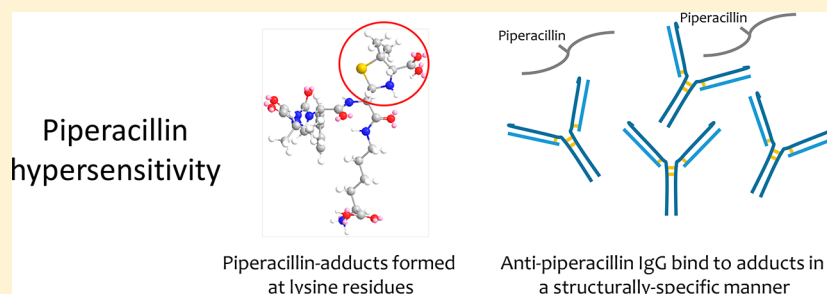


Assessment of Antipiperacillin IgG Binding to Structurally Related Drug Protein Adducts

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ABSTRACT: The risk of developing hypersensitivity to alternative antibiotics is a concern for penicillin hypersensitive patients and healthcare providers. Herein we use piperacillin hypersensitivity as a model to explore the reactivity of drug-specific IgG against alternative β -lactam protein adducts. Mass spectrometry was used to show the drugs (amoxicillin, flucloxacillin, benzyl penicillin, aztreonam, and piperacillin) bind to similar lysine residues on the protein carrier bovine serum albumin. However, the hapten-specific IgG antibodies found in piperacillin hypersensitive patient plasma did not bind to other β -lactam protein conjugates. These data outline the fine specificity of piperacillin-specific IgG antibodies that circulate in patients with hypersensitivity.

β -Lactam antibiotics contain a β -lactam ring that is critical for their antibacterial activity. These drugs are used either alone or in combination with β -lactamase inhibitors, which serve to conserve pharmacological activity and extend their spectrum. β -Lactam antibiotics have been implicated in a plethora of cutaneous hypersensitivity reactions that vary in severity and pathophysiology. Since all β -lactam antibiotics contain the same reactive strained ring structure that is susceptible to nucleophilic attack by lysine residues on protein it is likely that similar adducts are formed in patients that might act as neo-epitopes. Thus, inadvertent activation of the adaptive immune system in hypersensitive patients due to cross-reactivity is an important clinical concern.

Piperacillin is a β -lactam antibiotic commonly used to combat respiratory tract infections in patients with cystic fibrosis. Delayed-type hypersensitivity reactions occur at a higher frequency in patients with cystic fibrosis when compared to the general population with an incidence of up to 30% reported in some studies.¹ Thus, piperacillin hypersensitivity represents a relevant model to study the role of the adaptive immune system in the disease pathogenesis. We have recently found that T-cells from hypersensitive patients are activated with piperacillin albumin adducts following protein processing by antigen presenting cells.^{2–4} T-cell cross-reactivity with different drugs was not observed.⁵ In recent studies, we have also detected piperacillin hapten-specific B-cells in the peripheral blood of hypersensitive patients.⁶ Stimulation of

the B-cells *in vitro* resulted in the secretion of immunoglobulin (IgG) antibodies and the same antibodies were found to circulate in patient plasma.⁶ In the current study, structurally diverse β -lactam bovine serum albumin (BSA) adducts were synthesized, characterized, and used to explore whether IgG antibodies from patients react with different β -lactam structures (Figure 1). BSA was selected as a protein carrier since it contains a similar number of lysine residues to human serum

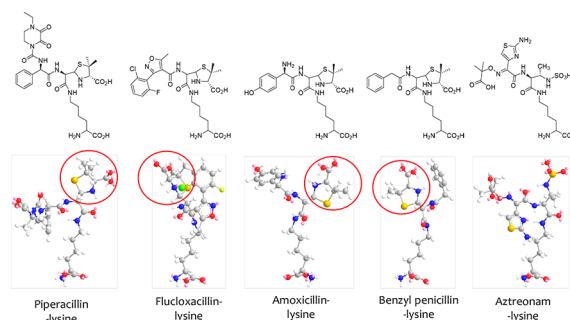


Figure 1. Structure of the β -lactam haptens bound to lysine residues on BSA. Red circles show masking of the thiazolidine ring by the isoxazole group in flucloxacillin.

