BACTERIAL PATHOGENICITY

Channel-forming leucotoxins from *Staphylococcus aureus* cause severe inflammatory reactions in a rabbit eye model

J. A. SIQUEIRA, C. SPEEG-SCHATZ,* F. I. S. FREITAS,† J. SAHEL,* H. MONTEIL† and G. PRÉVOST†

Instituto Hilton Rocha, Ave Anel DaSerra 1335, Mangabeiras, Caixa postal 700, Belo Horizonte 302010-090, Brazil, *Clinique Ophthalmologique des Hôpitaux Universitaires de Strasbourg, 1 Place de l'Hôpital, F-67000 Strasbourg and †Institut de Bactériologie de la Faculté de Médecine, Université Louis Pasteur, 3 rue de Koeberlé, F-67000 Strasbourg, France

Panton-Valentine leucocidin arises from the combination of one S component (LukS-PV) with one F component (LukF-PV), whereas γ -haemolysin comprises two S components (HlgA and HlgC) with one F component HlgB. The intravitreal injection of rabbit eye with the six combinations (S + F) of channel-forming leucotoxins produced by *Staphylococcus aureus* ATCC 49775 induced acute inflammatory reactions depending on time and doses of toxins. These reactions involved posterior chamber as well as anterior chamber and conjunctiva, eyelids and annexes. Histological examination confirmed the involvement of eye tissues and the disruption of the retinal barrier. The lesions began only 4 h after injections and persisted for at least 5 days. Clinical and biological effects of each leucotoxin were modulated by the speed of onset and intensity of inflammation and necrosis, leading to a functional classification according to the severity of the lesions (HlgA + LukF-PV > HlgA + HlgB $\geq$ LukS-PV + HlgB $\geq$ LukS-PV + LukF-PV > HlgC + HlgB $\geq$ HlgC + LukF-PV). Moreover, N-acetyl β -D glucosaminidase assays on crude extracts of vitreous revealed granules and granule secretions from polymorphonuclear cells with levels according the above classification. These results show that channel-forming leucotoxins have a very significant inflammatory activity. As most *S. aureus* strains produce two or even six leucotoxins depending on the production of Panton-Valentine leucocidin, these compounds could be considered to be virulence factors.

Introduction

Staphylococcal channel-forming leucotoxins are exotoxins consisting of two non-associated, but synergic class S and class F proteins [1, 2]. These toxins constitute a recently described protein family [3, 4], and two of them are produced from two different chromosomal loci of *Staphylococcus aureus* ATCC 49775. The first, encoding the Panton-Valentine leucocidin [3–6], consists of two co-transcribed genes, where the upstream one encodes a class S component (LukS-PV) and the downstream one a class F component (LukF-PV). The second locus encodes γ haemolysin, of which four molecular variants have been reported [3, 4, 7–12]. There is > 95% sequence

identity within the homologous proteins of these four variants. This second locus in fact consists of three genes, the first two encoding class S proteins (HlgA and HlgC), and the third encoding a class F protein (HlgB). The genes *hlgC* and *hlgB* are co-transcribed and *hlgA* constitutes an upstream open reading frame. As the four molecular variants of this second locus are constituted by two class S components (HlgA and HlgC), and by a single class F protein component (HlgB), each toxin of the γ -haemolysin group is, in fact, constituted of two different leucotoxins: HlgA + HlgB and HlgC + HlgB.

These toxins are known as membrane-directed toxins [13–15]; their targets are human host defence cells such as polymorphonuclear cells, monocytes and macrophages [2, 16]. It has been demonstrated by different methods that the first component that binds to target cells [14, 15] belongs to the structurally related class S proteins, thus allowing further inter-

action in an as yet undetermined stoichiometry, of a structurally related class F protein that is required to induce biological activity. Pantone-Valentine leucocidin (PVL) has only leucotoxic properties [16], whereas γ -haemolysin (HlgA + HlgB and HlgC + HlgB) has leucotoxic and also haemolytic properties. PVL-producing *S. aureus* strains are mostly associated with furuncles and *vice versa* [17, 18], but they represent only 2% of clinical hospital strains, whereas strains producing only γ -haemolysin represent 97% of clinical isolates [18]. In fact, PVL-producing strains produce not only PVL, but also γ -haemolysin, simultaneously [3], leading to the secretion of three class S components and two class F components. Moreover, the interchangeability inside the class S or class F components gives rise to the possibility of six combinations.

