Differential effects of β-lactams on human IFN-γ activity

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Objectives: To investigate whether a range of β-lactam antibiotics conjugate to and hence reduce the activity of IFN-γ, as has been shown for penicillin G. A selection of penicillins, cephalosporins, a monobactam (aztreonam), a β-lactamase inhibitor (clavulanic acid), a carbapenem (meropenem) and the non-β-lactam penicillin derivative α-penicillamine were tested for their effect on IFN-γ activity.

Methods: Following exposure to a range of concentrations of these compounds, for varying lengths of time, IFN-γ activity was assayed by induction of CD54 on the surface of the lung epithelial cell line A549, utilizing an ELISA.

Results: Clavulanic acid, cefotixin and cefaloridine were the most potent inhibitors of IFN-γ activity, followed by cefotaxime, ceftriaxone and phenoxyethylpenicillin. Amoxicillin was less inhibitory than penicillin G, whilst meropenem and aztreonam had the least effect and α-penicillamine had no effect. The modulatory effect of these compounds was not due to a direct effect on CD54 induction. Unlike freshly prepared drugs, aged preparations of penicillin G and clavulanic acid had no significant effect on IFN-γ activity.

Conclusions: β-Lactams differ in their capacity to modulate human IFN-γ activity. This finding may have implications for the immunomodulatory effects of β-lactams and for the design both of β-lactams that do not affect the immune system and those which may be used therapeutically to target cytokine action.

Keywords: cytokines, immunomodulation, drugs, modulation, interactions

Introduction

In addition to their antibacterial properties, β-lactams have immunomodulatory effects, which include inducing allergic responses, influences on phagocytic cell function, chemotaxis, lymphocyte proliferation, antibody and cytokine production and NK cell activity.1–5 To investigate the possible mechanisms by which β-lactams influence the immune system, we focused on their direct interaction with human cytokines. We have shown that benzylpenicillin (penicillin G) differentially conjugates to and reduces the activity of human IFN-γ, but does not have this effect on other cytokines tested.6 The conjugation of penicillin G to IFN-γ was concentration, time and temperature dependent and resistant to reducing conditions used for western blotting, indicating covalent conjugation.7 The effect of penicillin G on IFN-γ activity was also concentration, time and temperature dependent and occurred in the presence of serum.7 Given that IFN-γ inhibits the production of IL-4 (a cytokine which promotes IgE synthesis) by Th2 cells,8,9 these findings may explain the ability of penicillin G to cause IgE-mediated allergic responses in some patients. It may also explain the observation that antibiotic use in early childhood is associated with the development of atopy.10,11 Furthermore, it provides a rationale for a potential means to target human cytokines with β-lactams to modulate cytokine function in vivo.

In this study, we intended to determine whether other β-lactams could modulate IFN-γ activity. In addition, we addressed whether our results were due to a direct interaction of the parent drug with IFN-γ, or occurred as a result of accumulated breakdown products affecting the cytokine’s activity. Finally, we asked whether it was possible to relate the IFN-γ modulatory effects of these drugs to β-lactam structure. We chose a range of penicillins, cephalosporins, a monobactam, a β-lactamase inhibitor, a carbapenem and included α-penicillamine, which lacks a β-lactam ring.

Materials and methods

Drug–cytokine incubations prior to functional assays

Carrier-free recombinant human IFN-γ was purchased from Peprotech (London, UK).

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**β-Lactams and IFN-γ**

![Graph showing IFN-γ activity following exposure to β-lactams for 1 day (a) and 4 days (b).](image_url)

**Figure 1.** IFN-γ activity following exposure to drugs for (a) 1 day or (b) 4 days. Each drug (0.5–2 mg/mL) was incubated in RT2 with IFN-γ (50 ng/mL) at 37°C before assay at 2 ng/mL IFN-γ. Results represent means ± SEM of 3–5 experiments, each performed in triplicate. *P < 0.05, by ANOVA, for comparison with untreated IFN-γ. PEN G, penicillin G; AMP, ampicillin; PEN V, phenoxymethylpenicillin; CLD, cefaloridine; CTX, cefotaxime; CRO, ceftriaxone; FOX, cefoxitin; ATM, aztreonam; MEM, meropenem; CLA, clavulanic acid.

