Conclusive evidence of endotoxaemia in biliary obstruction

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Abstract

***Background*—Endotoxaemia is implicated in the pathophysiology of obstructive jaundice. The EndoCab enzyme linked immunosorbent assay (ELISA) is a novel assay which measures endogenous antibody (IgG) to the inner core region of circulating endotoxins (ACGA).**

***Aims*—To investigate the significance of endotoxaemia in biliary obstruction using the EndoCab assay and assess the specificity of the humoral response to endotoxin compared with an exogenous antigenic challenge (tetanus toxoid, TT).**

***Methods*—Three groups of adult male Wistar rats were studied: no operation, sham operation, and bile duct ligation for 21 days (BDL). In the second study, rats rats received prior immunisation with TT.**

***Results*—In the preliminary experiment, plasma ACGA was significantly increased in the BDL group (306.6 (18.3)% versus 119.9 (6.7)% and 105.2 (4.6)% in the sham and no operation groups, respectively; p<0.001). Although the mean endotoxin concentration in the BDL group was greater than that in the control groups this was not significant. There was a strong positive correlation between ACGA and endotoxin concentrations (p=0.0021). In the second study mean ACGA after 21 days of BDL was significantly elevated (267.1 (31.2)% versus 101.6 (21.2)% at baseline, p<0.0001). ACGA was unaffected in the other two groups. TT antibody concentrations fell in all three groups; only in the BDL group was the fall significant (97.6 (5.3)% versus 78.8 (4.2)% at baseline, p<0.05).**

***Conclusions*—The specific rise in ACGA supports the hypothesis that endotoxin has an integral role in the pathophysiology of obstructive jaundice. The production of anticore glycolipid antibodies specifically reflects systemic endotoxaemia in this model. The EndoCab assay provides a novel, sensitive, and specific method for endotoxin detection.**

(*Gut* 1998;**42**:293–299)

Keywords: biliary obstruction; endotoxaemia; EndoCab assay

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Accepted for publication 24 June 1997

Patients with obstructive jaundice undergoing surgical procedures have a significant risk of complications and death.[1–3] Gram negative sepsis constitutes the bulk of the morbidity and mortality, although renal dysfunction, coagulopathy, gastrointestinal haemorrhage, and impaired wound healing are well recognised.[4–6]

Many authors, using a variety of clinical and biochemical parameters, have attempted to identify those jaundiced patients most at risk; however there has been no universally accepted theory for the pathophysiology of complications seen in biliary obstruction.[7–9]

In 1970, systemic endotoxaemia was shown in jaundiced patients and was found to be associated with renal dysfunction.[10] Since then there has been much attention focused on the role of endotoxaemia in the pathophysiology of obstructive jaundice and other studies have shown increased levels of both portal and systemic endotoxins in biliary obstruction.[11–14] Although these initial studies were promising, two recent studies, using a more refined method of endotoxin detection, have not shown endotoxaemia in obstructive jaundice, casting doubt over its importance in the pathophysiology.[15 16]

Although there is considerable evidence to implicate endotoxaemia in the pathophysiology of biliary obstruction, data available on endotoxin concentrations in biliary obstruction are equivocal. Compounded by the inherent difficulties experienced by researchers using the *Limulus* amoebocyte lysate (LAL) chromogenic endotoxin assay there remains much contention over the role of endotoxaemia in the pathophysiology of biliary obstruction.

Scott and Barclay developed EndoCab, an enzyme linked immunosorbent assay (ELISA) which measures antibody produced to the highly conserved inner core region of circulating endotoxins.[17] This method of endotoxin detection has many advantages over the LAL assay which to date has been the gold standard method for endotoxin detection. This study investigates the efficacy of this novel assay in detecting systemic endotoxaemia in obstructive jaundice; its potential application in other septic conditions is discussed.

Materials and methods

EXPERIMENTAL DESIGN

Adult Wistar rats (250–300 g) from our breeding colony were housed in groups of three under constant temperature (22°C) and humidity with 12 hour dark/light cycles and allowed standard laboratory animal chow (Robert Morton and Co. Ltd, Ballymena, UK) and water ad libitum at all times throughout the experimental period.

