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DOI:

[10.1016/j.jim.2012.10.015](https://doi.org/10.1016/j.jim.2012.10.015)

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Document Version

Publisher's PDF, also known as Version of record

Citation for published version (Harvard):

O'shaughnessy, CM, Micoli, F, Gavini, M, Goodall, M, Cobbold, M, Saul, A & MacLennan, CA 2013, 'Purification of antibodies to O antigen of Salmonella Typhimurium from human serum by affinity chromatography', *Journal of Immunological Methods*, vol. 387, no. 1-2, pp. 199-210. <https://doi.org/10.1016/j.jim.2012.10.015>

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Research paper

Purification of antibodies to O antigen of *Salmonella* Typhimurium from human serum by affinity chromatographyColette M. O'Shaughnessy^{a,b,1}, Francesca Micoli^{a,1}, Massimiliano Gavini^a, Margaret Goodall^b, Mark Cobbold^b, Allan Saul^a, Calman A. MacLennan^{a,b,*}^a Novartis Vaccines Institute for Global Health, Via Fiorentina 1, 53100 Siena, Italy^b Medical Research Council Centre for Immune Regulation and Clinical Immunology Service, Institute of Biomedical Research, School of Immunity and Infection, College of Medicine and Dental Sciences, University of Birmingham, Birmingham, UK

ARTICLE INFO

Article history:

Received 14 August 2012

Received in revised form 9 October 2012

Accepted 11 October 2012

Available online 8 November 2012

Keywords:

Antibody

Salmonella

O-antigen

Serum

Affinity

Purification

ABSTRACT

Nontyphoidal *Salmonellae* (NTS) are a common cause of bacteraemia in children and HIV-infected adults in Sub-Saharan Africa. We have previously shown that antibodies play a key role in both bactericidal and cellular mechanisms of immunity to NTS, but found that high concentrations of antibody to *Salmonella* Typhimurium O antigen (OAg) in the serum of some HIV-infected African adults is associated with impaired killing of NTS. To further investigate the function of antibodies to the OAg of NTS, we developed a method to purify these antibodies from human serum by affinity chromatography. Purified *Salmonella* Typhimurium OAg was activated with adipic acid dihydrazide (ADH) via two different chemistries before linking to N-hydroxysuccinamide-Sepharose resin: one ADH molecule was introduced per OAg chain on its terminal 3-deoxy-D-manno-octulosonic acid sugar (OAg-ADH), or multiple ADH molecules were attached along the OAg chain after oxidation with sodium periodate (OAgoxADH). Both resulting columns worked well when tested with commercial polyclonal anti-O:4,5 antibodies from rabbit serum. Over 90% of the applied antibodies bound to the resin and 89% of these antibodies were then eluted as detected by ELISA. OAg-ADH was preferred as the method for OAg derivatisation as it does not modify the saccharide chain and can be applied to OAg from different bacteria. Both columns were able to bind OAg-specific antibodies in human serum, but antibody recovery was initially low. Different elution buffers were tested and different amounts of OAg-ADH were linked to the resin to improve the yield. Optimal recovery (51%) was obtained by loading 1 mg of activated OAg per ml of resin and eluting with 0.1 M glycine, 0.1 M NaCl pH 2.4. The column matrix could be regenerated following elution with no detectable loss in performance for over ten uses. This method offers the potential to purify antibodies to *Salmonella* OAg from polyclonal serum following vaccination or natural exposure to *Salmonella* and so investigate the functionality and diversity of the antibody response to OAg.

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1. Introduction

Nontyphoidal *Salmonellae* (NTS), including *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) and Enteritidis (*S. Enteritidis*) are a common cause of bacteraemia in children and HIV-infected adults in Sub-Saharan Africa (Morpeth et al., 2009; Reddy et al., 2010; Gordon et al., 2008). The case fatality rate for bacteraemia caused by NTS is 20–25% for children (Graham et al., 2000; Brent et al., 2006) and increases to 50% for children with NTS meningitis (Molyneux

Abbreviations: NTS, Nontyphoidal *Salmonella*; *S. Typhimurium*, *Salmonella enterica* serovar Typhimurium; LPS, lipopolysaccharide; OAg, O antigen; ADH, adipic acid dihydrazide; KDO, 3-deoxy-D-manno-octulosonic acid sugar; NHS, N-hydroxysuccinamide.

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et al., 2009). A mortality rate of 25–50% has been reported for HIV-infected adults with NTS bacteraemia (Gordon et al., 2002).

We have previously shown that antibodies play a key role in both bactericidal (MacLennan et al., 2008) and cellular (Gondwe et al., 2010) mechanisms of immunity to NTS. The highest incidence of NTS bacteraemia occurs in children aged between 3 and 24 months, when antibody levels are low (MacLennan et al., 2008). A decrease in cases is found in African children aged over 2 years, corresponding to the acquisition of antibody which is able to initiate both complement-dependent killing of *Salmonella* (MacLennan et al., 2008) as well as effective opsonisation and subsequent uptake by blood phagocytes (Gondwe et al., 2010), thus emphasising the importance of antibody. The targets of bactericidal and opsonic antibodies on invasive African NTS have not been fully defined. There is good support for the protective efficacy in mice of antibody against *Salmonella* outer membrane proteins, in particular OmpD (Gil-Cruz et al., 2009). There is also evidence that antibodies against the O antigen (OAg) of the lipopolysaccharide (LPS) molecule of *Salmonella* are protective (Jorbeck et al., 1981; Svenson and Lindberg, 1981). Passive transfer of monoclonal antibodies raised to smooth LPS, but not rough LPS that lacks the OAg chain, conferred significant protection in mice (Singh et al., 1996) and immunization with *S. Typhimurium* and *S. Enteritidis* OAg conjugates conferred protection to challenge (Simon et al., 2011; Watson et al., 1992). However, we have also found that HIV-infected African adults have impaired humoral immunity to NTS associated with dysregulated production of antibodies to the OAg of *S. Typhimurium* (MacLennan et al., 2010) and this warrants further investigation. Despite its probable importance, little is known about the natural immune response to LPS. The capacity to purify LPS-specific antibodies would, for example, be useful in analysing V region usage. Purification of *Salmonella* OAg-antibodies from polyclonal sera would allow further characterisation of both the functionality and specificity of these antibodies. This would facilitate the ongoing investigation of their potential protective and blocking effects in individuals immunised with OAg-based vaccines and in HIV-infected African adults.

Monoclonal and polyclonal antibodies are conventionally purified by affinity chromatography (Cuatrecasas, 1970; Jack, 1994; Huse et al., 2002), using the highly-specific nature of the interaction between antigen and antibody. The antigen is covalently attached to a solid support under conditions that retain antibody-binding capacity. Subsequently, when serum is passed over the antigen-bound column, only those molecules with specific affinity for the antigen are bound. After washing, the bound antibodies are eluted, thereby purifying them from the original sample. Although this method for recovering active antibodies is potentially selective, rapid and simple, allowing antibody purification in a single chromatographic step, the recovery is typically low (Casey et al., 1995; Cuatrecasas and Anfinsen, 1971). Optimised conditions need to be determined to permit efficient purification of the desired antibodies without altering their native structure (Narhi et al., 1997a).

Salmonella LPS consists of lipid A linked to the 3-deoxy-D-manno-octulosonic acid (KDO) terminus of the conserved core region, which in turn is linked to the serovar-specific OAg chain. The OAg chain is the immunodominant portion of the molecule and extends as a repeating polymer from the end of

the core region (Whitfield et al., 2003). In *S. Typhimurium*, the OAg repeat (O:4,5) consists of a trisaccharide backbone, with a branch of abequose, usually O-acetylated on C-2, which confers serogroup specificity (factor 4,5) (Fig. 1A) (Hellerqvist et al., 1969).

LPS detoxification is usually performed by acetic acid hydrolysis or by hydrazinolysis (Konadu et al., 1996), with the former commonly preferred as it retains the O-acetyl groups along the OAg chain. Acid hydrolysis cleaves the labile linkage between Lipid A and KDO leaving the OAg chain attached to the core region (Fig. 1A).

