-5775

Kudo I, Murakami M. Phospholipase A_2 enzymes. Prostaglandins Other Lipid Mediat 2002; 68-69, 3-58

Kugiyama K, Ota Y, Sugiyama S, Kawano H, Doi H, Soejima H, Miyamoto S, Ogawa H, Takazoe K, Yasue H. Prognostic value of plasma levels of secretory type II phospholipase A_2 in patients with unstable angina pectoris. Am J Cardiol 2000; 86: 718-722

Laine VJO, Grass DS, Nevalainen TJ. Protection by group II phospholipase A_2 against Staphylococcus aureus. J Immunol 1999; 162: 7402-7408

Lamberts, D. In: Smolen G, Thoft R (Eds.), The Cornea, Little, Brown and Company, Boston, MA, USA, 1994, pp 439-483.

Landreville S, Coulombe S, Carrier P, Gelb MH, Guérin SI, Salesse C. Expression of phospholipases A_2 and C in human corneal epithelial cells. Invest Ophthalmol Vis Sci 2004; 45: 3997-4003

Laouini D, Kawamoto S, Yalcindag A, Bryce P, Mizoguchi E, Oettgen H, Geha RS. Epicutaneous sensitization with superantigen induces allergic skin inflammation. J Allergy Clin Immunol 2003; 112: 981-987

Leistad L, Feuerherm AJ, Ostensen M, Faxvaag A, Johansen B. Presence of secretory group IIa and V phospholipase A2 and cytosolic group IValpha phospholipase A2 in chondrocytes from patients with rheumatoid arthritis. Clin Chem Lab Med 2004; 42: 602-610

Levin M, Quint PA, Goldstein B, Barton P, Bradley JS, Shemie SD, Yeh T, Kim SS, Cafaro DP, Scannon PJ, Giroir BP. Recombinant bactericidal/permeability-increasing protein ($rBPI_{21}$) as adjunctive treatment for children with severe meningococcal sepsis: a randomised trial. rBPI21 Meningococcal Sepsis Study Group. Lancet 2000; 356: 961-967

Levy O. Impaired innate immunity at birth: deficiency of bactericidal/permeability-increasing protein (BPI) in the neutrophils of newborns. Pediatr Res 2002a; 51: 667-669

Levy O. Therapeutic potential of the bactericidal/permeability-increasing protein. Expert Opin Investig Drugs 2002b; 11: 159-167

Levy O, Ooi CE, Weiss J, Lehrer RI, Elsbach P. Individual and synergistic effects of rabbit granulocyte proteins on Escherichia coli. J Clin Invest 1994; 94: 672-682

Levy O, Sisson R, Kenyon J, Eichenwald E, Macone A, Goldmann D. Enhancement of neonatal innate defense: effects of adding an N-terminal recombinant fragment of bactericidal/permeability-increasing protein (rBPI21) on growth and TNF-inducing activity of Gram-negative bacteria tested in neonatal cord blood ex vivo. Infect Immun 2000; 68: 5120-5125

Levy O, Zarember KA, Roy RM, Cywes C, Godowski PJ, Wessels MR. Selective impairment of TLR-mediated innate immunity in human newborns: neonatal blood plasma reduces monocyte TNF-alpha induction by bacterial lipopeptides, lipopolysaccharide, and imiquimod, but preserves the response to R-848. J Immunol 2004; 173: 4627-4634

Lister MD, Deems RA, Watanabe Y, Ulevitch RJ, Dennis EA. Kinetic analysis of the Ca2+-dependent, membrane-bound, macrophage phospholipase A_2 and the effects of arachidonic acid. J Biol Chem 1988; 263: 7506-7513

Madsen LM, Inada M, Weiss J. Determinants of activation by complement of group II phospholipase A_2 acting against Escherichia coli. Infect Immun 1996; 64: 2425-2430

Matsuda Y, Ogawa M, Sakamoto K, Yamashita S, Kanda A, Kohno M, Yoshida N, Nishijima J, Murata A, Mori T. Development of a radioimmunoassay for human group-II phospholipase A_2 and demonstration of postoperative elevation. Enzyme 1991; 45: 200-208

McCulley JP, Shine W. A compositional based model for the tear film lipid layer. Trans Am Ophthalmol Soc 1997; 95: 79-93

McNamara NA, Van R, Tuchin OS, Fleiszig SM. Ocular surface epithelia express mRNA for human beta defensin-2. Exp Eye Res 1999; 69: 483-490

Molloy MP, Bolis S, Herbert BR, Ou K, Tyler MI, van Dyk DD, Willcon MD, Gooley AA, Williams KL, Morris CA, Walsh BJ. Establishment of the human reflex tear two-dimensional polyacrylamide gel electrophoresis reference map: new proteins of potential diagnostic value. Electrophoresis 1997; 18: 2811-2815

Moreau JM, Girgis DO, Hume EB, Dajcs JJ, Austin MS, O'Callaghan RJ. Phospholipase A_2 in rabbit tears: a host defense against Staphylococcus aureus. Invest Ophthalmol Vis Sci 2001; 42: 2347-2354

Murakami M, Kudo I, Suwa Y, Inoue K. Release of 14-kDa group-II phospholipase A_2 from activated mast cells and its possible involvement in the regulation of the degranulation process. Eur J Biochem 1992; 209: 257-265

von der Möhlen MA, van der Poll T, Jansen J, Levi M, van Deventer SJ. Release of bactericidal/permeability-increasing protein in experimental endotoxemia and clinical sepsis. Role of tumor necrosis factor. J Immunol 1996; 156: 4969-4973

Nadziejko C, Finkelstein I. Inhibition of neutrophil elastase by mucus glycoprotein. Am J Respir Cell Mol Biol 1994; 11: 103-107

Nevalainen TJ, Eerola LI, Rintala E, Laine VJ, Lambeau G, Gelb MH. Time-resolved fluoroimmunoassays of the complete set of secreted phospholipases A_2 in human serum. Biochim Biophys Acta 2005; 1733: 210-223

Nevalainen TJ, Grönroos JM, Kallajoki M. Expression of group II phospholipase A_2 in the human gastrointestinal tract. Lab Invest 1995; 72: 201-208

Nevalainen TJ, Haapamäki MM, Grönroos JM. Roles of secretory phospholipases A_2 in inflammatory diseases and trauma. Biochim Biophys Acta 2000; 1488: 83-90

Nevalainen TJ, Kortesuo PT, Rintala E, Märki F. Immunochemical detection of group I and group II phospholipases A_2 in human serum. Clin Chem 1992; 38: 1824-1829

Newman SL, Gootee L, Gabay JE, Selsted ME. Identification of constituents of human neutrophil azurophil granules that mediate fungistasis against Histoplasma capsulatum. Infect Immun 2000; 68: 5668-5672

Ng V, Cho P, Wong F, Chan Y. Variability of tear protein levels in normal young adults: diurnal (daytime) variation. Graefes Arch Clin Exp Ophthalmol 2001; 239: 257-263

Nishijima J, Okamoto M, Ogawa M, Kosaki G, Yamano T. Purification and characterization of human pancreatic phospholipase A_2 and development of a radioimmunoassay. J Biochem (Tokyo). 1983; 94: 137-147

Nishimura H, Gogami A, Miyagawa Y, Nanbo A, Murakami Y, Baba T, Nagasawa S. Bactericidal/permeability-increasing protein promotes complement activation for neutrophil-mediated phagocytosis on bacterial surface. Immunology 2001; 103: 519-525

Nupponen I, Turunen R, Nevalainen T, Peuravuori H, Pohjavuori M, Repo H, Andersson S. Extracellular release of bactericidal/permeability-increasing protein in newborn infants. Pediatr Res 2002; 51: 670-674

Ooi CE, Weiss J, Doerfler ME, Elsbach P. Endotoxin-neutralizing properties of the 25 kD N-terminal fragment and a newly isolated 30 kD C-terminal fragment of the 55-60 kD bactericidal/permeability-increasing protein of human neutrophils. J Exp Med 1991; 174: 649-655

Ottino P, Finley J, Rojo E, Ottlecz A, Lambrou GN, Bazan HE, Bazan NG. Hypoxia activates matrix metalloproteinase expression and the VEGF system in monkey choroid-retinal endothelial cells: Involvement of cytosolic phospholipase A_2 activity. Mol Vis 2004; 10: 341-350

Patriarca P, Beckerdite S, Elsbach P. Phospholipases and phospholipid turnover in Escherichia coli spheroplasts. Biochim Biophys Acta 1972; 260: 593-600

Paulsen FP, Pufe T, Schaudig U, Held-Feindt J, Lehmann J, Schroder JM, Tillmann BN. Detection of natural peptide antibiotics in human nasolacrimal ducts. Invest Ophthalmol Vis Sci 2001; 42: 2157-2163

Pereira HA, Martin LE, Spitznagel JK. Quantitation of a cationic antimicrobial granule protein of human polymorphonuclear leukocytes by ELISA. J Immunol Methods 1989; 117: 115-120

Pereira HA, Spitznagel JK, Winton EF, Shafer WM, Martin LE, Guzman GS, Pohl J, Scott RW, Marra MN, Kinkade JM Jr. The ontogeny of a 57-Kd cationic antimicrobial protein of human polymorphonuclear leukocytes: localization to a novel granule population. Blood 1990; 76: 825-834

Peuravuori H, Korpela T. Pyrophosphatase-based enzyme-linked immunosorbent assay of total IgE in serum. Clin Chem 1993; 39: 846-851

Pruzanski W. Phospholipase A2: quo vadis? J Rheumatol 2005; 32: 400-402

Qu XD, Lehrer RI. Secretory phospholipase A_2 is the principal bactericide for staphylococci and other gram-positive bacteria in human tears. Infect Immun 1998; 66: 2791-2797

Radvanyi F, Jordan L, Russo-Marie F, Bon C. A sensitive and continuous fluorometric assay for phospholipase A_2 using pyrene-labeled phospholipids in the presence of serum albumin. Anal Biochem 1989; 177: 103-109

Rauniyar RK, Suzuma K, King AL, Aiello LP, King GL. Differential effects of bactericidal/permeability-increasing protein (BPI) analogues on retinal neovascularization and retinal pericyte growth. Invest Ophthalmol Vis Sci 2002; 43: 503-509

Redl B, Human tear lipocalin. Biochim Biophys Acta 2000; 1482: 241-248

Reichel PH, Seemann C, Csernok E, Schroder JM, Muller A, Gross WL, Schultz H. Bactericidal/permeability-increasing protein is expressed by human dermal fibroblasts and upregulated by interleukin 4. Clin Diagn Lab Immunol 2003; 10: 473-475

Reynolds LJ, Hughes LL, Dennis EA. Analysis of human synovial fluid phospholipase A_2 on short chain phosphatidylcholine-mixed micelles: development of a spectrophotometric assay suitable for a microtiterplate reader. Anal Biochem 1992; 204: 190-197

Reynolds LJ, Washburn WN, Deems RA, Dennis EA. Assay strategies and methods for phospholipases. Methods Enzymol 1991; 197: 3-23

Rietschel ET, Kirikae T, Schade FU, Mamat U, Schmidt G, Loppnow H, Ulmer AJ, Zahringer U, Seydel U, Di Padova F, Schreier M, Brade H. Bacterial endotoxin: molecular relationships of structure to activity and function. FASEB J 1994; 8: 217-225

Rintala E, Peuravuori H, Pulkki K, Voipio-Pulkki LM, Nevalainen T. Bactericidal/permeability-increasing protein (BPI) in sepsis correlates with the severity of sepsis and the outcome. Intensive Care Med 2000; 26: 1248-1251

Rosenthal MD, Gordon MN, Buescher ES, Slusser JH, Harris LK, Franson RC. Human neutrophils store type II 14-kDa phospholipase A_2 in granules and secrete active enzyme in response to soluble stimuli. Biochem Biophys Res Commun 1995; 208: 650-656

Rubin BB, Downey GP, Koh A, Degousee N, Ghomashchi F, Nallan L, Stefanski E, Harkin DW, Sun C, Smart BP, Lindsay TF, Cherepanov V, Vachon E, Kelvin D, Sadilek M, Brown GE, Yaffe MB, Plumb J, Grinstein S, Glogauer M, Gelb MH. Cytosolic phospholipase A_2-alpha is necessary for platelet-activating factor biosynthesis, efficient neutrophil-mediated bacterial killing, and the innate immune response to pulmonary infection: $cPLA_2$-alpha does not regulate neutrophil NADPH oxidase activity. J Biol Chem 2005; 280: 7519-7529

Saari KM, Aho V, Paavilainen V, Nevalainen TJ. Group II PLA_2 content of tears in normal subjects. Invest Ophthalmol Vis Sci 2001; 42: 318-320

Saari KM, Aine E, Posz A, Klockars M. Lysozyme content of tears in normal subjects and in patients with external eye infections. Graefes Arch Clin Exp Ophthalmol. 1983; 221: 86-88

Saari KM, Klockars M. Lysozyme Content in Tears of Patients with Various Inflammatory Diseases. In: Henkind P (ed) Acta: XXIV International Congress of Ophthalmology, San Francisco, October 31-November 5, 1982, vol. 2. Lippincott, Philadelphia, USA, 1983, pp 834-837

Sack RA, Tan KO, Tan A. Diurnal tear cycle: evidence for a nocturnal inflammatory constitutive tear fluid. Invest. Ophthalmol Vis Sci 1992; 33: 626-640

Said NA, Shoeir AT, Panjwani N, Garate M, Cao Z. Local and systemic humoral immune response during acute and chronic Acanthamoeba keratitis in rabbits. Curr Eye Res 2004; 29: 429-439

Sakata M, Sack RA, Sathe S, Holden B, Beaton AR. Polymorphonuclear leukocyte cells and elastase in tears. Curr Eye Res 1997; 16: 810-9

Santavuori SA, Kortesuo PT, Eskola JU, Nevalainen TJ. Application of a new monoclonal antibody for time-resolved fluoroimmunoassay of human pancreatic phospholipase A_2. Eur J Clin Chem Clin Biochem 1992; 29: 819-826

Santos AA, Browning JL, Scheltinga MR, Lynch EA, Brown EF, Lawton P, Chambers E, Dougas I, Benjamin CD, Dinarello CA, Wolff SM, Jacobs DO, Wilmore DW. Are events after endotoxemia related to circulating phospholipase A_2? Ann Surg 1994; 219: 183-192

Sathe S, Sakata M, Beaton AR, Sack RA. Identification, origins and the diurnal role of the principal serine protease inhibitors in human tear fluid. Curr Eye Res 1998; 17: 348-362

Schindler R, Marra MN, McKelligon BM, Lonnemann G, Schulzeck P, Schulze M, Oppermann M, Shaldon S. Plasma levels of bactericidal/permeability-increasing protein (BPI) and lipopolysaccharide-binding protein (LBP) during hemodialysis. Clin Nephrol 1993; 40: 346-351

Schumann RR, Zweigner J. A novel acute-phase marker: lipopolysaccharide binding protein (LBP). Clin Chem Lab Med 1999; 37: 271-274

Schädlich HR, Bühler M, Beger HG. Improved method for the determination of phospholipase A_2 catalytic activity concentration in human serum and ascites. J Clin Chem Clin Biochem 1987; 25: 505-509

Seal DV, McGill JI, Mackie IA, Liakos GM, Jacobs P, Goulding NJ. Bacteriology and tear protein profiles of the dry eye. Br J Ophthalmol 1986; 70: 122-125

Seilhamer JJ, Pruzanski W, Vadas P, Plant S, Miller JA, Kloss J, Johnson LK. Cloning and recombinant expression of phospholipase A_2 present in rheumatoid arthritic synovial fluid. J Biol Chem 1989; 264: 5335-5338

Sen DK, Sarin GS. Biological variations of lysozyme concentration in the tear fluids of healthy persons. Br J Ophthalmol 1986; 70: 246-248

Seydack M. Nanoparticle labels in immunosensing using optical detection methods. Biosens Bioelectron. 2005; 20: 2454-2469

Shakir KM. Phospholipase A_2 activity of post-heparin plasma: a rapid and sensitive assay and partial characterization. Anal Biochem 1981; 114: 64-70

Shimizu S, Tani Y, Yamada H, Tabata M, Murachi T. Enzymatic determination of serum-free fatty acids: a colorimetric method. Anal Biochem 1980; 107: 193-198

Shinohara H, Amabe Y, Komatsubara T, Tojo H, Okamoto M, Wakano Y, Ishida H. Group II phospholipase A_2 induced by interleukin-1 beta in cultured rat gingival fibroblasts. FEBS Lett 1992; 304: 69-72

Singer AG, Ghomashchi F, Le Calvez C, Bollinger J, Bezzine S, Rouault M, Sadilek M, Nguyen E, Lazdunski M, Lambeau G, Gelb MH. Interfacial kinetic and binding properties of the complete set of human and mouse groups I, II, V, X, and XII secreted phospholipases A_2. J Biol Chem 2002; 277: 48535-48549

Six DA, Dennis EA. The expanding superfamily of phospholipase A_2 enzymes: classification and characterization. Biochim Biophys Acta 2000; 1488: 1-19

