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Benzylpenicillin differentially conjugates to IFN-γ, TNF-α, IL-1β, IL-4 and IL-13 but selectively reduces IFN-γ activity

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SUMMARY

It is known that β-lactam antibiotics can conjugate to lysine and histidine residues on proteins *via* the carbonyl group of the opened β-lactam ring. However, it is not known which proteins these drugs target and there is little work addressing whether conjugation is preferential for some proteins over others or if conjugation has functional consequences for the protein. We have previously shown that the β-lactam antibiotic benzylpenicillin (BP) conjugates to IFN-γ and reduces its activity. This interaction demonstrates selectivity, as BP does not bind to IL-4. Here, we extend our study to include other Th1 and Th2 cell-associated cytokines and two cytokines associated with inflammatory responses. We demonstrate by Western blotting that BP also conjugates to IL-1β, IL-2, IL-5, IL-13 and TNF-α but not to IL-10. Densitometric analysis of leading cytokine bands on blots revealed that IFN-γ always gave more intense BP-positive bands than any other cytokine analysed. Cytokines pre-incubated with BP at 37°C in a protein-containing, serum-free medium were assayed for their biological activity. By *in vitro* bioassay, BP inhibited the ability of IFN-γ but not IL-1β or TNF-α to induce CD54 expression on epithelial cells. In addition, BP did not affect IL-4 or IL-13 inhibition of mast cell proliferation. When the pre-incubation temperature was reduced to 4°C, BP did not conjugate to IFN-γ or modulate its activity. BP retained its inhibitory effect on IFN-γ activity when 20% FCS was added to the pre-incubation medium. In conclusion, BP conjugates to some cytokines but not others and this does not appear to be related to primary protein structure. Furthermore, of the cytokines studied, conjugation only to IFN-γ is accompanied by inhibition of activity. This phenomenon is temperature dependent and occurs in the presence of serum. These findings provide further evidence for differential, direct drug–cytokine interactions. Such interactions may have therapeutic implications in terms of targeting cytokines to regulate their activity.

Keywords benzylpenicillin conjugation allergy β-lactam cytokine

INTRODUCTION

We have shown previously that the β-lactam antibiotic benzylpenicillin (BP) conjugates to IFN-γ and reduces its activity in a number of *in vitro* assays [1]. We also showed that BP does not bind to IL-4. Although generally nontoxic, β-lactams are one of the classes of drug most frequently associated with IgE-mediated allergy [2–5]. Our data led us to hypothesize that selective impairment of IFN-γ activity by β-lactams during the early phase of an immune response may favour the generation of Th2 over Th1 responses, thus leading to IgE production and allergy. Here, we extend our studies to question whether BP conjugates to and affects the activity of other cytokines, using conditions previously optimized for BP interactions with IFN-γ. We selected several cytokines to include Th1 (IL-2 and IFN-γ) and Th2 (IL-5, IL-10, IL-13 and IL-4) lymphocyte-associated cytokines and two cytokines that promote inflammation, TNF-α and IL-1β.

Western blotting revealed that BP bound in varying degrees to IFN-γ, IL-1β, IL-2, IL-5, IL-13 and TNF-α but did not bind to IL-4 or IL-10. Of interest, bands for BP conjugated to human IFN-γ were considerably more intense than those for murine IFN-γ, demonstrating interspecies heterogeneity. In bioassays for IFN-γ, IL-1β, TNF-α, IL-4 and IL-13 activity, BP affected only IFN-γ activity, showing that conjugation is not always associated with impairment of biological activity. Furthermore, the inhibitory

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effect of BP on IFN-γ activity does not occur when the drug and cytokine are incubated at 4, rather than 37°C, but does occur in the presence of 20% FCS.

METHODS

Cells and cytokines

A549 human lung epithelial cells (ECACC, Salisbury, UK) were cultured in DMEM containing 5% FCS. The human mast cell line HMC-1 was a generous gift from J.H. Butterfield [6] and was maintained by subculturing 1 : 8 weekly in IMDM + 5% FCS. Carrier-free recombinant human IFN-γ, IL-2, IL-5, IL-10, IL-4, IL-13, TNF-α, IL-1β and murine IFN-γ were purchased from Peprotech (London, UK).