Many approaches have been used to bind LPS or detoxified OAg from various bacteria to resins for use in affinity purification and, despite the high toxicity, CNBr-activated resin has been the most commonly employed (Stiller and Nielsen, 1983; Rodahl and Maeland, 1984). Girard and Goichot attached LPS to an aminohexyl-Sepharose resin by activation with benzoquinone (Girard and Goichot, 1981), while Fox and Hechemy tested epoxy-activated resin for the attachment of *Escherichia coli* LPS (Fox and Hechemy, 1978). The bound LPS showed low antibody binding capacity, which may be due to limited availability of antibody-binding sites on LPS resulting from steric hindrance and/or partial hydrolysis as a result of the alkaline pH used for the LPS coupling to the resin. Altman and Bundle showed that an epoxy-activated Sepharose-gel worked well with underivatized *Salmonella* Essen OAg, but OAg from other bacteria did not couple to this activated support (Altman and Bundle, 1994). They proposed the use of epoxy- or succinimide ester-activated Sepharose after OAg derivatisation with a 1,3-diaminopropane spacer.

For anti-NTS OAg antibody purification, affinity chromatography columns are not commercially available. We developed and compared two alternative strategies for inserting hydrazide groups into the OAg of *S. Typhimurium* prior to linking to commercially available N-hydroxysuccinamide (NHS)-Sepharose resin. The resulting affinity columns were tested for their ability to isolate antibodies from human serum against the OAg of LPS from *S. Typhimurium* D23580, a characteristic invasive African isolate of NTS (Kingsley et al., 2009).

2. Materials and methods

2.1. Chemicals

Unless otherwise stated, chemicals and reagents were from Sigma.

2.2. Commercial anti-O:4,5 antibody

Polyclonal monovalent anti-O:4,5 *Salmonella* Typhimurium antibodies raised in rabbits (Bio-Rad 59021C) were used as a source of purified antibodies. This antiserum was obtained by immunizing rabbits with selected strains of *Salmonella*. Monovalent sera were adsorbed in order to increase their specificity.

2.3. *S. Typhimurium* bacterial isolate

S. Typhimurium D23580, a well-characterized invasive clinical isolate of nontyphoidal *Salmonella* from Malawi (MacLennan et al., 2008; Kingsley et al., 2009) was used throughout the study. This strain is representative of 90% of

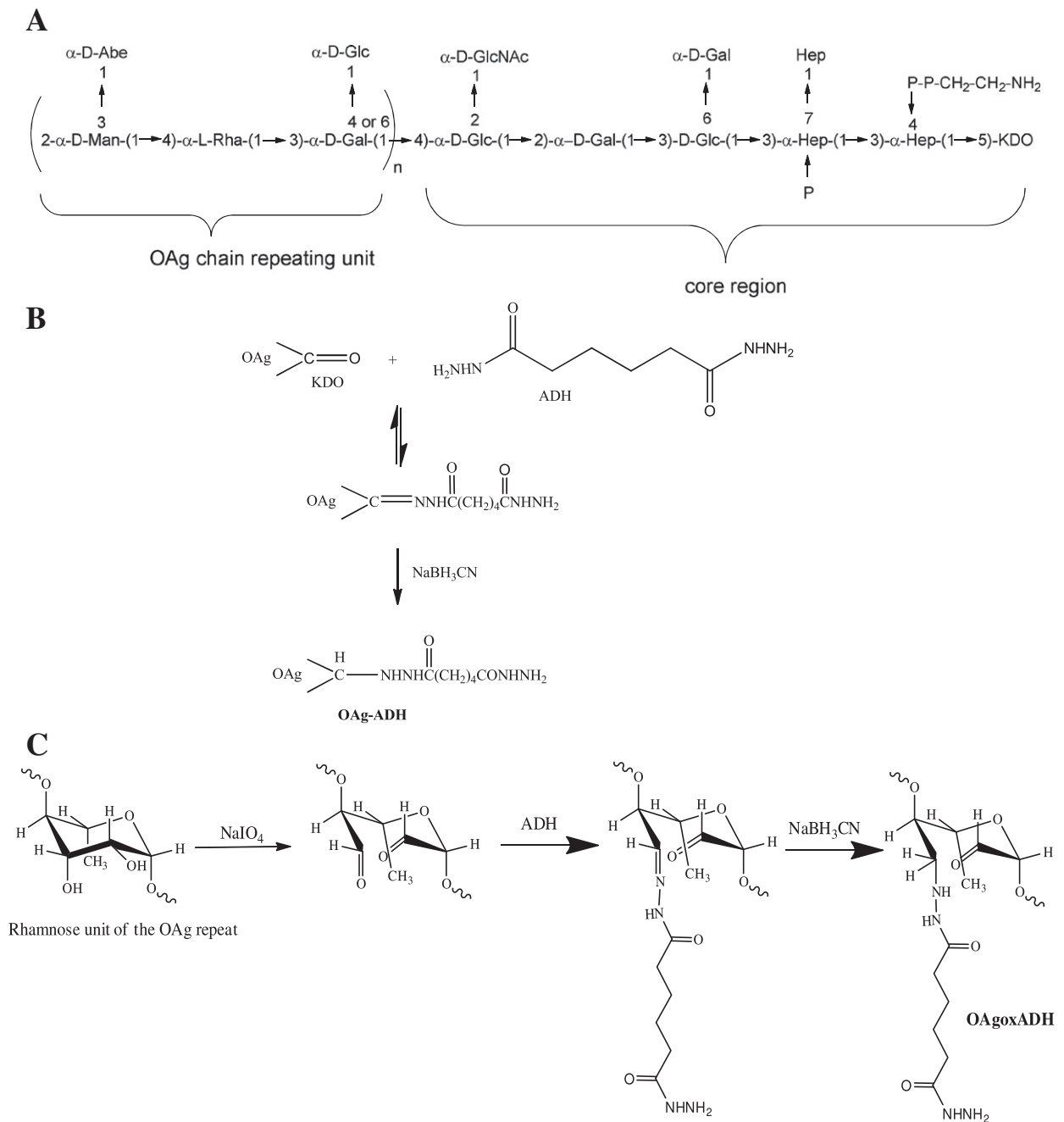


Fig. 1. Structure of *S. Typhimurium* OAg chain and core region following acid hydrolysis of LPS and reaction schemes for production of activated OAg. The structure of the *S. Typhimurium* OAg chain linked to the core region after acetic acid hydrolysis. Hydrolysis removes Lipid A leaving the OAg chain attached to the core. Core region structure taken from Jansson, et al [P.E. Jansson, A.A. Lindberg, B. Lindberg, R. Wollin, Structural studies on the hexose region of the core in lipopolysaccharides from Enterobacteriaceae, Eur. J. Biochem. 115 (1981) 571–7] (A). Reaction scheme for OAg-ADH: the ketone group of the terminus KDO is linked to ADH by reductive amination (B). Reaction scheme for OAgoxADH: diol groups are oxidised to aldehyde groups by NaIO₄. These aldehyde groups can then react with ADH by reductive amination (C).

invasive NTS isolates in Malawi. Bacteria were grown in a 7 l bioreactor (EZ-Control, Applikon) to an OD of 35 as previously described (Rondini et al., 2011).

2.4. OAg purification

The OAg was purified according to a new method developed at Novartis Vaccines Institute for Global Health. Briefly,

acid hydrolysis (2% acetic acid at 100 °C for 3 h) was performed directly on the bacterial fermentation culture and OAg was recovered from the supernatant by centrifugation. Lower molecular weight impurities were removed by tangential flow filtration (TFF), using a Hydrosart 30 kDa membrane. Protein and nucleic acid impurities were co-precipitated in citrate buffer 20 mM at pH 3. Proteins were further removed by ion exchange chromatography and nucleic acids

by precipitation adding 500 mM Na_2HPO_4 , EtOH and 5 M CaCl_2 , to give final concentrations of 18 mM, 24% and 200 mM respectively. OAg was recovered in water by a second TFF 30 kDa step.