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albumin, the major target for piperacillin binding in patients, and the native protein displays limited reactivity against piperacillin-specific IgG.⁶

Plasma from four hypersensitive patients was used to study IgG binding. Each patient developed maculopapular exanthema with or without drug-induced fever 3–7 days after initiation of piperacillin therapy. Blood samples were obtained with written consent and after approval by the Leeds East Ethics Committee. Peripheral blood mononuclear cells (PBMCs) were isolated using ficoll density centrifugation. The clinical diagnosis of piperacillin hypersensitivity was confirmed by *in vitro* assessment of T-cell proliferative responses using the lymphocyte transformation test. Lymphocytes from all four patients were stimulated to proliferate with piperacillin in a concentration-dependent manner (not shown).

To prepare adducts, drugs (amoxicillin, flucloxacillin, benzyl penicillin, aztreonam, and piperacillin) were incubated with BSA for 96 h at a protein/drug molar ratio of 1:50. Human serum albumin (HSA) could not be used to form adducts as high absorbance values were found in the ELISA with the unmodified protein. Ice-cold methanol was used to precipitate protein and the Bradford assay was used for protein quantification. Western blotting using antipenicillin (pen 9; ab69234; Abcam, Cambridge, UK) or antifleucloxacillin (custom-made by Eurogentec; Hampshire, UK) antibodies was performed to confirm β -lactam-protein binding; visible bands at 66 kDa were observed for all drug protein adducts. Adducts were digested with trypsin prior to mass spectrometric analysis. Samples were reconstituted in 2% acetonitrile/0.1% formic acid (v/v) and delivered into a QTRAP 5500 hybrid quadrupole-linear ion trap mass spectrometer (Sciex) by automated in-line LC using an established method.³ Multiple reaction monitoring (MRM) transitions specific for drug-modified peptides were selected as follows: the mass/charge ratio (m/z) values were calculated for all possible peptides with a missed cleavage at a lysine residue; to these were added the mass of the appropriate hapten; the parent ion masses were then paired with relevant fragment masses. MRM transitions were acquired at unit resolution in Q1 and Q3 quadrupoles to maximize specificity. They were optimized for collision energy and collision cell exit potential, and the dwell time was 20 ms. MRM survey scans were used to trigger enhanced product ion MS/MS scans of drug-modified peptides, with Q1 set to unit resolution, dynamic fill selected, and dynamic exclusion for 20 s. Total ion counts were determined from a second aliquot of each sample analyzed by conventional LC/tandem MS and were used to normalize sample loading. MRM peak areas were determined by MultiQuant 1.2 software (Sciex). Semi-quantitative epitope profiles were constructed by comparing the relative intensity of MRM peaks for each of the modified lysine residues within a sample and normalization of those signals across samples. Absolute quantification was not possible without synthetic peptides modified with drugs at each lysine group. Epitope profiles showed that a similar panel of lysine residues on BSA was modified with amoxicillin, benzyl penicillin, piperacillin, and flucloxacillin (Figure 2). Lysine residues on all four conjugates were haptenated at positions 4, 12, 211, 431, and 524. Three of the drugs modified lysine residues at positions 132, 136, and 523. Amoxicillin and benzyl penicillin alone modified lysine 20, 473, and 537, while lysine 535 was only modified with flucloxacillin. A more restricted binding profile was observed with aztreonam; modifications were only detected on lysine residues at positions 136, 211, and

