Antineutrophil cytoplasmic antibody (ANCA)-associated systemic vasculitis after immunisation with bacterial proteins

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Abstract

Objective

There is circumstantial evidence for a role for infections in the development of the small vessel vasculitides associated with antineutrophil cytoplasmic antibodies (ANCA). The aim of this study was to determine whether the immunisation of rats with bacterial proteins could result in circulating ANCA, T cells with specificity for ANCA antigens, and a systemic vasculitis.

Methods

Adult male Wistar rats were immunised with pasteurised sonicated S. aureus *(n=7),* E. coli *(n=8), purified protein derivative (PPD, n=5), myeloperoxidase (MPO, n=5) or phosphate-buffered saline (PBS, n=5), in complete and in incomplete Freund's adjuvant. ANCA were assayed by indirect immunofluorescent (IIF) examination of normal rat neutrophils, and in ELISAs using human proteinase 3 (PR3), MPO and bactericidal/permeability-increasing protein (BPI). The T cell response to PR3, MPO and BPI was assessed by a whole blood T cell proliferative assay* in vitro, *and by a delayed type hypersensitivity (DTH) response* in vivo. *Kidney and bowel were examined histologically for evidence of vasculitis and colitis.*

Results

One rat from each group immunised with S. aureus *or* E. coli *developed pauciimmune segmental glomerular sclerosis. The rat immunised with* E. coli *had additionally an arteritis affecting renal interlobular and gut vessels. This rat had circulating C-ANCA, that produced granular cytoplasmic neutrophil fluorescence with central accentuation, but the target antigen could not be determined in ELISAs using human PR3, MPO or BPI. In animals immunised with* S. aureus *or* E. coli, *there was no significant T cell proliferative or DTH response specific for human PR3, MPO or BPI.*

Conclusion

The development of ANCA and vasculitis in a rat immunised with bacterial proteins indicates that the relationship between infections and ANCA should be investigated further.

Key words

ANCA, cross-reactivity, epitopes, DTH, vasculitis.

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Abbreviations:
BPI: bactericidal/permeability increasing protein; C-ANCA and P-ANCA: cytoplasmic and perinuclear antineutrophil cytoplasmic antibodies;
CFA and IFA: complete and incomplete Freund's adjuvant;
DTH: delayed type hypersensitivity;
HSA: human serum albumin;
IIF: indirect immunofluorescence;
MPO: myeloperoxidase;
PBS: phosphate-buffered saline;
PPD: purified protein derivative;
PR3: proteinase 3.

Introduction

Antineutrophil cytoplasmic antibodies (ANCA) are autoantibodies directed against neutrophil granular enzymes (1). They can be demonstrated in most patients with Wegener's granulomatosis (1) and microscopic polyangiitis (2), when the target antigens are usually proteinase 3 (PR3) and myeloperoxidase (MPO) (3-5). ANCA are also common in individuals with inflammatory bowel disease (6,7) and cystic fibrosis (8), when the target antigens include bactericidal/permeability-increasing protein (BPI) (8-11).

There is circumstantial evidence for infections contributing to the development of ANCA and some ANCA-associated diseases. Such evidence includes the increased frequency of preceding bacterial lung infections in patients with Wegener's granulomatosis and microscopic polyangiitis (12-14); case reports of ANCA-associated systemic vasculitis occurring after appendicitis or a wound infection (15,16); the increased frequency of relapses in patients with Wegener's granulomatosis who have persisting nasal *S. aureus* (17); and the reduction in upper respiratory tract relapses with continuous trimethoprim-sulphamethoxazole (18). Bacterial contact may also contribute to the development of ANCA in patients with cystic fibrosis (11), and in inflammatory bowel disease.

The mechanisms that underly the putative relationship between infections and the development of ANCA and the ANCA-associated diseases are not known. Cross-reactivity between bacterial or viral protein sequences and target antigen epitopes has been suggested with a herpes simplex protein and U1RNP (19), *K. pneumoniae* nitrogenase and B27 (20), and the nucleocapsid protein of the vesicular stomatitis virus and the Ro antigen (21), among others. These target amino acid sequences have usually been defined by the binding of autoantibodies to synthetic overlapping peptides, and homology with bacterial proteins demonstrated from a database. However most ANCA antigen epitopes are conformational rather than linear (22,23), and homologous sequences defined in this way may be unimportant (24).