Snitko Y, Koduri RS, Han SK, Othman R, Baker SF, Molini BJ, Wilton DC, Gelb MH, Cho W. Mapping the interfacial binding surface of human secretory group IIa phospholipase A_2. Biochemistry 1997; 36: 14325-14333

Song CH, Choi JS, Kim DK, Kim JC. Enhanced secretory group II PLA_2 activity in the tears of chronic blepharitis patients. Invest Ophthalmol Vis Sci. 1999; 40: 2744-2748

Streckfus CF, Bigler LR. Saliva as a diagnostic fluid. Oral Dis. 2002; 8: 69-76

Takahashi M, Horiuchi Y, Tezuka T. Presence of bactericidal/permeability-increasing protein in human and rat skin. Exp Dermatol 2004; 13: 55-60

Talvinen KA, Kemppainen EA, Nevalainen TJ. Expression of group II phospholipase A_2 in the liver in acute pancreatitis. Scand J Gastroenterol 2001; 36: 1217-1221

Tan KO, Sack RA, Holden BA, Swarbrick HA. Temporal sequence of changes in tear film composition during sleep. Curr Eye Res 1993; 12: 1001-1007

Thuren T, Virtanen JA, Lalla M, Kinnunen PK. Fluorometric assay for phospholipase A_2 in serum. Clin Chem 1985; 31: 714-717

Thuren T, Virtanen JA, Somerharju PJ, Kinnunen PK. Phospholipase A_2 assay using an intramolecularly quenched pyrene-labeled phospholipid analog as a substrate. Anal Biochem 1988; 170: 248-255

Tobias PS, Soldau K, Iovine NM, Elsbach P, Weiss J. Lipopolysaccharide (LPS)-binding proteins BPI and LBP form different types of complexes with LPS. J Biol Chem 1997; 272: 18682-18685

Tobias PS, Soldau K, Ulevitch RJ. Isolation of a lipopolysaccharide-binding acute phase reactant from rabbit serum. J Exp Med 1986; 164: 777-793

Tutt R, Bradley A, Begley C, Thibos LN. Optical and visual impact of tear break-up in human eyes. Invest Ophthalmol Vis Sci 2000; 41: 4117-4123

Vadas P, Scott K, Smith G, Rajkovic I, Stefanski E, Schouten BD, Singh R, Pruzanski W. Serum phospholipase A_2 enzyme activity and immunoreactivity in a prospective analysis of patients with septic shock. Life Sci 1992; 50: 807-811

Valentin E, Lambeau G. Increasing molecular diversity of secreted phospholipases A_2 and their receptors and binding proteins. Biochim Biophys Acta 2000; 1488, 59-70

Valentin E, Singer AG, Ghomashchi F, Lazdunski M, Gelb MH, Lambeau G. Cloning and Recombinant Expression of Human Group IIF-Secreted Phospholipase A_2. Biochem Biophys Res Commun 2000; 279: 223-228

Walport MJ. Complement. First of two parts. N Engl J Med 2001; 344, 1058-1066

Weinrauch Y, Foreman A, Shu C, Zarember K, Levy O, Elsbach P, Weiss J. Extracellular accumulation of potently microbicidal bactericidal/permeability-increasing protein and p15s in an evolving sterile rabbit peritoneal inflammatory exudate. J Clin Invest 1995; 95: 1916-1924

Weiss J, Beckerdite-Quagliata S, Elsbach P. Determinants of the action of phospholipases A on the envelope phospholipids of Escherichia coli. J Biol Chem 1979; 254: 11010-11014

Weiss J, Elsbach P, Olsson I, Odeberg H. Purification and characterization of a potent bactericidal and membrane active protein from the granules of human polymorphonuclear leukocytes. J Biol Chem 1978; 253: 2664-2672

Weiss J, Elsbach P, Shu C, Castillo J, Grinna L, Horwitz A, Theofan G. Human bactericidal/permeability-increasing protein and a recombinant NH2-terminal fragment cause killing of serum-resistant gram-negative bacteria in whole blood and inhibit tumor necrosis factor release induced by the bacteria. J Clin Invest 1992; 90: 1122-1130

Weiss J, Inada M, Elsbach P, Crowl RM. Structural determinants of the action against Escherichia coli of a human inflammatory fluid phospholipase A_2 in concert with polymorphonuclear leukocytes. J Biol Chem 1994; 269: 26331-26337

Weiss J, Olsson I. Cellular and subcellular localization of the bactericidal/permeability-increasing protein of neutrophils. Blood 1987; 69: 652-659

Weiss J, Wright G, Bekkers AC, van den Bergh CJ, Verheij HM. Conversion of pig pancreas phospholipase A_2 by protein engineering into enzyme active against Escherichia coli treated with the bactericidal/permeability-increasing protein. J Biol Chem 1991; 266: 4162-4167

Westgard JO, Groth T, Aronsson T, de Verdier CH. Combined Shewhart-cusum control chart for improved quality control in clinical chemistry. Clin Chem 1977; 23: 1881-1887

White ML, Ma JK, Birr CA, Trown PW, Carroll SF. Measurement of bactericidal/permeability-increasing protein in human body fluids by sandwich ELISA. J Immunol Methods 1994; 167: 227-235

Wiese A, Brandenburg K, Carroll SF, Rietschel ET, Seydel U. Mechanisms of action of bactericidal/permeability-increasing protein BPI on reconstituted outer membranes of gram-negative bacteria. Biochemistry 1997; 36: 10311-10319

Wiik A. Delineation of a standard procedure for indirect immunofluorescence detection of ANCA. APMIS 1989; 6: 12-13

Wild D, Davies C. Components. In: Wild D (Ed.), The immunoassay handbook, MacMillan Press, London, UK, 1994, pp. 49-82

Wilde C. Subject preparation, sample collection and handling. In: Wild D (Ed.), The immunoassay handbook, MacMillan Press, London, UK, 1994, pp. 243-255

Willcox MD, Lan J. Secretory immunoglobulin A in tears: functions and changes during contact lens wear. Clin Exp Optom 1999; 82: 1-3

Willcox MDP, Morris CA, Thakur A, Sack RA, Wickson J, Boey W. Complement and complement regulatory proteins in human tears. Invest Ophthalmol Vis Sci 1997; 38: 1-8

Wilton DC. A continuous fluorescence displacement assay for the measurement of phospholipase A_2 and other lipases that release long-chain fatty acids. Biochem J 1990; 26: 435-439

Wolff, E, 1946. The muco-cutaneous junction of the lid margin and the distribution of the tear fluid. Trans Ophthalmol Soc UK 1946; 66: 291–308

Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 1990; 249: 1431-1433

Wright EM, Royston P. Calculating reference intervals for laboratory measurements. Stat Methods Med Res 1999; 8: 93-112

Yalow RS, Berson SA. Assay of plasma insulin in human subjects by immunological methods. Nature 1959; 184: 1648-1649

Yamada Y, Endo S, Kamei Y, Minato T, Yokoyama M, Taniguchi S, Nakae H, Inada K, Ogawa M. Plasma levels of type II phospholipase A_2 and nitrite/nitrate in patients with burns. Burns 1998; 24: 513-517

Yang HC, Mosior M, Johnson CA, Chen Y, Dennis EA. Group-specific assays that distinguish between the four major types of mammalian phospholipase A2. Anal Biochem 1999; 269: 278-288

Zhao MH, Jones SJ, Lockwood CM. Bactericidal/permeability-increasing protein (BPI) is an important antigen for anti-neutrophil cytoplasmic autoantibodies (ANCA) in vasculitis. Clin Exp Immunol 1995 99: 49-56

10. ORIGINAL PUBLICATIONS

Secretion of Group 2 Phospholipase A2 by Lacrimal Glands

Timo J. Nevalainen, Heikki J. Aho, and Heikki Peuravuori

Purpose. Tears are known to have antimicrobial properties. The authors investigated the presence of the antibacterial enzyme phospholipase A2 (PLA2) in tears and lacrimal glands.

Methods. The catalytic activity of PLA2 and the amount of pancreatic group 1 PLA2 and nonpancreatic group 2 PLA2 were measured in homogenates of eight human lacrimal glands from autopsied subjects and in tears from four healthy volunteers. The localization of PLA2s in lacrimal gland sections was studied by immunohistochemistry. Skeletal muscle was used as a control.

Results. The catalytic activity of PLA2 was significantly higher in lacrimal glands than in skeletal muscle. Immunochemical analysis showed significantly higher amounts of group 2 PLA2 in lacrimal gland than in skeletal muscle homogenates. Group 1 PLA2 was present in trace amounts only. The concentration of group 2 PLA2 in tears was high (1451.3 μg/l) compared to that in the serum of healthy individuals (3.7 μg/l). By immunohistochemistry, a granular reaction of group 2 PLA2 was localized in the glandular cells of lacrimal glands. The apical cytoplasm of many duct cells also was labeled.

Conclusions. The lacrimal glands secreted nonpancreatic group 2 PLA2, which most likely acts as an antiinfectious factor in tears. Invest Ophthalmol Vis Sci. 1994;35:417–421.

Phospholipase A2 (PLA2) catalyzes the hydrolysis of the acyl ester bond at the sn-2 position of phosphoglycerides. The enzyme is widespread in bacteria, plants, snake and bee venoms, and in mammalian cells and secretions.[1] Three human genes of PLA2s have been cloned: two coding 14-kDa secretory enzymes and one coding an 85-kDa intracellular PLA2.[2–5] Secretory PLA2s have been divided into two groups on the basis of the amino acid sequence of the enzyme proteins.[6] Group 1 PLA2s are found in mammalian pancreas. Snake venoms contain group 1 or group 2 PLA2s.[7] The human secretory nonpancreatic PLA2 present in synovial fluid may be classified to group 2.[3,4]

PLA2 has been postulated to play an important role in the inflammatory process.[8] The concentration of pancreatic group 1 PLA2 in serum is above normal in acute pancreatitis.[9] The concentration of nonpancreatic group 2 PLA2 is increased in, for example, sepsis[10] and rheumatoid arthritis.[11] The source of group 1 PLA2 found in serum is the pancreas, but the source of group 2 PLA2 in serum is unknown. Both epithelial and nonepithelial tissues contain group 2 PLA2.[12]

PLA2 found in inflammatory exudates is capable of destroying bacteria.[13] Because tears are known to have antimicrobial properties,[14] we analyzed the PLA2 contents of tears and lacrimal glands.

METHODS

Specimens

Lacrimal gland specimens of both right and left sides were obtained for PLA2 assays from eight autopsied subjects (two men and six women, aged 56 to 84 years, mean 72 years). Pieces of the main glands and the adjacent conjunctiva from four of these eight subjects as well as from four additional autopsied subjects were fixed in 10% buffered formalin for immunohistochemical analysis (three men and five women, aged 44 to 87 years, mean 71 years). Specimens from the pectoralis major muscle served as controls both in the biochemical and immunohistochemical analysis to ensure that the assays were working properly and that the measurements were reasonable. The skeletal muscle shows very low catalytic activity of PLA2 and low contents of both

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group 1 and group 2 PLA2s.[12] Because the samples came from routine autopsies 2 to 4 days after death, tissue integrity was verified by light microscopy with hematoxylin and eosin staining to rule out excessive autolysis. Tear specimens were obtained from four healthy female volunteers (aged 5 to 42 years, mean 29 years) capable of producing spontaneous emotional tears.

The principles of the World Medical Association Declaration of Helsinki were followed. An oral informed consent was obtained from the volunteers giving the tear specimens. The tissue specimens were obtained at routine clinical autopsies.

PLA2 Assays

The tissues were homogenized with an Ultra-Turrax (IKA Laboratechnik, Staufen, Germany) homogenizer in 0.01 M sodium phosphate buffer, pH 7.6 (1 g wet tissue per 5 ml buffer) and centrifuged at 10,000*g* for 1 hour at 4°C.

The catalytic activity of PLA2 (units per liter or units per gram protein, where units = millimoles substrate used per minute) was measured by using a mixture of 2-[^{3}H]palmitoyl phosphatidylcholine and unlabeled dipalmitoyl phosphatidylcholine as a substrate.[15]

The concentration of group 1 PLA2 was measured by a time-resolved fluoroimmunoassay using a monoclonal antibody to human pancreatic PLA2.[16] The concentration of group 2 PLA2 was measured by a time-resolved fluoroimmunoassay using a polyclonal rabbit antibody to recombinant human group 2 PLA2.[17] The results are expressed as concentrations (milligrams per liter) or as milligrams per gram protein.

The Mann-Whitney test was used for statistical analysis.

Immunohistochemistry

Sections of formalin-fixed paraffin-embedded tissue specimens were reacted with a monoclonal anti–group 1-PLA2 antibody[16] or a polyclonal rabbit anti–group 2-PLA2 antiserum.[17] The primary immunoreaction was localized according the avidin-biotin complex method[18] by using Vectastain avidin-biotin-peroxidase complex kits (Vector Laboratories, Burlingame, CA). Both primary antibodies were affinity purified by protein A. For controls, the primary antibodies were replaced by nonimmune rabbit serum or bovine serum albumin. The sections were counterstained lightly by hematoxylin.

RESULTS

The mean (± SEM) catalytic activity of PLA2 was 40.2 ± 4.4 U/l (6.2 ± 2.2 U/g protein) in the lacrimal gland homogenates and 0.25 ± 0.18 U/l (0.07 ± 0.05 U/g protein) in the skeletal muscle homogenates. The difference was significant ($P < 0.001$). The concentration of group 2 PLA2 was 2458.8 ± 623 μg/l (332.3 ± 50.4 μg/g protein) in the lacrimal glands and 38.3 ± 15.6 μg/l (10.1 ± 4.5 μg/g protein) in the muscle homogenates. The difference was significant ($P < 0.001$). In tear samples, the catalytic activity of PLA2 was 595.5 ± 152.6 U/l and the concentration of group 2 PLA2 1451.3 ± 78.0 μg/l. The concentrations of group 1 PLA2 were below the detection limit of the assay (0.28 μg/l[16]) in the lacrimal gland, muscle, and tear specimens. Therefore, the catalytic activity present in lacrimal homogenates and tears is due to the presence of group 2 PLA2.

The lacrimal glands showed normal histology (Fig. 1). Group 2 PLA2 was localized in the apical cytoplasm of the lacrimal gland cells by immunohistochemistry (Figs. 2 and 3). The apical granules in the acinar cells were most intensively labeled. In addition, some reaction was present in the apical parts of duct cells (Fig. 4). Diffuse weak staining was seen in some cells of the conjunctival epithelium. No staining was found in the myoepithelial cells of the ducts. The interacinar plasma cells and lymphocytes were also negative. Elastic fibers in the connective tissue and arterial walls showed some labeling. The sections of lacrimal glands did not contain immunoreactive group 1 PLA2 (Fig. 5). Neither group 1 nor group 2 PLA2 was found in the sections of skeletal muscle by immunohistochemistry.

DISCUSSION

The current study is the first demonstration of PLA2 in lacrimal glands and tears. We also localized group 2

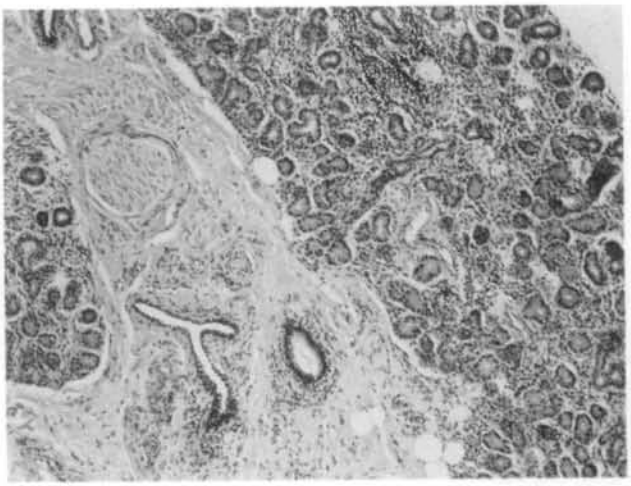

FIGURE 1. Normal structure of human lacrimal gland. Some lymphocytes are present between the acinar units. Two ducts and a nerve are seen in the connective tissue between two glandular lobules. Tissue from a 63-year-old man. (Hematoxylin and eosin, magnification ×90.)

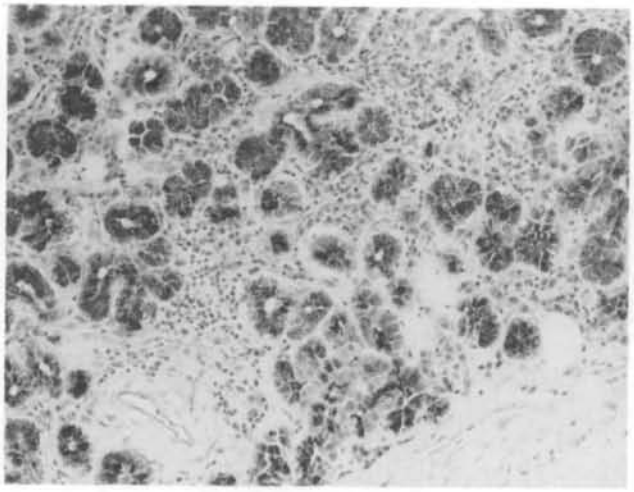

FIGURE 2. Immunohistochemical demonstration of group 2 PLA2 in the gland cells of the lacrimal gland. Tissue from a 63-year-old man. (Anti–group 2-PLA2 antibody, avidin-biotin-peroxidase complex, hematoxylin counter stain; magnification ×180.)