PVL (LukS-PV + LukF-PV) is one of the six possible combinations. The purified toxin has been shown to induce severe inflammatory lesions when injected intradermally into rabbit skin [19, 20], leading to capillary dilatation, chemotaxis, infiltration of polymorphonuclear leucocytes (PMNL), PMNL-cell caryorrhexis and finally tissue necrosis. In the same way, PVL was shown to induce the secretion of chemotaxis mediators from PMNLs, such as leucotriene B₄ [21] and interleukin (IL)-8 [22]. At the opposite end of the scale, the two pairs constituting γ -like haemolysin did not elicit tissue necrosis of rabbit skin, although they induced inflammatory lesions and mediators [23, 24].

The study of the biological activity of toxins on animal models is a prerequisite before injection of bacteria producing selected leucotoxins. However, the rabbit skin model is limited because the intradermal injection of *S. aureus* ATCC 49775 that produced PVL *in vitro* did not generate skin necrosis *in vivo*. It was not known whether this lack of necrosis was due to the inhibition of bacterial growth or to the inhibition of toxin production in rabbit skin. This led to the choice of another animal model with specific characteristics. First, the chosen model for bacterial infections should have a parallel in human pathology. Second, the staphylococci should have been demonstrated to grow and induce inflammatory lesions in this model. Third, the sampling procedure should be easy to do in order to obtain rapidly liquid samples in which inflammatory mediators could be quantified. Fourth, the injected toxins or bacteria should have a slow clearance, thus maximising biological effects and allowing an objective determination of bacterial growth. Fifth, some of the animal cells had to be sensitive to the action of the toxin *in vitro*. The model of the vitreous of the rabbit eye was chosen because it fulfilled most of the above criteria. Furthermore, after stimulation of the immune response, inflammatory cells which are normally scant inside the vitreous may accumulate in this area through the haemo-retinal

barrier. The ability of staphylococci to grow and to induce inflammatory lesions in the vitreous of rabbits has been reported previously [25, 26]. This model has also been used for studies of the biological activity of pneumolysin [27]. Before further experiments to establish their role in bacterial pathogenicity, it was necessary to determine the impact of each of these purified pairs of leucotoxins in the rabbit eye vitreous.

Materials and methods

Bacterial toxins

Protein components of channel-forming leucotoxins, i.e., LukS-PV, LukF-PV, HlgA, HlgC and HlgB were purified from *S. aureus* ATCC 49775 as described previously [3] and were diluted in sterile apyrogenic physiological medium. Each equimolar combination of proteins constituting leucotoxins was injected at doses of 3, 30, 300 or 3000 ng of each component per 50 μ l of physiological solution. The combinations injected were each of the six possible pairs of class S and class F components. Controls were apyrogenic physiological solution and each protein component injected alone.

Rabbits and injections

The experimental procedures were in accordance with the ARVO resolution on the use of animals in research. Permission (no. 04683) to experiment on animals was given by the French Ministry of Forests and Agriculture. Ten-week-old New Zealand White rabbits weighing 2.0–2.5 kg were given injections into the right eye after anaesthesia by intramuscular injection of 300 μ l of reconstituted Zoletil®-100 (Reading Laboratories, London). Atropine sulphate (Alcon Laboratories, Courbevoie, France) 0.3% (w/v) was then instilled into the rabbit eyes and 2 min later oxybuprocain hydrochloride (Chauvin Laboratories, Montpellier, France). Injections of 50 μ l of diluted leucotoxin were made through the *pars plana* into the vitreous with a 25-gauge needle, taking care to avoid touching the crystalline lens and injecting within the retina. This injection also required the slow removal of the needle, in order to avoid backward flow of toxin under the conjunctiva.

Clinical investigations

Eyes were observed clinically 4 h, 24 h and 48 h after injection. Clinical modifications of the posterior chamber were assessed by direct ophthalmoscopy (Heine) according to the criteria of Nussenblatt *et al.* [28]. Briefly, five increasing levels of severity of damage were defined: 0, normal eye without vitreous haze; 1, vitreous haze allowing observation of the optic nerve and retinal vessels; 2, vitreous haze still allowing observation of vessels and optic nerve, but with difficulty; 3, vitreous haze allowing observation of

the optic nerve only, its boundaries being blurred; 4, vitreous haze preventing observation of the optic nerve.