2.5. Activation of OAg with ADH

2.5.1. Selective activation of KDO

OAg was solubilized in 100 mM sodium acetate (AcONa) buffer pH 4.5 at a concentration of 40 mg/ml. Adipic acid dihydrazide (ADH), and then sodium cyanoborohydride (NaBH_3CN) were added as solids, both at a ratio of 1.2:1 by weight with respect to the OAg. The solution was mixed at 30 °C for 1 h before purifying by desalting against water on a G-25 column (HiTrap™ desalting column 5 ml or HiPrep™ 26/10 desalting column 53 ml, pre-packed with Sephadex™ G-25 Superfine; GE Healthcare). The derivatised OAg was indicated as OAg-ADH (Fig. 1B).

2.5.2. Random activation along the OAg chain after oxidation with sodium metaperiodate (NaIO_4)

OAg was solubilized in 0.1 M AcONa buffer pH 5 and 100 mM freshly prepared NaIO_4 added to give 6.25 mM NaIO_4 in the reaction mixture with OAg at a concentration of 10 mg/ml. The mixture was incubated for 2 h at room temperature in the dark, and then purified by desalting against water on a G-25 column. The oxidised OAg was dried in a SpeedVac vacuum centrifuge (Thermo SPD 131DDA) (room temperature, overnight, 500 mtorr), and then activated with ADH following the same procedure described above. The final product was indicated as OAgoxADH (Fig. 1C).

2.6. Analytical methods

The phenol sulphuric assay was used for total sugar content quantification (DuBois et al., 1956). OAg impurities were assessed by micro BCA (Bicinchoninic Acid) for protein content (using bovine serum albumin as a reference and following the manufacturer's instructions [Thermo Scientific]) and by UV spectroscopy for nucleic acids (at a wavelength of 260 nm assuming that a nucleic acid concentration of 50 µg/ml produces an OD_{260} of 1). The chromogenic kinetic LAL (Limulus Amoebocyte Lysate) Assay was used to measure endotoxin level (Charles River Endosafe-PTS instrument).

2.6.1. Size exclusion high pressure liquid chromatography (HPLC-SEC)

HPLC-SEC analysis was used to estimate the molecular size distribution of OAg populations. Samples were run on a TSK gel G3000 PWXL column (30 cm × 7.8 mm; particle size 7 µm; cod. 808021) with TSK gel PWXL guard column (4.0 cm × 6.0 mm; particle size 12 µm; cod. 808033) (TosohBioscience). The mobile phase was 0.1 M NaCl, 0.1 M NaH_2PO_4 , and 5% CH_3CN , pH 7.2 at a flow rate of 0.5 ml/min (isocratic method for 30 min). Void and bed volume calibration was performed with λ -DNA (λ -DNA molecular weight marker III 0.12–21.2 Kbp, Roche) and sodium azide (NaN_3 , Merck), respectively. OAg peaks were detected by differential refractive index (dRI). For kd determination, the following equation was used: $\text{kd} = (\text{Te} - \text{T0}) / (\text{Tt} - \text{T0})$ where: Te = elution time

of the analyte, T0 = elution time of the biggest fragment of λ -DNA and Tt = elution time of NaN_3 .

2.6.2. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

Rhamnose (Rha), galactose (Gal), glucose (Glc) and mannose (Man), each occurring once in the OAg chain repeating unit, and N-acetyl glucosamine (GlcNAc), sugars present in the core region only, were estimated by HPAEC-PAD after acid hydrolysis of the OAg to release the monosaccharides. Commercial monomer sugars were used for building the calibration curves. For Rha, Gal, Glc and Man quantification, OAg samples, diluted to have each sugar monomer in the range 0.5–10 µg/ml, were hydrolyzed at 100 °C for 4 h in 2 M TFA. These hydrolysis conditions were optimal for release of all monomers without their degradation. For GlcNAc quantification, OAg samples, diluted to a GlcNAc concentration of 0.5–10 µg/ml, were hydrolyzed at 100 °C for 6 h in 1 M TFA. After the hydrolysis, samples were chilled at 2–8 °C for about 30 min, dried by vacuum centrifuge overnight, reconstituted in water and filtered using 0.45 µm Acrodisc (Pall) filters prior to chromatographic analysis.

HPAEC-PAD was performed with a Dionex ICS3000 equipped with a CarboPac PA10 column (4 × 250 mm) coupled with PA10 guard column (4 × 50 mm). Separation of the sugars was performed with a flow rate of 1 ml/min eluting in a gradient from 10 mM NaOH to 18 mM NaOH over 20 min. After washing for 20 min with 100 mM AcONa in 28 mM NaOH, the column was re-equilibrated with 10 mM NaOH for 20 min. The effluent was monitored using an electrochemical detector in the pulse amperometric mode with a gold working electrode and an Ag/AgCl reference electrode. The Dionex standard quadruple-potential waveform for carbohydrates was used. The resulting chromatographic data were processed using Chromeleon software 6.8. Calibration curves were built for each sugar monomer (0.5–10 µg/ml). The standards were hydrolysed and analysed in the same way as the samples. For GlcNAc, glucosamine (GlcN) was the species detected by HPAEC-PAD after hydrolysis.

2.6.3. Proton nuclear magnetic resonance (^1H NMR) spectroscopy

^1H NMR analysis was performed to estimate the O-acetylation level. It was also used to confirm the identity of the OAg samples (typical signals of the OAg chain can be detected, confirming the presence of the characteristic sugars) and in particular for calculating the molar ratio of Rha to abequose (Abe) by comparing the integrals of the two peaks corresponding to Rha-H6 and Abe-H6.

The dried OAg sample was subsequently solubilized in deuterium oxide (D_2O) and transferred to a 5 mm NMR tube. A first spectrum was collected in D_2O and a second one after de-O-acetylation achieved by adding sodium deuteroxide (NaOD) to a final 200 mM concentration and heat treatment (37 °C for 2 h for complete de-O-acetylation). The first ^1H NMR spectrum was recorded to ensure the absence of impurities at the same chemical shift of the acetate anion released after de-O-acetylation of the sample that would interfere with the quantification of the O-acetyl content. O-acetylation level was quantified by comparing acetate (released after treatment with NaOD) and Rha-H6 peaks, and expressed as molar % of

O-acetyl with respect to OAg chain repeating units (based on Rha present only in the OAg chain at one sugar per repeating unit).

NMR experiments were recorded at 25 °C on Varian VNMR5-500 spectrometer, equipped with a Pentaprobe. Acquisition time of 5 s, relaxation delay of 15 s and number of scans of 64 was set for the acquisition of the spectra. For data acquisition and processing VNMRJ ver. 2.2 rev. C and Mestrenova 6.1 (Mestrelab Research) were used respectively. 1-D proton NMR spectra were collected using a standard one-pulse experiment. Chemical shifts were referenced to hydrogen deuterium oxide (HDO) at 4.79 ppm (1 H).

2.6.4. KDO quantification by semicarbazide/HPLC-SEC method

OAg samples were analysed by HPLC-SEC after derivatisation with semicarbazide to quantify α -ketoacid present at the terminus KDO. This reaction was performed as a slight modification of the semicarbazide assay for α -ketoacids determination (Macgee and Doudoroff, 1954). OAg samples and KDO standards (100 μ l of total volume in water), with a C=O concentration between 15.7 nmol/ml and 156.7 nmol/ml, were added to 100 μ l of semicarbazide solution (100 mg semicarbazide hydrochloride + 90.5 mg of sodium acetate anhydrous in 10 ml of water). Sample blanks were prepared by adding 100 μ l of sodium acetate (90.5 mg of sodium acetate anhydrous in 10 ml of water) to 100 μ l of the OAg samples at the same concentration used for the analysis. All samples and standards were heated at 50 °C for 50 min and then analysed by HPLC-SEC (80 μ l injected), on a TSK gel G3000 PWXL column with guard column in 0.1 M NaCl, 0.1 M NaH₂PO₄, 5% CH₃CN, pH 7.2 mobile phase at the flow rate of 0.5 ml/min (isocratic method for 30 min). Detection was done at 252 nm. The area under the peak corresponding to the OAg after derivatisation with semicarbazide was corrected with the area of the corresponding blank and the amount of KDO calculated with the calibration curve built with the areas of KDO standards at 252 nm.