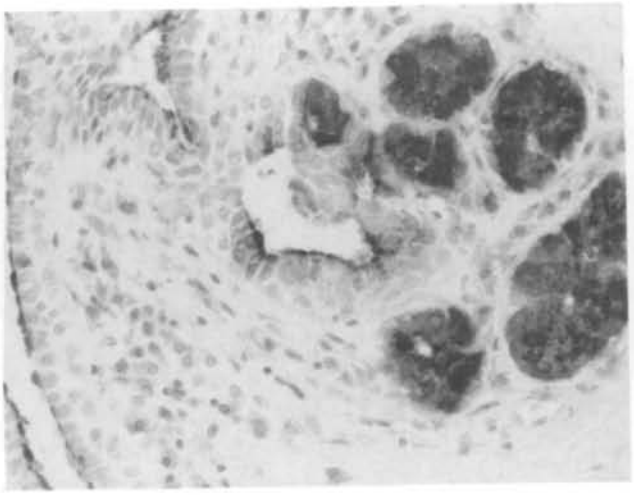

FIGURE 4. Details of the glandular and ductal localization of group 2 PLA2 in the lacrimal gland. Myoepithelial cells and interstitial lymphocytes are devoid of immunoreaction. Tissue from a 63-year-old man. (Anti–group 2-PLA2 antibody, avidin-biotin-peroxidase complex, hematoxylin counter stain; magnification ×360.)

PLA2 by immunohistochemistry in lacrimal gland acinar cells, ducts and conjunctiva. This, however, does not necessarily mean that PLA2 is synthesized also in the ductal or conjunctival cells, because absorption from the tear fluid cannot be ruled out by morphologic observation. The latter explanation seems feasible especially in conjunctival epithelium, which was only weakly and focally stained by the anti–group 2-PLA2 antibody. In the current study, only trace amounts of pancreatic type group 1 PLA2 were found in lacrimal glands, tears and skeletal muscle.

Group 2 PLA2 has been found in the secretory granules of the Paneth cells of the intestinal mucosa, glandular cells of the prostate, parotid glands, amnionic epithelial cells, chondrocytes and in the matrix of both articular and extraarticular cartilage.[12,19] High concentrations of group 2 PLA2 have been detected in synovial fluid[20] and seminal plasma.[21]

The catalytic activity of PLA2 and the concentration of group 2 PLA2 were significantly higher in the homogenates of lacrimal glands than skeletal muscle, which was used as a non–PLA2-secreting control in the current study. The current values obtained from

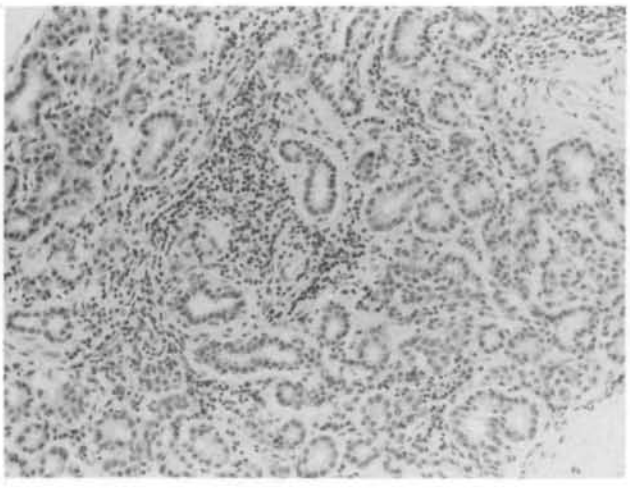

FIGURE 3. A control section of lacrimal gland after omission of the primary antiserum. The gland cells do not show any immunoreaction. Tissue from a 63-year-old man. (Avidin-biotin-peroxidase complex without primary antiserum, hematoxylin counter stain; magnification ×180.)

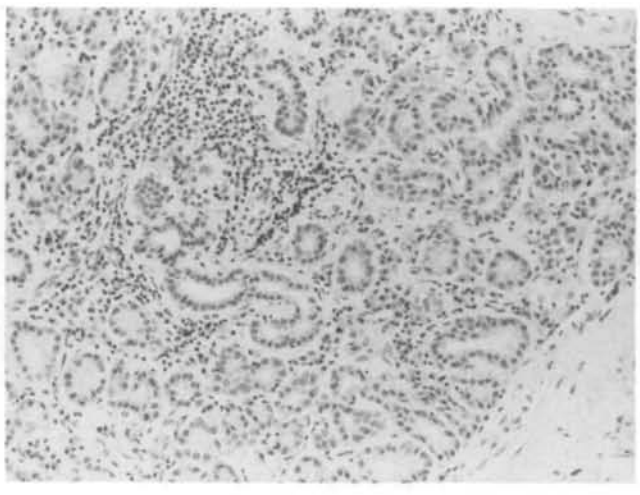

FIGURE 5. Immunohistochemical staining for pancreatic group 1 PLA2 in the lacrimal gland. There is no immunoreaction in this section. Tissue from a 63-year-old man. (Anti–group 1-PLA2 antibody, avidin-biotin-peroxidase complex, hematoxylin counter stain; magnification ×180.)

autopsy material do not necessarily represent the activity in fresh tissue because some enzyme activity might be lost during postmortal autolysis. A biochemical and immunohistochemical analysis of PLA2 in several human autopsy tissues was performed in our laboratory.[12] When compared to the values of that report, lacrimal glands seem to contain more group 2 PLA2 (average 332.3 μg/g protein) than small intestine (23.8 to 47.8 μg/g protein), parotid gland (14.0 μg/g protein), prostate (50.6 μg/g protein), pancreas (1.1 μg/g protein), and cartilage (121.9 μg/g protein). However, it may be misleading to compare the exact values between homogenates of different organs because the density of PLA2 containing cells is variable. Most epithelial cells of the lacrimal glands contain group 2 PLA2, but, for example, the Paneth cells are found only in small clusters at the base of intestinal crypts. The pancreatic acinar cells contain an entirely different type of PLA2 (group 1 PLA2[12]) than the lacrimal gland acinar cells (group 2 PLA2).

The concentration of group 2 PLA2 increases above normal in serum in various inflammatory diseases including sepsis,[10] bacterial, viral, and protozoan infections[22,23] and acute pancreatitis.[24] The cellular source and function of group 2 PLA2 in blood plasma and other body fluids are unknown. A hepatocyte cell line was found to release group 2 PLA2 into the culture medium when stimulated with cytokines. It was therefore postulated that circulating group 2 PLA2 might be derived from the liver and represent an acute phase protein.[25]

A role in the degradation of bacteria has been proposed for the PLA2 found in inflammatory exudate.[13] Both the acinar and ductal cells of lacrimal glands contain secretory granules[26,27] similar to the protein secreting glands of digestive organs. Paneth cells of the intestinal mucosa contain both group 2 PLA2[12] and lysozyme (muramidase).[28] It has been known for a long time that lysozyme has a broad spectrum of antibacterial properties,[29] and therefore it has been suggested that the function of Paneth cells is to regulate the intestinal flora by the degradative action of lysozyme on the glycopeptides of bacterial cell wall.[30,31]

Tears contain several proteins,[32] many of which have antimicrobial properties.[14] Lactoferrin, lysozyme, betalysin, complement and secretory immunoglobulin A are the most important proteins responsible for the defense against microbes.[33] In the current study, we found group 2 PLA2 in tears by a specific immunoassay. The origin of the enzyme is obviously the lacrimal gland acinar cells although active transport from the plasma analogous to that for the secretory component uptake cannot be excluded. However, it is important to notice that the concentration of group 2 PLA2 in tears (1451.3 μg/l) markedly exceeds the level found in normal serum (3.7 μg/l[17]). On the other hand, the concentration of group 2 PLA2 in seminal plasma (15 000 μg/l[34]) is an order of magnitude higher than in tears. Acinar and ductal cells of lacrimal glands contain lysozyme, and the granular in munohistochemical distribution of this enzyme[35] co relates well with the distribution of group 2 PLA2 : seen in the current study. Lysozyme and group PLA2 most probably are produced by the same cells i lacrimal glands. PLA2 is capable of hydrolysis of mer brane phospholipids[1,13] and it is therefore feasible t postulate that PLA2 found in tears acts in concert wit lysozyme to degrade bacteria.

In summary, we found considerable amounts (group 2 PLA2 in tears of healthy individuals and loca ized the enzyme in the gland cells of lacrimal glands b immunohistochemistry. The most likely function (PLA2 in tears is the defense against infection.

Key Words

lacrimal gland, group 1 phospholipase A2, group 2 pho pholipase A2, tears, time-resolved fluoroimmunoassay

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References

1. van den Bosch H. Intracellular phospholipases A. *Bi chim Biophys Acta.* 1980;604:191–246.
2. Seilhamer JJ, Randall TL, Yamanaka M, Johnson, K Pancreatic phospholipase A2: Isolation of the huma gene and cDNAs from porcine pancreas and huma lung. *DNA.* 1986;5:519–527.
3. Seilhamer JJ, Pruzanski W, Vadas P, et al. Cloning ar recombinant expression of phospholipase A2 prese in rheumatoid arthritic synovial fluid. *J Biol Che* 1989;264:5335–5338.
4. Kramer RM, Hession C, Johansen B, et al. Structu and properties of a human non-pancreatic phospho pase A2. *J Biol Chem.* 1989;264:5768–5775.
5. Clark JD, Lin L-L, Kriz RW, et al. A novel arachidon acid-selective cytosolic PLA2 contains a Ca^{2+}-depe dent translocation domain with homology to PKC ar GAP. *Cell.* 1991;65:1043–1051.
6. Heinrikson RL, Krueger ET, Keim PS. Amino ac sequence of phospholipase A2-a from the venom (*Crotalus adamanteus*: A new classification of phospho pases A2 based upon structural determinants. *J Bi Chem.*1977;252:4913–4921.
7. van den Bosch H, Aarsman AJ, van Schaik RH Schalkwijk CG, Nejs FW, Sturk A. Structural and e zymological properties of cellular phospholipases A *Biochem Soc Trans.* 1990;18:781–785.
8. Vadas P, Pruzanski W. Biology of disease: Role of s cretory phospholipase A2 in the pathobiology of d ease. *Lab Invest.* 1986;55:391–404.

9. Nevalainen TJ, Eskola JU, Aho AJ, Havia VT, Lövgren TN-E, Näntö V. Immunoreactive phospholipase A2 in serum in acute pancreatitis and pancreatic cancer. *Clin Chem.* 1985;23:897–904.
10. Green JA, Smith GM, Buchta R, et al. The circulating phospholipase A2 activity associated with sepsis and septic shock is indistinguishable from that associated with rheumatoid arthritis. *Inflammation.* 1991;15: 355–367.
11. Smith GM, Ward RL, McGuigan L, Rajkovic IA, Scott KF. Measurement of human phospholipase A2 in arthritis plasma using a newly developed sandwich ELISA. *Br J Rheumatol.* 1992;31:175–178.
12. Nevalainen TJ, Haapanen TJ. Distribution of pancreatic (group 1) and synovial-type (group 2) phospholipases A2 in human tissues. *Inflammation.* 1993;17: 453–464.
13. Wright GW, Ooi CE, Weiss J, Elsbach P. Purification of a cellular (granulocyte) and an extracellular (serum) phospholipase A2 that participate in the destruction of *Escherichia coli* in a rabbit inflammatory exudate. *J Biol Chem.* 1990;265:6675–6681.
14. Records RE. The tear film. In: Tasman W, Jaeger EA, eds. *Duane's Foundations in Clinical Ophthalmology.* Vol. 2. Philadelphia: JB Lippincott; 1991(chap 3):1–22.
15. Schädlich HR, Bühler M, Beger HG. Improved method for the determination of phospholipase A2 catalytic activity concentration in human serum and ascites. *J Clin Chem Clin Biochem.* 1987;25:505–509.
16. Santavuori SA, Kortesuo PT, Eskola JU, Nevalainen TJ. Application of a new monoclonal antibody for time-resolved fluoroimmunoassay of human pancreatic phospholipase A2. *Eur J Clin Chem Clin Biochem.* 1992;29:819–826.
17. Nevalainen TJ, Kortesuo PT, Rintala E, Märki F. Immunochemical detection of group 1 and group 2 phospholipases A2 in human serum. *Clin Chem.* 1992;38:1824–1829.
18. Hsu SM, Raine L, Fanger H. The use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem.* 1981;29:577–580.
19. Nevalainen TJ, Märki F, Kortesuo PT, Grütter M, DiMarco S, Schmitz A. Synovial-type (group 2) phospholipase A2 in cartilage. *J Rheumatol.* 1993;20: 325–330.
20. Stoner CR, Reik LM, Donohue M, Levin W, Crowl RM. Human group 2 PLA2: Characterization of monoclonal antibodies and immunochemical quantitation of the protein in synovial fluid. *J Immunol Methods.* 1991;145:127–136.
21. Takayama K, Hara S, Kudo J, Inoue K. Detection of 14 kDa group 2 phospholipase A2 in human seminal plasma. *Biochem Biophys Res Commun.* 1991;178:1505–1511.
22. Rintala EM, Nevalainen TJ. Group 2 phospholipase A2 in sera of febrile patients with microbiologically or clinically documented infections. *Clin Infect Dis.* 1993;17:864–870.
23. Vadas P, Keystone J, Stefanski E, Scott K, Pruzanski W. Induction of circulating group 2 phospholipase A2 expression in adults with malaria. *Infect Immun.* 1992;60:3928–3931.
24. Nevalainen TJ, Grönroos JM, Kortesuo PT. Pancreatic and synovial-type phospholipases A2 in serum in severe acute pancreatitis. *Gut.* 1993;34:1133–1136.
25. Crowl RM, Stoller TJ, Conroy RR, Stoner CR. Induction of phospholipase A2 gene expression in human hepatoma cells by mediators of the acute phase response. *J Biol Chem.* 1991;266:2647–2651.
26. Egeberg J, Jensen OA. The ultrastructure of the acini of the human lacrimal gland. *Acta Ophthalmol.* 1969;47:400–410.
27. Iwamoto T, Jakobiec FA. Lacrimal glands. In: Tasman W, Jaeger EA, eds. *Duane's Foundations in Clinical Ophthalmology.* Vol. 1. Philadelphia: JB Lippincott; 1991(chap 30):1–21.
28. Speece AJ. Histochemical distribution of lysozyme activity in organs of normal mice and radiation chimeras. *J Histochem Cytochem.* 1964;12:384–391.
29. Fleming A. On a remarkable bacteriolytic element found in tissues and secretions. *Proc R Soc Biol (Lond).* 1922;93:306–317.
30. Erlandsen SL, Chase DG. Paneth cell function: Phagocytosis and intracellular digestion of intestinal microorganisms. I. *Hexamita muris. J Ultrastruct Res.* 1972;41:296–318.
31. Erlandsen SL, Chase DG. Paneth cell function: Phagocytosis and intracellular digestion of intestinal microorganisms. II. Spiral micro-organism. *J Ultrastruct Res.* 1972;41:319–333.
32. Janssen E, van Bijsterreld O. Origin and biosynthesis of human tear fluid proteins. *Invest Ophthalmol Vis Sci.* 1983;24:623–630.
33. Mondino BJ. Host defence against bacterial and fungal disease. In: Tasman W, Jaeger EA, eds. *Duane's Foundations in Clinical Ophthalmology.* Vol. 2. Philadelphia: JB Lippincott; 1991(chap 45):1–10.
34. Nevalainen TJ, Meri K-M, Niemi M. Synovial-type (group 2) phospholipase A2 in human seminal plasma. *Andrologia.* 1993;25:355–358.
35. Gillette TE, Greiner JV, Allansmith MR. Immunohistochemical localization of human tear lysozyme. *Arch Ophthalmol.* 1981;99:298–300.

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CLINICAL INVESTIGATION

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Diurnal variation in group IIa phospholipase A_2 content in tears of contact lens wearers and normal controls

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Abstract *Purpose:* To study the diurnal rhythm in group IIA phospholipase A_2 ($GIIAPLA_2$) content of tears and the effect of the wearing time of soft contact lenses (CL) on the content of $GIIAPLA_2$ in tears. *Methods:* The $GIIAPLA_2$ content of tears was measured by a time-resolved fluoroimmunoassay in 22 healthy controls at 8 a.m., noon, 4 p.m. and 8 p.m. and in 20 CL wearers at 4 p.m. 1–2 days before using CLs and after 4 h (at noon), 8 h (4 p.m.) and 12 h (8 p.m.) use of soft CLs. *Results:* The $GIIAPLA_2$ content of tears of healthy controls was 80.6±47.8 μg/ml (mean±SD). The $GIIAPLA_2$ content was lower at 8 a.m. than at noon (p=0.006) and higher at 4 p.m. than at 8 p.m. (*P*=0.003). There was no statistically significant difference in the $GIIAPLA_2$ content of tears between the CL wearers without CLs (69.47±31.2 μg/ml) and the normal subjects (92.3±48.2 μg/ml) measured at 4 p.m. Compared with healthy controls, the $GIIAPLA_2$ values in subjects wearing CLs were statistically significantly lower at noon (*P*=0.0001) and at 4 p.m. (*P*=0.0002). *Conclusion:* In normal subjects, the $GIIAPLA_2$ content of tears increased from 8 a.m. to noon and decreased from 4 p.m. to 8 p.m. The use of CLs for 4 h and 8 h caused a decrease in the $GIIAPLA_2$ content of tears. This difference was not seen at 4 p.m. the day when the CL wearers did not use CLs.