SDS-PAGE and Western blotting

Cytokines were incubated at 10 μg/ml, as previously optimized for visualization for Western blot and amido black staining [1], with or without BP at a final concentration of 5 mg/ml in PBS at 37°C, unless otherwise stated. After overnight incubation, 5× loading buffer (50% glycerol (v/v), 10% SDS (w/v), 100 μM DTT in 50 mM TRIS-HCl) was added 1 : 5 to each sample and 30 μl then loaded onto SDS-10% PAGE vertical slab gels (Hoefer Mighty Small apparatus Amersham, Bucks, UK), each gel including molecular weight markers. Gels were run in duplicate (30 mA/gel for 2 h) and proteins transferred electrophoretically by semidry blotter (Biometra, Berks, UK) to nitrocellulose membranes (Hybond ECL, Amersham). To detect BP conjugation, one blot was incubated in 1 : 5000 rabbit anti-BP antibody followed by 1 : 25 000 peroxidase-labelled goat antirabbit IgG and developed in ECL™ reagent (Amersham) as previously decribed [7]. IFN-γ was included as an internal reference in all experiments and other cytokines were related to IFN-γ as a ratio of band intensity, following densitometric analysis using the 'Image' programme (National Institutes of Health, USA). Ratios of test cytokine to IFN-γ band intensity were calculated for leading bands (verified by molecular weight) and for the total intensity of all bands in the lane. For analysis of BP effects on cytokine multimer formation, blots were incubated with polyclonal antibody to IFN-γ, TNF-α or IL-2 (Peprotech), at 0·2 or 0·4 μg/ml and visualized in the same way. The second blot from each experiment was stained for total protein in 0·1% amido black to confirm protein transfer.

Drug-cytokine incubations prior to functional assays

Cytokines (100 ng/ml) were incubated at 37°C, unless otherwise stated, for 4 days, with or without BP at a final concentration of 2 mg/ml. The medium used was RPMI-1640 containing either 2% TCH™ serum replacement (RT2: ICN, Thame, UK) to give a final protein concentration of 0·65%, or various concentrations of FCS. Solutions of BP (Sigma, Poole, UK) were made up fresh for each experiment in RT2 or serum containing RPMI-1640 and filter-sterilized. As controls, cytokines were incubated alone for 4 days and BP was added immediately before bioassay, as previously described [1].

Assay for IL-4 and IL-13 activity

IL-4 and IL-13 each inhibit the proliferation of the human mast cell line HMC-1 [8]. HMC-1 cells were seeded into 96-well plates with RT2, BP, IL-4 or IL-13 preparations to give concentrations of 2, 10 or 50 ng/ml, in replicates of four. After 4–6 days incubation at 37°C, 5% CO_2, ^{3}H-thymidine (0·5 μCi/well) was added for the final 4 h of culture, the cells harvested and incorporation of radioactivity measured (PerkinElmer Life Sciences, Cambridge, UK).

Assay for IL-1β*, TNF-*α *and IFN-*γ *activity*

IL-1β, TNF-α and IFN-γ each induce expression of CD54 on A549 human lung epithelial cells [9]. A549 cells were grown to confluence in 96-well plates before the appropriate medium, BP or cytokine preparations were added in triplicate. After overnight incubation, CD54 expression was measured by ELISA. Medium was removed and cells incubated for 30 min at room temperature in Cellfix (Becton Dickinson, Oxford, UK). Plates were then washed with PBS + 0·05% Tween 20 (Sigma) (PBS-T) before addition of mouse IgG1 anti-CD54 monoclonal antibody (Pharmingen, Oxford, UK, 1/10 000) or isotype control (at the same final concentration) in PBS plus 2% goat serum plus 2% FCS (PBS-D) (100 μl/well). After 1 h at room temperature, plates were washed in PBS-T and HRP-labelled F(ab)$_2$ goat antimouse-IgG (Caltag, Silverstone, UK) 1/4000 or EnVision™+/HRP (DAKO, Ely, UK) 1/50 in PBS-D (100 μl/well) added for 1 h at room temperature. After further washing, 2–2′-Azinobis(3-ethylbenzthiazoline-sulphonic Acid) (ABTS: Sigma) or 3,3′,5,5′-tetramethylbenzidine (TMB: Sigma) (100 μl) was added to each well. The TMB reaction was stopped with 100 μl 1 M H_2SO_4 and absorbance read at 450 nm with a reference of 630 nm on an automatic plate reader (MRX; Dynatech Instruments, Torrance, CA, USA). For ABTS absorbance was read at 405 nm.