EXPERIMENT 1

Rats were assigned to one of three groups: no operation (n=39), bile duct ligation (BDL) (n=50), and sham operation (n=40). At the

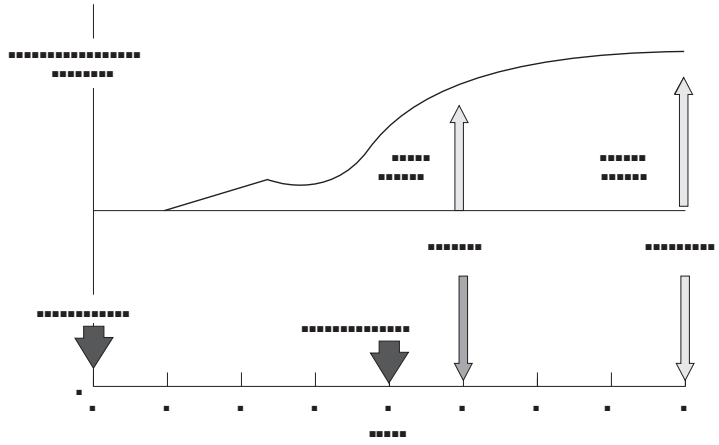

Figure 1 Summary of the important steps in experiment 2. The standard antibody response to TT is depicted in the upper frame.

end of the study period animals were deeply anaesthetised, and venous blood was collected by cardiac puncture in glass endotoxin free tubes, which were placed on ice and then centrifuged for 15 minutes at 2000 *g* at 4°C. Plasma samples were aliquoted and stored at −70°C for assay of bilirubin, endotoxin, and endogenous antibody (IgG) to the inner core region of circulating endotoxins (ACGA).

EXPERIMENT 2

Initially, 60 animals were actively immunised by intramuscular injection with 4 IU of tetanus toxoid (TT) (Pasteur Merieux); one month later they received a secondary intramuscular challenge with 4 IU of TT. Two animals were sacrificed prior to the secondary challenge and on every third day thereafter for 81 days. Plasma was collected and stored for tetanus antibody (TAB) assay.

Subsequently, 30 rats were actively immunised in similar fashion. Ten days following secondary challenge they were randomised to one of three groups: BDL, sham operation, or no operation. At this time 1 ml of blood was collected by cardiac puncture and stored for TAB and ACGA assay. After 21 days all animals were sacrificed and blood was collected for TAB and ACGA assay (fig 1). Results were expressed as the percentage rise or fall in the antibody concentrations from randomisation at time 0 (T_0) to the end of the study period 21 days later (T_{21}).

Table 1 ACGA and endotoxin concentrations in BDL rats and control groups

Model	*Endotoxin (pg/ml)*	*ACGA [IgG] (% control)*
No operation	3.4 (2.6)	105.2 (4.6)
Sham operation	12.4 (8.6)	119.9 (6.7)
BDL	21.3 (15.2)	306.6 (18.3)*

Data expressed as mean (SEM).
*$p < 0.0001$.

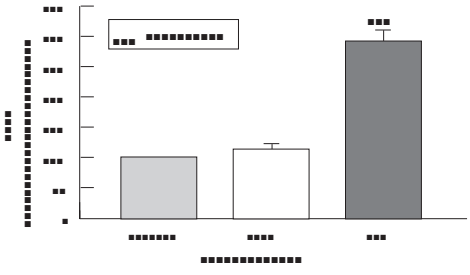

Figure 2 Concentrations of anticore glycolipid antibody (IgG) in the three animal groups after 21 days.

OPERATIVE PROCEDURES

The method described by Lee was used for bile duct ligation.[18] Briefly, via a 1 cm incision in the upper abdomen, the common bile duct was mobilised, doubly ligated using 5–0 silk, and divided. Sham operated rats had the bile duct mobilised but not ligated. All abdominal incisions were closed in two layers using 4–0 chromic catgut. All procedures were performed observing strict asepsis under general anaesthesia established using an intramuscular cocktail of ketamine 6 mg/100 g (Parke-Davis Veterinary, Gwent, UK) and xylazine 0.7 mg/100 g (Bayer UK Ltd, Bury St Edmunds, UK).

ASSAYS

Bilirubin

Bilirubin concentrations were assayed using a standard biochemical technique and expressed in μmol/l.