Introduction

Tears contain several antimicrobial proteins, including lysozyme, lactoferrin, betalysin, complement, secretory immunoglobulin A (sIgA), and phospholipase A_2 (PLA_2) [11, 15, 18, 21, 22]. We observed two specialized cell types in the main and accessory lacrimal glands, one synthesizing group IIA phospholipase A_2 ($GIIAPLA_2$) and the other synthesizing lysozyme [1]. Lysozyme was present in the secretory granules of most acini, whereas $GIIAPLA_2$ was seen in a minority of acinar cells, primarily in the central parts of lobules in the main and accessory lacrimal glands [1].

PLA_2 is a lipolytic enzyme that catalyses the hydrolysis of the sn–2 ester bond of phosphoglycerides [25]. The PLA_2 superfamily consists of 11 groups (I–XI) of PLA_2s, which in turn are divided into several subgroups [25]. $GIIAPLA_2$ is a 14-kDa secretory PLA_2 present in cell types and body fluids, e.g. in synovial fluid, seminal plasma, and tear fluid [1, 16, 18, 25, 29]. Human $GIIAPLA_2$ has been implicated in host-defense responses due to its antibacterial activity, consistent with its affinity for anionic bacterial membranes [20, 30]. The $GIIAPLA_2$ content of tears in 122 normal subjects was 54.5±33.9 μg/ml, which is one of the highest amounts of $GIIAPLA_2$ reported in human secretions [23]. It was highest in the age group 20–29 years (81.6±32.0 μg/ml) and the concentration decreased with increasing age. The $GIIAPLA_2$ content of tears was higher in patients with keratoconjunctivitis sicca than in normal subjects [3]. However, the concentration was normal in patients with senile cataract and in patients with primary open-angle glaucoma [2].

Several previous studies have reported diurnal variations in the concentrations of tear solutes, e.g. tear lysozyme values were significantly elevated from 9 a.m. to 12 a.m. and reduced during the sleep period from midnight to 3 a.m. [8]. In contrast, tear IgA, calcium and total protein levels showed no significant diurnal rhythm [8, 9, 19].

There is a great deal of uncertainty about the effect of wearing of contact lenses (CL) on antimicrobial tear film proteins in the healthy eye. According to some studies, the daily use of CLs has no effect on the amount of cells [10], total protein content [6, 10, 26], the content of sIgA [14], lysozyme [5, 14], albumin [5, 14] or lactoferrin [5] in tears. Furthermore, some studies have reported the tear sIgA content to increase due to the daily use of CLs [13, 24, 27] and another to decrease [12]. IgA isotype-specific antibodies reactive with *Escherichia coli* and *Staphylococcus epidermidis* have been reported to be present at lower levels in CL wearers than in normal subjects. In addition, the composition of tear proteins during the overnight use of CLs has been reported to change: both the total protein content and the C3, C4 and sIgA content of tears decreased after the overnight use of CLs [26].

No data are available on the diurnal rhythm of $GIIAPLA_2$ in tears or the effect of the wearing time of soft CLs on the content of $GIIAPLA_2$ in tears. The present study focused on these questions.

Material and methods

Subjects

The $GIIAPLA_2$ content of tears was measured in both eyes of 20 healthy CL wearers, 4 men and 16 women, whose age ranged from 19 to 34 (22.6±3.2, mean±SD) years. The subjects had used soft CLs for 5.2±3.1 years. As normal controls, we studied 22 subjects, 6 male and 16 female (age 24.8±2.0 years), who did not use CLs. The principles of the World Medical Association Declaration of Helsinki were followed. Written consent was obtained from each subject giving the tear specimen. The study protocol was approved by the local ethics committee.

Collection of tears

Nonstimulated tears were collected into disposable 5-µl microcapillaries (Microcaps 5µl, Drummond Scientific, Broomall, PA, USA) under a Haag–Streit 900 biomicrosope in a dark room using minimal slit-lamp illumination. Tears were collected from both eyes of the CL wearers and from the left eye of the normal subjects. The samples were gathered without topical anesthesia from the marginal tear strip of the lower lid near the lateral canthus, care being taken not to irritate the conjunctiva, cornea, or lid margin. From 0.15 µl to 5 µl of tear fluid was collected, and the volume was measured using a calibrating scale beside the microcapillary. In all cases, the collection time was limited to 5 min. The tear samples were diluted from 1:300 to 1:1100 with physiological saline and kept frozen at −70°C until assayed.

The tear samples were collected from the CL wearers at 4 p.m. after a break of at least 16 h without CLs. After 1 or 2 days, the same subjects applied the CLs to their eyes at 8 a.m. and they were studied after 4, 8 and 12 h use of CLs (at noon, 4 p.m. and 8 p.m.). To determine the normal diurnal variation in the $GIIAPLA_2$ content of tears, tear samples were collected from the normal controls at 8 a.m., noon, 4 p.m. and 8 p.m.

Phospholipase A2 assay

The concentration of $GIIAPLA_2$ in tear fluid was measured by a time-resolved fluoroimmunoassay using a polyclonal rabbit antibody to recombinant human $GIIAPLA_2$ [17]. The results are expressed as micrograms per milliliter.

Statistical analysis

The baseline measurements in the healthy controls and the CL wearers without CLs at 4 p.m. were conducted using the Wilcoxon rank sum test. The diurnal variation in the $GIIAPLA_2$ content of tears was compared between the healthy controls and the subjects wearing CLs by using analysis of variance for repeated measurements. The mean change of the $GIIAPLA_2$ content studied within the control group was also analyzed by means of repeated-measures analysis of variance. All P values lower than 0.05 were considered statistically significant. The results are expressed as mean±SD.

Results

The mean $GIIAPLA_2$ content of tears in healthy controls was 80.6±47.8 µg/ml. The concentration increased between 8 a.m. (69.9±37.5 µg/ml) and noon (95.2±48.2 µg/ml) (P=0.006), and decreased between 4 p.m. (92.3±48.2 µg/ml) and 8 p.m. (64.9±41.2 µg/ml) (P=0.003) (Fig. 1). There was no statistically significant difference in the $GIIAPLA_2$ content of tears between the CL wearers without CLs and the normal controls at 4 p.m.(P=0.286). The $GIIAPLA_2$ content of tears of CL

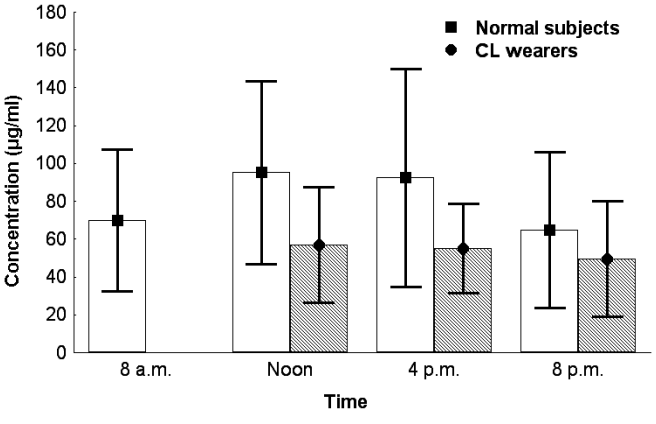

Fig. 1 Group IIA phospholipase A2 concentration (mean±SD) of tears in normal subjects and in contact lens (CL) wearers at 8 a.m., noon, 4 p.m. and 8 p.m.

Table 1 Diurnal variation in the concentration (mean±SD, μg/ml) of group IIA phospholipase A_2 in tear fluid of healthy controls and contact lens (*CL*) wearers

Group	Time			
	8 a.m.	noon	4 p.m.	8 p.m.
Controls	69.9±37.5	95.2±48.2*	92.3±48.2**	64.9±41.2
CL wearers with CLs		56.3±30.3	54.1±24.3	49.7±30.5
CL wearers 1–2 days before using CLs			69.5±31.2	

* p=0.006 vs. controls 8 a.m., p=0.0001 vs. CL–wearers with CLs at noon; ** p=0.003 vs. controls 8 p.m., p=0.0002 vs. CL–wearers with CLs 4 p.m.

wearers was 56.3±30.3 μg/ml at noon, 54.1±24.3 μg/ml at 4 p.m. and 49.7±30.5 μg/ml at 8 p.m. The $GIIAPLA_2$ values in CL wearers at noon (*P*=0.0001) and 4 p.m. (*P*=0.0002) were statistically significantly lower than the values in the healthy controls at the same time of day. There was no statistically significant difference in the $GIIAPLA_2$ content of tears between the CL wearers and the healthy controls at 8 p.m. (*P*=0.109) (Table 1).

Discussion

In this study, the mean concentration of $GIIAPLA_2$ in tears of healthy controls was 80.6±47.8 μg/ml, which is consistent with the $GIIAPLA_2$ content of tears in 20- to 29-year-old healthy subjects (81.6±32.0 μg/ml) reported in our earlier study [23]. In normal subjects in the present study, tear $GIIAPLA_2$ showed a discernible diurnal rhythm. The concentration increased between 8 a.m. (69.9±37.5 μg/ml) and noon (95.2±48.2 μg/ml) (*P*=0.006), was significantly elevated from noon to 4 p.m., and decreased between 4 p.m. (92.3±48.2 μg/ml) and 8 p.m. (64.9±41.2 μg/ml) (*P*=0.003) (Table 1). Similarly, Horwitz et al. showed tear lysozyme to be elevated between 9 and 12 a.m [8].

The current results showed that in CL wearers who had used their CLs for 5.2±3.1 years, after a break of at least 16 h without CLs, the $GIIAPLA_2$ content of tears was within the normal range (Table 1). This observation indicates that even long-term use of CLs does not cause any permanent changes in the $GIIAPLA_2$ content of tears. However, the CL wearers with CLs had statistically significantly lower $GIIAPLA_2$ content in tears at noon and at 4 p.m. than the healthy controls. In our earlier study, we observed that the $GIIAPLA_2$ content of reflex tears was lower than of nonstimulated tears [4]. A small transient lowering effect on the $GIIAPLA_2$ content of tears may also be associated with the absorption of $GIIAPLA_2$ into the CLs.

$GIIAPLA_2$ is the principal agent responsible for the ability of tears to kill a broad spectrum of gram-positive bacteria. Only 1.1 ng/ml of $GIIAPLA_2$ sufficed to kill *Listeria monocytogenes* and 15–80 ng/ml of $GIIAPLA_2$ to kill *Staphylococcus aureus* [20]. *Micrococcus luteus* was killed by 0.3 μg/ml of $GIIAPLA_2$ [20]. CL wearers may occasionally develop conjunctival inflammation or infectious keratitis with the corneal infection ranging from small peripheral ulcers to large suppurative central ulcers [7]. However, the results of the current study show that the levels of $GIIAPLA_2$ in tears after 4–12 h of CL wear were about 50 μg/ml and thus at least 150 times higher than the highest reported level required for effective killing of gram-positive bacteria [20]. This may offer an explanation for the observation that, in apparently normal eyes, the wearing of soft CLs does not introduce pathogenic bacteria into the eye and does not change the surface environment to allow the growth of pathogenic organisms [28].

In summary, we found that the $GIIAPLA_2$ content of tears in normal subjects was lower at 8 a.m. than at noon and higher at 4 p.m. than at 8 p.m. The $GIIAPLA_2$ was statistically significantly lower in contact lens wearers at noon and 4 p.m. than in control subjects.

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References

1. Aho HJ, Saari KM, Kallajoki M, Nevalainen TJ (1996) Synthesis of group II phospholipase A2 and lysozyme in lacrimal glands. Invest Ophthalmol Vis Sci 37:1826–1832
2. Aho VV, Nevalainen TJ, Paavilainen V, Saari KM (2002) Group II phospholipae A2 content of tears in patients with senile cataract and primary open-angle glaucoma. Eur J Ophthalmol 12:40–43
3. Aho VV, Nevalainen TJ, Saari KM (2002) Group IIA phospholipase A2 content of tears in patients with keratoconjunctivitis sicca. Graefes Arch Clin Exp Ophthalmol 240:521–523

4. Aho VV, Nevalainen TJ, Saari KM (2002) Group IIA phospholipase A2 content of basal, nonstimulated and reflex tears. Curr Eye Res 24:224–227
5. Carney FP, Morris CA, Willcox MDP (1998) Effect of hydrogel lens wear on the major tears proteins during extended wear. Aust N Z J Ophthalmol 25:36–38
6. Claudon-Eyl V, Baguet J (1994) Electrophoretic patterns of human denaturated tear proteins and glycoproteins from normal subjects and soft contact lens wearers. Adv Exp Biol Med 350:411–416
7. Groos EB (1999) Complications of contact lenses. In: Tasman W, Jaeger EA (eds) Duane's clinical ophthalmology, vol 4. Lippincott, Philadelphia, pp 1–21
8. Horwitz BL, Christensen GR, Ritzmann SR (1987) Diurnal profiles of tear lysozyme and gamma A globulin. Ann Ophthalmol 10:75–80
9. Huth SW, Miller MJ, Leopold IH (1981) Calcium and protein in tears: diurnal variation. Arch Ophthalmol 99:1628–1633
10. Ichijima H, Cavanagh HD (1994) Effects of rigid lens extended wears on lactate dehydrogenase activity and isozymes in rabbit tears. Cornea 13:429–434
11. Kijlstra A, Kuizenga A (1994) Analysis and function of the human tear proteins. Adv Exp Biol Med 350:299–308
12. Kijlstra A, Polak BCP, Luyendijk L (1992) Transient decrease of secretory IgA in tears during rigid gas permeable contact lens wear. Curr Eye Res 11:123–126
13. Mannucci LL, Pozzan M, Fregona I, Secchi AG (1984) The effect of extended wear contact lenses on tear immunoglobulins. CLAO J 10:163–165
14. McCellan KA, Cripps AW, Clancy RL, Billson FA (1998) The effect of succesful contact lens wear on mucosal immunity of the eye. Ophthalmology 105:1471–1477
15. Mondino BJ (1991) Host defence against bacterial and fungal disease. In: Tasman W, Jaeger EA (eds) Duane's clinical ophthalmology, vol 2. Lippincott, Philadelphia, pp 1–11
16. Nevalainen TJ, Haapanen TJ (1993) Distribution of pancreatic (group I) and synovial-type (group II) phospholipases A2 in human tissues. Inflammation 17:453–464
17. Nevalainen TJ, Kortesuo PT, Rintala E, Märki F (1992) Immunochemical detection of group 1 and group 2 phospholipases A2 in human serum. Clin Chem 38:1824–1829
18. Nevalainen TJ, Aho HJ, Peuravuori H (1994) Secretion of group 2 phospholipase A2 by lacrimal glands. Invest Ophthalmol Vis Sci 32:417–421
19. Ng V, Cho P, Wong F, Chan Y (2001) Variability of tear protein levels in normal young adults: diurnal (daytime) variation. Graefes Arch Clin Exp Ophthalmol 239:257–263
20. Qu X-D, Lehrer RJ (1998) Secretory phospholipase A2 is the principal bactericide for staphylococci and other gram-positive bacteria in human tears. Infection Immunity 66:2791–2797
21. Records RE (1991) The tear film. In: Tasman W, Jaeger EA (eds) Duane's clinical ophthalmology, vol 2. Lippincott, Philadelphia, pp 1–22
22. Saari KM, Aine E, Parviainen MT (1985) Determination of protein content in ocular fluids by high-performance gel filtration chromatography. In: O'Connor GR, Chandler JW (eds) Advances in immunology and immunopathology of the eye. Masson, New York, pp 258–262
23. Saari KM, Aho VV, Paavilainen V, Nevalainen TJ (2001) Group II phospholipase A2 content of tears in normal subjects. Invest Ophthalmol Vis Sci 42:318–320
24. Saini JS, Dhar S, Sharma A (1992) Role of tear secretory IgA in hard contact lens-related changes in the tarsal conjunctiva. Can J Ophthalmol 27:299–301
25. Six DA, Dennis EA (2000) The expanding superfamily of phospholipase A2 enzymes: classification and characterization. Biochim Biophys Acta 1488:1–19
26. Stapleton F, Willcox P, Morris CA, Sweeney DF (1998) Tear changes in contact lens wearers following overnight eye closure. Curr Eye Res 2:183–188
27. Temel A, Kazokoglu H, Taga Y, Orkan AL (1991) The effect of contact lens wear on tear immunoglobulins. CLAO J 17:69–71
28. Tragakis MP, Brown SI, Pearce DB (1973) Bacteriologic studies of contamination associated with soft contact lenses. Am J Ophthalmol 75:496
29. van den Bosch H, Aarsman AJ, van Schaik RHN, Schalkwijk CG, Neijs FW, Sturk A. (1990) Structural and enzymological properties of cellular phospholipases A2. Biochem Soc Trans 18:781–785
30. Weinrauch Y, Elsbach P, Madsen LM, Foreman A, Weiss J (1996) The potent anti-Staphylococcus aureus activity of a sterile rabbit inflammatory fluid is due to a 14-kD phospholipase A2. J Clin Invest 97:250–257

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Group IIA phospholipase A2 content of tears in patients with atopic blepharoconjunctivitis

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Abstract *Background:* To determine the concentration of group IIA phospholipase A_2 ($GIIAPLA_2$) in tears of patients with atopic blepharoconjunctivitis (ABC), and to compare it with the $GIIAPLA_2$ concentration of tears in age-matched healthy controls. *Methods:* The diagnosis of ABC was confirmed with a positive skin prick test and the presence of atopic dermatitis in lids. Conjunctival brush cytology was taken, and the cells including eosinophils, neutrophils, lymphocytes, squamous epithelial cells, columnar epithelial cells, metaplastic changes and the goblet cells were calculated separately. The $GIIAPLA_2$ concentration of tears was measured with a time-resolved fluoroimmunoassay in 29 patients with ABC (mean age 36.3±12.7 years) and 29 normal subjects (mean age 37.0±12.0 years). *Results:* The $GIIAPLA_2$ concentration of tears in patients with ABC was 43.8± 33.0 μg/ml, and in normal subjects it was 67.1±23.3 μg/ml. The difference was statistically significant (p=0.0018). The concentration of $GIIAPLA_2$ of tears was lowest in the subgroup of patients with ABC and dry eye (25.8 ±23.6 μg/ml), whereas it was only slightly decreased in patients with ABC and normal tear secretion (56.6±33.3 μg/ml). The difference between these two subgroups was statistically significant (p=0.011). There was no statistically significant correlation between the $GIIAPLA_2$ concentration of tears and the quantity of different conjunctival cells gathered by the brush cytology. However, an almost significant correlation was found between the $GIIAPLA_2$ concentration in tears and conjunctival eosinophils. *Conclusions:* The results indicate that in patients with ABC the $GIIAPLA_2$ content of tears was decreased, without any dependence on the quantity of different conjunctival cells.