The aim of the study described here was to determine whether bacterial proteins from *S. aureus* or *E. coli* could directly induce ANCA,ANCA antigen-specific T cells, and the ANCA-associated diseases. Adult male Wistar rats were immunised with pasteurised sonicated preparations of *S. aureus* or *E. coli*, and the animals were then tested for antibody and T cell responses against the major ANCA target antigens, PR3, MPO and BPI, and examined for histological evidence of vasculitis and colitis.

Materials and methods

Animals

Adult male Wistar rats were immunised with pasteurised sonicated *S. aureus* ATCC 25923 (n=7), *E. coli* ATCC 25922 (n=8), purified protein derivative (PPD, CSL, Melbourne, n=5), MPO (Calbiochem, n=5) or phosphate-buffered saline (PBS, n=5). *S. aureus* and *E. coli* were prepared from single colonies that had been incubated overnight at 37°C in Luria broth (Tryptone (DIFCO) 10 g/l, yeast extract (DIFCO) 5g/L, NaCl 9 g/l) with vigorous shaking. The bacterial cells were then sonicated at 300 Hz for one minute, pasteurised by heating to 60°C for one hour, and washed and resuspended in 0.9% NaCl. The protein concentration was determined using a Genequant spectrophotometer (Pharmacia Biotec) at 260 nm.

Each injection contained 10 mg of bacterial proteins, 200 U of PPD or 10 mg MPO, diluted in sterile PBS to a total volume of 250 µl, and emulsified in an equal volume of Incomplete Freund's Adjuvant (IFA, Sigma). Rats were initially immunised subcutaneously at about 10 day intervals (x5), without anaesthetic. However preliminary experiments showed that none of the rats injected with MPO developed MPO-ANCA or an MPO-specific T cell proliferative response, and subsequent immunisations were performed using Complete Freund's adjuvant (CFA, x3), and IFA (x3). Ten days after the final immunisation, the rats were bled, and delayed type hypersensitivity (DTH) testing performed. The rats were killed

upon completion of these experiments. This project had the approval of the Animal Ethics Committee of the Austin and Repatriation Medical Centre.

ANCA

Two ml of blood was collected from the tail veins of the rats into heparinised tubes, and plasma was prepared from 1 ml by centrifugation at 1500 g for 5 minutes, and then stored at -20° C.

Plasma samples were assayed for ANCA by IIF using normal rat peripheral blood smears fixed in ethanol. Smears were incubated with 1/10 dilutions of plasma in PBS for 30 minutes, washed and then incubated with 1/100 FITC-conjugated rabbit antirat IgG (Silenus) for 30 minutes, before further washing and examination using a fluorescent microscope. Sera from patients with active systemic vasculitis that produced cytoplasmic or perinuclear fluorescence on human blood smears prepared and fixed in the same way were included in the run. Fluorescence of these smears was however developed with a 1/100 FITC-conjugated sheep antihuman $F(ab)'_2$ IgG (AMRAD, Australia).

Plasma were also assayed for PR3-, MPO- and BPI-ANCA by ELISAs. Human PR3 (Wieslab, 10 µg/ml), MPO (12.5 µg/ml), both diluted in acetate buffer (50 mM sodium acetate, 100 mM, NaCl at pH 6.0), and BPI (Wieslab) 7 µg/ml in 0.05M carbonate buffer, pH 9.6, were coated to microtitre plates (Costar) by incubation at 4°C for 18 hours. These proteins produced single bands on polyacrylamide gel electrophoresis. Coated and uncoated plates were then blocked with phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (Calbiochem) and 0.05% Tween 20 (Sigma) at 37°C for one hour. Rat plasma were tested at a 1/100 dilution in blocking buffer, and alkaline phosphatase-conjugated goat-anti-rat IgG (Silenus) was also diluted 1/500 in blocking buffer. Samples were tested in duplicate and the mean values calculated after the binding to antigen-free coated plates had been subtracted. The normal range was determined from the mean plus 3 standard deviations of 15 normal rat plasma.