Endotoxin

Endotoxin concentrations were assayed using the quantitative *Limulus* amoebocyte lysate chromogenic assay (Coatest endotoxin, Kabi Diagnostica, Molndal, Sweden) and expressed in pg/ml. The samples were pretreated by a 10-fold dilution in pyrogen free water and heat treatment for five minutes at 85°C to negate the effects of plasma inhibitory factors on the assay. Endotoxin present in the plasma converts a proenzyme to an active enzyme which acts on a chromogenic substrate producing a colorimetric change, detectable spectrophotometrically at an absorbance wavelength of 405 nm.

Anticore glycolipid antibody concentrations

The relative concentration of antibodies to the core glycolipid region of lipopolysaccharide was measured using an ELISA (EndoCab, Celltech, Slough, England). This technique, originally described by Scott and Barclay, used microtitre plates coated with a cocktail of four rough endotoxin strains complexed with polymyxin B sulphate. Prediluted samples were incubated with the solid phase, and bound rat IgG detected using a specific antirat IgG-peroxidase conjugate (Serotec Ltd, Oxford, UK). The results are expressed as a percentage of the mean control value obtained from a large pool of normal rats.

Tetanus antibody

Tetanus antibody assays were performed using a standard in-house ELISA technique.

STATISTICAL ANALYSIS

Data analysis was performed on an Olivetti M300–30 microprocessor using Arcus professional software (Iain Buchan, Oxford, UK). The tests used were the Student's *t* test, and the Shapiro-Wilk, Kruskal-Wallis, Mann-Whitney

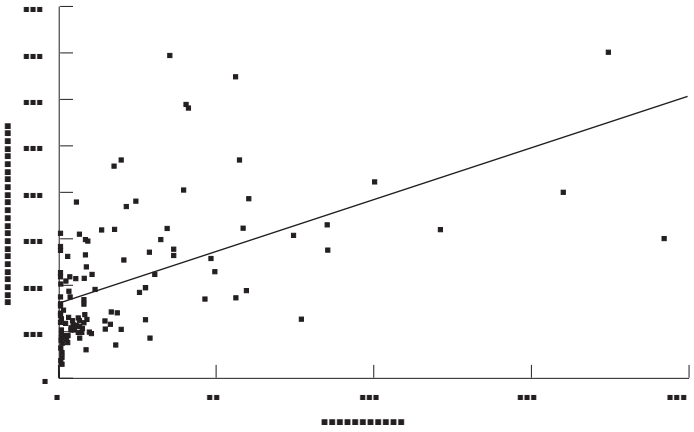

Figure 3 Correlation between ACGA and endotoxin concentrations.

Table 2 Humoral antibody response to exogenous challenge with TT and endogenous endotoxin in jaundiced animals and control groups

Model	*ACGA (T_0) (% control)*	*ACGA (T_{21}) (% control)*	*TT (T_0) (% control)*	*TT (T_{21}) (% control)*
No operation (n=8)	105.7 (9.3)	111.2 (19.2)	98.3 (5.2)	84.7 (5.9)
Sham operation (n=8)	113.9 (10.1)	109.8 (11.8)	96.5 (8.4)	82.4 (7.4)
BDL (n=14)	101.6 (21.2)	267.1 (31.2)*	97.6 (5.3)	78.8 (4.2)†

Data expressed as mean (SEM).
*p < 0.0001
† p <0.5.

U, Wilcoxon rank sum, and Spearman rank tests for correlation with statistical significance accepted at the 5% level.

Results

Table 1 shows that the concentrations of ACGA were normally distributed in rats undergoing sham operation or no operation (W=0.975, Shapiro-Wilk test for normality). In rats undergoing bile duct ligation for three weeks there was an approximately threefold increase in the concentrations of ACGA (IgG) when compared with sham operated rats (p<0.0001, Mann-Whitney U test) (fig 2). Endotoxin values were sporadically elevated in the BDL group, but never consistently so as to reach statistical significance. Despite this, ACGA and endotoxin concentrations correlated positively in this experimental model (p=0.0021, Spearman rank test) (fig 3).

In experiment 2, where the humoral antibody response to exogenous challenge with TT and endogenous endotoxin was measured, the standard response for IgG and IgM production to TT in the normal group of rats was observed (fig 4). After randomisation to the three groups, there was no significant difference between the ACGA or TAB concentrations in the three groups at T_0 (table 2). Over the course of the experiment there was no significant change in the ACGA and TAB concentrations in the control groups. In the BDL group there was a significant rise in the concentration of ACGA (p<0.0001, paired *t* test) (fig 5) and a significant decrease in TAB production compared with control groups (p=0.018, paired *t* test) (fig 6).

