

# Detection of drug-responsive B lymphocytes and antidrug IgG in patients with $\beta$ -lactam hypersensitivity

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## Keywords

B lymphocytes;  $\beta$ -lactam antibiotics; drug hypersensitivity; IgG.

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## Abstract

**Background:** Delayed-type  $\beta$ -lactam hypersensitivity develops in subset of patients. The cellular immunological processes that underlie the drug-specific response have been described; however, little is known about involvement of the humoral immune system. Thus, the aim of this study was to utilize piperacillin hypersensitivity as an exemplar to (i) develop cell culture methods for the detection of drug-specific B-cell responses, (ii) characterize drug-specific IgG subtypes and (iii) assess reactivity of IgG antibodies against proteins modified to different levels with piperacillin haptens.

**Methods:** IgG secretion and CD19<sup>+</sup>CD27<sup>+</sup> expression on B cells were measured using ELISPOT and flow cytometry, respectively. A piperacillin–BSA adduct was used as an antigen in ELISA antibody binding studies. Adducts generated using different ratios of drug to protein were used to determine the degree of conjugation required to detect IgG binding.

**Results:** B cells from hypersensitive patients, but not controls, were stimulated to secrete IgG and increase CD27 expression when cultured with soluble piperacillin. A piperacillin–BSA adduct with cyclized and hydrolysed forms of the hapten bound to eight lysine residues was used to detect hapten-specific IgG 1–4 subclasses in patient plasma. Hapten inhibition and the use of structurally unrelated hapten–BSA adducts confirmed antigen specificity. Antibody binding was detected with antigens generated at piperacillin/BSA ratios of 10:1 and above, which corresponded to a minimum epitope density of 1 for antibody binding.

**Conclusion:** These data show that antigen-specific B lymphocytes and T lymphocytes are activated in piperacillin-hypersensitive patients. Further work is needed to define the role different IgG subtypes play in regulating the iatrogenic disease.

Cystic fibrosis is a lethal autosomal recessive condition that leads to abnormal airway epithelial ion transport through mutations in a membrane-bound transporter. Recurrent infection develops as a consequence of mucus accumulation

in the lungs. Repeated courses of long-term  $\beta$ -lactam antibiotic are the cornerstone for management of respiratory exacerbations, but unfortunately their application is restricted due to delayed-type hypersensitivity reactions. Reactions develop at a higher frequency when compared to the general population (>30% of adult patients with cystic fibrosis experience  $\beta$ -lactam hypersensitivity) (1, 2). Patients present with rashes, fixed drug eruptions, arthralgia and drug fevers.

$\beta$ -Lactam antibiotics interact with and bind covalently to specific lysine residues on protein generating an antigen that may activate cellular immune responses in susceptible patients. We have recently focused on three commonly used

## Abbreviations

APC, allophycocyanin; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; HBSS, Hank's balanced salt solution; HSA, human serum albumin; MALDI, matrix-assisted laser desorption/ionization; MRM, multiple reaction monitoring; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PE, phycoerythrin.

drugs in patients with cystic fibrosis, piperacillin, meropenem and aztreonam and found that each forms a distinct haptenic structure on albumin resulting in activation of drug-specific CD4<sup>+</sup> T lymphocytes isolated from hypersensitive patients (3–5). T-cell cross-reactivity with the different drugs was not observed. The absence of detectable drug-specific T cells in tolerant patients exposed to several drug courses suggests that T cells are directly involved in the disease pathogenesis.

Drug–protein adducts might also activate B cells promoting hapten-specific immunoglobulin production (6–8). Once activated B cells differentiate, they are able to acquire a memory phenotype characterized by expression of cell surface receptors such as CD27<sup>+</sup> (9). Memory B cells reside primarily in peripheral blood and secrete immunoglobulin following re-exposure to antigen. *In vitro* activation of memory B cells following mitogen or specific antigen stimulation can be visualized using an IgG ELISPOT or increases in CD27 expression (10, 11). Antigen-specific memory B cells can be detected *ex vivo* in the presence or absence of circulating serum IgG; hence, it is important to measure memory B-cell activation alongside serum antibodies to obtain a more detailed analysis of the antigen-specific humoral immune response.

The role of humoral processes – specifically the activation of B cells and involvement of IgG antibodies in piperacillin hypersensitivity – has not been delineated. Thus, the objectives of this study were to (i) develop cell culture methods for the detection of B-cell responses including IgG subclasses in piperacillin-hypersensitive patients and (ii) characterize piperacillin protein binding and assess the relationship between hapten density and antibody binding.

## Methods

### Tissue culture reagents and antibodies

Culture medium for B-cell assays consisted of RPMI-1640 supplemented with 10% foetal bovine serum (FBS), 0.001% 2-mercaptoethanol, 100 mM L-glutamine, 100 g/ml penicillin and 100 U/ml streptomycin. FBS and human AB serum were purchased from Gibco, Life Technologies (Paisley, UK), and Innovative Research (Novi, MI, USA), respectively. All other culture reagents were purchased from Sigma-Aldrich (Poole, UK). CD3-fluorescein isothiocyanate (FITC), CD19-allophycocyanin (APC) and CD27-phycoerythrin (PE) antibodies used for flow cytometry were purchased from BD Biosciences (Oxford, UK).

### Patients' details

Patients were divided into three groups: piperacillin-hypersensitive ( $n = 3$ ), piperacillin-tolerant ( $n = 3$ ) and piperacillin-naïve ( $n = 3$ ) individuals. Hypersensitive patients developed maculopapular exanthema and in two cases drug-induced fever 2–9 days after the initiation of piperacillin therapy. The number of courses prior to the reactions ranged from 3 to 9. The analyses were conducted 3–8 years after the reactions subsided. Patients were defined as hypersensitive following clinical diagnosis and a positive lymphocyte transformation

test. Skin testing was not performed as previous studies show only 14% positivity in piperacillin-hypersensitive patients with cystic fibrosis. Provocation tests are contraindicated. Tolerant patients had previously been exposed to several piperacillin courses with no noted adverse effects. Naïve patients had never been exposed to the drug. Written informed consent was obtained from all patients, and the study was approved by the Leeds East Ethics Committee.

### Peripheral blood mononuclear cell isolation and T-cell activation studies

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood using Lymphoprep density gradient separation media (Axis Shield, Dundee, UK). Cells were suspended in B-cell culture medium and processed as outlined below or T-cell culture medium for the assessment of drug antigen-specific T-lymphocyte proliferative responses using the lymphocyte transformation test (12). Antigen-specific T-cell proliferative responses are presented as a stimulation index (SI; cpm in drug-treated cultures / cpm in control cultures), with an SI value  $\geq 2$  accepted as a positive response.