ANCA antigen-specific DTH T cell response

Rats were anaesthetised with isoflurane and the thickness of the dorsal pinna of the ear measured to 0.01 mm with an engineer's spring micrometer. Then 10 µg of PR3 in a total volume of 10 µl PBS was injected into the same spot using a 30 gauge needle. Twenty-four hours later, the rats were anaesthetised and the thickness of the pinna remeasured. After any swelling had subsided, the procedure was repeated for MPO, BPI and human serum albumin (HSA). Results were analysed using Fisher's exact probability test.

ANCA antigen-specific T cell response using a whole blood proliferative assay (25)

The T cell proliferative assay was performed in sterile round bottomed microtitre plates (Greiner, Labortechnik), with 20 µl of whole blood, 160 µl complete RPMI-10 (Trace Biosciences, containing 10% gentamicin, 0.4% L-glutamine (Calbiochem) and 0.04% β-mercaptoethanol), together with antigen in each well. The antigens tested were PR3, MPO, BPI and lactoferrin (Sigma) (all 10 µg). The response to individual antigens in each rat were assayed at least in triplicate, both before and after immunisation as described above.

The plates were incubated in a cell culture incubator with a humidified atmosphere of 5% CO_2 for 6 days. On the sixth day, 20 ml of complete RPMI-10 (without βmercaptoethanol) containing 1 mCi methyl^{3}H-thymidine (Amersham) with a specific activity of 25 Ci/mmol was added to each well. The T cell proliferative response was measured by the incorporation of methyl-^{3}H- thymidine into the cells after a further 6 hour incubation.

Cells were harvested using a multicell harvester (Silter mate 196 Packard harvester). The supernate was aspirated, the cells lysed and the incorporated radioactive level released onto filter papers (Packard) that were dried at 65°C. Each filter dot was then placed in a polyethylene scintillation counting vial containing 1 ml tetrahydrofuran (Sigma) and 5 ml scintillant. Counting took place in a scintillation counter (Packard).

The stimulation index (SI) for individual wells was calculated by dividing the counts in the wells containing antigen by the median value of the counts in the wells that had no antigen (background). A positive response was defined as a value ≥ 3.0. Results were analysed using Analysis Of Variance (ANOVA).

Histological examination of kidneys and large bowel

Kidneys and large bowel were obtained from rats injected with *S. aureus* (n = 3), *E. coli* (n = 4), PPD (n = 4), MPO (n = 5) and PBS (n = 2). Whole kidneys and any suspicious areas of the gut were fixed in formalin, stained with haematoxylin and eosin, and examined by a histopathologist. Some kidneys were further examined for immune deposits by electron microscopy. Formalin blocks were dewaxed and post-fixed in 1.5% osmium tetroxide and embedded in Spurr's resin; thin sections were cut on a LBK Ultrotome, and the grids were examined in a JEOL 1200 EX transmission electron microscope.

Results

ANCA (Table I, Fig. 1)

None of the rats immunised with *S. aureus* developed ANCA demonstrated by neutrophil fluorescence, or in the ELISAs using human PR3 or MPO. Four of these rats (4/7, 57%) developed BPI-ANCA, but antibody levels were all low (OD < 0.2). One animal immunised with *E. coli* developed cytoplasmic neutrophil fluorescence (C-ANCA) (Fig. 1A), but the antigen specificity could not be determined in the ELISAs. None of these rats developed BPI-ANCA. Four of the 5 rats (80%) immunised with MPO developed MPO-ANCA, and 3 of the 5 immunised with PPD (60%) developed low but positive levels of MPO-ANCA (OD < 0.2).

In vivo *DTH response* (Table II)

None of the rats immunised with *S. aureus* or *E. coli* developed a DTH response to human PR3, MPO or BPI that could be demonstrated by a significant increase in ear thickness after

Table I. PR3-,MPO and BPI-ANCA response in rats immunised with *S. aureus, E. coli* etc.