RESULTS

Benzylpenicillin conjugates to IFN-γ*, IL-1*β*, IL-2, IL-5, IL-13 and TNF-*α *but not IL-4 or IL-10*

Western blot analysis revealed that BP conjugated to seven out of the nine cytokines analysed, as shown in Fig. 1. Blots were aligned with amido black tranfers that contained molecular weight markers and the molecular weight of the bands determined. Using this method, it was evident that the leading bands visualized on Westerns with anti-BP antibodies corresponded to the molecular weight of a single molecule of each cytokine. The trailing bands represent multiples of this molecular weight. Multimers were observed on Western blots of all positive cytokines except for TNF-α (Fig. 1). Each cytokine was analysed in this way three times and, using densitometric analysis, ratios to IFN-γ determined for primary bands (monomers for all except IL-5, which is an interdigitating dimer, 10) and for total intensity of all bands, as described in Materials and Methods. Considering the primary bands, none of the cytokines gave a ratio of signal intensity of 1 or more, when compared to IFN-γ (Table 1). The mean ratios for IL-5, IL-13 and TNF-α were similar to each other, IL-2 being lower, whilst IL-1β was higher. BP bound minimally to IL-4 and not at all to IL-10 under these conditions and bound much less to murine compared to human IFN-γ. For every cytokine tested, protein transfer was confirmed by amido black staining of parallel blots. Ratios were also calculated for the total binding to all the bands in each lane. Here, the cytokines fell into three distinct groups: IL-5, which was equivalent to IFN-γ, IL-1β, IL-2, TNF-α and IL-13, which were similar to each other but less than IFN-γ and IL-5; and murine IFN-γ, IL-4 and IL-10, had the lowest ratios (Table 1). Comparison of these ratios of Western blot band intensities with the lysine and histidine content of the cytokines does not reveal any relationship (Table 1). For example, TNF-α, which contains only 6 lysine and 3 histidine residues, is conjugated relatively

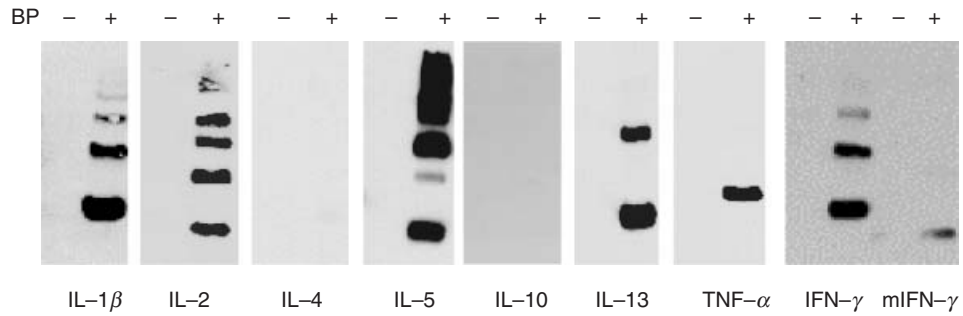

Fig. 1. BP conjugation to cytokines. Cytokines, each at 10 μg/ml in PBS, were incubated with or without 5 mg/ml BP overnight at 37°C. Samples were analysed by SDS-PAGE and Western blotted with rabbit polyclonal anti-BP antibody. In each case, one blot representative of three is shown. m, murine.