2.6.5. NH₂ quantification and calculation of OAg derivatisation with the linker ADH

The trinitrobenzene sulfonic acid (TNBS) colorimetric method (Palmer and Peters, 1969; Satake et al., 1960) was used for total NH₂ group quantification. 6-aminohexanoic acid was used as the standard for NH₂ quantification on underivatized OAg samples, while ADH was used as the standard for NH₂ quantification after OAg derivatisation with ADH. The amount of hydrazide groups introduced linking ADH was calculated by subtracting the number of NH₂ groups already present on the un-derivatized OAg sample and the number of free NH₂ groups, detected as free ADH by reverse phase high performance liquid chromatography (RP-HPLC) (Micoli et al., 2012) from the total NH₂ groups by TNBS. Selective activation on the terminal KDO was calculated as the percentage of moles of linked ADH per moles of GlcNAc (present as a unique sugar in the core region, Fig. 1), indicating the percentage of activated OAg chains. Random activation with ADH after oxidation was expressed as the percentage of moles of ADH per moles of Rha (present as one sugar per OAg repeating unit; Fig. 1).

2.7. Derivatized OAg coupling to NHS-Sepharose

Immobilization of the derivatized OAg samples, both OAg-ADH and OAgoxADH, on NHS-Sepharose was performed according to the manufacturer's instructions (GE Healthcare). Briefly, OAg-ADH or OAgoxADH was dissolved in coupling buffer (5–10 mg/ml; 0.5 M NaCl, 0.2 M NaHCO₃, pH 8.3). A HiTrap™ NHS-activated HP 1 ml column was washed with 1 mM HCl (6 column volumes) and dissolved activated OAg was added to the column and incubated overnight at 4 °C. The column was then washed with 0.5 M ethanolamine, 0.5 M NaCl pH 8.3 (6 column volumes) to block unreacted sites followed by 0.1 M AcONa, 0.5 M NaCl pH 4 (6 column volumes). Washing with 0.5 M ethanolamine, 0.5 M NaCl pH 8.3 was repeated (6 column volumes) and the column was left at 4 °C for 4 h. 0.1 M AcONa, 0.5 M NaCl pH 4, 0.5 M ethanolamine, 0.5 M NaCl pH 8.3 and again 0.1 M AcONa, 0.5 M NaCl pH 4 were passed through the column (6 column volumes for each buffer). The column was stored in 0.05 M Na₂HPO₄, 0.1% NaN₃ pH 7.0 at 4 °C. A syringe was used for all wash steps. Flow through and wash solutions were analysed by phenol sulphuric assay to calculate the amount of sugar linked to the resin.

2.8. Sera preparation

Blood containing anti-*Salmonella* antibodies was venesected from a healthy adult and left to clot at 22 °C for 4 h before separating by centrifugation at 4 °C and freezing in aliquots at –80 °C. Ethical approval for the use of human serum in this study was granted by the Life and Health Sciences Ethical Review Committee of the University of Birmingham. Informed written consent was obtained prior to venesection.

2.9. Ammonium sulphate precipitation of serum proteins

Ammonium sulphate was added as a solid to 1 ml of human serum to give a final concentration of 0.5 g/ml and the mixture was placed on ice for 5 min. The serum was centrifuged at 4 °C, 3300×g for 5 min and the supernatant discarded. The precipitate was washed twice with 1 ml 0.5 g/ml ammonium sulphate. The pellet was solubilized in 0.3 ml PBS and dialysed overnight against PBS at 4 °C.

2.10. Anti-*Salmonella* Typhimurium OAg antibodies purification by affinity chromatography

NHS HiTrap columns with activated OAg were equilibrated with PBS (6 column volumes) before applying the serum protein solution to the column and incubating overnight at 4 °C. Columns were then washed with PBS (6 column volumes), followed by 50 mM NaH₂PO₄, 500 mM NaCl pH 7.2 (6 column volumes). Bound antibodies were eluted in 5 column volumes of elution buffer, collecting fractions of 0.5 ml each. 0.1 M glycine, 0.1 M NaCl at pH 2.4, 2.6, 2.8 and 3.0; 20% ethanol; 4 M MgCl₂ in 10 mM Tris base pH 7; 8 M urea; and 100 mM Tris base pH 9 were tested as elution buffers. Following elution with glycine buffers using a pH 2.4–3.0, the pH was adjusted to 7.0 with 2 M Tris pH 9. Individual eluate fractions were analysed for protein content by measuring absorption at 280 nm. After elution, all eluates were dialysed overnight against PBS at 4 °C.

Purified antibodies were stored at 4 °C. Retention of antibodies on columns was investigated by applying 1% SDS to columns and analysing SDS-eluates by SDS-PAGE. Columns were washed with PBS and stored in 0.05 M Na₂HPO₄, 0.1% NaN₃ pH 7.0 at 4 °C.

2.11. ELISA for anti-*Salmonella* Typhimurium OAg antibody

96-well flat bottom plates (NUNC Maxisorp) were incubated with 100 µl per well of 5 µg/ml TLR-grade smooth LPS from *S. Typhimurium* (Alexis Biochemicals) or 15 µg/ml *S. Typhimurium* OAg, overnight at 4 °C. After coating, plates were washed with PBS 0.05% Tween and incubated with 200 µl blocking buffer (PBS 1% BSA) per well for 1 h at 37 °C. Plates were washed again with PBS 0.05% Tween and incubated for 1 h at 37 °C with 100 µl serial dilutions of antibody solution diluted with PBS 1% BSA 0.05% Tween. Following further washes, 100 µl of goat-antihuman alkaline-phosphatase-labelled IgG (Southern Biotech) was added to each well and plates were incubated for 1 h at 37 °C. Plates were washed to remove unbound antibody and bound antibody detected using 100 µl *p*-Nitrophenyl phosphate solution. The reciprocal of the concentration of antibody giving an OD of 1 at λ 405 nm was calculated. This value was plotted as the anti-*Salmonella* Typhimurium IgG concentration. The recovery of purified antibody was determined by multiplying the anti-*Salmonella* Typhimurium IgG ELISA concentration of each eluate by the appropriate dilution factor and dividing their sum by the anti-*Salmonella* IgG concentration for the human serum protein solution bound to the column. This value was expressed as the percentage recovery of purified anti-OAg antibody.

3. Results

3.1. OAg purification, activation with ADH and coupling to NHS-Sepharose

OAg from *S. Typhimurium* D23580 was purified by acetic acid hydrolysis of bacterial fermentation broth with the direct release of OAg into the supernatant. This avoids the need for hot phenol LPS extraction followed by LPS detoxification (Simon et al., 2011; Konadu et al., 1996; Watson et al., 1992). Purified OAg contained 0.4% protein, 0.15% nucleic acid (w/w with respect to total sugar content), with an endotoxin level <0.01 UI/µg. The O-acetyl content was 142% (expressed as molar ratio of OAc groups to Rha) which is likely to represent O-acetylation of Rha, as well as Abe, as previously described following lysogenisation of *S. Typhimurium* with bacteriophages A3 and A4 (Wollin et al., 1987). This is an unusual and interesting finding which may distinguish African invasive *S. Typhimurium* isolates from those found elsewhere and could impact on the polyclonal antibody response to *S. Typhimurium* OAg in Africa. Analysis by HPLC-SEC (dRI) revealed the presence of two main populations with different average MW, with kd values of 0.18 and 0.30 respectively. Analysis of the two separated populations by HPAEC-PAD indicated an average number of repeating units per OAg chain of 71 and 25 respectively, calculated from the molar ratio of Rha to GlcNAc (basic structure of OAg and core region of *S. Typhimurium* LPS shown in Fig. 1A). GlcNAc quantification was in good agreement with KDO quantification, confirming

the presence of one KDO per OAg chain. The molar ratios of Man, Gal, Glc and Abe to Rha were respectively 1.05, 1.08, 0.41 and 1.04. OAg contained 24.1% NH₂ groups pre-derivatisation with ADH (expressed as molar ratio % of NH₂ groups to GlcNAc), probably as pyrophosphoethanolamine residues in the core region (Fig. 1A).