Antigen	Pre-immunisation	After immunisation with S. aureus (n=7)	E. coli (n=8)	PPD (n=5)	MPO (n=5)	PBS (n=5)
PR3	0/30 (0%)	0/7 (0%)	0/8 (0%)	0/5 (0%)	1/5 (20%)	0/5 (0%)
MPO	0/30 (0%)	0/7 (0%)	0/8 (0%)	*3/5 (60%)	4/5 (80%)	0/5 (0%)
BPI	nd	*4/7 (57%)	0/8 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)

*OD < 0.20
PPD:purified protein derivative; MPO: myeloperoxidase; PBS: phosphate buffered saline; nd: not done. Peripheral blood from each rat was assayed in duplicate. Positive results bound at greater than mean plus 3SD of the binding of 15 normal rats.

subcutaneous injection of these antigens. However, there was an increase in ear thickness in response to MPO in rats immunised with MPO ($p < 0.05$), or with PPD ($p < 0.05$), which correlated with the finding of MPO-ANCA in these rats.

In vitro *T cell proliferative response* (Table III)
The rats immunised with *S. aureus* or *E. coli* did not demonstrate a significantly more frequent T cell proliferative response to PR3, MPO or BPI (where tested) compared with the response after immunisation with lactoferrin (Table III). However T cells from rats immunised with MPO responded more often to MPO than to lactoferrin ($p < 0.05$).

Histological appearance of kidneys and bowel (Fig. 1 B-D)
One rat of the 3 rats immunised with *S. aureus* in whom histology was performed had segmental glomerular sclerosis (Fig. 1B), but no evidence of

