Comparison of the \textit{in vitro} effects of amoxicillin and ampicillin on the polymorphonuclear neutrophil respiratory burst

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Objectives: The aim was to compare the \textit{in vitro} effects of amoxicillin and ampicillin on the oxidative metabolism of polymorphonuclear neutrophils (PMNs).

Methods: Superoxide radical anion production by PMNs, stimulated or not by various exogenous stimulants and in contact with increasing antibiotic concentrations, was measured using spectrophotometric methods.

Results: Whereas a pro-oxidative action of amoxicillin on PMNs was obtained without exogenous stimulation or with opsonized zymosan (OZ), the $\text{O}_2^-$ production by PMNs incubated with ampicillin did not increase significantly.

Conclusions: This amoxicillin pro-oxidative effect could be due to the activation of the PMN NADPH oxidase, to its induction by a membrane effect or via the OZ pathway. It probably reinforces amoxicillin intrinsic bactericidal action and might partly explain the severe rashes sometimes occurring with amoxicillin treatment.

Keywords: oxidative, superoxide anions, aminopenicillins

Introduction

The phagocytic capacity of polymorphonuclear neutrophils (PMNs) and their production of reactive oxygen species (ROS) have a bactericidal effect during the ‘respiratory burst’ in the inflammatory process to help the host to counter infections. However, a misbalanced ROS production, if the inflammatory reaction is deficient or races out of control, can then be harmful: it is even involved in the pathophysiology of numerous diseases.\textsuperscript{1} PMNs can also play a role in some cutaneous lesions, drug-induced or not, being recruited within skin inflammation.\textsuperscript{2} Besides, skin reaction cases were reported after an intake of aminopenicillins within infectious mononucleosis\textsuperscript{3} or as signs of allergy.\textsuperscript{4} It is thus interesting to try and give an explanation for this in keeping with PMN oxidative metabolism.

In this study, the aim was to evaluate the influence of two aminopenicillins, amoxicillin and ampicillin, on the oxidative metabolism of PMNs. Thus, ROS production by PMNs in contact with increasing antibiotic concentrations was estimated. A medicine can have a direct effect on ROS produced by PMNs during oxidative stress or can indirectly interact with the PMN oxidative stress pathways. So, we evaluated the direct antibiotic action on ROS in models without cells and in cellular models, the \textit{in vitro} antibiotic effects on ROS production by PMNs stimulated or not with phorbol-12-myristate-13-acetate (PMA), $N$-formyl-Met-Leu-Phe (fMLP) or opsonized zymosan (OZ). Thus, the cellular/acellular mechanism(s) of a possible antibiotic/PMN oxidative metabolism interaction can be explained.

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Materials and methods

Reactive compounds were from Sigma-Aldrich (St-Quentin Fallavier, France). PMNs were obtained and purified (lymphocytes <2 ± 0.8% and erythrocytes <0.3 ± 0.1%) from fresh heparinized venous blood, according to Cabanis et al.5 Separate experiments were carried out on different days on blood freshly collected each day from different donors (n = 32). After a Histopaque density gradient centrifugation, erythrocytes were removed by hypotonic haemolysis, and PMNs were washed, suspended in an appropriate buffer (pH 7.4) and counted. PMN viability (always >95%) was assessed by the Trypan Blue exclusion test.

The antibiotic cytotoxicity towards PMNs was also estimated by this test, and the pH of the incubation mixtures was checked for the antibiotic concentrations tested, ranging from 0 to 500 mM.

Superoxide anion (O2-) generation by PMNs stimulated with OZ, fMLP or PMA or without any exogenous stimulation was evaluated according to Cohen and Chovanice6 after incubation for 30 min with increasing concentrations of the antibiotic. PMNs (1 × 10^6) stimulated with 1.6 nmol PMA, 100 nmol fMLP and 500 mM cytochalasin B or OZ (0.03 mg/mL) released O2- that reduced ferricytochrome C (ferC). Supernatant absorbances were measured with a Kontron Uvikon 933 spectrophotometer (Vancouver, Canada) at a wavelength of 550 nm against reference cuvettes. Absorbances were converted into O2- concentrations (nmol/mL) using the ferC extinction coefficient (ε_{550nm} = 2.11 × 10^{-5} μM^{-1}/cm).

In the acellular model, the O2- amount, similar to that released by 1 × 10^6 stimulated PMNs, was generated by the hypoxanthine–xanthine oxidase system, and the ferC reduction was measured according to Aruoma et al.7 The results were expressed as in the cellular experiment.

The data from six to eight individual experiments for both acellular and cellular models were analysed using SUPERANOVA statistical software (Abacus Concepts, Berkley, CA, USA). O2- concentration values were processed by one-way analysis of variance (antibiotic concentration factor). When Fisher’s global test was significant at the 0.05 level, pairwise comparisons were performed by a posteriori Fisher’s protected least significant difference (PLSD) test (P < 0.05). Distinct homogeneous groups of means are indicated by the letters a and b for amoxicillin (P = 0.01). No significant effect was shown for ampicillin.

Results

No significant PMN mortality was found at the antibiotic concentrations tested, enabling the validation of the cellular dosages. The incubation mixture pH was stable compared with that of antibiotic-free controls (difference not greater than 0.03, variation was observed for 500 mM ampicillin).

The O2- measurements in the presence of amoxicillin or ampicillin without any exogenous PMN stimulation showed a significant pro-oxidative effect for the concentrations higher than 1 mg/L (P = 10^{-5}, n = 8) for amoxicillin (Figure 1) and no significant O2- production increase in the presence of ampicillin. A significant pro-oxidative effect at the 0.05 level was only shown for amoxicillin for the concentrations above 1 mg/L (P = 0.01, n = 8) with PMNs stimulated with OZ (Figure 2). No significant amoxicillin or ampicillin effects were detected on PMN O2- production when PMA or fMLP was used.