Table 1. Conjugation of BP to cytokines relative to IFN-γ

Cytokine	MW	Amino acid residues per molecule		Relative band intensity	
		Lysine	Histidine	Leading band Mean ± SD	Total Mean ± SD
IFN-γ	16·7	20	2	1	1
IL-1β	17	15	1	0·82 ± 0·06	0·66 ± 0·15
IL-2	15·4	11	3	0·21 ± 0·59	0·59 ± 0·33
IL-4	14·9	12	5	0·05 ± 0·05	0·06 ± 0·05
IL-5*	25	16	6	0·68 ± 0·11	1·03 ± 0·14
IL-10	18·6	13	3	0	0
IL-13	12·5	7	3	0·67 ± 0·16	0·51 ± 0·14
TNF-α	17·4	6	3	0·66 ± 0·14	0·46 ± 0·09
mIFN-γ	15·6	10	3	0·24 ± 0·27	0·16 ± 0·19

Cytokines were incubated at 10 μg/ml with or without 5 mg/ml of BP in PBS. After overnight incubation at 37°C, samples were analysed by SDS-PAGE and Western blotted with rabbit polyclonal anti-BP antibody. IFN-γ was included as an internal reference in all experiments and other cytokines were related to IFN-γ as a ratio of band intensity, following densitometric analysis. Ratios of test cytokine to IFN-γ were calculated for monomer bands (determined from molecular weight) and for the total intensity of all bands in each lane. Results represent the mean ± standard deviation of three experiments. MW, molecular weight in kD. m, murine. *dimer, see text and [10].

strongly by BP, whereas IL-10, with 13 lysines and 3 histidines is negative.

Since Western blots for BP conjugation revealed bands corresponding to multimers of cytokine molecules (Fig. 1), we determined whether BP induced the formation of these multimers, using polyclonal anticytokine antibody in place of anti-BP antibodies. IFN-γ, TNF-α and IL-2 each gave the same band pattern on Westerns blots whether or not they had been incubated with BP (Fig. 2). Therefore, BP did not affect the aggregation of these cytokines. Of interest, although TNF-α could be visualized as multimers on SDS-PAGE using an anti-TNF-α polyclonal antibody (Fig. 2), only the monomeric form of this cytokine was positive for BP (Fig. 1).

BP does not affect IL-4 or IL-13 activity

IL-4 and IL-13 were each incubated in RT2 at 37°C at 100 ng/ml for 4 days with or without BP (2 mg/ml), conditions previously optimized for experiments with IFN-γ [1]. The cytokines were then added to HMC-1 cells and their effect on proliferation measured. Both IL-4 and IL-13 caused a concentration-dependent inhibitory effect on HMC-1 proliferation which was significant at 10 and 50 ng/ml (Fig. 3a and b, respectively). Pre-treatment of either of these two cytokines with BP did not affect their activity (Fig. 3a,b). Likewise, addition of BP to these cytokines immediately before bioassay had no effect on their activity. In addition, BP added to the bioassays at the same final concentration as in these samples had no effect (data not shown).

BP affects IFN-γ but not TNF-α or IL-1β activity

TNF-α, IL-1β and IFN-γ were each incubated in RT2 at 37°C at 100 ng/ml for 4 days with or without BP (2 mg/ml), conditions previously optimized for experiments with IFN-γ [1]. TNF-α, IL-1β and IFN-γ each induced CD54 expression on A549 cells, IL-1β being the most potent (Fig. 4a-c). Pre-treatment of either IL-1β or TNF-α with BP, or addition of BP immediately before assay, had no effect their activity (Fig. 4a and b, respectively). In contrast, IFN-γ pre-incubated with BP had a significantly reduced activity compared to IFN-γ incubated alone or to IFN-γ to which BP was

