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Preliminary study of mucosal IgA in the equine small intestine: specific IgA in cases of acute grass sickness and controls

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Summary

Reasons for performing study: **There is much evidence to suggest that group III *Clostridium botulinum* (types C and D) are involved in the aetiology of equine grass sickness (EGS). Antibodies have been detected previously in the blood and high levels associated with resistance to disease. Specific mucosal antibodies in the gastrointestinal (GI) tract are likely to be important in protection, and this study was performed to ascertain if such antibodies could be detected and if their levels were related to disease state.**

Objectives: **To develop a method for quantifying IgA antibodies to *C. botulinum* types C and D in the GI tract of horses and to relate antibody levels to disease status.**

Methods: **Samples of tissue (n = 25: 6 duodenum, 7 jejunum and 12 ileum) were taken from acute grass sickness (AGS) cases and from control horses (n = 12; 4 samples from each site) at *post mortem*. They were extracted with the detergent saponin in the presence of protease inhibitors and assayed for total IgA, for specific IgA against botulinum neurotoxins types C and D (BoNT/C or BoNT/D), and against surface antigens of a BoNT/C negative strain of *C. botulinum* type C (SA) and of *Clostridium tetani* (TetSA), as a control. Specific IgA was expressed as percentage total IgA.**

Results: **Compared to controls, significantly higher levels of specific IgA against BoNT/C were detected in the jejunum (P = 0.04) and ileum (P = 0.02) of AGS cases. Similarly, higher specific levels against BoNT/D were demonstrated in duodenum (P = 0.01) and jejunum (P = 0.02). Significantly higher levels of IgA against SA were demonstrated only in duodenal samples (P = 0.01).**

Conclusions: **Levels of IgA antibody to BoNTs in control horses were at near undetectable levels, suggesting no recent exposure to toxins. In AGS cases, significantly higher levels of specific IgA were detected predominantly in jejunum and ileum.**

Potential relevance: **If specific IgA is protective then any successful vaccine for EGS should induce a mucosal response.**

Introduction

Equine grass sickness (EGS) is a primary dysautonomia of equids that is generally fatal and currently unpreventable. The aetiology of the disease remains unproven, although substantial circumstantial evidence has been demonstrated that supports the hypothesis that EGS is caused by a toxicoinfection with *Clostridium botulinum* types C and D. This includes toxin detection from clinical samples (Hunter *et al.* 1999); analysis of serum IgG (Hunter and Poxton 2001; McCarthy *et al.* 2004a) and mucosal IgA from cases and controls (Hunter 2001); and epidemiological studies (McCarthy *et al.* 2004a,b).

It has been suggested that a vaccine that induced antibodies and immunological memory against the toxins and cell-surface antigens of *C. botulinum* may be protective (Hunter and Poxton 2001; McCarthy *et al.* 2004a). To date, the studies of antibody levels in EGS cases and controls have concentrated on serum (systemic) IgG antibodies. Mucosal immunity in the GI tract is possibly of more importance than systemic immunity, but this has not been extensively investigated. The aims of this study were, therefore, to develop a method to measure specific IgA in the gut tissue of the horse, and to compare levels of specific IgA against the neurotoxins and cell surface antigens of *C. botulinum* types C and D between acute grass sickness (AGS) cases and controls with the objective of studying the natural disease.

Materials and methods

Samples

In 2004, samples from 7 AGS cases, subjected to euthanasia and necropsied within 36 h of diagnosis, had samples taken from the small intestine: duodenum, jejunum and ileum. Four other horses, subjected to euthanasia for reasons other than GI problems, were sampled *post mortem* in the same way as the 7 AGS cases and were referred to as non-AGS controls. In addition, ileal samples were taken from 5 AGS cases that underwent *post mortem* examination in 2003. A summary of the samples is as follows: duodenum 6 cases and 4 controls; jejunum 7 and 4; ileum 12 and 4. The

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samples consisted of tied-off pieces of intestine that were frozen at -80ºC until processing. Just prior to extraction squares of tissue (approximately 2 x 2 cm) were excised from the frozen tissue using sterile scalpels and weighed.