Two different chemistries were used for inserting reactive hydrazide groups into the OAg prior to linking to commercially available NHS-Sepharose. For one method, the KDO sugar at the end of the core region was linked through reductive amination to one ADH molecule, thus producing OAg-ADH (Fig. 1B). With the second method, OAg underwent an oxidative step prior to activation with ADH (Fig. 1C). Diol moieties are susceptible to oxidation with NaIO₄, producing aldehyde groups along the length of the OAg chain that can then react with ADH by reductive amination. In contrast to the first method, the OAg chain was modified with multiple ADH linkers introduced along the saccharide chain. All of the OAg-ADH preparations were characterized by a sugar recovery greater than 80%, with more than 80% of OAg chains activated and <2% (in moles) of free to linked hydrazide groups. We confirmed the absence of dimer and aggregate formation with the reaction condition used by analysing the OAg-ADH using HPLC-SEC. This showed the presence of one peak with the same kd value as the underivatized OAg. The OAgoxADH preparation was characterized by a total sugar recovery of 73%, with 20% activation (molar % linked ADH to Rha) and <2% free to linked hydrazide groups. In theory, the presence of more than one ADH linker per OAg chain in OAgoxADH could favour the OAg binding to the NHS-Sepharose. However the binding capacity was found to be 3.7 mg of OAgoxADH and 4.3 mg of OAg-ADH per ml of resin.

3.2. Evaluating the antigenic integrity of derivatised OAg

The prepared affinity columns were tested with a commercially-available preparation of purified polyclonal rabbit anti-*Salmonella* Typhimurium O:4,5 antibodies to determine if the hydrolysis and activation of OAg with ADH had impaired the antigenic structure of the OAg. 3.7 and 4.3 mg of OAgoxADH and OAg-ADH respectively were linked to NHS-Sepharose columns and 300 µl of O:4,5 antibodies (with an antibody concentration corresponding to 1666 ELISA units) were applied to each column. 92% of the antibodies bound to the OAg-ADH column (Fig. 2A) and 96% bound to the OAgoxADH column (Fig. 2B) with the remaining applied antibodies detected in the flow through and subsequent wash fractions. 89% and 90% of bound antibodies were eluted with 0.1 M glycine, 0.1 M NaCl pH 3 buffer from the OAg-ADH and OAgoxADH columns respectively.

3.3. OAg-ADH column with human serum

Following the previous result confirming the functional antigenic integrity of both forms of derivatised OAg bound to NHS-Sepharose, we applied a protein preparation concentrated from human serum containing polyclonal anti-*Salmonella* antibodies to both columns. The proteins had been precipitated from human serum using ammonium sulphate in order to reduce the presence of contaminants that could interfere with the interactions between OAg on the columns and corresponding

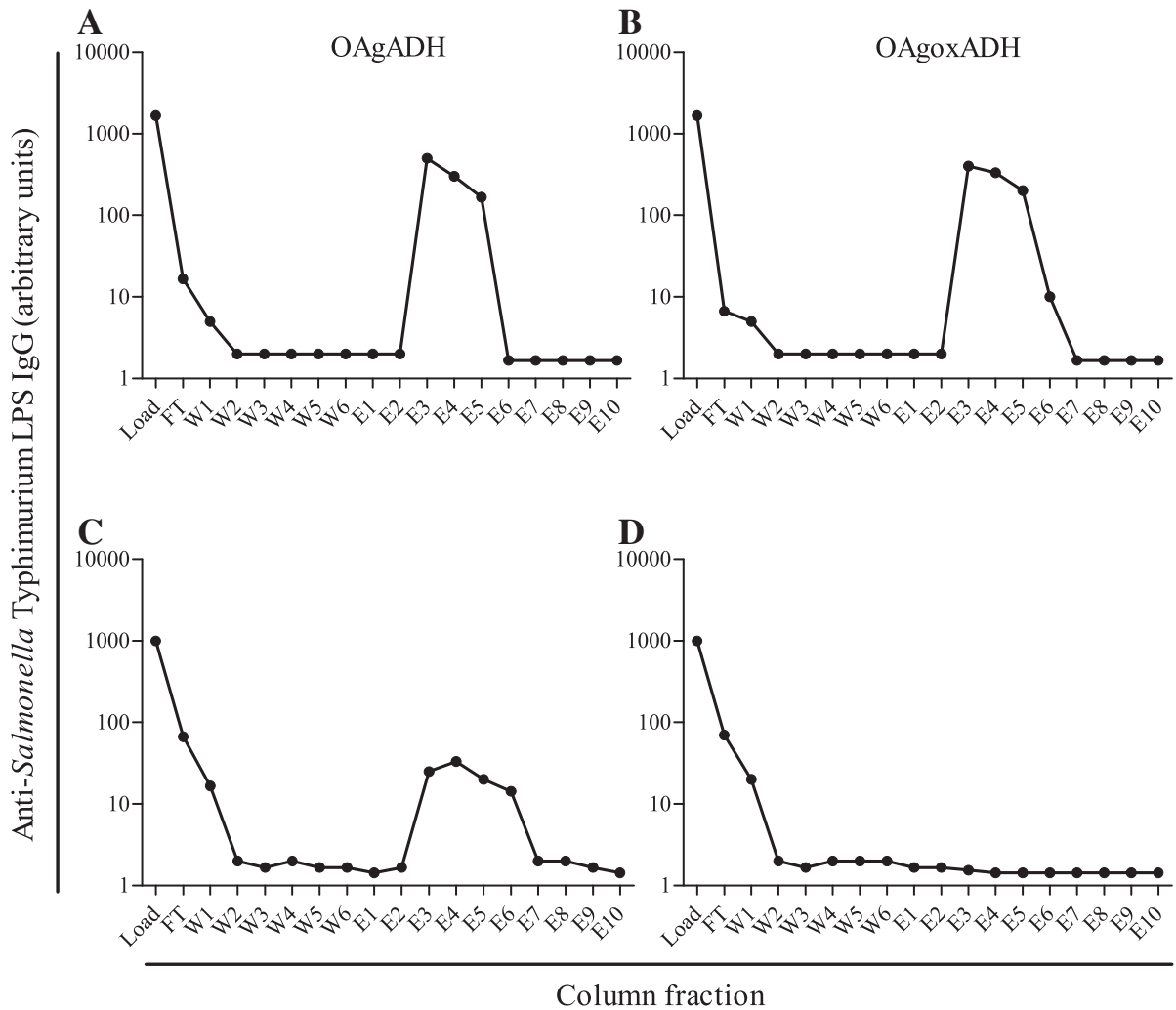


Fig. 2. Activated OAg from *S. Typhimurium* D23580 coupled to NHS-Sepharose can reversibly bind antibody. ELISA profile for the binding and elution of anti-*Salmonella* Typhimurium O:4,5 IgG antibody from an OAg-ADH (A) and OAgoxADH NHS-Sepharose column (B). 300 μ l of anti-*Salmonella* Typhimurium O:4,5 rabbit antiserum (Biorad), with an antibody concentration corresponding to 1666 ELISA units, was applied to the column (load). Flow through (FT) containing unbound antibody was collected, and the column washed with 6 ml PBS (W1–3), followed by 6 ml 50 mM NaPi, 500 mM NaCl pH 7.2 (W4–6). Bound antibodies were eluted with 5 ml 0.1 M glycine, 0.1 M NaCl pH 3 in 500 μ l fractions (E1–10). 300 μ l of ammonium sulphate-precipitated human serum proteins with an anti-*Salmonella* Typhimurium antibody concentration corresponding to 1000 ELISA units was loaded on an OAg-ADH (C) and an OAgoxADH NHS-Sepharose column (D) and washing and elution performed as above. All fractions were collected and the concentration of anti-LPS IgG antibody in each fraction was tested by ELISA for antibody to LPS. Reciprocal of concentration of antibody giving an OD of 1 at λ 405 nm is plotted against column fraction collected.