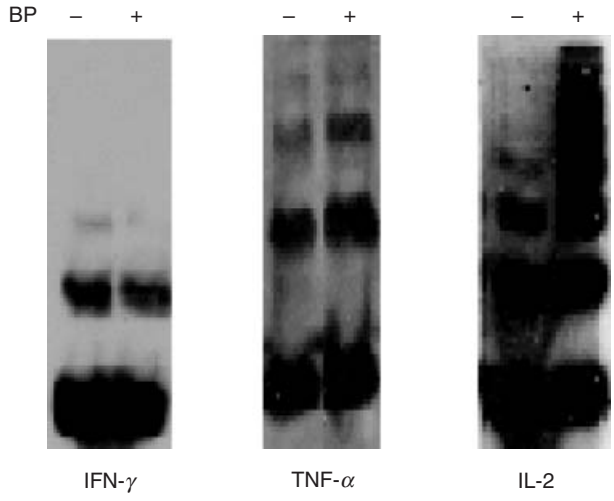

Fig. 2. BP has no effect on cytokine oligomerization. IFN-γ, IL-2 and TNF-α, each at 10 μg/ml in PBS, were incubated with or without 5 mg/ml BP overnight at 37°C. Samples were analysed by SDS-PAGE and Western blotted with the appropriate polyclonal anti-cytokine antibody.

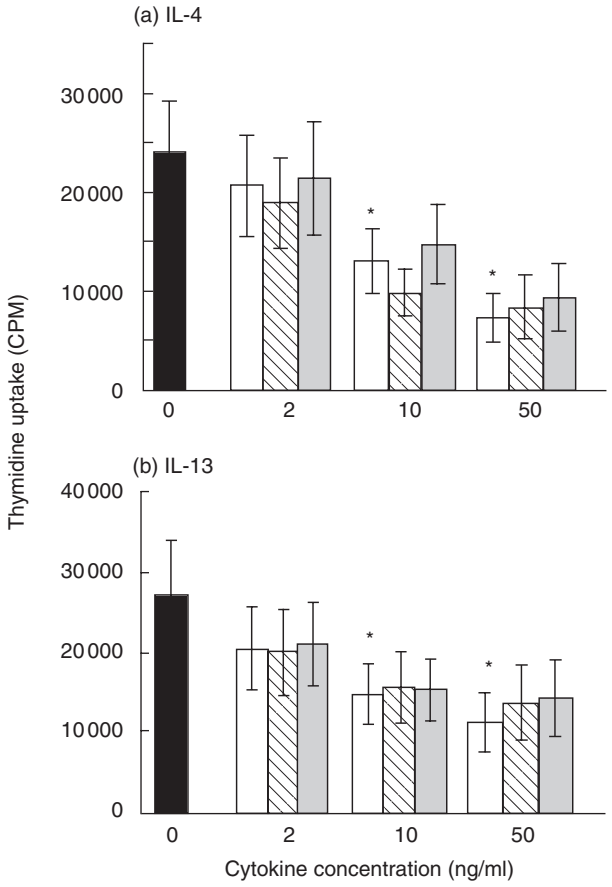

Fig. 3. Effect of BP on the ability of (a) IL-4 or (b) IL-13 to inhibit HMC-1 cell proliferation. Each cytokine (100 ng/ml) was incubated in RT2 with or without BP (2 mg/ml) for 4 days at 37°C, then each preparation was assayed at 2, 10 or 50 ng/ml. Proliferation was measured as uptake of ^{3}H-thymidine after cells had been incubated for 4–6 days with the IL-4 or IL-13 preparations. Results represent mean ± SEM of 4 experiments, each performed in replicates of at least 4. *$P < 0{\cdot}05$, by paired Student's *t*-test, for comparison of cells cultured with or without cytokine. ■ No cytokine, □ Cytokine incubated 4 days alone, ▨ Cytokine incubated 4 days + 2 mg/ml BP, ▩ Cytokine incubated 4 days alone, 2mg/ml BP added immediately before assay.

added immediately before assay (Fig. 4c). BP-treated IFN-γ preparations assayed at 2 and 10 ng/ml had a reduced activity of 71 and 38%, respectively, compared to untreated IFN-γ. BP alone did not affect CD54 expression (data not shown).