Introduction

Atopic blepharoconjunctivitis (ABC) is a chronic and disabling eye disease characterized by type I hypersensitivity reactions, often high immunoglobulin E (IgE) levels, and multiple positive skin prick tests (SPT) [7]. Raised levels of inflammatory mediators including histamine, tryptase, plasmin, eosinophilic cationic proteins, leukotrienes and prostaglandin D2 have been reported in the tear fluid of ABC patients [7]. The conjunctival cytology of ABC patients is characterized by eosinophils, lymphocytes, neutrophils and occasional monocytes and plasma cells [7]. The occurrence of *Staphylococcus aureus* in the conjunctiva and lid margin is a constant finding in patients with ABC [7, 12]. Little is known of the antimicrobial proteins of tear fluid in patients with ABC.

Nevalainen et al. were the first to report the presence of phospholipase A_2 (PLA_2) in the human main lacrimal gland and tears [16]. We observed two specialized cell types in the main and accessory lacrimal glands, one synthesizing group IIA phospholipase A_2 ($GIIAPLA_2$)

and the other synthesizing lysozyme [1]. GIIAPLA$_2$ was present in the secretory granules, primarily in the central parts of lobules in the main and accessory lacrimal glands [1]. GIIAPLA$_2$ is a lipolytic enzyme that belongs to the PLA$_2$ superfamily, which consists of 12 groups (I-XII) of PLA$_2$s [20]. The GIIAPLA$_2$ catalyses the hydrolysis of the sn-2 ester bond of phosphoglycerides located primarily on cell membranes of gram-positive bacteria [20]. Consequently, it is capable of killing a broad spectrum of gram-positive bacteria [8, 10, 11, 14, 17]. It has only recently been realized that GIIAPLA$_2$ is one of the most important antibacterial enzymes in tears, especially against *Staphylococcus aureus* [10, 11, 14, 17].

The GIIAPLA$_2$ concentration of tears in normal subjects (54.5±33.9 μg/ml) was one of the highest amounts of GIIAPLA$_2$ reported in human secretions [19]. The concentration decreased with increasing age [19], showed a discernible diurnal rhythm [6] and was statistically, significantly lower in reflex than in basal tears [3]. The GIIAPLA$_2$ concentration of tears was elevated in patients with keratoconjunctivitis sicca [4], whereas in patients with senile cataract or primary open-angle glaucoma the concentration was normal [2].

The purpose of this study was to determine the content of GIIAPLA$_2$ in tear fluid of patients with ABC and to compare the levels with the presence of different cells of conjunctival cytology.

Material and methods

Subjects

Altogether, we included in this study 58 subjects—22 men and 36 women, with the age ranging between 10 and 63 years (mean 36.7±12.6). The principles of the World Medical Association Declaration of Helsinki were followed. Informed consent was obtained from each subject giving the tear specimen. The study protocol was approved by the ethics committee of the University of Turku and the Turku University Hospital.

We studied 29 patients with ABC, 11 men and 18 women, with the age ranging from 10 to 61 years (mean 36.3±12.7) (Table 1). All patients had typical symptoms of ABC, including intense itching, lacrimation, conjunctival discharge and redness. The diagnosis of ABC was confirmed with a positive SPT, the presence of chronic atopic dermatitis in lids, face and elsewhere in the body, and by using conjunctival brush (Accellon Multi Biosampler) cytology. Fourteen of the 29 patients (48%) had dry eyes verified by Schirmer's test or decreased tear film breakup time (BUT <10 s).

Table 1 Number (No.) and age (mean±SD) of patients with atopic blepharoconjunctivitis (*ABC*) and of age-matched healthy controls

ABC	Men	Women	Total
No.	11	18	29
Age (years)	35.8±15.3	36.7±11.4	36.3±12.7
Controls			
No.	11	18	29
Age (years)	35.9±14.4	37.7±10.6	37.0±12.0

All patients with ABC were treated by intermittent preservative-free chromoglycate eye drops for lubrication. The treatment was stopped in all patients 3 days before the examination. All patients with ABC showed bacterial cultures that were almost constantly positive for *Staphylococcus aureus* at levels of 1+ to 3+.

As normal age-matched controls, we studied 29 non-atopic subjects, 11 men and 18 women, with the age ranging from 20 to 63 years (mean 37.0±12.0) (Table 1). None of the control subjects wore contact lenses, and all of them revealed normal findings in a routine ophthalmological examination.

Conjunctival cytology

Conjunctival brush (Accellon Multi Biosampler) cytology was taken from 21 patients with ABC from the lower and upper lid tarsal conjunctiva of the eye after the collection of tear fluid. The sample was fixed with alcohol and stained after the Papanicolau method. The cells including eosinophils, neutrophils, lymphocytes, squamous and columnar epithelial cells, metaplastic changes and goblet cells were calculated [9].

Collection of tears

The values of GIIAPLA$_2$ were assessed in tear samples from one eye of each subject. A nonstimulated tear sample of 5 μl was taken using disposable 5 μl microcapillaries (Microcaps 5 µl, Drummond Scientific, Broomall, PA, USA) under a Haag-Streit 900 biomicroscope. The samples were gathered from the marginal tear strip of the lower lid near the lateral canthus, with care being taken not to irritate the conjunctiva, cornea, or lid margin. Tears were immediately transferred into Eppendorf tubes, placed on dry ice and kept at −70 °C until analyzed.

GIIAPLA$_2$ assay

The concentration of GIIAPLA$_2$ in tear fluid was measured by a highly monospecific time-resolved fluoroimmunoassay (TR-FIA) using a polyclonal rabbit antibody to recombinant GIIAPLA$_2$ [15]. The results are expressed as micrograms per milliliter (µg/ml).

Statistical analysis

Statistical analyses were performed using SAS for Windows version 8.2. After tests for normality, the difference in the GIIAPLA$_2$ content between the patients with ABC and the healthy controls, and the effect of age on the GIIAPLA$_2$ concentration of tears was analyzed using the generalized linear models procedure. Difference in the GIIAPLA$_2$ content of tears between ABC patients with dry eyes and ABC patients with normal tear secretion rate was tested with a two-way *t*-test. Linear regression analysis with stepwise selection model was used to determine the correlation of the GIIAPLA$_2$ concentration of tears with all the different conjunctival cells (eosinophils, neutrophils, lymphocytes, squamous epithelial cells, columnar epithelial cells and goblet cells) separately and with all the inflammatory cells (eosinophils, neutrophils and lymphocytes) together. Pearson's correlation was determined between the GIIAPLA$_2$ concentration of tears and the quantity of eosinophils. All the p values lower than 0.05 were considered statistically significant. The results are expressed as mean±SD.

Table 2 Group IIA phospholipase A_2 concentration (μg/ml) (mean±SD) in tear fluid of 29 patients with atopic blepharoconjunctivitis (ABC) and in 29 age-matched healthy controls

	Men	Women	Total
ABC	45.2±40.1	43.0±29.1	43.8±33.0*
Controls	64.8±24.3	68.5±23.3	67.1±23.3

*p=0.0018 vs controls (analyzed using generalized linear models procedure)

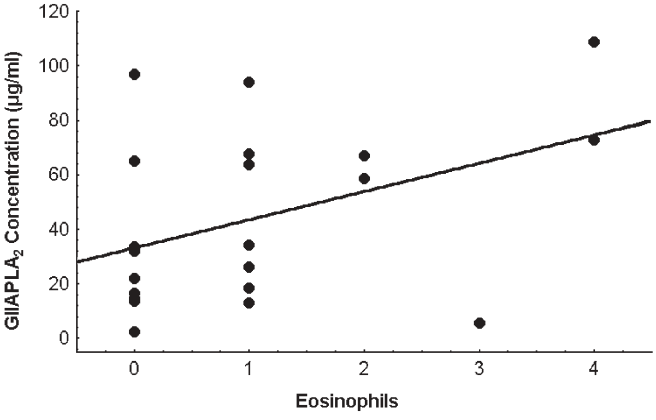

Fig. 1 Correlation between the concentration (µg/ml) of group IIA phospholipase A_2 ($GIIAPLA_2$) in tears and the quantity of conjunctival eosinophils in patients with atopic blepharoconjunctivitis

Results

The $GIIAPLA_2$ content of tears was 43.8±33.0 µg/ml in patients with ABC and 67.1±23.3 µg/ml in normal subjects (Table 2). Analysis using the generalized linear models procedure showed that the difference between these two test groups was statistically significant (p=0.0018) and that a decrease in the concentration of $GIIAPLA_2$ occurred with increasing age (p=0.025). The mean concentration of $GIIAPLA_2$ in tears was 25.8 ±23.6 µg/ml in patients with ABC and dry eye, and 56.6±33.3 µg/ml in ABC patients with normal tear secretion rate. The difference between these groups was statistically significant (p=0.011). Linear regression analysis did not reveal any statistically significant (p<0.05) correlations between the $GIIAPLA_2$ content of tears and the different conjunctival cells gathered by the brush cytology. Conjunctival eosinophilia was present in 15 patients of 21 (71%). An almost significant correlation was found between the $GIIAPLA_2$ concentration of tears and the quantity of conjunctival eosinophils (linear regression analysis p=0.07; Pearson's R=0.41, p=0.06; Fig. 1).

Discussion

This is the first study to show that $GIIAPLA_2$ concentration of tears decreases in patients with ABC (p=0.0018). We have previously shown that the concentration of $GIIAPLA_2$ decreases in tears of older subjects [19] and contact lens wearers [6], in reflex tears [3], and early in the morning and in the evening [6]. Similarly, the $GIIAPLA_2$ concentration of tears decreased two days after a PRK operation, although 1 week postoperatively it was higher than preoperatively [5]. In the present study, nonstimulated tear samples were taken from middle-aged ABC patients who did not wear contact lenses and from age-matched control subjects. It is important to note that almost one-half (48%) of the patients in the present study had dry eyes. In our previous study, however, the $GIIAPLA_2$ concentration of tears in patients with keratoconjunctivitis sicca increased markedly [4]. Thus, the decrease in the $GIIAPLA_2$ concentration of tears in patients with ABC found in this study could not be explained by any of the factors mentioned above, and may be associated more directly with atopy. Similarly, the lysozyme concentration of tears decreased in patients with atopy [18].

Several studies have shown that the occurrence of *Staphylococcus aureus* in the conjunctiva and lid margin is a constant finding in patients with ABC [12], as was seen also in our patients. Continuous infection in the conjunctiva may stimulate the secretion of antimicrobial proteins of tears, which in longstanding hypersecretion may cause an increased consumption of antimicrobial proteins of tears and, in this way, a decrease in the $GIIAPLA_2$ content of tears. The present results showed that the group of ABC patients with dry eye had significantly lower concentration of $GIIAPLA_2$ in tears than those ABC patients with a normal tear secretion. In the former group, $GIIAPLA_2$ concentration in tears may decrease due to nearly constant occurrence of *Staphylococcus aureus* infection and insufficient secretion capacity of the lacrimal glands, whereas in the latter group, $GIIAPLA_2$ concentration of tears was only slightly lower but still in the normal range.

The results showed no statistically significant correlation between the $GIIAPLA_2$ content of tears and the different conjunctival cells. However, there was an almost significant correlation between the $GIIAPLA_2$ content of tears and conjunctival eosinophils. The superantigen produced by *Staphylococcus aureus* is known to stimulate the occurrence of conjunctival eosinophils [13]. Thus, nearly constant occurrence of *Staphylococcus aureus* may decrease the concentration of $GIIAPLA_2$ in tears of ABC patients, but in those patients with an increased number of conjunctival eosinophils, the inflammation may also stimulate the production of $GIIAPLA_2$ in tears.

The results of the current study showed that the levels of $GIIAPLA_2$ in tears of patients with ABC were below the normal values, but they are still considerably above

the level required for effective killing of gram-positive bacteria [17]. However, in the patients with ABC and dry eye, the $GIIAPLA_2$ concentration of tears may occasionally be insufficient for effective kill of gram-positive bacteria. Therefore, the results of the present study suggest that the therapy for ABC must also be effective against staphylococci. In another study we have tested the effect of tacrolimus ointment applied on the lids of patients with ABC and found not only an anti-inflammatory effect but also a good antibacterial efficacy, especially against *Staphylococcus aureus* (unpublished observation).

In summary, we conclude that the concentration of $GIIAPLA_2$ in tears of patients with ABC decreased, and that there was no correlation between the $GIIAPLA_2$ content of tears and the different conjunctival cells, except eosinophils, which showed a nearly significant correlation with $GIIAPLA_2$ content of tears.