### B-cell activation studies

Solutions of CpG-DNA mitogen stimulation media and piperacillin were prepared in B-cell culture medium and transferred to 48-well culture plates containing  $1 \times 10^6$  PBMC per well. Final concentrations of CpG-DNA and piperacillin were 1.5  $\mu\text{g}/\text{ml}$  and 2 mM, respectively. Cells in the plates were cultured for 5 days at 5%  $\text{CO}_2/37^\circ\text{C}$ . PBMC were harvested on day 5 for IgG ELISPOT analysis and assessment of CD19/CD27 expression by flow cytometry. Culture supernatant was harvested for the assessment of secreted IgG by ELISA.

Cells were stained with anti-CD3, anti-CD19 and anti-CD27 antibodies and analysed using a Canto II flow cytometer (BD Biosciences) to assess the activation status of B cells. A minimum of 50 000 cells were acquired using forward and side scatter characteristics. Data were analysed using Cyflogic software (CyFlo, FL, USA). IgG release measured by ELISPOT utilized the method developed by Crotty et al. (13). Spot-forming units representing secreted IgG were visualized and analysed using an AID ELISPOT plate reader (Autoimmun Diagnostika, DE).

### ELISA for quantification of total and drug-specific IgG in patient plasma and cell culture supernatant

Total IgG was measured by ELISA using a goat anti-human IgG antibody. To quantify antipiperacillin-specific IgG, the plates were coated overnight at  $4^\circ\text{C}$  with the piperacillin–BSA adducts (20  $\mu\text{g}/\text{ml}$ ; in 0.05 M phosphate buffer, pH 7.2; 100  $\mu\text{l}/\text{well}$ ). After washing, patient plasma or culture supernatant was added for 1 h. IgG subclasses were quantified using horseradish peroxidase-labelled mouse anti-human IgG1–4 (Invitrogen, Paisley, UK) and NOR-01 human serum standard for IgG subclasses (Nordic immunology, Susteren, Netherlands).

### Preparation of piperacillin–BSA adducts

To prepare adducted proteins, piperacillin was incubated with BSA in PBS at drug/protein ratios of 1:1, 5:1, 10:1, 20:1, 50:1 and 100:1 for 24–96 h at 37°C, pH 7.4. Incubations containing BSA alone were prepared and processed in the same way to generate an unmodified negative control. The extraction procedure is available in the Data S1.

### Mass spectrometric analysis of piperacillin–BSA adducts

Trypsin was added to the piperacillin–BSA adducts, and the tubes were incubated at 37°C for 24 h. Piperacillin binding was initially quantified using matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. Subsequent analysis used a QTRAP 5500 hybrid quadrupole-linear ion trap mass spectrometer (ABSciex) to analyse relative levels of piperacillin binding at each modified lysine residue. 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 masses of the appropriate haptens (cyclized piperacillin, 517 amu; hydrolysed piperacillin, 535 amu); the parent ion masses were then paired with a fragment mass of 160 ( $[M+H]^+$  of cleaved thiazolidine ring) and/or a fragment mass of 106 ( $[M+H]^+$  of cleaved benzylamine group). 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.

## Results

### Lymphocyte transformation test

Preliminary experiments utilized the lymphocyte transformation test to determine whether T cells from piperacillin-hypersensitive and piperacillin-tolerant patients, as well as

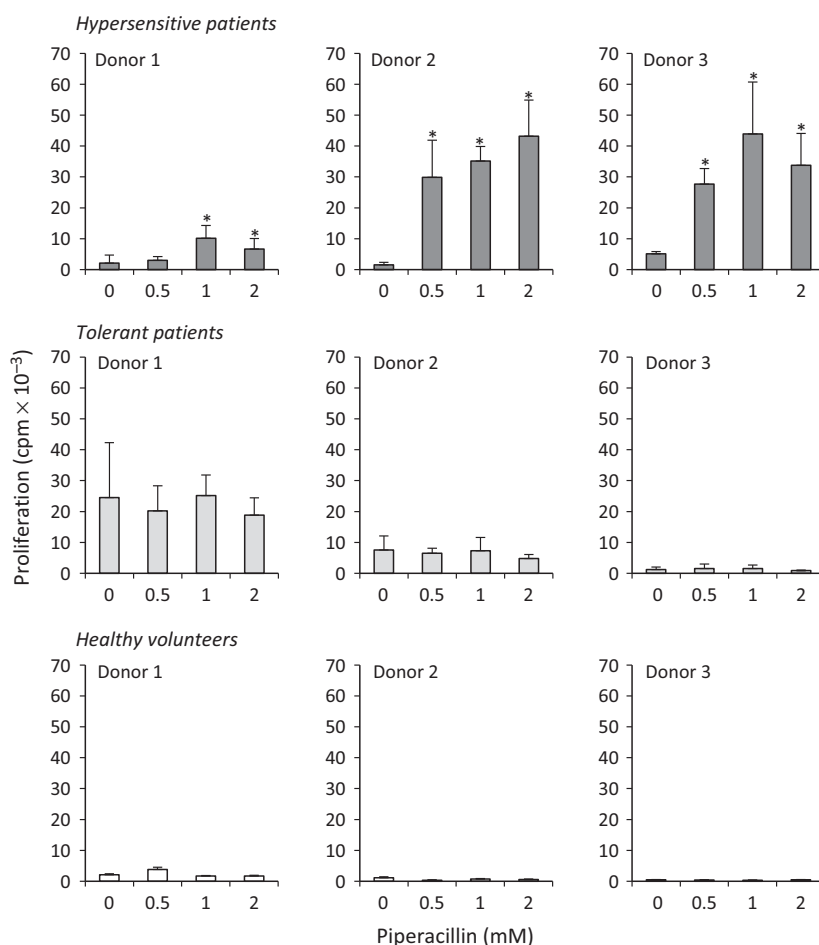


**Figure 1** Piperacillin-specific proliferation of PBMC from hypersensitive and tolerant patients and healthy volunteers. PBMC ( $1.5 \times 10^5$  cells in 100  $\mu\text{l}$ ) were incubated with graded concentrations of piperacillin (0.5–2 mM in 100  $\mu\text{l}$ ) in 96-well U-bottom plates. Plates were incubated at 37°C under an

atmosphere of 5%  $\text{CO}_2$  for 5 days. [ $^3\text{H}$ ]-thymidine (0.5  $\mu\text{Ci}$ /well) was added for the final 16 h of incubation and T-cell proliferation measured using scintillation counting with a beta counter. The data were analysed by Student's t-test with  $P < 0.05$  considered significant.

piperacillin-naïve volunteers, are stimulated to proliferate *in vitro* in the presence of the drug. Proliferative responses above control values were observed when PBMC from all three hypersensitive patients were cultured with piperacillin. Piperacillin-specific responses were dose dependent with the strongest responses detected at concentrations of 1–2 mM. The proliferative response tapered off at concentrations of 4 mM and above (results not shown). Thus, 1–2 mM piperacillin was selected for all subsequent experiments. PBMC from piperacillin-tolerant patients and piperacillin-naïve volunteers were not activated in response to piperacillin (Fig. 1).