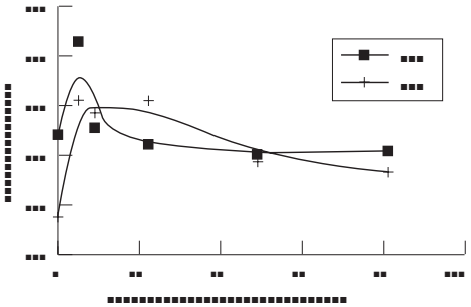

Figure 4 Standard humoral antibody response (IgG and IgM) of adult male Wistar rats to tetanus toxoid over an 81 day period.

Discussion

MECHANISMS PROPOSED FOR ENDOTOXAEMIA IN BILIARY OBSTRUCTION

Endotoxaemia is strongly implicated in the complications common to jaundiced patients undergoing surgery.[19] The pathogenic mechanism of systemic endotoxaemia in biliary obstruction is postulated to be the result of a disturbance in the homoeostatic environment of the gut-liver axis. The gastrointestinal tract provides the largest source of Gram negative bacteria in the mammalian body and the integrity of the gut mucosal barrier prevents the passage of bacteria, in either the vegetative form or as endotoxins, into the portal blood stream. Under the influence of various physiological insults such as ischaemia, obstruction, haemorrhage, infection, trauma, burns, parenteral nutrition, and some drugs, gut mucosal integrity is compromised, permitting the passage of indigenous enteric bacteria and endotoxins into sites which are normally sterile—a term which is coined bacterial translocation. Four factors have been implicated in the pathogenesis of this phenomenon: immunological impairment, direct gut mucosal injury, overgrowth of intestinal flora, and endotoxaemia per se.[20] Although the exact mechanism of bacterial translocation is not known it has been shown to occur consistently in obstructive jaundice.[21–23]

Furthermore, 85% of the body's mononuclear phagocytic cells reside in the sinusoids of the liver; their major role is to sequester and eliminate foreign material such as endotoxins from the portal bloodstream, hence protecting the systemic circulation from the gamut of physiological perturbations associated with systemic endotoxaemia. There is a large volume of clinical and experimental evidence showing depression of mononuclear phagocytic function and specifically Kupffer cell clearance capacity in obstructive jaundice.[13 24–27] Although the mechanism of depressed Kupffer cell phagocytic function is not clear various hypotheses have been derived. Direct biochemical toxicity from high systemic bile acid concentrations, high intraductal biliary pressure, portal-systemic shunting of blood, reduction in major histocompatibility complex (MHC) class II surface antigen expression, and autocrine effects of inflammatory cytokines locally have all been implicated.[28–30]

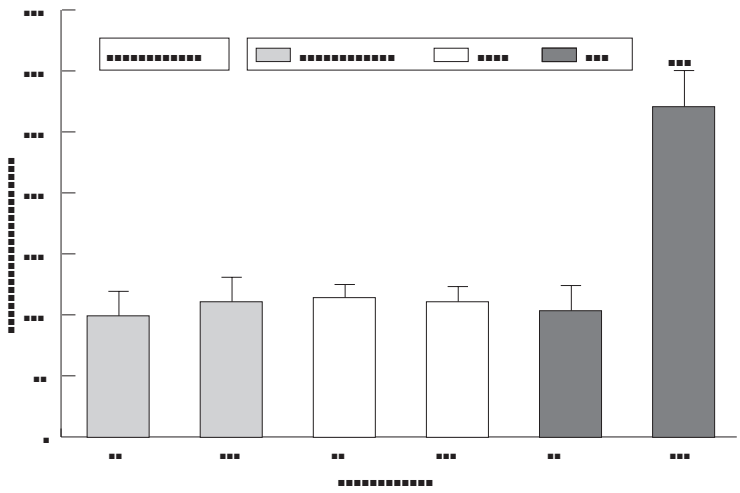

Figure 5 Change in plasma ACGA concentrations in the three animal groups over a 21 day period.