The clinical investigation of the anterior chamber and its annexes also resulted in the distinction of five increasing levels of severity of the lesions: 0, normal eye with no physical damage; 1, a slight conjunctival hyperaemia located around the site of injection; 2, the presence of conjunctival hyperaemia involving at least half of the surface and associated with scant discharge, but without haze in the anterior chamber. There was no clinical change in the eyelids, nor in the cornea, nor in the anterior chamber, at inflammatory levels 1 and 2. If clinical damage increased (stages 3 and 4), the clinical description was completed with details of the eyelids: blepharitis and oedema. The importance of secretions, partial or total damage to the cornea and the corneal state (haze, ulceration) were also noted, as well as the state of the anterior chamber (hypopyon, hyphema, fibrin). Level 3 showed moderate secretions, slight blepharitis, total conjunctival hyperaemia involving all the eyeball, perikeratic injection, chemosis, and a slight haze of the anterior chamber, still allowing observation of the iris. At stage 4, again, total conjunctival hyperaemia, blepharitis and oedema, chemosis and secretions were found. The secretions were more obvious and these features were associated with a significant haze of the anterior chamber which prevented observation of the iris. Hypopyon and phtisis occurred only occasionally at this stage. Clinical observations were made for all rabbits used either for histological examinations or glucosaminidase assays.

Histopathological observations

After clinical examination, anaesthetised rabbits were killed immediately by intracardiac injection of pentobarbital (Sanofi, Libourne, France) 1.5 ml/kg, before immediate enucleation of the eyeball. Eyes were fixed in formaldehyde 10% (v/v) for at least 48 h, embedded in paraffin, and then cut in 5- μ m thick sections. Before microscopic observation ($\times 400$), sections were stained with haematoxylin and eosin.

Microscopic observation included the cornea, anterior chamber, iris, vitreous space, retina and sclera. The structure of tissues, presence of cell infiltrate and local necrosis of tissues were recorded. According to the histological modifications observed, five histological levels were defined: 0, corresponded to a normal eye without blood cell in the vitreous and without damage to the tissues; 1, the presence of a few erythrocytes in the vitreous without leucocytes and without tissue necrosis; 2, characterised by an inflammatory infiltrate in the vitreous of up to 300 inflammatory cells per microscopic field ($\times 400$), without necrosis; congestion of the iris and of the choroid were also noticed. At level 3 haemorrhage in the vitreous was still moderate but there were between 300 and 800 inflammatory

cells per microscopic field. There was also partial necrosis of the retina and severe congestion of the iris and the choroid. Level 4 showed severe haemorrhage and congestion of the iris and the choroid. Additionally, inflammation of the posterior chamber with > 800 cells/field occurred, and also involved the anterior chamber, iris, ciliary body and choroid. Necrosis extended to the whole retina and was often associated with partial necrosis of the corneal endothelium, iris, ciliary body and choroid. Independent histological examinations were made as two independent series.

The two clinical scores and the histological one recorded for each inflammatory reaction were summed to give an 'inflammatory score' (IS) for each eye. Thus, the IS reflects the sensitivity of the rabbit eye 4, 24 or 48 h after injection of a given leucotoxin at a given dose into the vitreous.

N-acetyl β -D glucosaminidase assays

Vitreous samples (300 μ l) containing inflammatory cells were harvested from killed rabbits 24, 48 and 72 h after injections and were diluted in Triton X-100 0.1%. After centrifugation for 5 min at 0°C and 10 000 g cell debris was discarded. Lysate supernates were diluted five-fold and 10-fold in PBS (Dulbecco). Enzymic reactions were initiated [14] by mixing 50 μ l of diluted lysate and 50 μ l of 1 mM *p*-nitrophenyl-N-acetyl- β -D-glucosaminide solubilised in 0.2 M sodium citrate, pH 4.5, at room temperature. Reactions were stopped after 30 min and 1 h by the addition of 100 μ l of 1 M Tris-HCl, pH 9.0. Optical density was recorded at 405 nm. Assays were performed twice on given samples as four independent series from different rabbits.

Clinical and histological observations and glucosaminidase assays were performed in double-blinded fashion.