antibodies, and to concentrate the antibodies. 300 μ l of resulting protein solution (with an antibody concentration corresponding to 1000 ELISA units) were applied to each 1 ml column. A high proportion (>75%) of the antibodies applied to the columns in the serum protein solution bound to the column as shown by the low signal in the ELISA for OAg antibodies in the flow through and wash fractions (Fig. 2C and D). For the OAg-ADH column (Fig. 2C), elution with 0.1 M glycine, 0.1 M NaCl pH 3 and immediate neutralisation with 2 M Tris pH 9, resulted in a recovery of only 14% of the bound antibodies. For the OAgoxADH column, only 2% of the bound antibodies were eluted under the same conditions (Fig. 2D). The same results were obtained whether the ELISA was performed coating the plates with purified OAg from *S. Typhimurium* D23580 or commercial LPS, confirming that the antibodies detected were against the OAg

and not the lipid A of LPS. Given that LPS is commercially available, this was used to coat the plates for all subsequent ELISA.

Only 50% of the antibodies bound to the resin when either 300 μ l or 900 μ l of whole serum (without performing serum precipitation with ammonium sulphate) were applied to the OAg-ADH column (data not shown). 900 μ l corresponded to a similar total amount of antibodies loaded after concentrating the serum 2.5-fold with ammonium sulphate. When human serum was precipitated using ammonium sulphate but not concentrated during this step, and loaded on the OAg-ADH column then, although the binding of antibody to the column was high, the recovery of antibody using 0.1 M glycine, 0.1 M NaCl pH 3.0 was only 5%. A 10-fold concentration of the serum, instead of 2.5-fold, loading 300 μ l with a concentration

of 6666 ELISA units of antibodies on the column, allowed a recovery of 15%, but was difficult to reproduce without high loss of antibodies and was not used for further experiments.

Despite the apparent binding of the majority of human anti-LPS antibody to both columns, the recovery of these antibodies was very low, particularly with OAgoxADH. Eluted fractions which lacked anti-LPS antibodies by ELISA also lacked protein content according to absorption measurements at 280 nm. This suggests that the poor recovery of antibodies was not an artefact resulting from a lack of antibody functionality due to the elution buffers used. Considering that the binding capacity of OAg–ADH to NHS-Sepharose was not lower than OAgoxADH, that only one step is required for its synthesis, and that this derivatisation method can be generally applied to OAg from different bacteria independent of its structure, the OAg–ADH column was selected for performing further experiments.

3.4. Elution under acidic conditions is optimal

With the aim of improving the recovery of antibody from OAg–ADH columns, different elution buffers were tested. Eight 1 ml OAg–ADH NHS columns were prepared, each with an equal amount of OAg–ADH linked to the Sepharose (3.5 mg/column), using the same batch of activated OAg. Precipitated human serum proteins from the same donor as in the previous experiments (with an antibody concentration corresponding to 1300 ELISA units), were loaded onto each column. The relative amount of unbound antibodies was very low and comparable for all eight columns (Fig. 3A–B).

Glycine at acidic pH is commonly used as an elution buffer (Narhi et al., 1997a, 1997b). We therefore tested 0.1 M glycine, 0.1 M NaCl at pH 3, pH 2.8, pH 2.6 and pH 2.4 (Fig. 3A) which gave elutions containing 9%, 16%, 12% and 26% of the bound antibodies respectively. We also examined the effect of using 20% ethanol, 4 M MgCl₂ in 10 mM Tris base pH 7, 8 M urea and 100 mM Tris base pH 9 (Fig. 3B) as elution buffers which yielded 7%, 18%, 8% and 1% of the bound antibodies respectively.

Polyclonal antibodies can be involved in different interactions and have different affinities for the same antigen. Consequently they may require different conditions to disrupt the antigen–antibody interaction. To determine if the recovery of human polyclonal antibodies could be improved by combining the two most efficient elution buffers tested, one OAg–ADH column was loaded with precipitated human serum proteins (antibody concentration corresponding to 1300 ELISA units) with sequential elution steps with 10 ml 0.1 M glycine, 0.1 M NaCl pH 2.4, and 10 ml 4 M MgCl₂ in 10 mM Tris pH 7 and with washing with 6 ml PBS between each step (Fig. 3C). Elution with 0.1 M glycine, 0.1 M NaCl pH 2.4 recovered 28% of the bound antibody but no further antibody was removed with MgCl₂.

Glycine was also the optimal elution buffer when 300 µl of commercial anti-O:4,5 antibodies (antibody concentration corresponding to 1666 ELISA units) was loaded onto the OAg–ADH column. Eluting with 4 M MgCl₂ in 10 mM Tris pH 7, only 44% of bound antibodies were removed (Fig. 3D). Passing 3 ml 8 M urea and then 3 ml 20% ethanol through the same column, no antibodies were removed whereas 3 ml 0.1 M glycine, 0.1 M NaCl pH 2.4 eluted a further 31% of bound antibodies (Fig. 3D).

3.5. Decreasing the amount of activated OAg coupled to NHS-Sepharose increases the recovery of purified antibody

To investigate how the ratio of OAg coupled to NHS-Sepharose in a column affects the recovery of purified antibodies, 3.5 mg, 1 mg and 0.5 mg of OAg–ADH were immobilised on 1 ml NHS-Sepharose columns. Equal amounts of precipitated human serum proteins (antibody concentration corresponding to 1200 ELISA units) were applied to each column and 80% of loaded antibodies were retained by each column, regardless of the amount of OAg linked to the matrix (Fig. 4A–C).

Elution of antibodies bound to the column with 1 mg of OAg–ADH linked, with 0.1 M glycine, 0.1 M NaCl pH 2.4 resulted in an increased recovery of purified antibodies (Fig. 4B) of 51% compared to 26% for the original 3.5 mg OAg–ADH column (Fig. 4A), while the yield decreased to 19% for the column with the lowest amount of OAg–ADH (Fig. 4C). Applying 1% SDS to the column at the end of the experiments and analysing the SDS-eluate by SDS-PAGE revealed no protein bands. This suggests that large amounts of antibody had not been retained on the column following the various elutions.

4. Discussion

This study describes a new approach for the purification of antibodies specific to *S. Typhimurium* OAg from human serum by affinity chromatography.

We successfully coupled purified activated OAg from invasive African *S. Typhimurium* D23580 to NHS-Sepharose. As the key intermediate step to this process, two different procedures were tested for introducing hydrazide groups onto the OAg. In one case (OAg–ADH; Fig. 1B), the OAg chain was linked via the KDO unit at the end of the core region, proximal to the OAg, to a single ADH molecule. This method, which is similar to the method proposed by Altman and Bundle (Altman and Bundle, 1994), does not alter the available epitopes along the saccharide chain and can be applied to OAg obtained from different bacteria regardless of their structure. Reductive amination with ADH is a more rapid process (1 h) than that required to link an amino group (5 days) (Altman and Bundle, 1994), and was characterized by a high OAg recovery and percentage of activation. The other derivatised antigen, OAgoxADH (Fig. 1C), underwent prior oxidation resulting in multiple ADH molecules linked to the OAg chain that could potentially enhance the binding capacity of the OAg to the NHS-Sepharose. This procedure modifies the OAg chain structure with possible implications for antibody binding and can only be applied to OAg containing diol groups which are susceptible to oxidation with sodium metaperiodate. Both OAg–ADH and OAgoxADH columns bound and gave a similar recovery of commercial rabbit anti-*Salmonella* O:4,5 antibodies. However there was a greater recovery of purified antibodies from the human serum for the OAg–ADH column compared to the OAgoxADH column. Considering also that the binding efficiency is not lower and OAg–ADH requires only one step of synthesis, this method of activation was selected for optimising the antibody extraction process.