#### Activation of B lymphocytes following piperacillin and mitogen treatment

PBMC isolated from each patient group were cultured with either mitogen or piperacillin for 5 days, after which CD19 and CD27 expression and IgG secretion were measured. A significant increase in cells staining positive for CD19 and CD27 was observed in all patients following mitogen treatment ( $P < 0.05$ ; Fig. 2). In contrast, an expansion of memory B cells (CD19+CD27+) with piperacillin was only observed with PBMC from hypersensitive patients.

PBMC ( $4\text{--}50 \times 10^3$ ) from piperacillin-naïve volunteers were used in preliminary experiments to determine the optimum conditions under which *in vitro* secretion of IgG from isolated B cells could be detected using ELISPOT. An

increase in the number of IgG-secreting cells was observed at each cell number when the mitogen-treated cells were compared to the negative control (results not shown). Experiments conducted with  $2 \times 10^4$  PBMC produced the most consistent results, and as such, this cell number was used in all subsequent experiments with PBMC from piperacillin-naïve, piperacillin-tolerant and piperacillin-hypersensitive patients. As observed in the initial experiments, an increase in the number of IgG-secreting cells was observed with PBMC from piperacillin-naïve volunteers and piperacillin-tolerant and allergic patients when mitogen-treated cells were compared to the negative control. Piperacillin-treated PBMC from hypersensitive patients also showed an increase in IgG secretion. In contrast, an increase in IgG secretion was not observed with PBMC from piperacillin-tolerant patients and healthy volunteers (Fig. 3A and B). Allergic patient 3 donated blood on four separate occasions over an 18-month period, and although the number of spot-forming units varied in each experiment, piperacillin consistently stimulated an increase in IgG secretion when compared with the negative control (Fig. 3C).

#### Piperacillin-specific IgG in hypersensitive patient plasma

Unmodified BSA was found to show low absorbance values, and hence, piperacillin–BSA adducts were generated, characterized and used for the detection of piperacillin-specific IgG.

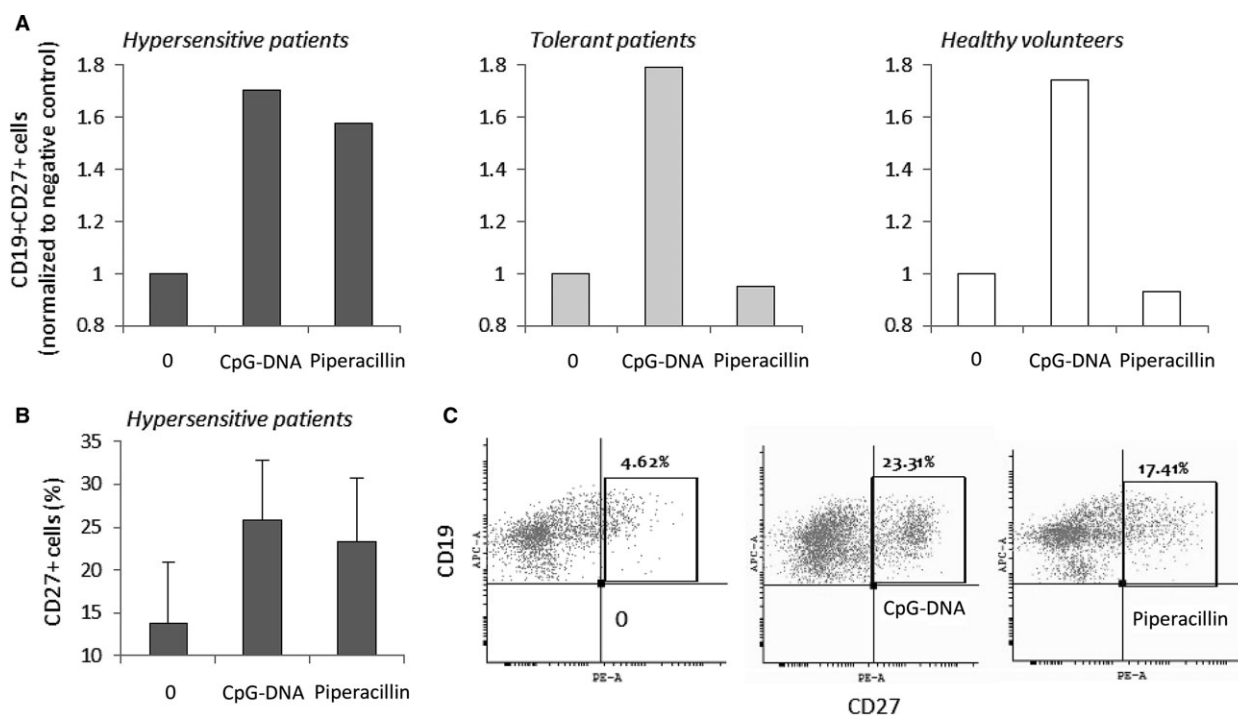


**Figure 2** CD27+ expression on piperacillin-treated B cells of hypersensitive and tolerant patients and healthy volunteers. PBMC ( $1 \times 10^6/1$  ml) were cultured in 24-well flat-bottomed plates with piperacillin (1–2 mM) and CpG-DNA (1.5  $\mu$ g/ml) for 5 days. CD27+ expression was measured by flow cytometry. (A) Comparison of

normalized results from hypersensitive and tolerant patients and healthy volunteers. (B) Number of hypersensitive patient CD19+ cells that express CD27 with and without treatment. (C) Representative flow cytometry images.