Treatment of equine GI tissue for IgA extraction

Antibody extraction from tissue was achieved with saponin as described by Bergquist *et al.* (2000) with some modifications. Frozen tissue (approximately 3 g) was removed aseptically and incubated for 8 h at 4ºC in PBS containing 2% saponin (w/v), soybean protease trypsin inhibitor (1 mg/ml), EDTA; 0.05 mol/l, Tween 20 (0.05%), phenylmethylsulphonylfluoride (2 mmol/l), sodium azide (0.2 mg/ml) and 4% fetal calf serum (v/v); 2 ml were added per gram of tissue. Samples were vortexed for 30 s and, after incubation, were agitated using sterile forceps, vortexed for 30 s and then the tubes placed in a sonicating water bath for 2 min. After further vortexing, samples were centrifuged at 15,000 ***g*** for 5 min, and supernatants collected and stored at -70ºC until used. All reagents and samples were kept on ice between steps.

Antigens

Toxoid complex from types C1 and D were purchased from Metabiologics Inc[1]. Surface antigens (SA) were extracted from whole washed cells with 10 mmol/l EDTA as described previously (Poxton 1984) and stored at -20ºC. The strains used were *C. botulinum* type C (NCTC 3732) and *C. tetani* (NCTC 5413) that were both negative by neurotoxin gene PCR; the *C. botulinum* type C strain was also negative by capture-antigen ELISA (A. Heffron, personal communication). The protein concentration of extracts was determined as described by Lowry *et al.* (1951).

ELISA to detect total IgA

A standard curve of equine IgA was constructed using a Bethyl Laboratories Equine IgA Quantification kit[2] as per the manufacturer's instructions. Reference serum was diluted in PBS-TG (0.05 mol/l phosphate buffer with 2% teleostean gelatin[3] and 0.1% Tween 20, pH 7.4; 200 µl/well) to concentrations recommended by the manufacturers. Samples were diluted 1 in 1000. Samples and reference sera were assayed in quadruplicate at 100 µl/well and incubated overnight at room temperature. Plates were washed 6 times between steps with ELISA wash buffer (1 x PBS BR14a tablet[3]/l, 15 mmol/l NaCl, 2 mmol/l KCl, Tween 20 0.05%) pH7.3 between each stage.

Goat anti-equine IgA alkaline phosphate conjugate[1] was diluted 1 in 10,000 in PBS-TG and added 100 µl/well. Plates were incubated for 4 h at 37ºC with shaking. Alkaline phosphatase substrate tablets (Sigma 104-105[3], p-nitrophenyl phosphate 5 mg/ml) were diluted 1 in 10000 with substrate solvent (0.05 mol/l sodium carbonate solution, pH 9.8, with 1 mmol/l magnesium chloride) to give a concentration of 1 mg/ml. Plates were then incubated at room temperature for 60 min and read with an Anthos plate reader at 405 nm referenced at 620 nm.

ELISA to detect specific IgA

Surface antigens (SAs) were diluted to 30 µg/ml in 0.05 mol/l sodium carbonate buffer (pH 9.6, 0.02% w/v sodium azide).

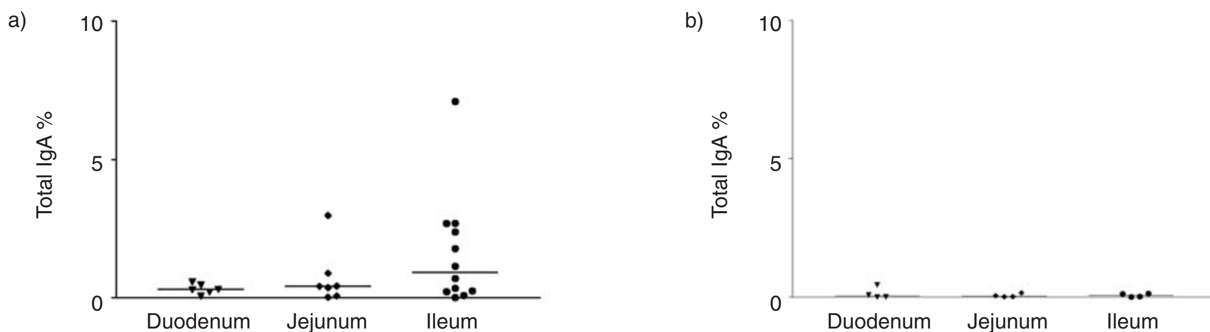

Fig 1: Graphs showing the range of specific IgA found along the GI tract against BoNT/C in (a) AGS cases and (b) non-AGS controls. Horizontal lines depict the median. Significant differences were found between cases and controls in jejunum samples (P = 0.04) and ileum samples (P = 0.02), but not duodenum samples (P = 0.17).