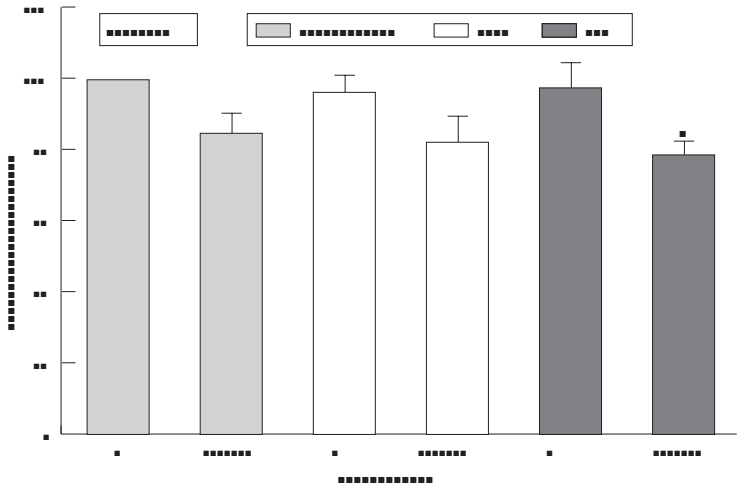

Figure 6 Change in plasma TAB concentrations in the three animal groups over a 21 day period.

EVIDENCE FOR AN ENDOTOXIN MEDIATED CYTOKINE RESPONSE

Obstructive jaundice is a condition with protean systemic manifestations. Although endotoxin is implicated in the development of these multisystemic complications, it is not intrinsically poisonous but its effect depends largely on the response of the host. The multiple organ dysfunction seen in obstructive jaundice is most likely an indirect effect of endotoxin, stimulating cytokine release from cells of the mononuclear phagocytic system resulting in a systemic inflammatory response typified by microcirculatory disruption, decreased oxygen delivery, and ultimately the multiple organ dysfunction syndrome (MODS). There is mounting evidence implicating the systemic cytokine response in the pathophysiology of obstructive jaundice.[31]

Three ways are postulated by which endotoxins may spur macrophages to produce inflammatory mediators. Primarily, LPS binds with a circulating LPS binding protein (LBP) and this complex docks with a receptor known as CD14 on the surface of the macrophage which subsequently instructs the nucleus to produce a cytokine response. It is possible that CD14 issues no signals but instead facilitates activation of the cell through direct complexing of LPS with a second surface receptor. Alternatively LPS may activate certain receptors directly without help from LBP or CD14.[32 33]

Endotoxin has been shown to cause renal impairment due to glomerular and peritubular fibrin deposition in jaundiced patients.[34] Greve *et al* showed, in an experimental study in germ free rats, that endotoxin was directly responsible for depression in cell mediated immunity.[16] Parenteral endotoxin administration in rats has been shown to produce gastric erosions.[35] Endotoxin affects the clotting cascade at various sites causing disseminated intravascular coagulation, but is equally damaging in producing increased fibrinolysis.[36] Impaired wound healing with its incumbent sequelae, namely dehiscence and incisional hernia formation, are well recognised in obstructive jaundice and although impaired nutritional status and the presence of malignancy are implicated, endotoxaemia may also be influential.[37]

EVIDENCE FROM STUDIES USING ANTIENDOTOXIN STRATEGIES

There is evidence from some studies using specific antiendotoxin strategies in obstructive jaundice; however this is not conclusive. The results from prospective clinical trials on preoperative biliary decompression by external or internal means on postoperative morbidity and mortality have been equivocal, nevertheless experimental studies have shown beneficial effects of internal biliary decompression on reducing systemic endotoxaemia.[38] Bile acids have antibacterial effects and have a direct detergent effect on the LPS molecule. It is hypothesised that their absence from the gastrointestinal tract in biliary obstruction removes the constraint on the indigenous microflora, resulting in overgrowth of bacteria and consequently bacterial translocation. In both experimental and clinical studies performed to date, where bile acids have been administered enterally, the results are inconclusive. Kocsar *et al* showed that administration of bile acids in jaundiced rats significantly reduced mortality after administration of endotoxin.[39] Cahill reported that preoperative oral bile salt administration in jaundiced patients prevented systemic endotoxaemia and consequently reduced the incidence of postoperative renal failure.[40] In a subsequent prospective randomised controlled clinical trial by Thompson *et al* in 1986, oral administration of ursodeoxycholate in jaundiced patients had no significant benefit in terms of systemic endotoxaemia, renal function, or postoperative outcome.[41] Gawley *et al* showed that sodium deoxycholate administration in jaundiced patients reduced systemic endotoxin concentrations but potentiated renal failure.[42] The results of oral bile salts, endotoxaemia, and postoperative outcome are conflicting and no definitive conclusion can be drawn regarding their association or efficacy.