Effect of temperature on BP conjugation to IFN-γ

BP conjugated to IFN-γ at 37°C but not at 4°C, as revealed by Western blotting (Fig. 5)

Influence of temperature and serum on the inhibitory action of BP on IFN-γ *activity*

When BP and IFN-γ were pre-incubated together at 37°C, BP inhibited IFN-γ activity. However, if the pre-incubation temperature was reduced to room temperature or 4°C, BP did not influence IFN-γ activity (Fig. 6). The inhibitory action of BP on IFN-γ activity was retained when pre-incubation was performed in medium containing 20% or 2% FCS (Fig. 6).

DISCUSSION

Here we demonstrate that the β-lactam antibiotic BP conjugates differentially to cytokines. Band intensity for primary bands on Western blots was greatest for IFN-γ, followed by IL1-β, IL-5, IL-13 and TNF-α, less for IL-2 and murine IFN-γ, very weak for IL-4 and no bands could be detected for IL-10. TNF-α resolved as multimers on PAGE but BP was only visualized as binding to the monomeric form, suggesting that only this form of TNF-α contains sites accessible for BP conjugation. If this is the case, it is not surprising that BP does not modulate TNF-α activity in our *in vitro* assays, as the active form of this cytokine is a trimer [11]. In contrast, the other cytokines to which BP bound displayed positive monomeric and oligomeric bands.

Until now the only human protein to which BP conjugation has been well characterized is serum albumin, in which lysine residues are the sites of binding [12] but other protein targets for BP are poorly defined. The major interaction between BP 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 [12–14]. Comparison of the primary amino acid sequence of these cytokines shows that the extent of BP binding is not determined purely by the quantity of lysine and histidine residues in the polypeptide. Further analysis of the structural features of these cytokines reveals that many of them (IL-2, IL-4, IL-5, IL-10, IL-13 and IFN-γ) share a common four-helical bundle topology [15–17], whilst IL-1β and TNF-α belong to the β-trefoil and β-jellyroll families, respectively [15,18,19]. IL-1β, IL-2, IL-4 and IL-13 exist as monomers [15,17,18], IFN-γ, IL-5 and IL-10 as dimers [15,16,20] whilst TNF-α exists predominantly as a trimer [11,19]. However, there is no correlation between the structural groupings of these proteins and their susceptibility to conjugation by BP. Of interest, the cytokine with a structure most similar to IFN-γ is IL-10, to which BP does not conjugate at all. Therefore, the chemical environment of individual lysine and histidine residues within each protein and the relative availability of these