Statistical procedures

Statistical analysis was performed with the Statistical Analysis System program (SAS Institute, Cary, NC, USA). The non-parametric tests of Kruskal-Wallis and Wilcoxon-Mann-Whitney were used for comparison of the data obtained from clinical and histological observations, and the χ^2 test for values obtained from N-acetyl β -D-glucosaminidase assays; $p < 0.05$ results were considered statistically significant.

Results

Time and dose-dependent reactions

During these experiments, apyrogenic physiological solution and doses of 3000 ng of each protein alone were injected into rabbit eyes as controls. Each control

induced reaction level 1 in posterior and anterior chambers as well as reaction level 1 for histological observation, 4, 24 or 48 h after injection. The reaction obtained with two individual proteins was significantly different from that obtained with the corresponding tested pair ($p < 0.001$), demonstrating the synergy between leucotoxin components. This reaction was assumed to be due to the injection only. No noticeable clinical or histological differences were observed following identical injections in different rabbits.

As listed in Table 1, with doses of 3 or 30 ng of leucotoxins (S and F combinations), the inflammatory reactions were not different from those of the negative controls, at 4, 24 or 48 h, except for a dose of 30 ng each of HlgA + LukF-PV, which was enough to generate a haze in the posterior chamber within 48 h, with the optic nerve being barely visible (stage 3). The latter difference was statistically significant ($p < 0.001$) from observations with the rest of the pairs tested. For all the pairs of S and F components, equimolar doses of 300 ng and 3000 ng induced significant and different time-dependent reactions. These reactions began to be noticeable only 4 h after injection, except for the two pairs containing 300 ng of the component HlgC, when the reaction was delayed. The latter feature was confirmed in each of the clinical and histological observations, and was hence evidenced by the IS. As measured at different times following injections, the effects of toxins were progressive up to 48 h, except for some pairs such as HlgA + LukF-PV and HlgA + HlgB, where the maximal effects (IS = 12) were reached 24 h after injection of equimolar doses of 3000 ng of the components ($p < 0.01$). This maximal damage (IS = 12) was reached within 48 h or less with at least 3000 ng for four of the six combinations of S and F components, being significantly different from the damage generated by the two pairs including HlgC within 48 h ($p < 0.005$).

Observation at 15 days after injection with 300 and 3000 ng each of LukS-PV + LukF-PV (PVL) revealed that the clinical lesions were stable only between 48 h and 5 days. The reactions decreased slowly in the anterior chamber during the following days. For example, after injection of equimolar doses of 300 ng and 3000 ng of PVL, the inflammatory lesions in the anterior chamber changed from stages 3 and 4 to stages 2 and 3, respectively, between days 5 and 15.

With the injection of an equimolar dose of 3000 ng of PVL, the reaction score of the posterior chamber (stage 4) was stable from 2 to 15 days after injection, whereas it decreased (from stage 4 to stage 3) following the injection of an equimolar dose of 300 ng of PVL during the same period. For these rabbits, histological examination at day 15 after injection revealed that healing of all the necrotic tissues had begun.

Biological activity of channel-forming leucotoxins in the rabbit eye

An equimolar dose of 300 ng of the channel-forming leucotoxins appeared to be that which induced the most variable and progressive intensities of clinical reactions, according to the time of observation. It appeared that the pairs that included HlgA were the most potent, those with HlgC were responsible for less acute inflammatory reactions and those with LukS-PV had an intermediate activity. In addition, the combination HlgA + HlgB + HlgC, which would occur naturally, if the three components constituting γ -haemolysin were produced by most *S. aureus* strains in equimolar concentrations, was studied. Time and dose-dependent responses were also noted for this combination. The maximal damage, represented by total retinal necrosis, was observed 48 h after the injection of 3000 ng of each of the three components (IS_{3000ng} 4, 24, 48 h = 7, 11, 12, respectively). The severity of the lesions and the rapidity of their formation were intermediate between those induced by the pairs HlgA + HlgB and HlgC + HlgB, probably because HlgB had to be distributed among the two class S components, HlgC and HlgA, in order to form respective channels. However, the two-fold increase of dose of HlgB did not induce appreciable variation ($p > 0.05$) of the inflammatory response if compared with the above.

In such a way, a classification of the leucotoxins was assessed. The combination HlgA + LukF-PV was the most efficient pair. Eyes given an equimolar dose of 3000 ng of this combination demonstrated the highest inflammatory score only 24 h after injection, as did HlgA + HlgB. Furthermore, only this pair when injected at an equimolar dose of 30 ng was responsible for a significant inflammatory reaction after 48 h. In addition, this was the only pair to induce corneal ulceration and cataract associated with hypopyon and necrosis of the ciliary body, the choriocapillary cells and of the whole retina.