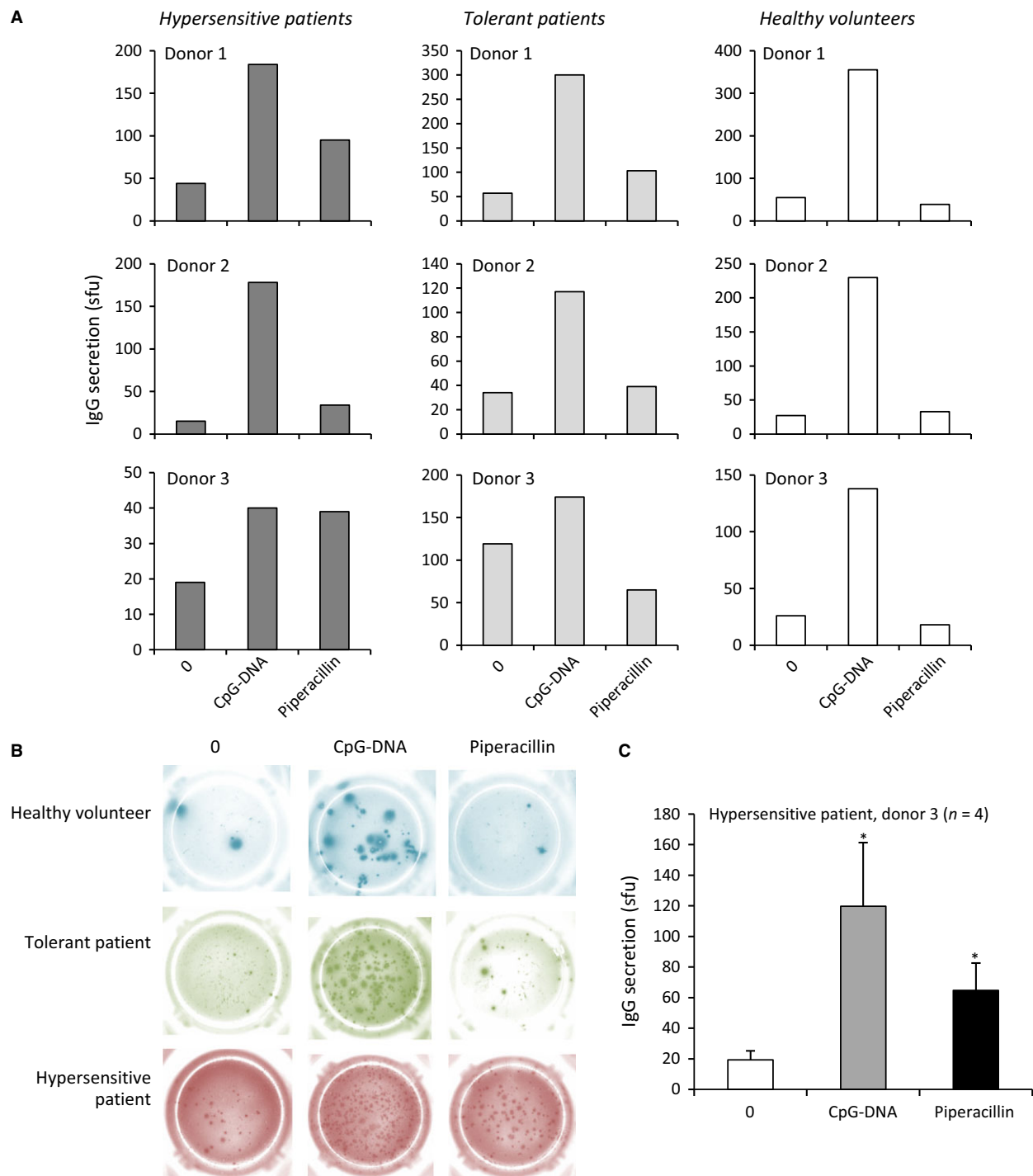


**Figure 3** Piperacillin-specific IgG secretion from B cells of hypersensitive and tolerant patients and healthy volunteers. PBMC ( $1 \times 10^6/1$  ml) were cultured in 24-well flat-bottomed plates with piperacillin (1–2 mM) and CpG-DNA (1.5  $\mu$ g/ml) for 5 days. (A) ELISPOT plates were precoated with anti-human IgG incubated overnight at 4°C. PBMC were harvested and  $5 \times 10^4$  transferred to each well and incubated for 48 h. ELISPOT plates were developed according to the manufacturer's instructions. Data were analysed

using an AID ELISPOT reader. Bar charts show results from individual patients. (B) Representative images from a tolerant and hypersensitive patient and a healthy volunteer. (C) Reproducibility of the ELISPOT data using hypersensitive patient 3 PBMC isolated from four separate blood donations over a two-year period. The data were analysed by Student's t-test with  $P < 0.05$  considered significant.