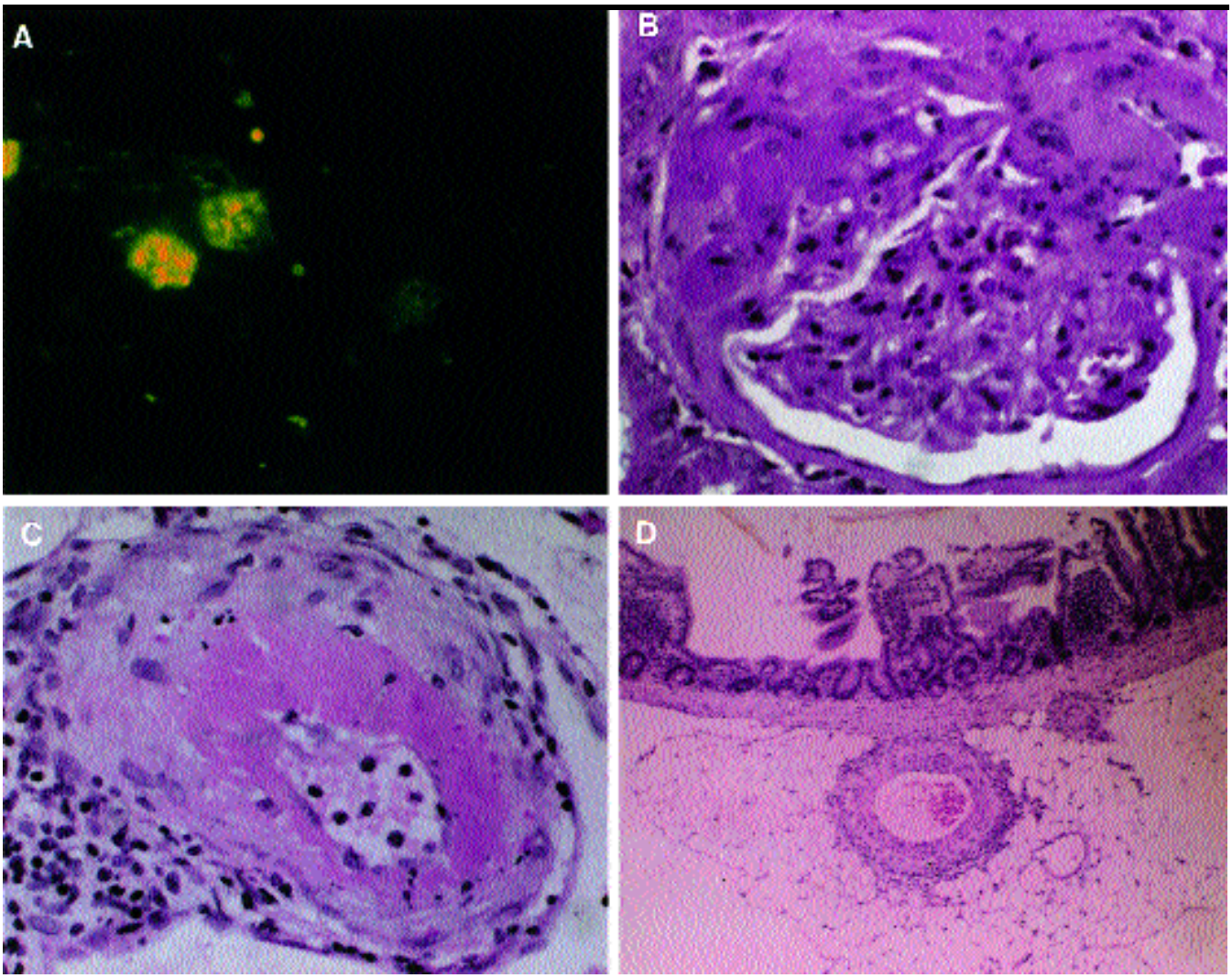

Fig. 1. (**A**) IIF of rat peripheral blood smear showing diffuse cytoplasmic neutrophil fluorescence with central accentuation; (**B**) segmental sclerosis of rat glomerulus after immunisation with *S. aureus*; (**C**) necrotising arteritis with leucocytoclastic debris of small muscular extrarenal artery after immunisation with *E. coli*; and (**D**) panarteritis of gut vessel in rat after immunisation with *E. coli*.

Table II. DTH response to PR3,MPO and BPI in rats immunised with *S. aureus, E. coli* etc.

	After immunisation with				
Antigen	*S. aureus* (n = 7)	*E. coli* (n = 8)	PPD (n = 5)	MPO (n = 5)	PBS (n = 5)
PR3	0.64	0.93	0.94	0.84	0.93
MPO	0.18	0.35	0.42*	0.60*	0.16
BPI	0.58	0.44	0.79	0.63	0.35
HSA	0.17	0.21	0.29	0.17	0.22

*p < 0.05
PPD: purified protein derivative; MPO: myeloperoxidase; PBS: phosphate buffered saline; PR3 proteinase 3; BPI: bactericidal/permeability-increasing protein; HSA: human serum albumin.
The response was measured as an increase in ear thickness 24 hours after the subcutaneous injection of antigen (mean, in mm).

Table III. T cell proliferative response to neutrophil antigens in rats immunised with *S. aureus, E. coli* etc.

		After immunisation with				
Antigen	Pre-immunisation	*S. aureus* (n = 7)	*E. coli* (n = 8)	PPD (n = 5)	MPO (n = 5)	PBS (n = 5)
PR3	nd	1/6 (17%)	nd	nd	nd	nd
MPO	2/120 (2%)	5/21 (24%)	2/24 (8%)	4/15 (27%)	*8/15 (53%)	1/15 (7%)
BPI	nd	nd	1/6 (17%)	nd	nd	nd
LF	5/120 (4%)	5/21 (24%)	4/24 (17%)	3/15 (20%)	2/15 (13%)	0/15 (0%)

*p < 0.05
PPD: purified protein derivative; MPO: myeloperoxidase; PBS: phosphate buffered saline; PR3: proteinase 3; BPI: bactericidal/permeability-increasing protein; LF: lactoferrin.

immune deposits on ultrastructural examination. There were no vascular lesions in the bowel of this animal. This rat did not have an antibody or an *in vivo* or *in vitro* T cell response to any of the ANCA antigens tested.

One of the 4 rats immunised with *E. coli* in whom histology was performed also had pauciimmune segmental glomerular sclerosis. In addition there was a necrotising arteritis affecting a renal interlobular vessel, and panarteritis of a gut vessel (Fig. 1 C,D). No granuloma were present. This animal had C-ANCA, with moderately-strong granular cytoplasmic fluorescence and central accentuation (Fig. 1 A). The antigen specificity could not, however, be determined in ELISAs using human PR3, MPO or BPI, and there was no *in vivo* or *in vitro* T cell response to the same antigens.

None of the animals examined had any histological evidence of an inflammatory colitis, and none of the control animals had any histological abnormalities in the kidney or bowel.