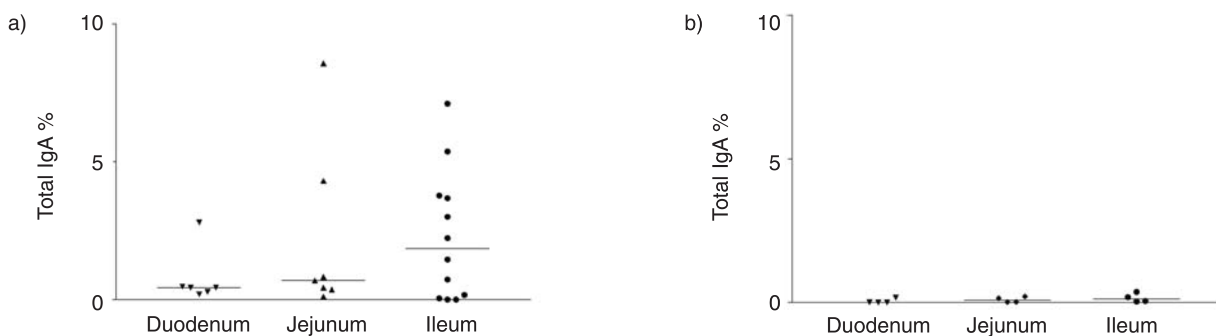

Fig 2: Graphs showing the range of specific IgA found along the GI tract against BoNT/D in (a) AGS cases and (b) non-AGS controls. Significant differences were found between cases and controls in duodenum samples (P = 0.01) and jejunum samples (P = 0.02), but not between ileum samples (P = 0.16).

Botulinum neurotoxin C1 and type D toxoid complexes were diluted to 5 µg/ml. They were added to Nunc Polysorb plates[4] (100 µl/well) and incubated overnight at room temperature for SAs and 4ºC for toxoids.

All plates were washed 6 times between each stage and blocked with PBS-TG for 90 min with PBS-TG (200 µl/well) at 37ºC, with the exception of both surface antigen ELISAs, where blocking was found to be unnecessary. Plates were used straight away or stored in sealed plastic bags at -20ºC for up to one week. Samples were diluted in PBS-TG 1 in 4, 1 in 8 or 1 in 16 to fall within the standard curve of total IgA. Samples were added at 100 µl/well in quadruplicate. Conjugate and colour development was as described in the previous section.

Controls and statistical analysis

Blank wells, consisting of coating buffer were included on each plate. Negative controls, consisting of PBS-TG in place of sample, were included on the plates to control for nonspecific binding of conjugate to the antigen.

A number of saponin extracts were titrated to identify an intraplate control and the optimal dilutions for specific IgA against all antigens. Optimal dilution was at 1 in 8 for the majority of samples although some were repeated at 1 in 4 and 1 in 16.

All statistical analyses were performed on Graphpad Prism V3.03[5]. This software tests for Gaussian distribution using a variation of the Kolmolgorov and Smirnov test. Normality testing could not be carried out on the control horse samples due to their small number, and the nonparametric Mann-Whitney U test was therefore employed to test for differences between cases and control medians. Significance was set at a P≤0.05.

Results

IgA was detected in all specimens of tissue. The levels of total IgA in the jejunum were the highest with a range of 659–5044 µg/ml (per 0.5 g tissue) in AGS cases and 1148–6664 µg/ml in the controls. In the duodenum IgA levels ranged from 566–2074 µg/ml in AGS cases and 683–2852 µg/ml in controls, and in the ileum from 274–4127 µg/ml (AGS cases) and 876–1579 µg/ml (controls). In all tissues the levels between cases and controls were not statistically different.