IfN-γ (50 ng/mL) was incubated at 37°C for 1 or 4 days, or as otherwise stated, with or without β-lactams, each at final concentrations of 0.25, 0.5, 1 and 2 mg/mL. β-Lactams were obtained as follows: benzylpenicillin, β-lactamase, ampicillin, phenoxymethylpenicillin (penicillin V), cefotaxime, cefaloridine and cefoxitin from Sigma (Poole, UK); ceftriaxone from Roche Products Limited (Herts, UK); aztreonam from Bristol-Myers Squibb (Dublin, Ireland); and clavulanic acid from Mast Laboratories (Bootle, UK). Solutions of β-lactams were made up freshly for each experiment in RPMI-1640 containing 2% TCH serum replacement (RT2: ICN, Thame, UK), to give a final protein concentration of 0.65% and filter-sterilized. As controls, IFN-γ was incubated alone for 4 days and β-lactams added immediately before bioassay, as previously described.²

**Bioassay for IFN-γ activity**

The biological activity of IFN-γ was assayed by its ability to induce the adhesion molecule CD54 on human lung epithelial cells, as previously described.² The effect of each drug on IFN-γ was calculated as a percentage of untreated IFN-γ activity.

**Statistics**

Data are presented as the means ± SEM of several experiments and statistical analyses are by Student’s t-test or ANOVA with Bonferroni correction, as stated.

**Results**

**Reduction of IFN-γ activity following exposure to β-lactams**

IFN-γ was incubated for 24 h with each drug at 0.25–2 mg/mL before the cytokine’s activity was measured (Figure 1a). D-Penicillamine (not shown) and aztreonam had no effect on IFN-γ activity. Penicillin G, ampicillin and cefaloridine at 2 mg/mL reduced IFN-γ activity. Clavulanic acid was inhibitory down to 0.25 mg/mL and cefoxitin to 0.5 mg/mL, whilst penicillin V, cefaloridine and cefotaxime were effective at 2 and 1 mg/mL. The degree of inhibition by meropenem at 2 mg/mL was less than that of the other drugs effective at this concentration. There was a significant difference between the effect of each drug on IFN-γ (P = 0.0001).

**Time dependence of the effect of β-lactams on IFN-γ activity**

When the co-incubation time was increased from 1 to 4 days, the inhibitory effects of the drugs on IFN-γ activity became more pronounced (Figure 1b). For example, inhibition by 0.25 mg/mL cefaloridine goes from 40% at 1 day to 85% after 4 days. The IC₂₅ of each drug is given in Table 1. This shows that the rank order of inhibitory activity for the β-lactams was: meropenem < aztreonam < ampicillin < penicillin G < penicillin V < cefotaxime = ceftriaxone < cefoxitin = clavulanic acid < cefaloridine. Again, d-penicillamine had no inhibitory effect on IFN-γ activity (not shown). There was a significant difference between the effect of each drug on IFN-γ (P = 0.0001). Drugs incubated alone for 4 days in RT2 at 37°C and added to IFN-γ immediately prior to assay were without effect on IFN-γ activity (not shown), demonstrating that the drugs did not directly modulate CD54 induction.

**Effect of both penicillin G and clavulanic acid on IFN-γ activity is due to parent compound**

As we have shown in Figure 1(a and b), increasing the incubation time resulted in enhanced inhibition of IFN-γ activity by β-lactams.
we show that other β-lactams can modulate IFN-γ activity and that they differ in potency. Clavulanic acid, cefaloridine and cefoxitin were the most potent inhibitors of IFN-γ activity, followed by cefotaxime, ceftriaxone and penicillin V. Amoxicillin was less effective, whilst meropenem and aztreonam had least effect. D-Penicillamine, which lacks a β-lactam ring, had no effect on IFN-γ activity.

Our data showed that preparations of penicillin G and clavulanic acid aged for 4 days had no significant effect on IFN-γ activity. Although we did not have the opportunity to perform a chemical analysis of the breakdown products of the parent compounds, these results indicate that the effect of time on the interactions was probably due to increasing interaction of the parent drugs with the cytokine rather than formation and subsequent interaction of a breakdown product(s).