One of the main caveats when selecting and producing an affinity column is that modifications to the structure of the target antigen can occur during activation or coupling of the

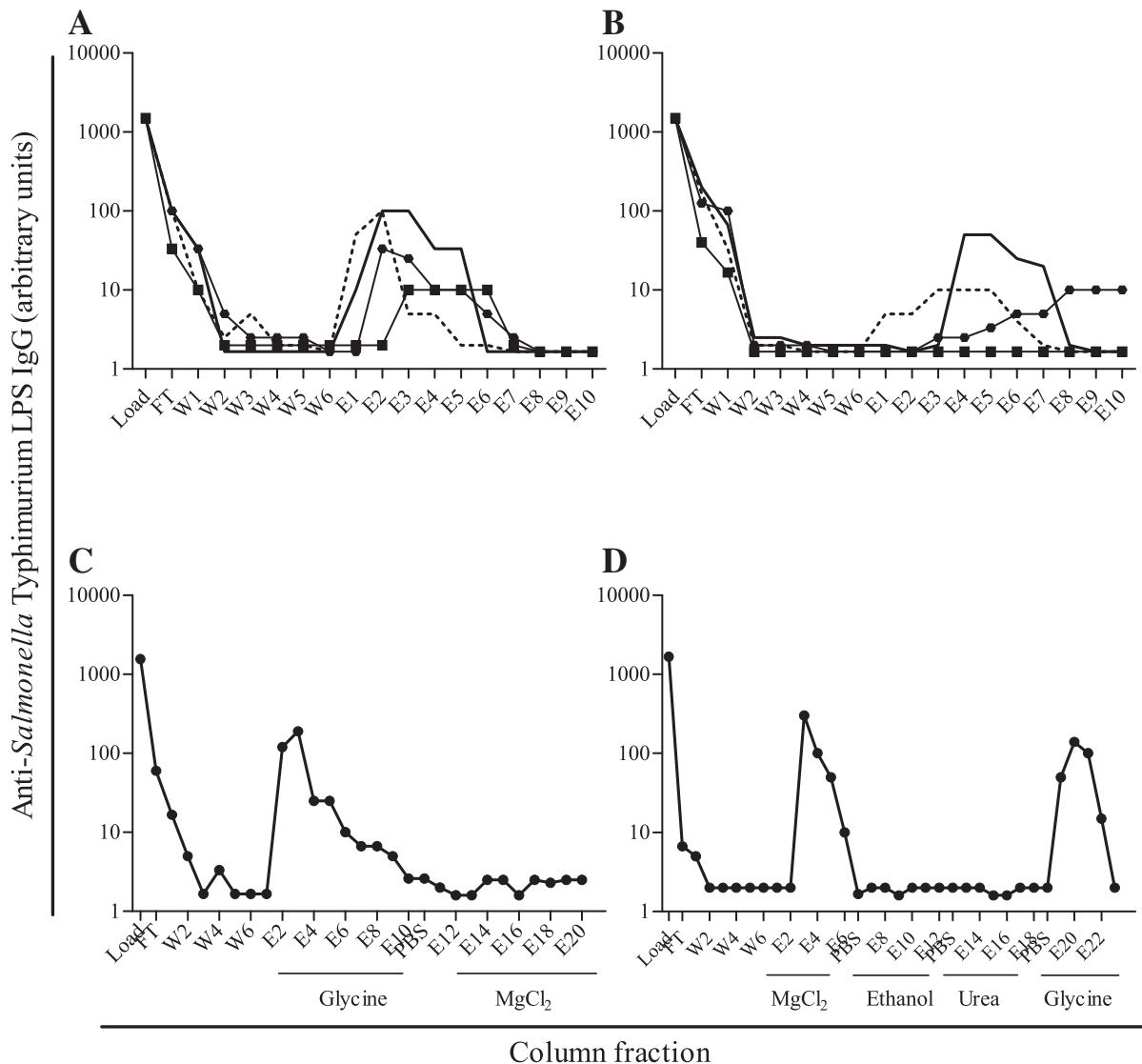


Fig. 3. Ability of different buffers to elute bound anti-OAg IgG antibodies from human or rabbit serum. Eight OAg-ADH-NHS-Sepharose columns were loaded with 300 μ l of ammonium sulphate-precipitated human serum proteins with an anti-*Salmonella* Typhimurium antibody concentration corresponding to 1300 ELISA units (load) and the flow through (FT) collected. Each column was washed with 6 ml PBS (W1–3), 6 ml 50 mM NaPi, 500 mM NaCl pH 7.2 (W4–6). 5 ml of different elution buffers were passed through each column: (A) 0.1 M glycine, 0.1 M NaCl pH 3 (squares), 0.1 M glycine, 0.1 M NaCl pH 2.8 (dotted line), 0.1 M glycine, 0.1 M NaCl pH 2.6 (circles), 0.1 M glycine, 0.1 M NaCl pH 2.4 (solid line), (B) 100 mM Tris base pH 9 (squares), 20% ethanol, (dotted line), 4 M MgCl₂ in 10 mM Tris base pH 7 (solid line), 8 M urea pH 7 (circles) and the eluates collected and neutralised if required (E1–10). (C) OAg-ADH column was loaded with 300 μ l precipitated human serum with an IgG antibody concentration corresponding to 1250 ELISA units, and bound antibody was eluted with 10 ml 0.1 M glycine, 0.1 M NaCl pH 3, followed by 10 ml 4 M MgCl₂ in 10 mM Tris base pH 7 after washing with 6 ml PBS. (D) 300 μ l of anti-*Salmonella* Typhimurium O:4,5 rabbit antiserum (Biorad), with an antibody concentration corresponding to 1666 ELISA units, was applied to the OAg-ADH column (load), flow through (FT) containing unbound antibody collected, and the column washed with 6 ml PBS (W1–3), followed by 6 ml 50 mM NaPi, 500 mM NaCl pH 7.2 (W4–6). Bound antibody was sequentially eluted with 3 ml 4 M MgCl₂ in 10 mM Tris base pH 7; then 3 ml 20% ethanol; 3 ml 8 M urea pH 7; and 3 ml 0.1 M glycine, 0.1 M NaCl pH 3 with 6 ml PBS passed through the column between buffers. Eluates were collected in 500 μ l fractions (E1–10). Concentration of anti-OAg IgG antibody in each fraction was tested by ELISA using LPS coated plates. Reciprocal of concentration of antibody giving an OD of 1 at λ 405 nm is plotted against column fraction collected.

ligand to the chromatography matrix, and the affinity of that antigen for its corresponding antibodies is frequently reduced (Fox and Hechemy, 1978). Testing both OAg-ADH and OAgoxADH columns with purified polyclonal antibodies specific for O:4,5 of *S. Typhimurium* raised in rabbits, and then with polyclonal antibodies from human serum, we verified that both immobilised antigens were able to bind antibodies (more than 90% of the applied antibodies bound

for the commercial anti-*Salmonella* O:4,5 antiserum and more than 75% for human serum). This finding suggested that the method used for OAg extraction and purification, and the subsequent activation with ADH did not alter the antigenic determinants present on the molecule.