IgA that reacted specifically with the neurotoxins or surface antigens was readily detected in most of the samples from the AGS cases, with median levels in the ileum being highest with maximum levels of 64, 56, 24 and 74 µg/ml for BoNT/C, BoNT/D, and surface antigens from *C. botulinum* and *C. tetani* (TetSA), respectively. Levels in the controls were at the lower limit of detection: with 2 exceptions all contained less than 3 µg/ml, with levels of the IgA to TetSA reaching 10 µg/ml in the duodenum and 22 µg/ml in jejunum. The results are summarised in Figures 1–4 where the levels of specific IgA are expressed as a percentage of total IgA and the statistical differences are given in the legends. The most striking differences are seen for the levels of IgA against BoNT/C and BoNT/D between the cases and controls (Figs 1, 2). The levels in the samples from all of the controls are only just detectable. However, in the corresponding samples from the cases the IgA levels are generally much higher, with those to BoNT/C from the jejunum and ileum, and to BoNT/D from the duodenum and jejunum being significantly higher than the controls. The IgA levels from the other 2 tissues (to BoNT/C in duodenum and to BoNT/D in ileum) appear visibly higher in the figure but do not reach significance, with P = 0.17 and 0.16, respectively. This can be attributed to the small numbers of samples affecting the calculation.

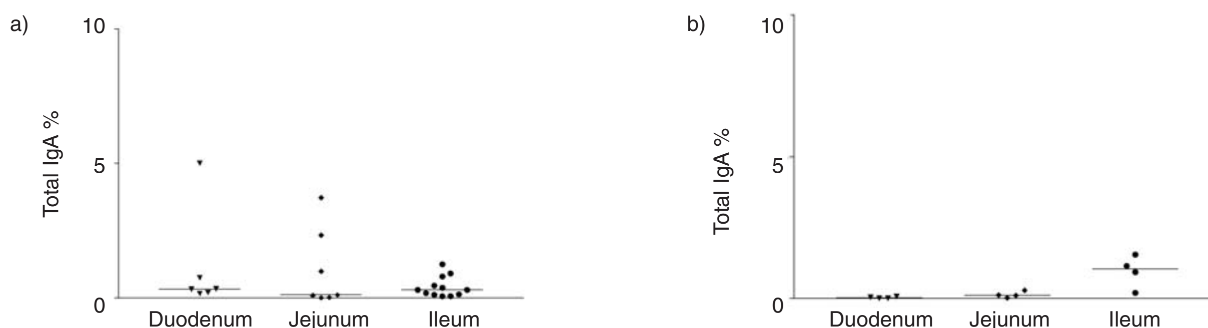

Fig 3: Graphs showing the range of specific IgA found along the GI tract against C. botulinum *surface antigens in (a) AGS cases and (b) non-AGS controls. Differences were found between cases and controls in duodenum samples (P = 0.01) and approached significance between ileum samples (P = 0.08). No significant difference was demonstrated between jejunum samples.*

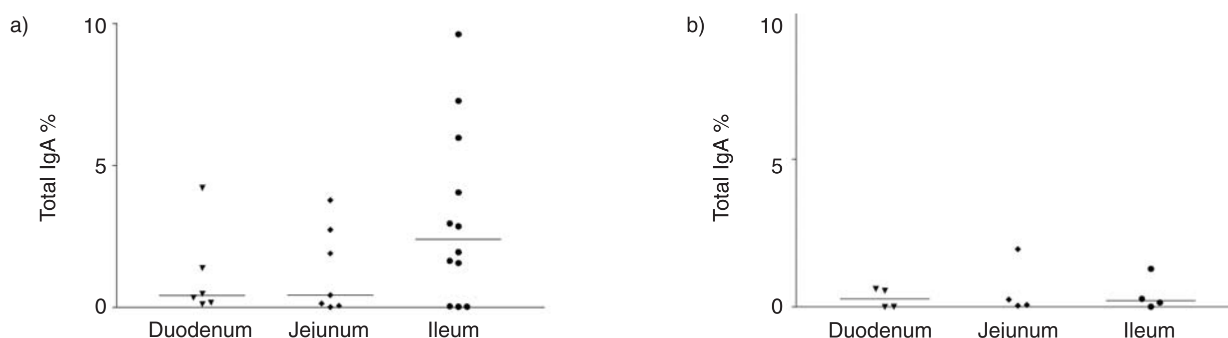

Fig 4: Graphs showing the range of specific IgA found along the GI tract against C. tetani *surface antigens in (a) AGS cases and (b) non-AGS controls. No significant differences were found between cases and controls, although the difference between ileum samples approached significance (P = 0.08).*

The results of the determinations of specific IgA antibodies to surface antigens were less substantial than those of the neurotoxins with specific antibodies being detected more in the controls than in cases; the opposite to levels detected to the neurotoxins. A significant difference was observed between specific IgA levels against SA in duodenal samples (P = 0.02) and approaching significance in ileal samples (P = 0.08), controls having higher specific IgA levels. This was not demonstrated in jejunal samples despite the highest upper range found in cases. The levels of IgA to TetSA approached significance in ileal samples (P = 0.08), again with the higher levels being detected in the controls.