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References

1. Aho HJ, Saari KM, Kallajoki M, Nevalainen TJ (1996) Synthesis of group II phospholipase A2 and lysozyme in lacrimal glands. Invest Ophthalmol Vis Sci 37:1826–1832
2. Aho VV, Nevalainen TJ, Paavilainen V, Saari KM (2002) Group II phospholipase A2 content of tears in patients with senile cataract and primary open-angle glaucoma. Eur J Ophthalmol 12:40–43
3. Aho VV, Nevalainen TJ, Saari KM (2002) Group IIA phospholipase A2 content of tears in basal, nonstimulated and reflex tears. Curr Eye Res 24:224–227
4. Aho VV, Nevalainen TJ, Saari KM (2002) Group IIA phospholipase A2 content of tears in patients with keratoconjunctivitis sicca. Graefes Arch Clin Exp Ophthalmol 240:521–523
5. Aho VV, Holopainen JM, Tervo T, Moilanen JA, Nevalainen TJ, Saari KM (2003) Group IIA phospholipase A_2 content in tears of patients having photorefractive keratectomy. J Cataract Refract Surg 29:2163–2167
6. Aho VV, Paavilainen V, Nevalainen TJ, Peuravuori H, Saari KM (2003) Diurnal variation in group IIA phospholipase A2 content in tears of contact lens wearers and normal controls. Graefes Arch Clin Exp Ophthalmol 241:85–88
7. Bielory L (2000) Allergic and immunologic disorders of the eye. Part II: ocular allergy. J Allergy Clin Immunol 106(6):1019–1032
8. Harwing SSL, Tan L, Qu X-D, Cho Y, Eisenhauer PB, Lehrer RI (1995) Bactericidal properties of murine intestinal phospholipase A_2. J Clin Invest 95:603–610
9. Kari O (1988) Atopic conjunctivitis. A cytologic examination. Acta Ophthalmol (Copenh) 66:381–386
10. Koduri RS, Grönroos JO, Laine VJ, Le Calvez C, Lambeau G, Nevalainen TJ, Gelb MH (2002) Bactericidal properties of human and murine groups I, II, V, X, and XII secreted phospholipases A_2. J Biol Chem 8:5849–5857
11. Laine VJ, Grass DS, Nevalainen TJ (1999) Protection by group II phospholipase A_2 against *Staphylococcus aureus*. J Immunol 162:7402–7408
12. Laouini D, Kawamoto S, Yalcindag A, Bryce P, Mizoguchi E, Oettgen H, Geha RS (2003) Epicutaneous sensitization with superantigen induces allergic skin inflammation. J Allergy Clin Immunol 112(5): 981–987
13. Lubbe J (2003) Secondary infections in patients with atopic dermatitis. Am J Clin Dermatol 4(9): 641–654
14. Moreau JM, Girgis DO, Hume EBH, Dajcs JJ, Austin MS, O'Callaghan RJ (2001) Phospholipase A2 in rabbit tears: A host defense against *Staphylococcus aureus*. Invest Ophthalmol Vis Sci 42:2347–2354
15. Nevalainen TJ, Kortesuo PT, Rintala E, Märki F (1992) Immunochemical detection of group 1 and group 2 phospholipases A2 in human serum. Clin Chem 38:1824–1829
16. Nevalainen TJ, Aho HJ, Peuravuori H (1994) Secretion of group 2 phospholipase A2 by lacrimal glands. Invest Ophthalmol Vis Sci 32:417–421
17. Qu X-D, Lehrer RJ (1998) Secretory phospholipase A_2 is the principal bactericide for staphylococci and other gram-positive bacteria in human tears. Infect Immun 166:2791–2797
18. Saari KM, Klockars M (1983) Lysozyme content in tears of patients with various inflammatory diseases. In: Henkind P (ed) Acta: XXIV International Congress of Ophthalmology, San Francisco, October 31–November 5, 1982, vol. 2. Lippincott, Philadelphia, pp 834–837
19. Saari KM, Aho VV, Paavilainen V, Nevalainen TJ (2001) Group II phospholipase A2 content of tears in normal subjects. Invest Ophthalmol Vis Sci 42:318–320
20. Six DA, Dennis EA (2000) The expanding superfamily of phospholipase A_2 enzymes: classification and characterization. Biochim Biophys Acta 1488:1-19

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Time-resolved fluoroimmunoassay for bactericidal/permeability-increasing protein

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BACTERICIDAL/permeability-increasing protein (BPI) is a cationic antimicrobial protein produced by polymorphonuclear leukocytes, that specifically interacts with and kills Gram-negative bacteria. BPI competes with lipopolysaccharide-binding protein (LBP) secreted by liver cells into blood plasma for binding to lipopolysaccharide (LPS) and thus reduces the proinflammatory effects of LPS. We have developed a time-resolved fluoroimmunoassay for BPI and measured the concentration of BPI in human serum and plasma samples. The assay is based on a rabbit antibody against recombinant BPI. This antibody specifically adheres to polymorphonuclear leukocytes in immunostained human tissues. The difference in the serum concentration of BPI between unselected hospitalized patients with and without an infection was statistically significant. The mean concentration of BPI in serum samples was 28.3 μg/l (range 1.64–132, S.D. 26.8, $n=83$). In contrast, there was no difference between the two groups in the BPI levels in plasma samples. For all individuals tested, BPI levels were consistently higher in plasma samples compared to the matched serum samples. The mean concentration of BPI in plasma samples was 52.3 μg/l (range 0.9–403, S.D. 60.6, $n=90$). There was a positive correlation between the concentration of BPI and the white blood cell count as well as between the BPI concentration and C-reactive protein (CRP) in serum samples. In conclusion, the present study demonstrates that BPI can be quantified reliably by time-resolved fluoroimmunoassay in human serum samples.

Key words: Bactericidal/permeability-increasing protein, ELISA, Polymorphonuclear leukocytes, Serum/plasma protein, Time-resolved fluoroimmunoassay

Introduction

The bactericidal/permeability-increasing protein (BPI) is a cationic antimicrobial protein produced by polymorphonuclear leukocytes. BPI specifically interacts with and kills Gram-negative bacteria. BPI binds to the lipopolysaccharide (LPS) component of the outer membrane of Gram-negative bacteria, increases membrane permeability to hydrophobic substances and causes irreversible loss of bacterial cell homeostasis.[1–6] BPI competes with lipopolysaccharide-binding protein (LBP) secreted by liver cells into the blood plasma for binding to LPS.[7–10] In this way BPI reduces the proinflammatory effects of LPS.[11–19]

Because there is potential anti-infectious therapeutic use for recombinant BPI,[20–25] a sensitive assay which can measure BPI in body fluids is needed. The purpose of the present study was to develop a time-resolved fluoroimmunoassay (TR-FIA) for the measurement of the concentration of BPI in human serum.

Materials and Methods

Instrumentation: Time-resolved fluorescence was measured with an Arcus fluorometer (Wallac, Turku, Finland). The plate washer (Wellwash) and plate shaker (Delfia Plateshake) used in the fluoroimmunoassay were from Denley (Billinghurst, England) and Wallac (Turku, Finland), respectively. Data were handled with MultiCalc data management software (Wallac, Turku, Finland).

Serum and plasma samples: Serum and plasma samples were collected from unselected hospital-

ized patients with and without an infection (42 women and 48 men). The average age was 61 years (range 14–93 years). Samples were stored frozen at −20°C until assayed.

BPI standards: The BPI cDNA was cloned and expressed in a Chinese hamster ovary cell line as described elsewhere.[26] BPI standards were prepared from recombinant human BPI (kindly donated by Dr Marian Marra, Incyte Pharmaceuticals, Inc., Palo Alto, CA, USA) stock solution into assay buffer (Wallac, Turku, Finland) to give five concentrations (4.07, 9.76, 48.8, 122 and 305 µg/l).

Preparation of antibodies to recombinant human BPI: Antiserum to recombinant BPI was raised in a rabbit. The rabbit was immunized four times at 3-week intervals subcutaneously with 0.05–0.2 mg of human recombinant BPI (Incyte, Palo Alto, CA, USA) in Freund's complete adjuvant at the first immunization and in Freund's incomplete adjuvant on later occasions. Serum was collected 2 weeks after the last booster injection.

Labelling of anti-BPI antibody: Protein A-purified anti-recombinant BPI antibody was labelled with an isothiocyanate derivative of a europium chelate (Eu^{3+}-*N*-(*p*-*iso*thiocyanatobenzyl)-diethylene-tri-amine-N^1,N^2,N^3,N^3-tetra-acetate) by using an Eu-labelling kit (Wallac, Turku, Finland) according to the manufacturer's instructions.

Time-resolved fluoroimmunoassays: For the TR-FIA, microtitre plates were coated overnight with protein A-purified anti-BPI antibody (25 µg/ml in 50 mmol/l Tris-HCl, pH 7.75/0.15 mmol/l NaCl/0.05% NaN_3, 200 µl/well) treated with three volumes of HCl/water (125 µl of 11.6 M HCl in 50 ml of water) for 5 min. Coated plates were washed two times and 25 µl of BPI standard (0, 4.07, 9.76, 48.8, 122 and 305 µg/l) or sample were pipetted into the wells containing 175 µl of assay buffer. After 1 h incubation, with shaking, at room temperature and washing six times, Eu-labelled anti-BPI antibody (2.5 µg/ml in assay buffer, 200 µl/well) was added. The washing step was repeated after 1 h and 200 µl of enhancement solution (Wallac, Turku, Finland) was added. Fluorescence was measured after a further 5 min shaking and 10 min standing. Microtitre plates were from Eflab (Helsinki, Finland) and assay buffer for TR-FIA was from Wallac (Turku, Finland).

Immunostaining: Sections of formalin-fixed, paraffin-embedded human tissues from the files of the Department of Pathology, University of Turku were reacted with an IgG fraction of polyclonal rabbit anti-BPI antiserum, and the primary immunoreaction was localized as described previously[27] by using a Vectastain ABC kit (Vector Laboratories, Burlinghame, CA, USA) according to manufacturer's instructions. The intensity of immunostaining improved when the sections were heated for 2 × 5 min in a microwave oven before staining. For controls, the primary antibody was replaced by preimmune rabbit serum. The sections were counterstained by haematoxylin.

Statistical analysis: Student's *t*-test and Pearson's linear regression were used for statistical analysis.

Results

The mean concentration of BPI in plasma ($n = 90$) was 52.3 µg/l (range 0.9–403, S.D. 60.6) and in serum samples ($n = 83$) 28.3 µg/l (range 1.64–132, S.D. 26.8). The linear range for the BPI standard curve was 5–500 µg/l (Fig. 1). The detection limit of the assay was 1.6 µg/l corresponding to the mean ± 3 S.D. of the zero standard (blank) fluorescence counts. The difference in the serum concentration of BPI between unselected hospitalized patients with and without infection was statistically significant ($p < 0.0001$, Fig. 2). The mean concentration of BPI in serum samples for all measured patients was 28.3 µg/l (range 1.64–132, S.D. 26.8, $n = 83$). In contrast, there was no difference between the two groups in plasma samples. For all individuals tested, BPI

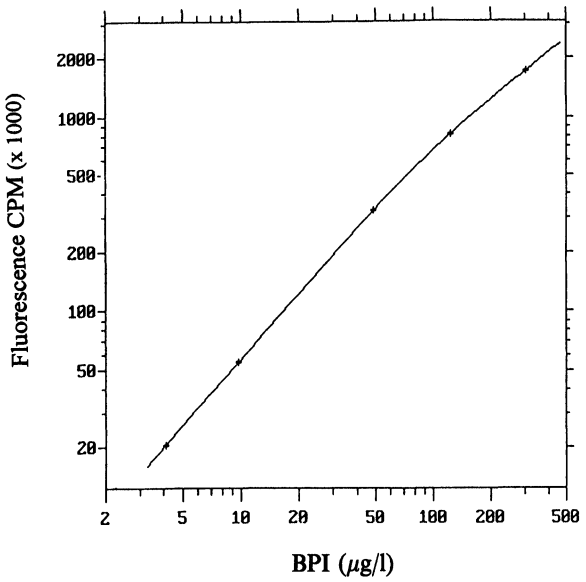

FIG. 1. The standard curve of time-resolved fluoroimmunoassay for BPI three separate assays.

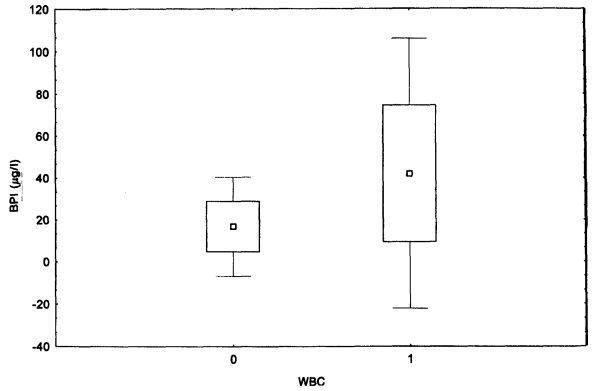

FIG. 2. Difference in the BPI concentrations between unselected hospitalized patients without an infection (group 0, $n=45$) and patients with an infection (group 1, $n=38$, $p < 0.0001$). Student's *t*-test was used for statistical analysis.

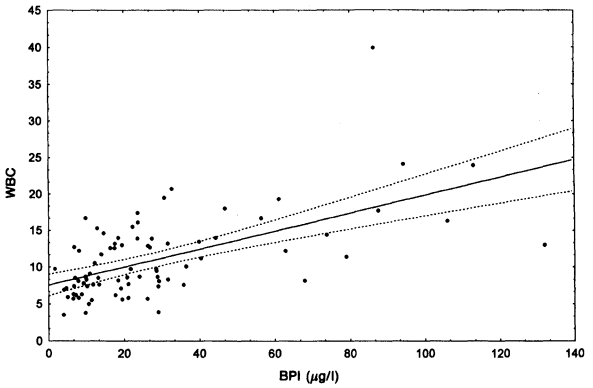

FIG. 3. Linear regression with 95% confidention intervals between blood white cell count (WBC, $\times 10^9$/l) and serum BPI concentration ($r=0.589$, $p < 0.0001$, $n=83$).

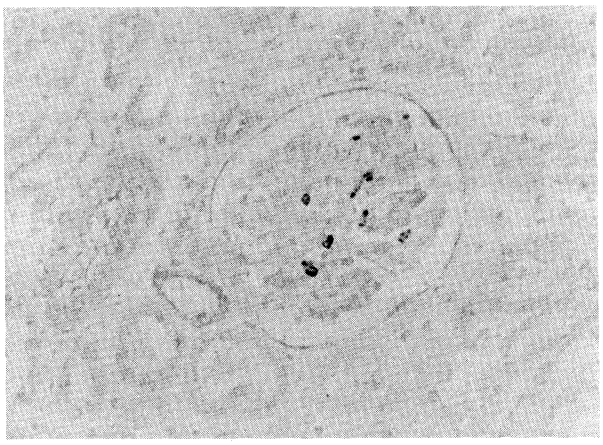

FIG. 4. Immunoreaction for BPI in polymorphonuclear leukocytes in the vascular compartment of a glomerulus of human kidney. Anti-BPI antibody. Avidin-biotin-peroxidase complex, haematoxylin counterstaining. Magnification 330×.

levels were consistently higher in plasma samples compared to the matched serum samples. The mean concentration of BPI in plasma samples was 52.3 µg/l (range 0.9–403, S.D. 60.6, $n = 90$).

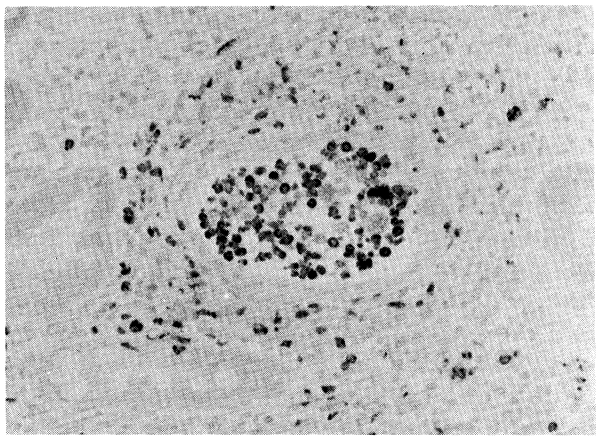

FIG. 5. Immunoreaction for BPI in polymorphonuclear leukocytes in the vascular compartment of colonic mucosa. Anti-BPI antibody. Avidin-biotin-peroxidase complex, haematoxylin counterstaining. Magnification 330×.

There was a positive correlation between the concentration of BPI in serum and the white blood cell count ($r = 0.589$, $p < 0.0001$, $n = 83$) (Fig. 3). There was also a positive correlation between serum BPI and CRP levels ($r = 0.39$, $p < 0.05$, $n = 59$). However, in plasma samples there was no correlation between BPI and white blood cell count or CRP. Intense immunoreaction was seen in polymorphonuclear leukocytes at numerous locations, e.g. in the vascular compartment of kidney glomeruli (Fig. 4) and colonic mucosa (Fig. 5). Control sections reacted with preimmune serum were devoid of immunoreaction.

Discussion

The present paper describes a new immunoassay using time-resolved fluorescence technology for measuring the concentration of BPI. An enzyme immunoassay (ELISA) for determining the concentration of BPI was recently developed by White and coworkers.[28] The mean concentration of BPI in serum as measured by the ELISA[28] and the current TR-FIA are very similar (27.1 µg/l and 28.3 µg/l, respectively). As determined by the current TR-FIA, there was a positive correlation between the concentration of BPI and the white blood cell count as well as between the serum BPI and CRP values. A statistically significant difference was found in the serum BPI levels between patients with and without manifest infections by the current TR-FIA. Furthermore, the presence of BPI in polymorphonuclear leukocytes was confirmed by immunohistochemistry in the current study. Thus, the serum concentration of BPI seems to reflect the intensity of the inflammatory process in the body.

The mean concentration of BPI in heparinized plasma samples was markedly higher than that in serum samples as determined by the current assay. However, no correlations was found between BPI values and white blood cell counts in plasma samples. Furthermore, BPI concentrations varied randomly in plasma samples when ammonium-heparin, sodium citrate and EDTA were used as anticoagulants in preliminary tests (data not shown). Thus, the detection of BPI in plasma calls for further studies.

In conclusion, the concentration of BPI can be measured reliably in human serum by time-resolved fluoroimmunoassay.