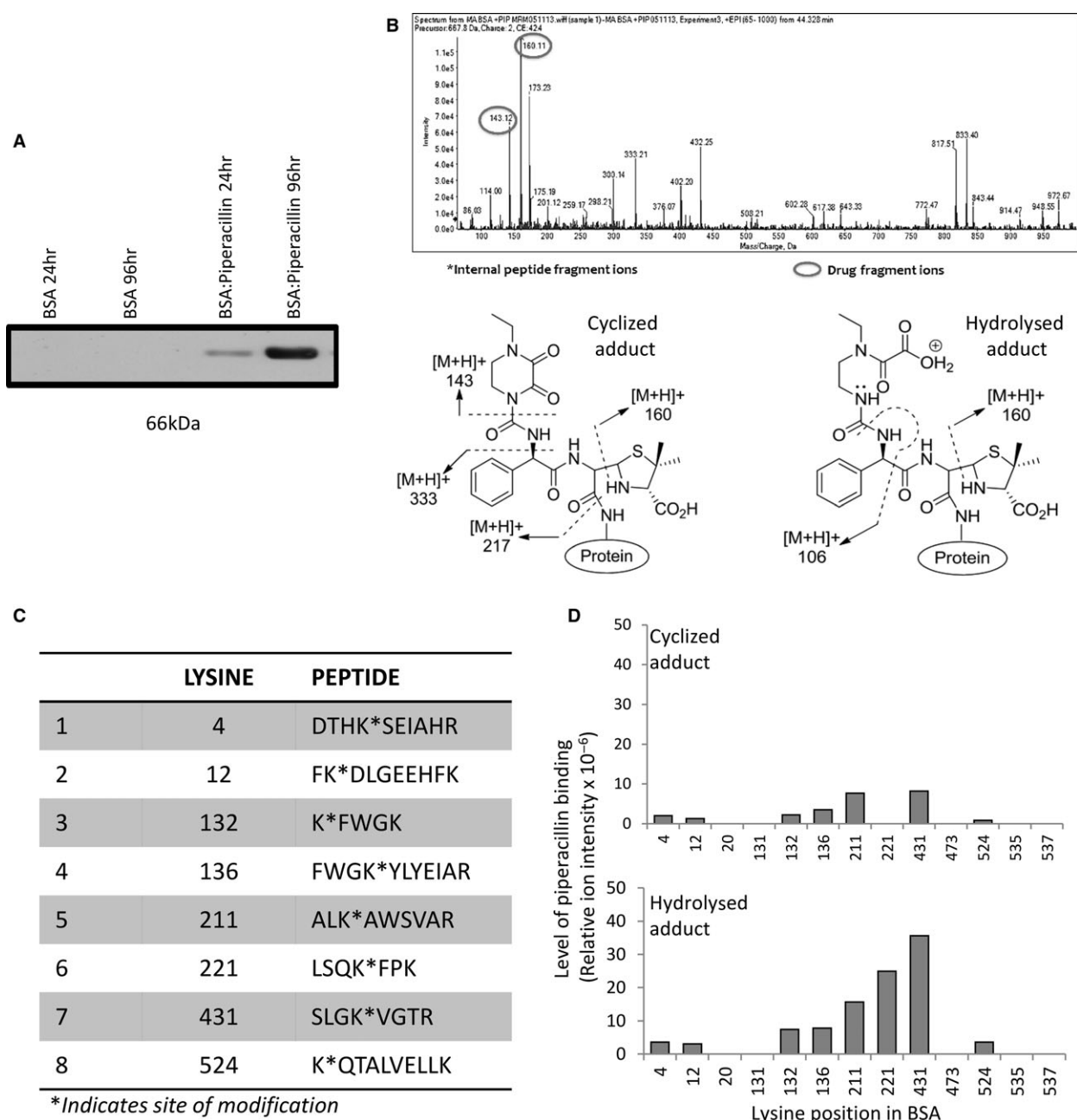


**Figure 4** Characterization of the piperacillin–BSA antigen. Piperacillin and BSA were incubated at a molar ratio of 50:1 for 24 or 96 h at 37°C. Unmodified drug was removed prior to analysis using immunochemical and mass spectrometric methods. (A) Unmodified and piperacillin-modified BSA were run on SDS-PAGE and blotted onto a nitrocellulose membrane. The membrane was blocked with 2.5% milk and incubated overnight at 4°C with a monoclonal mouse antipenicillin antibody. After washing, the membrane was incubated with goat anti-mouse HRP-conjugated secondary antibody prior to ECL development with photographic film. (B) Representative MRM spectral image of a BSA peptide containing a

piperacillin-modified lysine residue and chemical structure of the cyclized and hydrolysed forms of the piperacillin hapten bound covalently to BSA. Spectral images show piperacillin modification on Lys190 showing with the characteristic fragment ions at  $m/z$  160 and 143. (C) Table showing the tryptic peptide sequences containing lysine residues in BSA modified by piperacillin. Mass spectrophotometry was used to characterize the sites of modification. (D) Epitope profile showing the lysine residues of BSA modified with the cyclized and hydrolysed piperacillin haptens. Graphs show all 13 piperacillin binding sites in HSA.