Discussion

Earlier investigations of the systemic immune response demonstrated that horses with EGS possessed lower levels of specific IgG (as measured by OD level) to BoNT/C and to surface antigens than control horses (Hunter and Poxton 2001). A large epidemiological study was carried out at The University of Liverpool Veterinary School (McCarthy *et al.* 2004a,b) that built on this initial study. The findings supported the earlier study and led the authors to propose that low antibody levels to surface antigens enable proliferation of *C. botulinum* type C/D in the gut leading to toxin production. Immune responses to these organisms are probably more important at the gastrointestinal (GI) level than in the systemic circulation, but little is known or has been published on GI immunity in the horse. Preliminary investigations into IgA detection in EGS cases (Hunter 2001) were limited by a small number of samples and a lack of reagents. Investigations into feline dysautonomia (which we have proposed to have a similar aetiology to EGS) demonstrated significantly higher levels of specific IgA mucosal (ileum) antibodies against both BoNT/C and SA (Nunn *et al.* 2004). Hunter (2001) suggested that the presence of higher levels of IgA in cases also indicates a degree of exposure to the organism prior to the onset of clinical signs and, therefore, toxin production may be a cause of gut stasis rather than an effect.

In the present study, relatively low specific IgA levels were demonstrated against surface antigens of *C. botulinum* type C in both cases and controls, with controls showing a higher level in the ileum. This had also been demonstrated for the surface antigens of *C. novyi* type A (Hunter 2001), an organism phenotypically highly similar to *C. botulinum* type C. The high degree of cross-reactivity previously demonstrated between the surface antigens of these organisms (Poxton 1984) may account for this; *C. novyi* has been hypothesised to be part of the normal flora of the equine GI tract and as such may induce a degree of immune tolerance from the host. However, higher levels of systemic IgG against SA are associated with protection from EGS (McCarthy *et al.* 2004a). The relatively high levels of specific IgA against the surface antigens of *C. tetani* may be attributed to the high levels of clostridia found in GI stasis (Garrett *et al.* 2002).

Increased levels of specific IgA in AGS cases compared to controls may appear to contradict existing evidence of lower specific systemic IgG in cases compared to controls (Hunter *et al.* 2001; McCarthy 2004a). However, IgA levels in the gut tissue are much more likely to reflect current exposure to an agent than IgG in the system (which reflects past exposure) as IgA has a short half-life in the gut and mucosal immunological memory is also short (Pierce and Cray 1982).

This preliminary study indicates that the cases investigated exhibited higher levels of specific IgA against BoNT/C and D and surface antigens of the botulinum organisms than non-AGS controls. Statistical testing was, however, compromised by the small number of controls - the main weakness of this study - but obtaining such specimens is difficult.

It should be stressed that this study was performed on horses with acute grass sickness, which, by definition, has a short duration and fatal outcome. The levels of specific IgA detected to the neurotoxins at *post mortem* are likely to have been produced during the preclinical phase of the disease and have not reached protective levels. Mucosal IgA antibodies to surface antigens may have been pre-existing as they are detectable in some of the controls. However, levels of antibodies in the GI tract of these cases have not been able to prevent disease. Whether it is possible to provoke mucosal antibodies in the GI tract that are protective may have important implications for any future vaccine.

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Manufacturers' addresses

[1]Metabiologics Inc., Madison, Wisconsin, USA.
[2]Bethyl Laboratories, Inc., Montgomery, Texas , USA.
[3]Sigma-Aldrich Company Ltd., Poole, Dorset, UK.
[4]Fisher Scientific, Loughborough, Leicestershire, UK.
[5]GraphPad Software, San Diego, California, USA.

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