References

1. Gray PW, Flaggs G, Leong SR, Gumina RJ, Weiss J, Ooi CE, Elsbach P. Cloning of a human neutrophil bactericidal protein. *J Biol Chem* 1989; **264:** 9505–9509.
2. Ooi CE, Weiss J, Elsbach P, Frangione B, Mannion B. A 25-kDa NH_2-terminal fragment carries all the antibacterial activities of the human neutrophil 60-kDa bactericidal permeability-increasing protein. *J Biol Chem* 1987; **262:** 14891–14894.
3. Elsbach P, Weiss J. Phagocytosis of bacteria and phospholipid degradation. *Biochim Biophys Acta* 1988; **947:** 29–52.
4. Weiss J, Elsbach P, Shu C, Castillo J, Grinman J, Horwitz A, Theofan G. The human bactericidal/permeability-increasing protein and a recombinant NH2-terminal fragment cause killing of serum resistant gram-negative bacteria. *J Clin Invest* 1992; **90:** 1122–1130.
5. Weiss J, Beckerdite-Quagliata S, Elsbach P. Determinants of the action of phospholipases A2 on the envelope phospholipids of *Escherichia coli*. *J Biol Chem* 1979; **254:** 11010–11014.
6. Forst S, Weiss J, Maraganoe JM, Heinrikson RL, Elsbach P. Relation between binding and the action of phospholipases A2 on *Escherichia coli* exposed to the bactericidal/permeability-increasing protein of neutrophils. *Biochem Biophys Acta* 1987; **920:** 221–225.
7. Wilde CG, Seilhamer JJ, McGrogan M, *et al.* Bactericidal/permeability-increasing protein and lipopolysaccharide (LPS)-binding protein. *J Biol Chem* 1994; **269:** 17411–17416.
8. Tobias PS, Ulevitch RJ. Lipopolysaccharide-binding-protein (LBP) and CD14 in lipopolysaccharide-dependent macrophage activation. *Immunobiol* 1993; **187:** 227–232.
9. Haziot A, Tsuberi BZ, Goyert SM. Neutrophil CD14: biochemical properties and role in the secretion of tumor necrosis factor-alpha in response to lipopolysaccharide. *J Immunol* 1993; **150:** 15556–15565.
10. Ischii Y, Wang Y, Haziot A, Del Vecchio PJ, Goyert SM, Malik AB. Lipopolysaccharide-binding-protein and CD14 interaction induces tumor necrosis factor-alpha generation and neutrophil sequestration in lungs after intratracheal endotoxin. *Canc Res* 1993; **73:** 15–23.
11. Heumann D, Gallay P, Betz-Corradin S, Barras C, Baumgartner JD, Glauser MP. Competition between bactericidal/permeability-increasing protein and lipopolysaccharide-binding-protein for binding to monocytes. *J Infect Dis* 1993; **167:** 1351–1357.
12. Dentener WA, van Asmuth EJ, Francot GJ, Marra MN, Buurman WA. Antagonistic effects of lipopolysaccharide-binding-protein and bactericidal/permeability-increasing protein on lipopolysaccharide (LPS) induced cytokine release by mononuclear phagocytes. Competition for binding to LPS. *J Immunol* 1993; **151:** 4258–4265.
13. Ooi CE, Weiss J, Doerfler ME, Elsbach P. Endotoxin neutralizing properties of the 25-kD N-terminal fragment and a newly isolated 30-kD C-terminal fragment of the 55-60 kD bactericidal/permeability-increasing protein of human neutrophils. *J Exp Med* 1991; **174:** 649–655.
14. Marra MN, Thornton MB, Snable JL, Wilde CG, Scott RW. Endotoxin binding and neutralizing properties of recombinant bactericidal/permeability-increasing protein and monoclonal antibodies HA-1A and E5. *Crit Care Med* 1994; **22:** 559–565.
15. Gazzano-Santoro H, Meszaros K, Birr C, *et al.* rBPI23, a recombinant fragment of bactericidal/permeability-increasing protein and lipopolysaccharide-binding-protein for binding to lipopolysaccharide and gram-negative bacteria. *Infect Immun* 1994; **62:** 1185–1191.
16. Corradin SB, Heumann D, Gallay P, Smith J, Manel J, Glauser MP. Bactericidal/permeability-increasing protein inhibits induction of macrophage nitric oxide production by lipopolysaccharide. *J Infect Dis* 1994; **169:** 105–111.
17. Ammons WS, Kung AH. Recombinant amino-terminal fragment of bactericidal/permeability-increasing protein prevents hemodynamic responses to endotoxin. *Circ Shock* 1993; **41:** 176–184.
18. Kohn FR, Ammons WS, Horwitz A, Grinman L, Theofan G, Weickmann J, Kung AH. Protective effect of a recombinant amino-terminal fragment of bactericidal/permeability-increasing protein in experimental endotoxemia. *J Infect Dis* 1993; **168:** 1307–1310.
19. Meszaros K, Parent JB, Gazzano-Santoro H, *et al.* A recombinant amino-terminal fragment of bactericidal/permeability-increasing protein inhibits the induction of leucocyte responses to lipopolysaccharide. *J Leukoc Biol* 1993; **54:** 558–563.
20. Fisher Jr CJ, Marra MN, Palardy JE, Marchbanks CR, Scott RW, Opal SM. Human neutrophil bactericidal/permeability-increasing protein reduces mortality rate from endotoxin challenge: a placebo-controlled study. *Crit Care Med* 1994; **22:** 553–558.
21. Gazzano-Santoro H, Parent JB, Grinman L, *et al.* High-affinity binding of the bactericidal/permeability-increasing protein and a recombinant amino-terminal fragment to the lipid-A region of lipopolysaccharide. *Infect Immun* 1992; **60:** 4754–4761.
22. Marra MN, Wilde CG, Collins MS, Snable JL, Thornton MB, Scott RW. The role of bactericidal/permeability-increasing protein as a natural inhibitor of bacterial endotoxin. *J Immunol* 1992; **148:** 532–537.
23. Weiss J, Wright G. Mobilization and function of extracellular phospholipase A2 in inflammation. *Adv Exp Med Biol* 1990; **275:** 103–113.
24. Tobias PS, Soldau K, Ulevitch RJ. Identification of a lipid A binding site in the acute phase reactant lipopolysaccharide-binding-protein (LBP). *J Biol Chem* 1989; **264:** 10867–10871.
25. Mathison J, Tobias P, Wolfson E, Ulevitch RJ. Regulatory mechanisms of host responsiveness to endotoxin (lipopolysaccharide). *Pathobiology* 1991; **59:** 185–188.
26. Wilde CG, Seilhamer M, McGrogan N, *et al.* Bactericidal/permeability-increasing protein and lipopolysaccharide (LPS)-binding protein. *J Biol Chem* 1994; **269:** 17411–17416.
27. Hsu SM, Raine L, Fanger H. The use of avidin–biotin–peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem* 1981; **29:** 577–580.
28. White ML, Ma JK, Birr CA, Trown PW, Carroll SF. Measurement of bactericidal/permeability-increasing protein in human body fluids by sandwich ELISA. *J Immun Methods* 1994; **167:** 227–235.

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Bactericidal/permeability-increasing protein in lacrimal gland and in tears of healthy subjects

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Abstract *Background:* To determine the expression of bactericidal/permeability-increasing protein (BPI), a novel antimicrobial molecule, in the main lacrimal gland and its content in tears of young healthy subjects. *Methods:* BPI concentration of tears was measured in 42 healthy volunteers, 13 men and 29 women, with ages ranging from 22 to 30 (mean 24.7±2.1) years by a time-resolved fluoroimmunoassay (TR-FIA). Immunohistochemical analysis was made to localize BPI in lacrimal gland and conjunctiva of eight autopsied subjects, two men and six women, with the age range from 44 to 87 (mean 72.3±14.9) years. *Result:* The mean concentration of BPI in tears was 27.8±29.5 μg/l, and it decreased with an increase in tear flow rate ($P<0.0001$). There was no statistically significant difference in BPI content of tears between the genders. BPI was immunohistochemically seen in outer basal epithelial cells of intralobular and excretory ducts, squamous and basal cells of conjunctiva as well as faintly in myoepithelial cell layer of acini. The presence of BPI in the lacrimal gland and in the tear fluid was verified by Western blotting. *Conclusions:* The results indicate that outer basal epithelial cells of lacrimal gland ducts contain BPI, which occurs in a relatively high concentration in tears. BPI may have a substantial antibacterial role in human tears.

Introduction

The non-specific defense mechanisms of the eye against outer bacterial infections include the blinking action of the lids, the flushing action of tears, and natural antimicrobial components of the tear film. Human tears contain several natural antimicrobial proteins including groupIIA phospholipase A_2 ($GIIAPLA_2$), lysozyme, lactoferrin, defensins and gammaglobulins [2, 10, 11, 17, 18, 20]. It has been proposed that $GIIAPLA_2$ is principally responsible for the ability of tears to kill a broad spectrum of gram-positive bacteria, notwithstanding the presence of lysozyme and lactoferrin in much higher concentrations [23, 26]. The $GIIAPLA_2$ content of tears was one of the highest reported in human secretions [26]. The concentration decreased with an increase in age [26], showed a discernible diurnal rhythm [6] and was statistically significantly lower in reflex than in basal tears [4]. The $GIIAPLA_2$ content of tears was elevated in patients with keratoconjunctivitis sicca [5] and decreased in subjects wearing contact lenses [6], whereas in patients with senile cataract or primary open-angle glaucoma the concentration was normal [3]. Despite its efficacy in relation to gram-positive organisms, $GIIAPLA_2$ has little or no activity against Gram-negative bacteria due to the outer lipopolysaccharide capsule of these organisms. However, studies have reported the bactericidal activity of $GIIAPLA_2$ against gram-negative organisms in the presence of bactericidal/permeability-increasing protein (BPI) [9].

BPI is a 55-kD membrane-associated protein in the azurophil granules in human neutrophils [7], but also found to a lesser extent in eosinophils. Recently it was discovered to be produced by mucosal epithelial cells [8]. BPI was detected in human nasolacrimal ducts by using the reverse transcription-polymerase chain reaction (RT-PCR) technique [22]. It is released into the phagosome, as well as into the systemic circulation, during neutrophil activation. BPI and its active recombinant form $rBPI_{21}$ rapidly kill many Gram-negative organisms, such as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudosomas aeruginosa* and *Neisseria meningitidis*, based on the electrostatic interactions between the cationic N-terminal half of BPI and the negatively-charged phosphate groups of lipopolysaccharide (LPS), lipid A moiety [28]. $rBPI_{21}$ has been in human clinical phase III trials for severe meningococcal sepsis [14], and is also undergoing evaluation in the treatment of Crohn's disease and proliferative retinal diseases such as diabetic retinopathy [24].

BPI has been shown to be present in human nasolacrimal ducts [22], but there are no data on the presence of BPI in the lacrimal gland or in the tear fluid. In this study, we did an immunohistochemical analysis to demonstrate and localize BPI in the main lacrimal gland and measured the concentration of BPI in tears of healthy subjects and showed that human tears contain a high concentration of BPI.

Materials and methods

Subjects

The concentration of BPI in tears was studied in 42 healthy volunteers, 13 men and 29 women with ages ranging from 22 to 30 (mean 24.7±2.1) years (Table 1). None of the subjects had any general diseases or eye diseases, or wore contact lenses. All of the subjects had normal visual acuity (at least 20/20) without or with spectacle correction. All of them revealed normal findings in a routine ophthalmological examination. Pieces of main lacrimal gland and the adjacent conjunctiva were obtained from eight autopsied subjects, two men and six women with ages ranging from 44 to 87 (mean 72.3±14.9) years. The tissue specimens were from cadavers with no known ocular disease. The principles of the World Medical Association Declaration of Helsinki were followed. Written informed consent was obtained from each healthy subject giving the tear specimen. The study protocol was approved by the Ethics Committee of the University of Turku and the Turku University Hospital.

Table 1 Number of patients (*n*), age of patients, tear flow rate, and concentration of bactericidal/permeability-increasing protein (*BPI*) in tears of normal subjects

	Men	Women	Total
n	13	29	42
Age (years)	25.7±2.5	24.3±1.8	24.7±2.1
Tear flow rate (μl/min)	4.5±2.6	11.9±16.2	9.1±13.2
BPI (μg/l)	26.8±19.7	28.2±33.3	27.8±29.5

Results are expressed as mean±SD

Collection of tears

The values of BPI were assessed in tear samples from the left eye of each healthy subject. Non-stimulated tears were collected using disposable 20 μl microcapillaries (Duran Ringcaps, Hirschmann Laborgeräte, Germany) under a Haag-Streit 900 biomicroscope. The samples were gathered from the marginal tear strip of the lower lid near the lateral canthus, with care being taken not to irritate the conjunctiva, cornea, or lid margin. The amount of tears collected varied between 0.5 and 20 (mean 17.0±5.8) μl. The tear collection time and the amount of tears collected were specified in 20 subjects to determine the tear flow rate (μl/min). In all cases, the collection time was limited to 5 min. The tear samples were kept frozen at −70°C until assayed.

BPI assay

BPI content in the tear samples was determined by using a time-resolved fluoroimmunoassay (TR-FIA) method published earlier [12], with some modifications. Briefly, the assay volume was reduced to 100 μl per well, sensitivity was increased, a new lower standard concentration (0, 1.22, 4.07, 9.76, 48.8 and 122 μg/l) was added, and the sample volume was reduced to 10 μl/well (originally 25 μl/well). The modified assay was carried out with 30 min incubation times. The results are expressed as μg/l.

Western blotting

The tissue samples of the lacrimal gland for Western blotting were homogenized with an Ultra-Turrax (IKA Laboratechnik, Staufen, Germany) homogenizer in 0.01 M sodium phosphate buffer, pH 7.6 (1 g wet tissue per 5 ml buffer) and centrifuged at 10,000 *g* for 1 h at 4°C. Samples from tissue homogenate supernatant and tear specimen were heated at 95°C in non-reducing electrophoresis buffer for 5 min with a dilution factor of 1:10. Electrophoresis was performed on MultiPhor system with homogeneous 15% SDS gels. Separated proteins were transferred to Hybond ECL nitrocellulose membrane by using MultiPhor II Nova Blot system according to the manufacturer's instructions. In identifying the blots, the same polyclonal

antibody for human recombinant BPI with a dilution factor of 1:1000 was used as in immunohistochemistry. Visualization was achieved by using ECL western blotting reagents and exposing the chemilumininescence to film. All reagents and equipment were purchased from Amersham Pharmacia, Uppsala, Sweden.

Immunohistochemistry

Sections of formalin-fixed paraffin-embedded tissues containing the lacrimal gland and the adjacent conjunctiva were digested with pepsin, 4 mg/ml in 10 nM HCl, and reacted with an IgG fraction (2.84 mg/ml, dilution 1:5000) of a rabbit anti-BPI antiserum [12]. For controls, the primary antibody was replaced by nonimmune rabbit serum. Other antibodies used were monoclonal antibody HHF 35 for muscle actin (Enzo, Farmingdale, N.Y., USA; dilution 1:50), monoclonal antibody for vimentin (clone V9, catalog no. 08-00520, Zymed Laboratories, San Francisco, Calif., USA; dilution 1:50), monoclonal antibody 34 beta E 12 for high molecular weight cytokeratins 1, 5, 10 and 14 (Enzo; dilution 1:5) and polyclonal antiserum for S-100 (Dako, Glostrup, Denmark; dilution 1:1000). The primary immunoreaction was localized by using a commercial biotin-avidin based detection systems (Vectastain ABC kit; Vector Laboratories, Burlingame, Calif., USA) according to the manufacturer's instructions. The sections were counterstained with hematoxylin.

Statistical analysis

Statistical analyses were performed using SAS for Windows. After tests for normality, the BPI values in tears between the genders were analyzed using Mann-Whitney *U*-test. In addition, Spearman Rank Order Correlation was determined between the tear flow rate and the concentration of BPI in tears of healthy subjects. All *P*-values lower than 0.05 were considered statistically significant. The results are expressed as mean±SD.

Results

The mean concentration (mean±SD) of BPI in tears of healthy subjects was 26.8±19.7 μg/l in men, 28.2±33.3 μg/l in women, and 27.8±29.5 μg/l in the total material (Table 1). There was no statistically significant difference in the BPI content of tears between the genders (P=0.454).

The tear flow rate was 4.5±2.6 μl/min in men, 11.9±16.2 μl/min in women, and 9.1±13.2 μl/min in the total material. The concentration of BPI decreased statistically significantly with an increase in the tear flow rate (Spearman R=−0.84, P<0.0001) (Fig. 1).