Discussion

Adult male Wistar rats were examined in this study because they produce a specific T cell response after MPO immunisation (25). Rats were immunised with common laboratory strains of *S. aureus* or *E coli. S. aureus* was used because of the increased relapse rate in patients with Wegener's granulomatosis who have nasal *S. aureus* (17), and because staphylococcal wound infections occasionally precipitate the onset of systemic vasculitis (16). Rats were immunised with *E. coli* as a bacterial control for *S. aureus*, and because of a possible relationship with inflammatory bowel disease. PPD contains mycobacterial proteins,and tuberculous lung infections occasionally precede Wegener's granulomatosis (12). The initial immunisations were in IFA rather than CFA (which contains *M. tuberculosis* proteins), but when no MPO-specific immune response could be demonstrated, rats were further immunised with antigen in CFA and subsequently IFA. It is unclear how much the response that eventuated depended on this unusual immunisation regimen. The outcome may have been similar using fewer immunisations all with CFA.

One rat from each group immunised with *S. aureus* or *E. coli* developed pauciimmune segmental glomerular sclerosis of a vasculitic nature. None of the animals from the other groups did. The rat immunised with *E. coli* had additionally an arteritis affecting renal interlobular and gut vessels. Gut vasculitis is uncommon in the human systemic vasculitides, but occurs in animal models. Bacterial infections have been shown to be important in the development of rat models of vasculitis, because pretreatment with broad spectrum antibiotics reduces severity of the disease (26,27). The rat immunised with *E. coli* that developed vasculitis also had C-ANCA, and this coincidence suggests that both the antibody and the histopathology had a common pathogenesis. Why so few animals developed ANCA and vasculitis after bacterial immunisation is not obvious. It is unlikely to be related to bacterial load and cannot be due to individual variation because the animals were inbred. Only a portion of the bowel, but nearly half the kidney was examined, making it unlikely that any vasculitic lesions were overlooked.

The C-ANCA observed in this animal produced an identical pattern to that seen with PR3 specificity, and contrasted with the dull cytoplasmic fluorescence that occurs with a polyclonal immunoglobulin response. The ANCA response was demonstrated by IIF of rat neutrophils, and in ELISAs using purified human antigens. There is some cross-reactivity between rat and human MPO (28) but this is not complete. Thus rats immunised with human MPO developed MPO-ANCA demonstrable by ELISA but not by IIF on rat neutrophils. The amount of cross-reactivity between rat and human PR3

is not known. The C-ANCA specificity in the rat described here could not be determined using the human proteins, PR3, MPO and BPI. This raises the possibility that the C-ANCA was specific for a rat protein. This would occur if the C-ANCA arose from an immunogenic response to rat neutrophil enzymes rather than because of homology between the neutrophil enzymes and bacterial proteins.

Most of the work published to date has emphasised the relationship of autoantibodies to bacterial and viral exposure. However there is good evidence that antigen-specific T cells are also important in the pathogenesis of the ANCA-associated systemic vasculitides and inflammatory bowel disease. This evidence includes the high affinity nature of ANCA that implies T cell help; the increased levels of circulating antigen-specific T cells and related cytokines in patients with active vasculitis (29, 30); the demonstration of T cell tissue infiltrates and granulomata in such patients (31); and the responsiveness of these diseases to T cell-specific treatments such as cyclosporine (32) and T cell-depleting antibodies (33).

We have evaluated the presence of an ANCA antigen-specific T cell response using 2 assays. The *in vitro* T cell proliferative assay used whole blood rather than isolated mononuclear cells, because of the rat's small blood volume and the number of antigens to be tested. This may have contributed to the lack of specificity of the assay. Lactoferrin acted as a control protein because it is not targetted by ANCA in systemic vasculitis, although it is recognised in inflammatory bowel disease and rheumatoid arthritis (34). The increased frequency of T cells for different ANCA antigens in all immunised groups suggested non-specific immunoreactivity possibly because of the adjuvant, or because of endotoxin contamination of the immunogen. There was no DTH response to PR3, MPO or BPI in rats after immunisation with *S. aureus* or *E. coli*. It is not clear whether the absence of a specific T cell response in these animals could be attributed to the lack of crossreactivity between rat and human neutrophil enzymes (which were used in testing) or to the insensitivity of the assays. However it should be noted that some rats immunised with human MPO developed MPO-ANCA and made an MPO-specific T cell response in both the *in vitro* and *in vivo* tests.

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