The potential of inflammation of three other combinations, i.e., LukS-PV + LukF-PV (PVL), LukS-PV + HlgB, HlgA + HlgB, were not distinguishable. For these leucotoxins, a maximal IS of 12 was reached by injecting 3000 ng dose of proteins constituting pairs, but only at 48 h after the injection. At the clinical level, these pairs were responsible for marked haemorrhage in the vitreous after injection of this dose. Secretions, oedema and blepharitis were significantly less pronounced than those obtained with the same doses of HlgA + LukF-PV and appeared later. These observations made the leucotoxin pair HlgA + LukF-PV significantly more effective than the three others listed earlier ($p < 0.01$). As an example, physiological solution containing 3000 ng each of LukS-PV + LukF-PV induced a strong inflammatory reaction (Fig. 1B; IS = 10) 24 h after injection, as compared with a normal eye (Fig. 1A; IS = 3). At this

Table 1. Inflammatory scores in rabbit eyes after intravitreal injection of channel-forming leucotoxins from *S. aureus*

Time (h)	Dose (ng) of each component	Reaction stage and inflammatory score after injection of							
		HlgA + HlgB	HlgC + HlgB	HlgA + HlgB	HlgC + HlgB	HlgA + LukF-PV	HlgC + LukF-PV	LukS-PV + LukF-PV	LukS-PV + HlgB
4	3	1* + 1† + 1‡ = 3§	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3
	30	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3
	300	1 + 2 + 2 = 5	1 + 1 + 1 = 3	2 + 1 + 2 = 5	1 + 1 + 1 = 3	2 + 1 + 2 = 5	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3
	3000	3 + 3 + 2 = 8	2 + 2 + 1 = 5	3 + 3 + 2 = 8	2 + 2 + 1 = 5	3 + 3 + 2 = 8	2 + 2 + 1 = 5	2 + 2 + 1 = 5	2 + 3 + 2 = 7
24	3	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3
	30	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3
	300	3 + 4 + 2 = 9	2 + 2 + 2 = 6	4 + 4 + 2 = 10	2 + 2 + 2 = 6	4 + 4 + 2 = 10	2 + 2 + 2 = 6	2 + 3 + 2 = 7	3 + 3 + 2 = 8
	3000	4 + 4 + 3 = 11	3 + 4 + 3 = 10	4 + 4 + 4 = 12	3 + 4 + 3 = 10	4 + 4 + 4 = 12	2 + 3 + 2 = 7	3 + 4 + 3 = 10	3 + 4 + 3 = 10
48	3	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3
	30	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 3 + 2 = 6	1 + 1 + 1 = 3	1 + 3 + 2 = 6	1 + 1 + 1 = 3	1 + 1 + 1 = 3	1 + 1 + 1 = 3
	300	3 + 4 + 3 = 10	2 + 3 + 2 = 7	4 + 4 + 3 = 11	2 + 3 + 2 = 7	4 + 4 + 3 = 11	2 + 3 + 2 = 7	3 + 4 + 3 = 10	4 + 3 + 3 = 10
	3000	4 + 4 + 4 = 12	3 + 4 + 3 = 10	4 + 4 + 4 = 12	3 + 4 + 3 = 10	4 + 4 + 4 = 12	2 + 3 + 2 = 7	4 + 4 + 4 = 12	4 + 4 + 4 = 12

3, 30, 300 or 3000: dose of each component (ng).

*Reaction stage in anterior chamber.

†Reaction stage in posterior chamber.

‡Histological reaction stage.

§Inflammatory score (see procedures for scoring in *Materials and methods*).

Fig. 1. Inflammatory reaction induced 24 h after intravitreal injection of 50 μ l of physiological and apyrogenic medium alone (A, C) or of 50 μ l of this medium containing an equimolar dose of 3000 ng of PVL (B, D). The rabbit eye inoculated with PVL (B) exhibits oedema, blepharitis, total conjunctival hyperaemia, chemosis and haze in the anterior chamber. The histological sections of the normal rabbit eye show the sclera with no infiltrated cells (C), whereas for the PVL-injected eye, the width of the sclera increased (D), the retina did not appear to be homogeneously margined, and numerous neutrophils infiltrated tissues.

time, oedema, blepharitis, total conjunctival hyperaemia, chemosis and haze in the anterior chamber were already developed in the PVL-injected eye and were directly observable. Although histological section of the normal rabbit eye showed the sclera with no infiltrated cells (Fig. 1C), the PVL-injected eye revealed that the width of sclera increased, whereas the retina did not appear homogeneously margined, but numerous neutrophils had infiltrated the tissues (Fig. 1D).