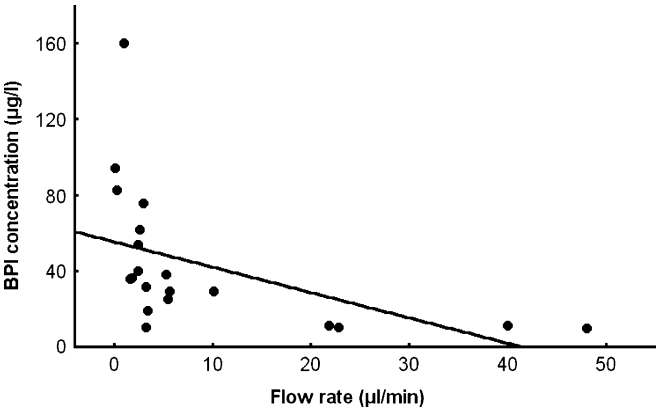

Fig. 1 Correlation between the concentration (μg/l) of bactericidal/permeability-increasing protein (*BPI*) in tears and the tear flow rate determined in 20 subjects (μl/min)

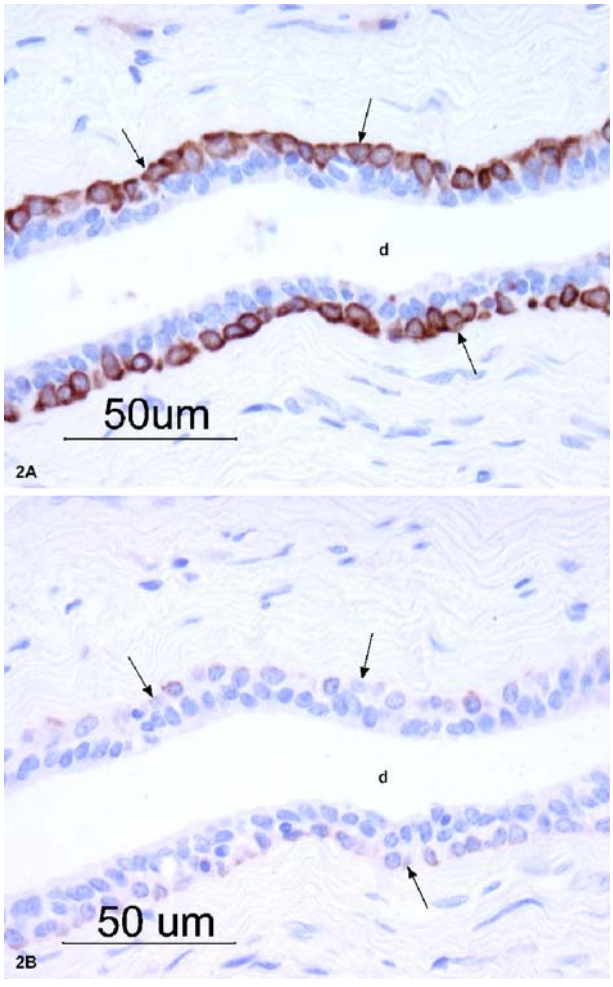

Fig. 2 **a** Positively stained basal epithelial cells (*arrows*) of excretory lacrimal gland duct (*d*) (BPI, hematoxylin counterstain, original magnification×380). **b** Negative control without immunoreaction (non-immune serum, hematoxylin counterstain, original magnification×380)

BPI immunoreactivity was detected in the cytoplasm of outer basal epithelial cell layers of all intercalated, intralobular, interlobular and excretory ducts (Fig. 2a) and also very faintly and focally in myoepithelial cells around acini (Fig. 3a).The staining pattern was identical in all of the eight subjects tested. Ductal basal cells and myoepithelium were negative with normal control serum (Figs 2b and 3b). Periacinar myoepithelial cells of spindle type were positive for muscle actin, vimentin and S-100. Moderate staining of variable intensity for cytokeratin 34 beta E 12 were present in acini, ducts and conjunctiva and also in some myoepithelial cells. Occasional dendritic melanocytic S-100 and vimentin positive cells were found in excretory ducts.

Western blot analysis of the homogenized tissue sample of the lacrimal gland showed one protein band with an approximate molecular weight of 55 kDa (Fig. 4a). BPI in the tear fluid specimen was also observed in Western blot analysis. However, the protein band visualized for the BPI in the tear fluid appeared with slightly elevated molecular weight (Fig. 4b).

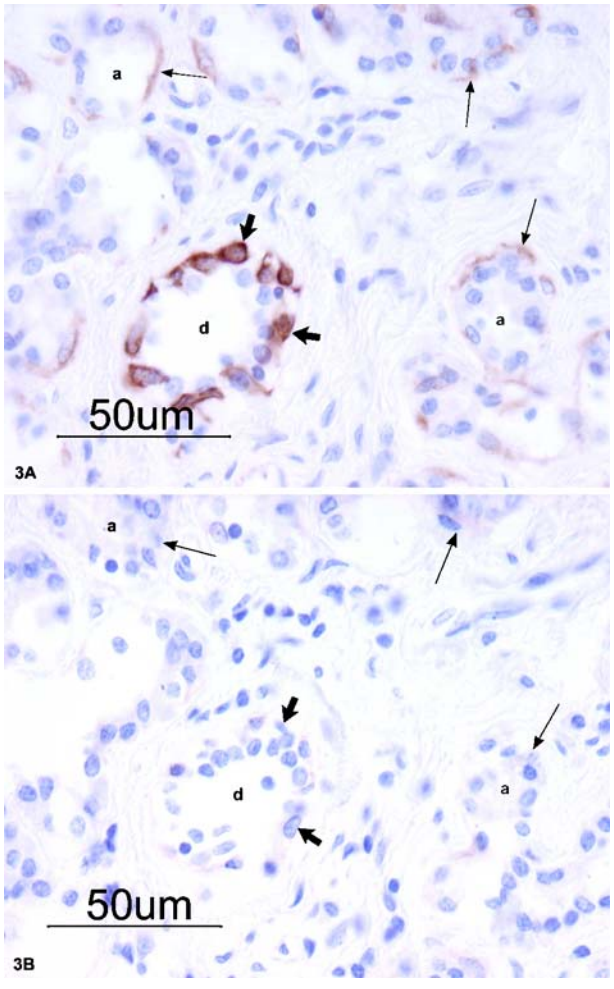

Fig. 3 **a** Elongated myoepithelial cells (*thin arrows*) around acini (*a*) are faintly labeled. Basal cells (*thick arrows*) of intraglandular ducts (*d*) are positive (BPI, hematoxylin counterstain, original magnification×380). **b** Negative control without immunoreaction (non-immune serum, hematoxylin counterstain, original magnification× 380)

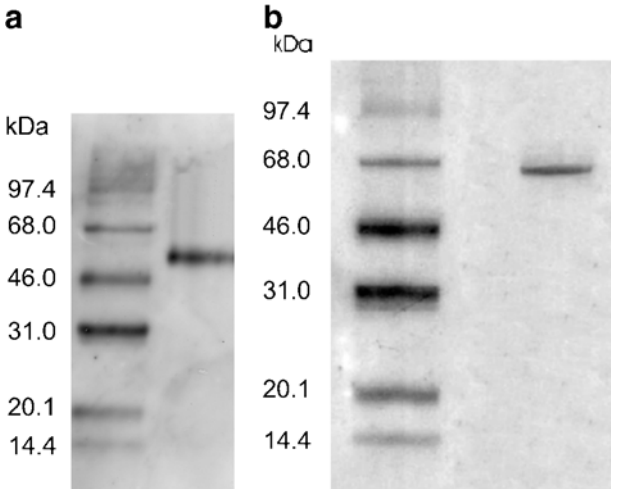

Fig. 4 **a** Western blot analysis with a polyclonal anti-Human recombinant antibody recognizing BPI from a homogenate of a healthy lacrimal gland (*right side*) compared with the molecular weight standards (*left side*). **b** Western blot analysis with a polyclonal anti-human recombinant antibody recognizing BPI from normal tear fluid (*right side*) compared with the molecular weight standards (*left side*)

Discussion

The majority of the natural antimicrobial proteins controlling infectious agents in the tear film and on the conjunctival and corneal surfaces are effective against Gram-positive bacteria only. Tears contain a-defensins and lactoferrin which have activity also against Gram-negative bacteria [11, 17]. This is the first study to show that human lacrimal gland and tears contain BPI, a novel antimicrobial substance, which is effective against a broad spectrum of Gram-negative bacteria.

In this study, we showed by immunohistochemistry that the cytoplasm of basal ductal epithelial cells of lacrimals glands contained BPI. The present results confirmed this finding also by Western blot analysis of the homogenized tissue of the lacrimal gland, showing one protein band with a molecular weight of 55 kDa published earlier for BPI [9]. This protein was not produced by the acini or inner surface epithelial cell layer of ducts. These findings suggest that the outer basal epithelial cell layers of all intercalated, intralobular, interlobular and excretory ducts and also some myoepithelial cells of the lacrimal gland may produce BPI. The first stage of BPI secreted by the basal layers of the ductal epithelium of the lacrimal gland may be modified

during its transport through the acini or inner surface epithelial cell layer of ducts. The salt content of the tear fluid and possible other components attached to the BPI protein may explain the mobility of the BPI of the tear fluid in the Western blot analysis.

The results of the present study showed that the mean concentration of BPI in tears of healthy subjects was 27.8± 29.5 μg/l (mean±SD). This concentration markedly exceeds the level found in normal plasma (7.3 μg/l) [21, 25]. The mean concentration of BPI of the tear fluid showed a very high SD similarly as observed earlier in the mean content of lysozyme [27] and $GIIAPLA_2$ [26]. This may be due to some variation of the reflex and basal tears in normal conditions, which may respectively decrease or increase the content of different antimicrobial proteins in tears [4, 26, 27]. The present results showed that there was no significant difference in the BPI content of tears between the genders similarly as earlier reported on the $GIIAPLA_2$ and lysozyme secretion [26, 27].

BPI is a potent cytotoxin that acts specifically against a broad spectrum of Gram-negative bacteria, including *Escherichia coli*, *Salmonella* species, *Shigella* species, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* [15, 16, 28]. Of the Gram-negative bacteria that cause keratitis in the US, *Pseudomonas aeruginosa* is the most common. *Klebsiella pneumoniae* and *Escherichia coli* can produce indolent corneal ulcers that are usually seen in debilitated or immunocompromised patients or in corneas with an underlying pathogenic condition. *Shigella* and *Salmonella* are rare causes of microbial keratitis in the US [1]. These bacteria are killed even by nanomolar (nmol/l) concentrations of BPI. Thus, the concentrations of BPI in tears found in the present study are sufficient for effective killing of Gram-negative bacteria. These findings suggest a substantial antibacterial role for BPI in human tears which together with the antibacterial effects of lysozyme [10, 27] and $GIIAPLA_2$ [2–6, 20, 23, 26] may add our understanding on the defense mechanisms of tears. On the basis of the present findings, we feel that the antibacterial defense functions of human tears are more effective than thought earlier.

The results of the current study revealed decreasing concentrations of BPI with an increase in the tear flow rate (Spearman $R=-0.84$, $P<0.0001$) (Fig. 1). This decrease in the BPI concentration could be explained by dilution of the BPI concentration in tear samples with increasing flow rate. This finding is in a good agreement with our previous results, that in reflex tears, the $GIIAPLA_2$ content of tears decreased progressively with stimulation of the tear secretion probably caused by a dilution of $GIIAPLA_2$ content during hypersecretion of reflex tears [4]. However, the results of this study showed that the tear flow rate was somewhat but not statistically significantly higher in women than in men but this did not have any decreasing effect on the BPI concentration of tears in women (Table 1). It has been previously reported that under the age of 30 the tear flow rate is higher in women that in men [19].

The results of the present study suggest that BPI present in human tears is secreted from the lacrimal gland. It is important to notice, however, that even nonstimulated tear samples may occasionally contain small amounts of polymorphonuclear (PMN) cells that are rich in BPI. Thus, a small part of the BPI measured in tears with TR-FIA may actually be derived from the PMN cells bursted open on freezing.

In summary, we conclude that the basal epithelial cell layer of extra- and intraglandular ducts contain BPI, the concentration of which in tears of healthy subjects was 27.8±29.5 μg/l (mean±SD), and that the BPI content decreased with an increase in the tear flow rate. These findings, reflected against the background of biological data we have of BPI, suggest a substantial antibacterial role for BPI in human tears.

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References

1. Abbott RL, Zegans M, Kremer PA (2004) Bacterial corneal ulcers. In: Tasman W, Jaeger EA (eds) Duane's clinical ophthalmology, vol. 4, 18. Lippincott Williams & Williams, pp 1–29
2. Aho HJ, Saari KM, Kallajoki M, Nevalainen TJ (1996) Synthesis of group II phospholipase A2 and lysozyme in lacrimal glands. Invest Ophthalmol Vis Sci 37:1826–1832
3. Aho VV, Nevalainen TJ, Paavilainen V, Saari KM (2002) Group II phospholipase A2 content of tears in patients with senile cataract and primary open-angle glaucoma. Eur J Ophthalmol 12:40–43
4. Aho VV, Nevalainen TJ, Saari KM (2002) Group IIA phospholipase A2 content of tears in basal, nonstimulated and reflex tears. Curr Eye Res 24:224–227
5. Aho VV, Nevalainen TJ, Saari KM (2002) Group IIA phospholipase A2 content of tears in patients with keratoconjunctivitis sicca. Graefe's Arch Clin Exp Ophthalmol 240:521–523
6. Aho VV, Paavilainen V, Nevalainen TJ, Peuravuori H, Saari KM (2003) Diurnal variation in group IIA phospholipase A2 content in tears of contact lens wearers and normal controls. Graefe's Arch Clin Exp Ophthalmol 241:85–88

7. Calafat J, Janssen H, Knol EF, Malm J, Egesten A (2000) The bactericidal/permeability increasing protein (BPI) is membrane-associated in azurophil granules of human neutrophils, and relocation occurs upon cellular activation. Acta Pathol Microbiol Immunol Scand 108:201–208
8. Canny G, Levy O, Furuta GT, Narravula-Alipati S, Sisson RB, Serhan CN, Colgan SP (2002) Lipid mediator-induced expression of bactericidal/permeability-increasing protein (BPI) in human mucosal epithelia. Proc Natl Acad Sci U S A 99:3902–3907
9. Elsbach P, Weiss J (1998) Role of the bactericidal/permeability-increasing protein in host defence. Curr Opin Immunol 10:45–49
10. Fleming A (1922) On a remarkable bacteriolytic element found in tissues and secretions. Proc R Soc Ser B 93:306–317
11. Haynes RJ, Tighe PJ, Harminder SD (1999) Antimicrobial defensin peptides of the human ocular surface. Br J Ophthalmol 83:737–741
12. Häggblom J-O, Jokilammi-Siltanen AB, Peuravuori H, Nevalainen TJ (1996) Time-resolved fluoroimmunoassay for bactericidal/permeability-increasing protein. Mediat Inflamm 5:47–50
13. Kuizenza A, van Haeringen NJ, Kijlstra A (1991) SDS-Minigel electrophoresis of human tears. Invest Ophthalmol Vis Sci 32:381–386
14. Levin M, Quint PA, Goldstein B, Barton P, Bradley JS, Shemie SD, Yeh T, Kim SS, Cafaro DP, Scannon PJ, Giroir BP (2000) Recombinant bactericidal/permeability-increasing protein (rBPI21) as adjunctive treatment for children with severe meningococcal sepsis: a randomised trial. rBPI21 Meningococcal Sepsis Study Group. Lancet 356:961–967
15. Levy O (2000) Antimicrobial proteins and peptides of blood: templates for novel antimicrobial agents. Blood 96:2664–2672
16. Levy O (2002) Therapeutic potential of the bactericidal/permeability-increasing protein. Expert Opin Invest Drugs 11:159–167
17. Masson PL, Heremans JF, Dive C (1966) An iron binding protein common to many external secretions. Clin Chim Acta 14:735
18. McClellan BH, Whitney CR, Newman LP, Allansmith MR (1973) Immunoglobulins in tears. Am J Ophthalmol 76:89–101
19. Milder B (1981) The lacrimal apparatus. In: Moses RA (ed) Adler's physiology of the eye, clinical application, 7th edn. CV Mosby Company, St Louis, pp 16–37
20. Nevalainen TJ, Aho HJ, Peuravuori H (1994) Secretion of group 2 phospholipase A2 by lacrimal glands. Investig Ophthalmol Vis Sci 32:417–421
21. Nupponen I, Turunen R, Nevalainen T, Peuravuori H, Pohjavuori M, Repo H, Andersson S (2002) Extracellular release of bactericidal/permeability-increasing protein in newborn infants. Pediatr Res 51:670–674
22. Paulsen FP, Pufe T, Schaudig U, Held-Feindt J, Lehmann J, Schröder JM, Tillmann BN (2001) Detection of natural peptide antibiotics in human nasolacrimal ducts. Invest Ophthalmol Vis Sci 42:2157–2163
23. Qu X-D, Lehrer RJ (1998) Secretory phospholipase A2 is the principal bactericide for staphylococci and other Gram-positive bacteria in human tears. Infect Immun 66:2791–2797
24. Rauniyar RK, Suzuma K, King AL, Aiello LP, King GL (2002) Differential effects of bactericidal/permeability-increasing protein (BPI) analogues on retinal neovascularization and retinal pericyte growth. Invest Ophthalmol Vis Sci 43:503–509
25. Rintala E, Peuravuori H, Pulkki K, Voipio-Pulkki L-M, Nevalainen T (2000) Bactericidal/permeability-increasing protein (BPI) in sepsis correlates with the severity of sepsis and the outcome. Intensive Care Med 26:1248–1251
26. Saari KM, Aho VV, Paavilainen V, Nevalainen TJ (2001) Group II phospholipase A2 content of tears in normal subjects. Invest Ophthalmol Vis Sci 42:318–320
27. Saari KM, Aine E, Posz A, Klockars M (1983) Lysozyme content of tears in normal subjects and in patients with external eye infections. Graefe's Arch Clin Exp Ophthalmol 221:86–88
28. Weiss J, Elsbach P, Shu C, Castillo J, Grinna L, Horwitz A, Theofan G (1992) Human bactericidal/permeability-increasing protein and a recombinant NH2-terminal fragment cause killing of serum-resistant gram-negative bacteria in whole blood and inhibit tumor necrosis factor release induced by the bacteria. J Clin Invest 90:1122–1130