The acellular models showed that there was no detectable direct effect of amoxicillin and ampicillin on O2-.
Discussion

In this study, we focused on the very start of the respiratory burst of PMNs placed in the presence of ampicillin or amoxicillin. Thus, among the ROS produced by PMNs, only O$_2^-$ production was investigated, in order to find any mode of action of PMN stimulation by ampicillin or amoxicillin. PMA and fMLP stimulate PMN oxidative metabolism via the direct (PMA) or indirect (via the phospholipase A$_2$ or phospholipase C pathways for fMLP) stimulation of NAPDH oxidase. The O$_2^-$ production induced by PMA and fMLP does not seem to be influenced either by amoxicillin or ampicillin. However, amoxicillin seemed to interact, more than ampicillin, with the PMN oxidative metabolism at another level. Indeed, a pro-oxidative effect was noticed either when PMNs were stimulated with OZ, which mimics the bacterial effect on them and activates the phagocytic pathway, or even more when PMNs were exposed to amoxicillin without any exogenous stimulation. Amoxicillin could act on a membrane receptor inducing the NADPH oxidase activation or on the membrane NADPH oxidase itself. It could also interfere in the OZ pathway. These results, proof of an enhancement of PMN activities by amoxicillin, corroborate those of Reato et al., showing, in particular, an overproduction of pro-inflammatory cytokines by PMNs induced with amoxicillin combined with clavulanic acid. Only amoxicillin showed a significant activation of O$_2^-$ production by PMNs without any other stimulants or in the presence of OZ. This last stimulation method proved to be weaker than that generated by both PMA and fMLP, which are very potent reactants and could erase any effect caused by amoxicillin. Such an effect of amoxicillin on O$_2^-$ production could have been detected only by using OZ, because a maximal or ceiling production of this ROS due to OZ alone was not reached. Thus, the effect of amoxicillin could be added and seen in our results as a concentration-dependent increase in O$_2^-$ production. We investigated more precisely the mechanism of action of amoxicillin on the PMN oxidative metabolism (data not shown). The aim was to determine whether amoxicillin interacted with the fMLP and/or OZ pathway(s), both of which can simultaneously cause PMN stimulation. In order to check whether amoxicillin used the way of fMLP by binding a membrane receptor for the signal to be transduced, experiments blocking the OZ pathway were carried out using cytochalasin B, but without adding fMLP. Thus, a potential receptor-mediated activation by amoxicillin alone could be shown. Our results (data not shown) revealed that cytochalasin B (without fMLP) inhibited the amoxicillin effect on PMNs, as the O$_2^-$ production was not statistically different from that of the non-stimulated PMNs. Therefore, the mode of action of amoxicillin probably involves the same pattern as that of OZ. A possible reinforcement of the protein kinase C (PKC) action that phosphorylates NAPDH oxidase or a direct action of amoxicillin on NAPDH oxidase itself had also to be considered. So, additional assays (data not shown) were performed: PMNs were exposed to amoxicillin (100 mg/L) and varying concentrations of bisindolylmaleimide VIII and H7, both inhibitors of PKC, to evaluate any direct stimulation of NAPDH oxidase by amoxicillin. For concentrations currently used to inhibit PKC (or higher), the O$_2^-$ production by PMNs was partially reversed and still remained statistically higher than that of non-stimulated PMNs. Amoxicillin probably reinforces the NAPDH oxidase activity by a way other than that of the strict phosphorylation by PKC.

Moreover, the statistically significant effect of amoxicillin on O$_2^-$ production was shown for the concentrations tested over 1 mg/L, which are compatible with the pharmacokinetic parameters of this antimicrobial agent. The average serum concentration measured by Eshelman and Spyker at peak after oral administration of a 500 mg capsule of amoxicillin to healthy volunteers was ~9 mg/L.

Amoxicillin is structurally different from ampicillin by the addition of a hydroxyl to the amino-benzyl group of its side chain. This could be responsible for a better recognition of PMNs by amoxicillin in comparison with ampicillin, necessary for the activation of a cellular pathway, hence the higher pro-oxidative effect shown here in vitro on PMNs. This latter effect probably reinforces the intrinsic bactericidal effect of amoxicillin.

It would be interesting to investigate end oxidative products, such as thiobarbituric-acid-reactive species or HOCl, and also intermediate ROS, such as hydrogen peroxide and the hydroxyl radical, to thoroughly determine the global impact of amoxicillin on the PMN burst cascade. Other methods could also be used, such as cytometric analysis of dihydrorhodamine (DHR)-stimulated PMNs or DHR-stimulated whole blood in the presence of ampicillin/amoxicillin.

The interaction shown between aminopenicillins and PMN oxidative metabolism, especially for amoxicillin, might help to partly explain the rashes that occur when aminopenicillins are given to patients with infectious mononucleosis.

Some authors suggest that these adverse skin reactions result from amoxicillin and ampicillin sensitization within infectious mononucleosis, due to immunogenic epitopes that are located on their side chain. An interaction of aminopenicillins, especially amoxicillin, with PMNs could contribute to initiate, sustain or enhance this specific immunity reaction, via the oxidative metabolism and the pro-inflammatory cytokine production of PMNs. Nevertheless, infectious mononucleosis or allergy is a very complex process, and it seems preferable to carry out supplementary investigations before accepting PMN activation by aminopenicillins as the cause of rashes.

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References