For the last two leucotoxin pairs containing HlgC – HlgC + HlgB and HlgC + LukF-PV – significant secretion from the eye was never induced. Further-

more, no significant necrosis of the tissues was evidenced and erythrocytes were not observed in the vitreous, whatever the dose tested and the time of observation after injection.

In other experiments, 300 ng of either LukS-PV + LukF-PV or HlgA + LukF-PV were instilled on the conjunctiva or injected under the conjunctiva of rabbits' eyes, and clinical observations were made 4, 24 and 48 h after injection. Instillation of these two pairs on the eye did not produce any detectable effect, but when injected under the conjunctiva they induced clinically observable stage 2 reactions in the anterior chamber at 4 and 24 h. However, these reactions

disappeared within 48 h. In the posterior chamber, stage 2 lesions were observed only with injection of the pair HlgA + LukF-PV and not with PVL. These lesions appeared at 4 h, and decreased to stage 1 at 24 h. Channel-forming leucotoxins were much less damaging for the rabbit eye when instilled on the conjunctiva or injected under it, than when injected into the posterior chamber of the eye.

Relationships between clinical and histological observations

During the experiments, the inability to observe the optic nerve (stage 4 in the posterior chamber) was always associated with complete retinal necrosis (stage 4 at histological examination). The observation of a hypopyon in the anterior chamber (stage 4) always corresponded to the presence of fibrin on histopathological examination and always occurred 48 h after injection of 3000 ng each of HlgA + LukF-PV and HlgA + HlgB.

The dose of 3000 ng each of HlgA + LukF-PV in the vitreous induced ulceration of the cornea within 48 h. Histological data confirmed the latter clinical feature by the presence of polymorphonuclear cell infiltrates in the corneal stroma (stage 4), and by the adhesion of the same cells to the corneal endothelium. This adhesion process had already begun 24 h after the

injection. However, the state of the corneal endothelium could not be assessed because of damage to the tissue by formaldehyde.

N-acetyl- β -D glucosaminidase

N-Acetyl- β -D glucosaminidase activity in rabbit eye lysates was detectable 24 h after leucotoxin injection (Fig. 2). Results were expressed as OD_{405nm} units because rabbit enzyme was not available. However, under experimental conditions, 1 OD_{405nm} unit corresponded to 6×10^{-6} U of human placental N-acetyl- β -D glucosaminidase assuming that one unit hydrolyses 1 μ mole of the substrate in 1 min at 25°C. A dose of 30 ng each of HlgA + LukF-PV induced enzyme activity at levels of 0.75 SD 0.08 (arbitrary units) within 48 h and of 1.2 SD 0.1 within 72 h. For all leucotoxins, only the equimolar doses of 300 and 3000 ng induced significant amounts of enzyme within 24, 48 or 72 h after injection. Time- and dose-dependent reactions were also observed. Enzymic activities (48 and 72 h) obtained with the combination HlgA + LukF-PV were significantly different ($p < 0.01$) from those found with pairs containing HlgC. Potential of leucotoxins were in accordance with those determined by clinical and biological observations. However, if equimolar 300 ng doses showed a continuous increase, it seemed that a plateau was reached about 48 h after injections.

Fig. 2. N-Acetyl- β -D glucosaminidase activities (and SD as bar lines when no overlapping symbols) contained in 50 μ l of the four series of rabbit vitreous samples according to the time (12, 24, 48 or 72 h) following the injection of HlgA + LukF-PV (▼), HlgA + HlgB (○), HlgC + LukF-PV (□), HlgC + HlgB (●), LukS-PV + LukF-PV (■) and LukS-PV + HlgB (▽). Panel a: 300 ng of each protein; b: 3000 ng of each protein. One arbitrary unit (OD_{405nm}) corresponds to 6×10^{-6} U of the human placental enzyme.