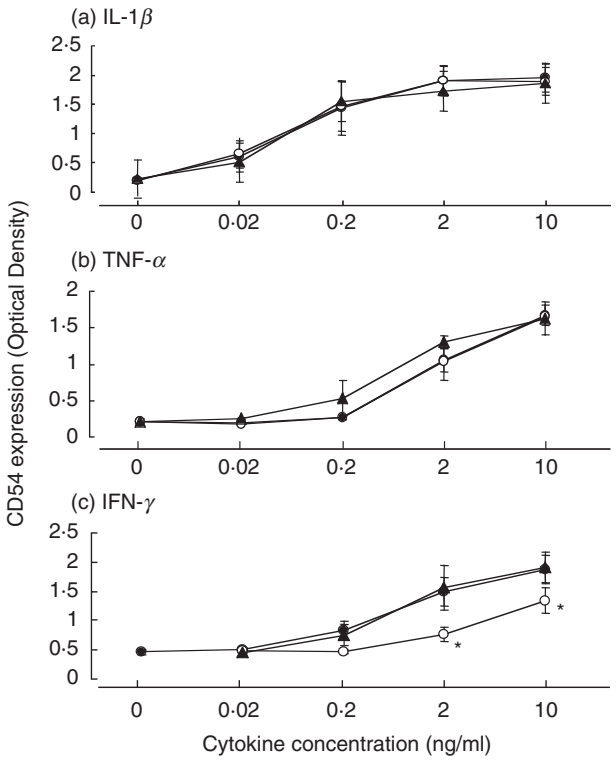

Fig. 4. Effect of BP on the ability of (a) IL-1β, (b) TNF-α or (c) IFN-γ to induce CD54 expression on A549 cells. Each cytokine (100 ng/ml) was incubated in RT2 with (○) or without (●) BP (2 mg/ml) for 4 days at 37°C, then each preparation was assayed at 0·02, 0·2, 2 or 10 ng/ml. Confluent layers of A549 cells in 96-well plates were incubated overnight with cytokine preparations. After fixing, CD54 expression was measured by ELISA. ▲ Cytokine incubated 4 days alone, 2 mg/ml BP added immediately before assay. Results represent mean ± SEM of 3 (IL-1β and TNF-α) or 4 (IFN-γ) experiments, each performed in triplictate. *$P < 0{\cdot}05$, by paired Student's *t*-test, for comparison of untreated to BP-treated IFN-γ.

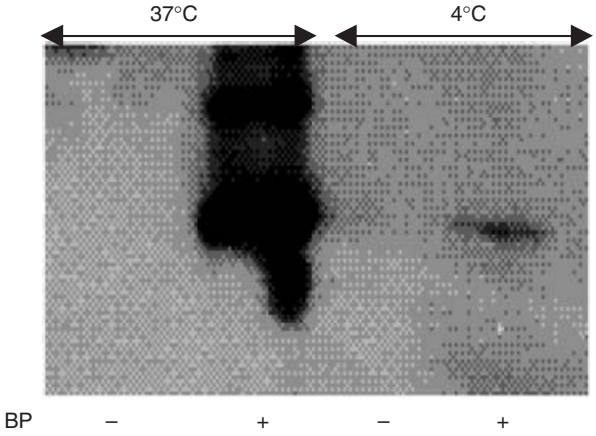

Fig. 5. Effect of temperature on BP conjugation to IFN-γ. IFN-γ, at 10 μg/ml in PBS, was incubated with or without 5 mg/ml BP overnight at 4 or 37°C. Samples were analysed by SDS-PAGE and Western blotted with rabbit polyclonal anti-BP antibody.

residues within the 3D structure of the molecule must determine whether or not BP will conjugate to them.

When five of these cytokines were assayed to see if BP modulates their *in vitro* biological activity, only IFN-γ was affected. Predictably, the activity of IL-4, to which BP does not conjugate, was unaffected. However, the activity of three cytokines to which BP does bind (IL-1β, TNF-α and IL-13) also remained unaltered. This may be a consequence of BP binding to functionally unimportant sites within these cytokine molecules. For IFN-γ the regions of the molecule important for receptor (residues 1–42, 108–124 and 130–132) and glycosaminoglycan (GAG: residues 125–131) binding are known [20–22]. These regions all contain histidine and/or lysine groups. In the case of TNF-α, corresponding regions have not yet been defined. Regions of IL1-β and IL-13 that bind to their receptors have been identified [23–25]. These are thought to be multiple single amino acid residues and peptide sequences in IL-1β [23,24], whereas, for IL-13, the areas predicted to be important in receptor binding are helices A, C and D [25]. Again, there are lysine and histidine residues within these regions. Therefore, there appears to be something unique in the interaction of BP with IFN-γ that results in the decreased activity of this cytokine.

The temperature at which BP is pre-incubated with IFN-γ had a marked effect on its ability to conjugate to and inhibit the activity of the cytokine. This supports the notion that the modulation of IFN-γ activity by BP is due to a chemical interaction. In the presence of 20% FCS during the pre-incubation of BP with IFN-γ, the drug mediated a significant reduction the biological activity of the cytokine, supporting our hypothesis that BP may regulate IFN-γ activity under physiological conditions.