When human serum proteins were applied to the columns, the step of ammonium sulphate precipitation (Baines and Thorpe, 1992; Page and Thorpe, 2002) was introduced before

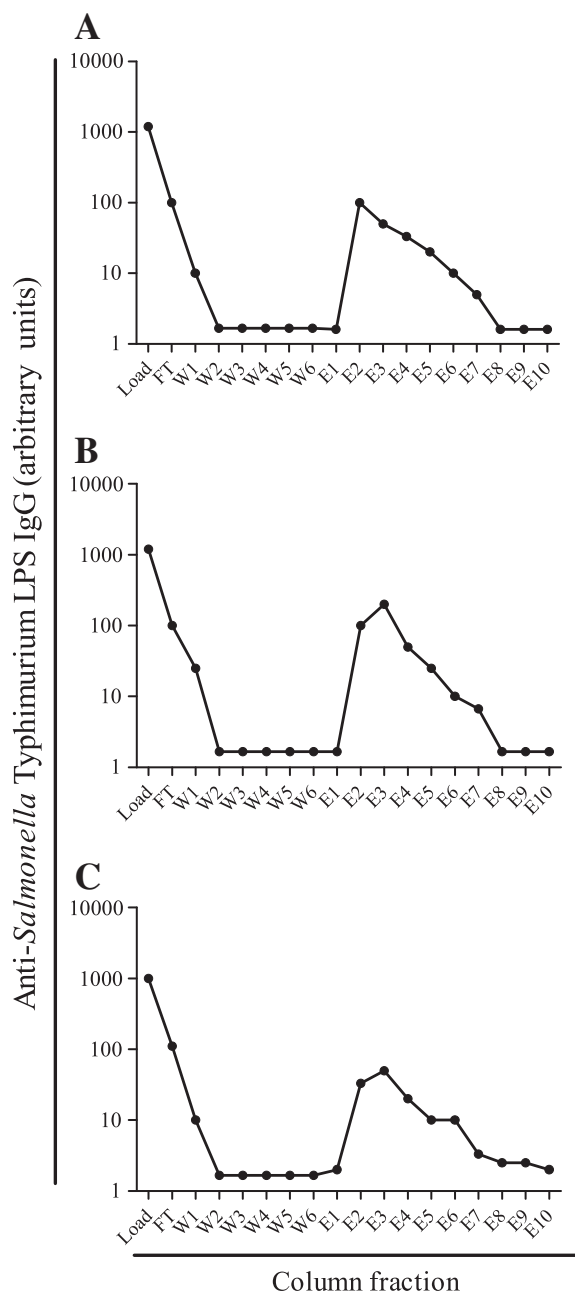


Fig. 4. Variation in amount of activated OAg immobilised to NHS-Sepharose affects recovery of bound antibody from affinity columns. 300 μ l ammonium sulphate-treated human serum containing anti-*Salmonella* Typhimurium antibodies corresponding to 1200 ELISA units was applied to three OAg-ADH columns each with varying concentration of OAg-ADH linked to 1 ml NHS-Sepharose: 3.5 mg/ml (A), 1 mg/ml (B), 0.5 mg/ml (C) and the flow through (FT) was collected. Each column was washed with 6 ml PBS (W1–3), 6 ml 50 mM NaPi, 500 mM NaCl pH 7.2 (W4–6) and bound antibody was eluted with 5 ml 0.1 M glycine, 0.1 M NaCl pH 2.4 (E1–10). Concentration of anti-OAg IgG antibody in each fraction was tested by ELISA using LPS coated plates. Reciprocal of concentration of antibody giving an OD of 1 at λ 405 nm is plotted against column fraction collected.

loading the serum on the affinity column in order to concentrate the antibodies and remove a large number of contaminants such as lipids and nucleic acids which could interfere with binding.

Using purified polyclonal anti-*Salmonella* Typhimurium O:4,5 antibodies raised in rabbits, elution with 0.1 M glycine, 0.1 M NaCl pH 3 was successful, allowing 89% of antibody recovery (Fig. 2A and B). With human serum, only 14% of antibodies were eluted from the OAg-ADH column (Fig. 2C) and 2% from the OAgoxADH column (Fig. 2D), using the same buffer. Differences in the avidity of human and rabbit antibodies, particularly for carbohydrate epitopes, may underlie the high recovery of polyclonal rabbit antibodies and lower comparative recovery of human antibodies with our columns.

Sera contain many polyclonal antibodies which recognize and bind different epitopes on the same antigen with different binding affinities. Antigen-antibody binding involves many weak interactions, including hydrogen bonds, van der Waals forces, ionic and hydrophobic interactions (Smith-Gill et al., 1982; Sakurabayashi, 1995; Mukkur, 1984; Smith-Gill, 1996). Therefore effective elution of polyclonal antibodies may require several different elution conditions. Glycine at acidic pH is commonly used to elute antibodies from antigen-affinity column, but there are other possible solvents for this purpose involving the use of alkaline pH, changes in ionic strength, use of chaotropic salts (that disrupt the structure of water and reduce hydrogen bonds and weaken hydrophobic interactions), denaturants or organic buffers (Yarmush et al., 1992; Jack, 1994). Testing glycine elution buffers at different pH, pH 2.4 was the most effective (Fig. 3A), but recovery of antibodies was still low (26%). Different buffers were then tested: 20% ethanol to investigate the effect of an organic solvent, 100 mM Tris pH 9 as alkaline buffer, 8 M urea as a denaturant and 4 M MgCl_2 to raise the ionic strength of the solvent, with an accompanying weak chaotropic effect. The highest recovery with an alternative buffer was obtained with 4 M MgCl_2 (18%; Fig. 3B). However, the yield was still lower than that with 0.1 M glycine, 0.1 M NaCl pH 2.4 (26%; Fig. 3A), and 4 M MgCl_2 was not as effective as glycine at removing commercial anti-*Salmonella* Typhimurium O:4,5 antibodies either (Fig. 3D). To understand whether MgCl_2 and glycine were removing different sub-populations of human antibodies, and in an attempt to increase the recovery, both buffers were used sequentially, but MgCl_2 was unable to elute any remaining bound antibody (Fig. 3C).

It is possible that the majority of antibodies bound to the column were successfully eluted, but that some did not fully renature and therefore were no longer able to bind to LPS in the ELISA. Even if the extracted antibodies refold in their native conformation because of immediate neutralisation and/or dialysis following elution (Narhi et al., 1997a, 1997b) we did not investigate the effect of the elution buffers on their conformation and so cannot exclude that an irreversible denaturation occurred. Nevertheless, our 280 nm absorption measurements of the column eluates indicated that those fractions which lacked anti-LPS antibodies by ELISA also lacked measurable protein content and thus were unlikely to contain significant amounts of denatured antibody. We verified that the ratio of antigen to antibody affected antibody elution. Reduction in the amount of OAg-ADH coupled to the resin from 3.5 mg to 1 mg per ml of resin, increased the recovery of purified antibody from 26% to 51% working with the same elution buffer (glycine pH 2.4). Decreasing the concentration of linked OAg-ADH further to 0.5 mg per ml of resin decreased the recovery to 19% (Fig. 4C). Differential

loading of the antigen on the resin could result in different OAg chain conformations and a different exposure of the epitopes and/or determine a different interaction with the corresponding antibodies.

The present study describes the successful coupling of OAg from *S. Typhimurium* to NHS-Sepharose in order to produce an affinity matrix that is capable of purifying specific antibodies from polyclonal human serum. The method can be applied to OAg from different bacteria and it does not modify OAg chain epitopes. Columns were filled with different OAg-ADH preparations with consistent results and columns were used at least 10 times with no deleterious effect on recovery of antibodies. This process could potentially be adapted for the purification of antibodies against other bacterial polysaccharides, as well as for the large scale purification of antibodies against *Salmonella* OAg. As such, the method will be useful for the investigation of the protective capacity of OAg antibodies with different fine specificities in the context of immunity to NTS, both in HIV-infected African adults and in individuals immunised with OAg-based vaccines.

Acknowledgements

We thank Simona Rondini for the extraction of OAg from *S. Typhimurium* D23580. We are grateful to Robert Heyderman and the Malawi-Liverpool-Wellcome Trust Clinical Research Programme for *S. Typhimurium* D23580. We thank Simona Rondini, Yunshan Goh and Adam Cunningham for helpful discussions. This work was supported by a PhD studentship from the Medical Research Council, UK (to CMO'S) and a Clinical Research Fellowship from GlaxoSmithKline (to CAM). The authors declare that they have no conflict of interest.

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