Although there have been reports that antibacterials have immunomodulatory effects,\(^1\) there has been little attempt to reveal any possible mechanisms underlying these phenomena. The only other example of a drug conjugating directly to a cytokine with subsequent modulation of cytokine activity is the work of Senter et al.,\(^12\) showing that N-acetyl-p-benzoquinone imine (NAPQI), a metabolite of acetaminophen, conjugates to and inhibits the activity of macrophage inhibitory factor (MIF). However, the β-lactams appear to be the only type of drug so far reported to be capable, in native non-metabolized form, of modulating cytokine activity as demonstrated in this and previous studies.\(^6,5\)

The interaction of penicillin G with proteins has been more extensively studied and is better understood than for the other β-lactams. The only human protein to which penicillin G conjugation has been well characterized is serum albumin, in which lysine residues are the sites of binding.\(^13\) The major interaction between Penicillin G and proteins is via the β-lactam ring, to form penicilloyl determinants. The amino acids that could be involved in this situation are lysine and histidine\(^1,13,14\) but it remains to be determined which particular amino acids are important in the modulation of IFN-γ activity by β-lactams. There was no obvious structural property that related to the potency of β-lactam inhibition of IFN-γ activity.

In conclusion, we have shown that β-lactams had differential effects on IFN-γ activity. For penicillin G and clavulanic acid, these effects are probably due to the action of the parent drugs rather than breakdown products. Our results may provide a mechanistic explanation for the ability of β-lactams (and other reactive drugs) to have regulatory effects on the immune system independently from their antibacterial properties. Having established the principle that a range of structurally related drugs can differentially modulate the activity of a human cytokine, this provides a basis for the design of β-lactams that do not interact with cytokines and a rationale for designing β-lactams that specifically interact with selected cytokines and growth factors, for use therapeutically in diseases caused by these peptides.

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**Transparency declarations**

No declarations were made by the authors of this paper.

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**Table 1. IC\(_{25}\) of β-lactams on IFN-γ activity**

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC(_{25}) (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin G</td>
<td>1.5</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>2</td>
</tr>
<tr>
<td>Penicillin V</td>
<td>1</td>
</tr>
<tr>
<td>Cefaloridine</td>
<td>&lt;0.25</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.8</td>
</tr>
<tr>
<td>Ceftriazone</td>
<td>0.75</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>0.3</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>&gt;2</td>
</tr>
<tr>
<td>Meropenem</td>
<td>&gt;2</td>
</tr>
<tr>
<td>Clavulanic acid</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Each drug was incubated with IFN-γ for 4 days before IFN-γ activity was measured by induction of CD54 on A549 cells. The concentration of drug which caused a 25% reduction in IFN-γ activity is given in mg/mL.

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**Figure 2.** Effect of ‘ageing’ on drugs’ ability to inhibit IFN-γ activity. Drugs were freshly made up or incubated in RT2 for 4 days before being added to IFN-γ (50 ng/mL) for at least a further 24 h at 37°C. Each preparation was then assayed at 2 mg/mL IFN-γ. Results represent means ± SEM of 3–7 experiments, each performed in triplicate. *Significant inhibition of IFN-γ activity, P < 0.01 by Student’s t-test. Pen G, penicillin G; CLA, clavulanic acid.

This may be either because conjugation of drugs to the cytokine is time-dependent, or may be due to the gradual formation of β-lactam breakdown products that subsequently inhibit IFN-γ activity. To answer this question, two of the drugs, penicillin G and clavulanic acid were incubated alone in solution for 4 days before being mixed with IFN-γ for at least a further 24 h. Freshly prepared drugs were also mixed with IFN-γ for at least 24 h and, as expected, reduced IFN-γ activity (Figure 2). In contrast, drugs aged for 4 days had no significant effect on IFN-γ activity (Figure 2).

**Discussion**

Having previously studied the interaction of penicillin G with human IFN-γ, we intended to determine whether other β-lactams were also able to affect IFN-γ activity, to ascertain if this was a unique property of penicillin G and to see if structural features of β-lactams could be related to their effect on this cytokine. Here
References