Discussion

Staphylococcal Panton-Valentine leucocidin and γ -haemolysin have been described recently [24] as members of a new family of bi-component exotoxins. Although most clinical *S. aureus* strains produce γ -haemolysin, only 2% of them also produce PVL. The latter strains are strongly associated with primary necrotising cutaneous infections in man, particularly with furuncles. The two toxins were reported to have distinct genetic locations and biological activities *in vitro* [3]. The simultaneous expression of PVL and γ -haemolysin by *S. aureus* strains such as ATCC 49775 would generate six combinations of a type S component with a type F component, which have been reported to have biological activity on human and rabbit PMNLs.

All channel-forming leucotoxins had strong inflammatory potential if injected in the vitreous of rabbit, thus mimicking the clinical signs of infectious endophthalmitis. The synergic effect of class S and class F components was observed once more and it was confirmed that each of the components alone had no damaging activity on the rabbit eye. The observed biological activities of leucotoxins were assumed to originate from the vitreous space as the two most potent pairs, when instilled on the conjunctiva or injected under this tissue, did not induce any significant effect. The same doses of the same toxins injected in the vitreous (e.g., 300 ng each of HlgA + HlgB or LukS-PV + LukF-PV led to maximal inflammatory effects within 48 h. During experiments, it was observed that injection of toxins such as HlgA + HlgB or HlgA + LukF-PV into the vitreous space were rapidly responsible for ulceration of the retina with significant cell recruitment. Therefore, cells sensitive to leucotoxins seem to exist within the vitreous, may be as the small number of macrophages normally present on the surface of the retina. It has been reported that platelet-activating factor may participate in the breakdown of the blood-retinal barrier [29]. Therefore, inflammatory cells sensitive to the leucotoxins remain to be characterised.

HlgA + LukF-PV was observed to be the most efficient leucotoxin pair because only 30 ng of each component induced a significant biological effect within 48 h. PVL-encoding genes are encountered in only 2% of clinical *S. aureus* strains, and are stable in these strains despite the constant presence of related genes (those encoding γ -haemolysin). However, PVL was reported earlier as the most dermo-necrotising leucotoxin for rabbit skin. The latter biological activity may illustrate the benefit for such strains of maintaining expression of the two relevant loci. Such observations may bring into question the specificity of each leucotoxin in relation to certain inflammatory cells. Glucosaminidase assays revealed

a delay between the presence of inflammatory cells observed by histology and the presence of a detectable enzymic activity. It was more likely associated with the beginning of tissue necrosis (stage 3 of histological examination). Therefore, infiltrated PMNLs would be activated further for the production of inflammatory mediators. As the different pairs induced various inflammatory reactions, it was not credible to parallel the glucosaminidase activity with the number of cells infiltrating the rabbit vitreous. Up to now, HlgC appears to be the least effective of the class S components tested, as none of the pairs that included this protein led to dramatic cytolytic levels or large inflammatory events. The simultaneous expression of *hlgA* and *lukF-PV* genes *in vivo* by *S. aureus* strains remains to be demonstrated.

If such toxins are produced, they should account significantly for the pathogenicity of *S. aureus* infections. However, the role of channel-forming leucotoxins in the pathogenicity of bacteria should be assessed in conjunction with other virulence factors frequently encountered in *S. aureus* strains, such as α -toxin. α -Toxin had been described as a major virulence factor in staphylococcal keratitis [30]. It appears from this work that some leucotoxins injected under the conjunctiva led to moderate reactions of the anterior posterior chambers, indicating that both α -toxin and leucotoxins may play roles in clinical manifestations of superficial eye infections. Moreover, recent work [31] showed that *agr*-defective (accessory gene regulator) strains had dramatically decreased pathogenicity in the rabbit eye.

Finally, the efficiency of a channel-forming leucotoxin was quite low if the class S component was HlgC. The nature of the class F component may also contribute to the efficacy of the leucotoxin, as the association of one of the two class F components (LukF-PV or HlgB) with HlgA induced lesions differing significantly from each other.

Host response to leucotoxins seemed to increase in progressive steps. These steps depended on the dose and on the efficiency of each combination. Assuming that all these six pairs probably have a similar catabolic turnover in this animal model (because they share 60–75% homology [3], the different effects of leucotoxins might be explained also by the relative affinity of the components, the specific kinetics of binding of class S components to cell ligands, or by the number of specific binding sites at the surface of target cells, depending on the toxin.

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