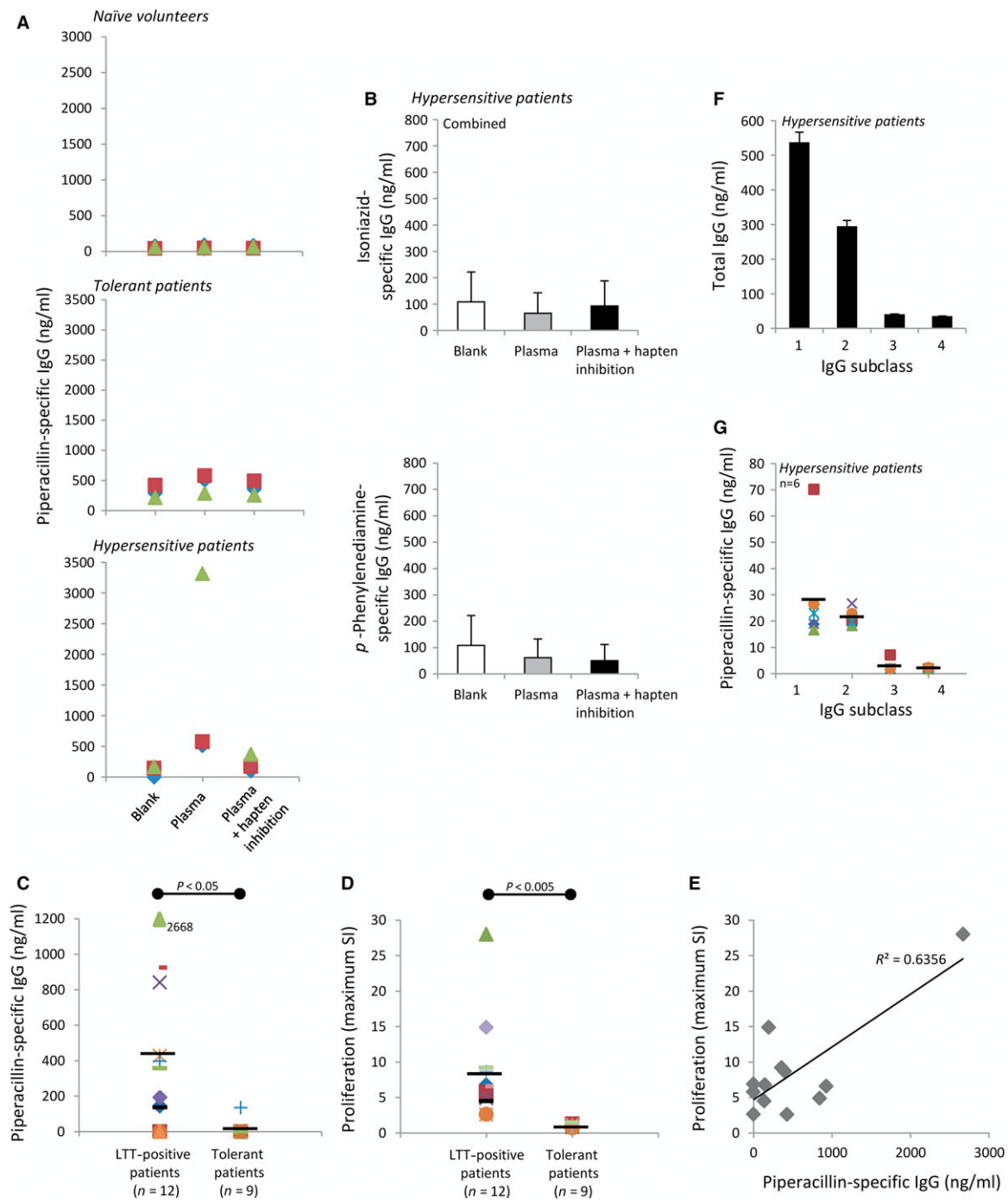


**Figure 5** Detection of piperacillin-specific IgG in plasma of hypersensitive and tolerant patients and healthy volunteers. (A) Detection of piperacillin-specific IgG (mean  $\pm$  SD) by ELISA from each patient group ( $n = 3$  per group). An aliquot of plasma was preincubated with an excess of piperacillin for the analysis of hapten inhibition. (B) Detection of IgG binding to structurally unrelated chemical and drug antigens using plasma from hypersensitive patients. Results presented as mean  $\pm$  SD ( $n = 3$  per group). (C) Expression of piperacillin-specific IgG in plasma of 12 lymphocyte transformation test-positive patients. Each data point shows ng/ml in patient plasma

with plasma + hapten inhibition subtracted. (D) Maximum lymphocyte transformation test result with PBMC from patients in (C). Each coloured symbol shows results from one patient. (E) Correlation of piperacillin-specific PBMC proliferation with detection of specific IgG in plasma. (F) Expression of total IgG subclasses in plasma of patients. (G) Expression of piperacillin-specific IgG subclasses in six hypersensitive patients. Colour coding does not refer to the same patients shown in (C) and (D). Data were analysed by Student's *t*-test to compare the difference between means with  $P \leq 0.05$  considered as significant.

SDS-PAGE and Western blot analysis of a piperacillin–BSA adduct, prepared using a 50:1 ratio of drug to protein, showed that drug binding was time dependent with 96 h shown to be the optimum time for adduct formation (Fig. 4A). Mass spectrometric analysis identified the characteristic fragment ions  $m/z$  160 and 143 (Fig. 4B). Piperacillin haptens were detected on eight of the 13 lysine residues modified in HSA (Fig. 4C). Relative levels of modification at each lysine residue are shown in Fig. 4D.

Antipiperacillin-specific IgG was detected in plasma of each hypersensitive patient at levels ranging from 500 to 3000 ng/ml (Fig. 5A). In each case, the addition of an excess of piperacillin to plasma prevented piperacillin–BSA IgG binding. Antipiperacillin-specific IgG was not detected in plasma from either drug-tolerant patients or drug-naïve volunteers.

*p*-Phenylenediamine–BSA adducts and isoniazid–BSA adducts were prepared according to the methods of Jenkinson et al. and Meng et al. (14, 15), respectively, to confirm the specificity of piperacillin-specific IgG. An increase in absorbance readings was not observed when piperacillin-hypersensitive patient plasma was added to ELISA plates coated with *p*-phenylenediamine–BSA adducts or isoniazid–BSA adducts (Fig. 5B).

To determine whether our findings with three hypersensitive patients are representative of piperacillin-hypersensitive patients in general, plasma from 12 lymphocyte transformation test-positive patients and nine drug-tolerant controls (Fig. 5D) was used to quantify piperacillin-specific IgG by ELISA. Piperacillin-specific IgG was detected in nine hypersensitive patients. In contrast, plasma from only one tolerant control displayed low levels of piperacillin-specific IgG (Fig. 5C). A weak correlation between lymphocyte proliferation and levels of piperacillin-specific IgG in plasma was observed (Fig. 5E); however, the data are somewhat skewed by data from one patient with the strongest lymphocyte proliferation data.

#### IgG subclass analysis in allergic patient plasma

There are four classes of IgG: IgG1 (60–65%; approximate abundance in humans), IgG2 (20–25%), IgG3 (5–10%) and IgG4 (4%) (16–19). IgG subclass analysis of total IgG in plasma from piperacillin-hypersensitive patients revealed the expected profile with antibody classes in the order of IgG1 > IgG2 > IgG3 > IgG4 (Fig. 5F). The piperacillin–BSA adduct was used as an antigen to assess the IgG subclasses with specificity for piperacillin. Antipiperacillin-specific IgG expression seemed to show a bias for IgG2 over other subclasses; IgG1 and IgG2 were expressed at approximately the same level in five of six hypersensitive patients (Fig. 5G). The ratio of piperacillin-specific IgG subclasses in one patient was similar to that seen with total IgG.

#### Hapten density-dependent binding of piperacillin-specific antibodies to BSA adducts

Piperacillin–BSA adducts with various hapten densities were synthesized by incubating piperacillin with BSA at molar

ratios of 1:1, 5:1, 10:1, 20:1, 50:1 and 100:1 for 96 h. SDS-PAGE analysis of the adducts revealed bands of increasing intensity at 66 kDa as the concentration of piperacillin was increased (Fig. 6A). The relative level of piperacillin binding at each of the eight modified lysine residues (Lys 4, 12, 132, 136, 211, 221, 431 and 524) is shown in Fig. 6B. The level of binding increased with increasing piperacillin concentration at each site of modification (Fig. 6B). However, the highest ion counts were consistently detected with the peptide containing Lys 431 (Fig. 6C). An increase in the total number of sites modified with piperacillin was not observed as the drug/protein ratio increased. MALDI-TOF analysis showed the corresponding masses of the different adducts generated. The mass values were used to estimate the density of the piperacillin hapten bound to BSA (Fig. 6D).

Plasma samples from the hypersensitive patients were then used to explore the influence of hapten density on piperacillin-specific IgG binding. A direct correlation between hapten density and IgG binding was observed ( $r^2 = 0.9574$ ). Piperacillin-specific IgG binding was observed with antigen generated using drug/protein ratios of 10:1 and above (Fig. 6E).