BP interaction with amino acid residues within a cytokine could have several possible outcomes. First of all, it could disrupt the secondary or tertiary structure of the molecule, causing functional modulation. Secondly, BP could bind to residues within the receptor-binding region of the molecule, affecting receptor engagement and therefore biological activity. Thirdly, conjugation to residues within GAG-binding regions of a cytokine could disrupt GAG-binding and have an effect not only on activity but also on biodistribution and half-life [26]. Our biological assays performed to date do not allow us to distinguish between any effect that BP may be having on receptor *versus* GAG-binding, as we have not determined the possible contribution of GAGs in these assays. However, it is known that many cytokines, including most of those in our study, do conjugate to GAGs and that this has important functional consequences [26–31].

If BP conjugates to cytokines *in vivo*, there could be several outcomes. One of these is the favouring of Th2 over Th1 lymphocyte responses due to the differential effects we have shown on IFN-γ over Th2 cytokine (IL-4 and IL-13) *in vitro* activity. It is possible that other haptens behave in a similar fashion, conjugating to and disrupting the activity of cytokines in a differential manner. Cytokines structurally modified by BP or other haptens may also form neoantigens, with various immunological consequences. In addition to our work showing that the commonly used drug BP can reduce IFN-γ activity, others have shown that a reactive metabolite of acetaminophen can covalently bind to and inhibit the function of MIF [32]. Thus, drug modulation of cytokine activity is not restricted to β-lactams and IFN-γ.

In conclusion, we have shown that BP differentially conjugates to cytokines and preferentially disrupts the activity of IFN-γ. These findings have implications for understanding possible

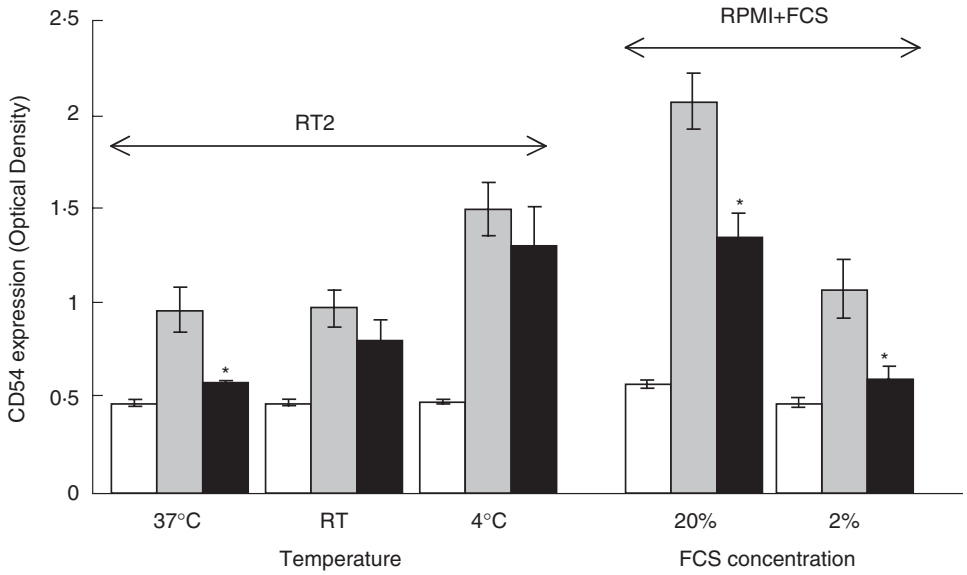

Fig. 6. Effect of temperature and serum on BP reduction of IFN-γ activity. IFN-γ was incubated, at 100 ng/ml, at different temperatures in RT2, or in varying concentrations of FCS for 4 days with or without 2 mg/ml BP. Confluent layers of A549 cells in 96-well plates were incubated overnight with each cytokine preparation at a final concentration of 2 ng/ml. After fixing, CD54 expression was measured by ELISA. Results represent mean ± SEM of 4 experiments, each performed in triplictate.*$P < 0{\cdot}01$ by paired Student's *t*-test, for comparison of untreated to BP-treated IFN-γ. □ medium alone, ▨ IFN-γ, ■ IFN-γ + BP.

mechanisms by which BP and other haptens cause allergy or disrupt immune regulation. They also have therapeutic implications, as they provide a rationale for the targeting and selective modulation of cytokine bioactivity.

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