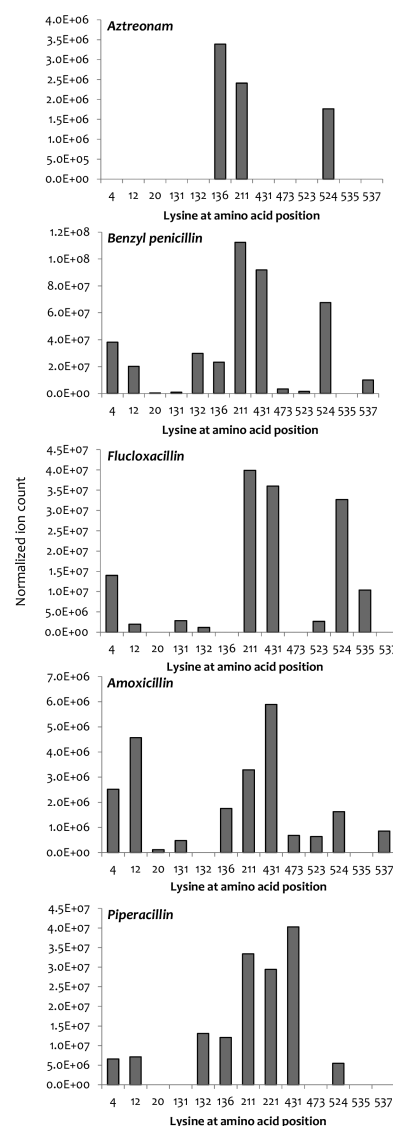


Figure 2. Epitope profile for piperacillin, amoxicillin, aztreonam, flucloxacillin, and benzyl penicillin BSA adducts. Adducts were generated by culturing BSA with the drugs at a 1:50 ratio for 96 h. Proteins were denatured, digested with trypsin, and desalted prior to characterization by mass spectrometry.

524. This may be due to the drugs inherent chemical reactivity or different noncovalent docking interactions with the protein leading to less favorable orientations for binding.

ELISA was used for the detection of antidrug IgG antibody levels in hypersensitive patient plasma. Plates were coated overnight at 4 °C with a 20 μ g/mL solution of drug BSA adducts. Piperacillin (1 mM) was added to certain wells for 16 h, prior to the addition of BSA adducts, for hapten inhibition. Following washing and blocking, a 1/10 dilution of plasma from the patients was added. Goat antihuman IgG, a horseradish peroxidase-labeled rabbit antigoat IgG and TMB substrate were used to visualize bound antipiperacillin IgG. Absorbance values were read at 490 nm using an automated plate reader (Dynatech MR600, Hendersonville, TN, USA).

Piperacillin IgG binding to piperacillin-modified-BSA was observed with plasma from all four patients (Figure 3). Antibody binding was reduced by greater than 85% when the antibody was preincubated with piperacillin, but not fluclo-

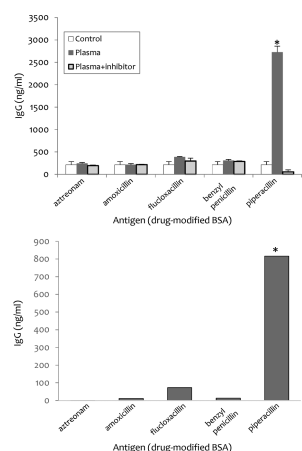


Figure 3. Detection of piperacillin IgG binding to different β -lactam BSA adducts using ELISA. (A) Results using plasma from the patient with highest levels of piperacillin-specific IgG are shown to illustrate control values and the effect of hapten inhibition. Data show mean $\pm$ SD hapten inhabitable antibody binding to the different BSA adducts. (B) Mean data showing hapten-inhabitable IgG values obtained with plasma from four piperacillin hypersensitive patients. Error bars have been omitted for clarity. Data were analyzed with the Students *t* test to compare differences between means.

acillin, in hapten inhibition experiments. Antibody binding was not observed when the piperacillin-BSA adduct was replaced with amoxicillin, aztreonam, or benzyl penicillin-BSA adducts, while very low levels of binding were detected with the flucloxacillin-BSA adduct (Figure 3).

Previous studies with T-cells from piperacillin hypersensitive patients revealed low levels of cross reactivity.^{4,5} Similarly, the extent of cross-reactivity between different classes of β -lactam antibiotic in the clinic and during skin testing (e.g., more than 70% of patients with hypersensitivity display negative responses to benzyl penicillin in patch tests) is low. Our recent discovery of piperacillin-specific IgG in hypersensitive patient plasma⁶ prompted us to investigate whether piperacillin-specific IgG bound to different β -lactam protein conjugates. Mass spectrometry revealed that the different drugs bound to a similar panel of lysine residues on the protein carrier BSA, albeit at different levels. However, the absence of piperacillin-specific IgG binding to amoxicillin, aztreonam, and benzyl penicillin adducts and the low levels of binding seen with the flucloxacillin adduct shows that the humoral immune response induced in hypersensitive patients is also highly structurally specific. IgG antibodies must interact with the penicilloyl structure as well as the side chain of piperacillin to confer such specificity. If confirmed in additional studies using patients with different forms of β -lactam hypersensitivity, our findings imply that the policy of avoiding all penicillins in hypersensitive patients may be overly conservative.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

BSA, bovine serum albumin; PBMC, peripheral blood mononuclear cells

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