#### Discussion

$\beta$ -Lactam antibiotics are a common cause of delayed-type hypersensitivity. Drug binding to protein lysine residues is believed to represent the principal initiating event for the activation of an immune response and tissue injury in susceptible patients. Mass spectrometry has previously been used to characterize the nucleophilic targets on HSA for  $\beta$ -lactam hapten binding in patients (5, 20, 21). Subsequently, synthetic  $\beta$ -lactam–HSA adducts with the drug hapten bound to the lysine residues modified *in vivo* were used as an antigen in *in vitro* T-cell assays. The adduct was found to activate PBMC and T-cell clones from hypersensitive, but not tolerant, patients to proliferate and secrete cytokines via a pathway dependent on protein processing by antigen-presenting cells (4, 5, 22). Hence, hapten-specific T cells and the effector molecules they secrete are thought to be responsible for the initiation and regulation of delayed-type  $\beta$ -lactam reactions.  $\beta$ -Lactam–protein adducts also activate a humoral response in hypersensitive patients; (6–8) however, investigation of the nature of the response has been knowingly or otherwise neglected. Thus, the objectives of this study were to characterize drug-specific B-cell responses in hypersensitive patients, analyse whether drug-specific IgG circulates in plasma and explore the relationship between hapten density and IgG binding. Piperacillin-hypersensitive patients with cystic fibrosis were selected as the study cohort because the piperacillin–HSA binding interaction and the drug hapten-specific cellular response have been characterized previously (3–5).

Initially, the lymphocyte transformation test was used to confirm the presence of drug-responsive T cells in hypersensitive patients. PBMC from all three hypersensitive patients were stimulated to proliferate *in vitro* in a concentration-dependent manner. In contrast, T cells from drug-tolerant

patients and drug-naïve volunteers were not activated with piperacillin. To detect a B-cell response to piperacillin, PBMC from each patient group were cultured with the drug for 5 days prior to the detection of specific B-cell activation markers by flow cytometry and IgG secretory profiles by ELISPOT. CpG-DNA was selected as a positive control because of its propensity to activate immature and mature B cells via TLR9 stimulation (23) leading to both cell proliferation and IgG production (24).

Memory B cells with the ability to synthesize and rapidly secrete immunoglobulins can be differentiated from their naïve counterparts by enhanced expression of CD27+ (9, 25). Thus, CD27 was used as a marker on CD19+ B cells to quantify the number of memory B cells after incubation of PBMC with piperacillin or CpG-DNA. Flow cytometric assessment of PBMC from hypersensitive patients showed an increase in expression of CD27+ on B cells in response to piperacillin (and mitogen) treatment. In contrast, a piperacillin-specific increase in CD27 expression was not observed on cells from tolerant patients or healthy donors. These data are in agreement with previous studies that show an increase in the number of memory B cells in patients infected with *Schistosoma Haematobium* (26). Piperacillin treatment of PBMC from hypersensitive patients also led to an increase in the secretion of IgG, visualized using a memory B-cell ELISPOT assay established by Crotty et al. (13). However, no difference in IgG secretion was observed with PBMC from tolerant patients and naïve volunteers after drug treatment. Collectively, these data indicate that piperacillin-responsive memory B cells circulate in the peripheral blood of hypersensitive, but not tolerant, patients for multiple years after the initial exposure.

ELISA has proved useful in both the detection and assessment of antibody responses against protein and drug antigens (6, 27, 28). A piperacillin-BSA adduct was generated and employed as an antigen in a hapten inhibition ELISA for unambiguous analysis of IgG specific to piperacillin. As described in our previous study using HSA as a protein carrier (5), piperacillin formed archetypal adducts on lysine residues of BSA through opening of the  $\beta$ -lactam ring. Moreover, an additional hapten structure was detected in which the 2,3-dioxopiperazine ring had undergone hydrolysis. Modification of eight lysine residues was detected on BSA under the experimental conditions used to generate an antigen (50:1 piperacillin/BSA, 96-h incubation) for

immunochemical detection of piperacillin-specific IgG. Each site of modification paralleled a piperacillin-modified lysine residue on HSA, further highlighting the acute specificity of the binding interaction to hydrophobic pockets in the protein that have previously been shown to be involved in the noncovalent docking of low molecular weight compounds (29, 30). Hapten-inhibitable antidrug antibodies specific to piperacillin were detected in plasma from the hypersensitive patients. These data suggest that the IgG circulating in hypersensitive patient plasma exhibits specificity for the piperacillin hapten. This was confirmed through (i) the generation of BSA adducts using structurally unrelated chemical (*p*-phenylenediamine) and drug (isoniazid) haptens and assessment of IgG binding and (ii) analysis of a larger patient cohort. IgG circulating in piperacillin-hypersensitive patients did not bind to either *p*-phenylenediamine-protein adducts or isoniazid-protein adducts. However, piperacillin-specific IgG was detected in nine of 12 piperacillin lymphocyte transformation test-positive patients, but only one of nine piperacillin-tolerant controls.

To explore the impact of the carrier protein on the detection of piperacillin-specific IgG, piperacillin-human serum albumin and piperacillin-lysozyme adducts were generated and characterized in terms of relative levels of lysine modification. Unfortunately, IgG quantification experiments were hindered by high levels of nonspecific binding associated with the use of the protein carrier alone. Thus, future studies should attempt to identify alternative protein carriers to determine the importance of the protein structure in antibody binding. Piperacillin hapten-specific IgG was not detected in plasma of naïve volunteers, whereas low levels were found in one tolerant control.

The previously described profile of total IgG subclasses (IgG1 > IgG2 > IgG3 > IgG4) was detected in hypersensitive and tolerant patient plasma. A similar analysis of piperacillin hapten-specific IgG showed a bias for IgG2 over other subclasses in five of six hypersensitive patients. An increased susceptibility to certain bacterial infections is related to a deficiency in IgG2, signifying a role for IgG2 in combating bacterial pathogens (31). Moreover, patients with immediate allergic reactions to food have been shown to have significantly raised levels of antigen-specific IgG2 (32).

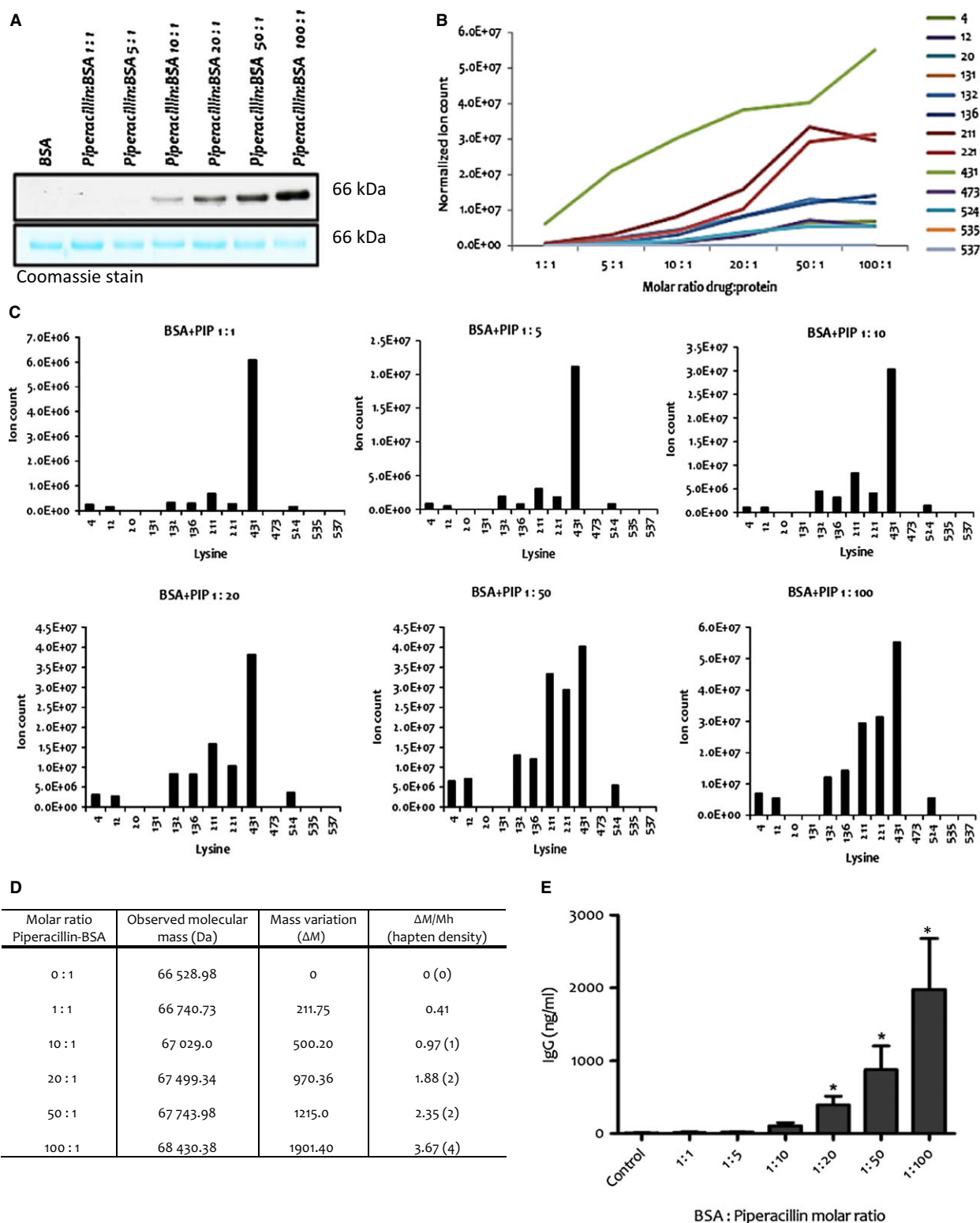
The ratio at which drugs and proteins are conjugated has previously been shown to influence the nature of the antibodies induced by the hapten, with an increase in epitope

**Figure 6** Piperacillin-specific IgG binding to antigens with different epitope profiles. Piperacillin and BSA were incubated at ratios of 1:1, 5:1, 10:1, 20:1, 50:1 and 100:1 (piperacillin/BSA) for 96 h at 37°C. Free drug was removed and adducts were characterized using immunochemical and mass spectrometric methods. (A) Western blot and an antipenicillin mouse monoclonal antibody were used to show the dose-dependent binding of piperacillin to BSA. (B) Concentration-dependent increase in piperacillin hapten binding (cyclized and hydrolysed forms combined) at each modified lysine residue of the piperacillin-BSA antigens generated using different molar ratios of drug to protein. (C) Epitope profiles of the

piperacillin-BSA antigen generated using different molar ratios of drug to protein. (D) Quantification of piperacillin-BSA antigen. The observed molecular mass values from the MALDI-TOF were obtained, and these values were used to determine the mass variations detected. The ratio of the variations to the molecular mass of piperacillin ( $\Delta M/M_h$ ) produced the hapten density. (E) IgG antibody binding to the different piperacillin-BSA antigens. Plasma from hypersensitive patients was incubated with the plate-bound antigens and the level of binding quantified using ELISA. Data were analysed by Student's *t*-test to compare the difference between means with  $P \leq 0.05$  considered as significant.

density usually bringing about an increase in the strength and specificity of the immune response. Therefore, the final component of our study was directed towards investigating the relationship between piperacillin hapten density and

antibody binding. A range of piperacillin–BSA adducts were generated at a drug/protein ratio of 1:1–100:1. MALDI-TOF analysis showed that the number of piperacillin molecules bound to BSA increased in a linear fashion with an



increase in the piperacillin/BSA ratio. Based on the molecular mass of the adducts, it was possible to estimate that the hapten density ranged from 0.4 to 3.7 (molecules of piperacillin bound covalently to each molecule of BSA). As expected, the relative level of binding increased at each modified lysine residue with increasing concentrations of piperacillin. Antibody binding was initially detectable using an adduct generated at a piperacillin/BSA ratio of 10:1, which corresponded to an epitope density of approximately 1. The extent of antibody binding then escalated in an incremental fashion with an increase in the epitope density ( $r^2 = 0.9574$ ).

To conclude, our data show the activation of B cells isolated from hypersensitive patients with piperacillin. The presence of circulating piperacillin-specific IgG was detected in nine of 12 patients with a positive lymphocyte transformation test, but only in one of nine tolerant controls. Thus, future studies should investigate how antibodies interact with T cells (i) during the pathogenic response and (ii) in patients undergoing desensitization with piperacillin. Furthermore, it would be interesting to explore whether the methods developed here could be used to detect other classes of piperacillin-specific antibody.

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## Conflicts of interest

The authors declare no competing financial interest.

## Author contributions

MOA, AS, JF and LF conducted the biological experiments. REJ and XM prepared the conjugates and conducted the mass spectrometric analyses. PW and DP collected the clinical samples. BKP and DJN designed the study. MOA and DJN analysed the data and drafted the manuscript. All authors critically reviewed the manuscript.

## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Data S1** Methods.

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