Large bowel irrigation reduces the intralumenal endotoxin load but Hunt *et al* could not show any significant benefit of large bowel preparation in jaundiced patients undergoing surgery.[43] Lactulose is well recognised for its antiendotoxin properties and it has been used successfully both experimentally and clinically in reducing endotoxin related postoperative complications.[44 45]

ENDOTOXIN AND THE LAL ASSAY

The endotoxin molecule is composed of an O-specific side chain which is specific to the Gram negative bacterial strain and is very variable. It is typically composed of 20–40 repeating units that include up to eight sugar molecules. The core oligosaccharide is divided up into the outer core which connects the O-specific side chain to the highly conserved inner core molecule; this consists of two unusual sugars, heptose which has seven carbon atoms and Kdo (3-deoxy-D-manno-2-octulosonic acid) which is common to all endotoxins and links the polysaccharide to the lipid A structure. The lipid A molecule is capable of producing harmful systemic disturbances but also has certain benefits, namely, increasing host resistance to infection and cancer.

Bang in 1956 was the first to recognise and report that lysate derived from the amoebocytes of the horseshoe crab *Limulus polyphemus* clotted in the presence of minute amounts of endotoxin.[46] The *Limulus* amoebocyte assay (LAL) for endotoxin, although initially a qualitative assay, was refined by Iwanga *et al* in 1978 who noted that the activated LAL proenzyme would cleave *p*-nitroalanine substrates; this principle was used to generate a colorimetric assay capable of accurate quantitation and increased sensitivity.[47] Despite this major improvement the LAL assay is plagued with other problems which limit its sensitivity and specificity. These are related principally to the presence of endogenous and exogenous inhibitory plasma factors (esterases, elastases, antithrombin III, heparin, and LBP).[48–51] Several methods have been used to inactivate or remove these but the preferred treatment is by dilution and heat treatment.[52 53] Other problems arise in collection, preparation, and storage of the plasma sample. Endotoxin is ubiquitous and exogenous contamination is a risk at all stages from sampling to completion of the assay. Endotoxin may be rapidly denatured if not kept at 4°C after sampling; however the LAL assay involves heat inactivation which denatures proteins bound to endotoxin. The results obtained may not accurately reflect the bioactivity of endotoxins. Recognition of endotoxin by the host involves several different receptor mechanisms, not all of which result in a biological response.[54] Variations in the relative availability of these receptors, such as lipopolysaccharide binding protein, may alter the response to endotoxin. These factors limit the sensitivity of the LAL assay to reflect systemic endotoxaemia accurately. Endotoxin is believed to be released intermittently and its half life is short; single sampling may therefore miss transient endotoxaemia making interpretation of results difficult. There may be batch to batch variability in the LAL substrate which may affect the reproducibility of the assay. Consequently, results of studies using the LAL assay may be variable and to date have always been prone to criticism.

ENDOCAB ASSAY DEVELOPMENT AND APPLICATION

The EndoCab ELISA was originally devised to screen blood donor plasma for high titre antibodies to endotoxin core which are cross reactive with endotoxins of a number of Gram negative bacterial species and strains. The initial aim was to recruit a panel of EndoCab high titre donors for plasmapheresis for hyperimmune antiendotoxin gammaglobulin preparation, used in passive immunotherapy of Gram negative sepsis. The final form of the EndoCab ELISA was comprised of an equimolar cocktail of an incomplete core rough LPS (R-LPS) from each of four species (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella aerogenes*, and *Salmonella typhimurium*). Each R-LPS preserved an intact inner core but did not express complete outer core. Complexing each R-LPS with polymyxin B increased the sensitivity of the ELISA considerably.[17]

IgG EndoCab is present at birth, and is probably maternal (transplacental). This gradually diminishes over the first three months; endogenous IgG EndoCab then begins to increase. The IgM EndoCab (endogenous) is virtually absent in the first month of life but increases gradually to around adult median levels by one year. EndoCab continues to develop in children. By six to seven years of age the IgG EndoCab stabilises at the adult median concentration. The IgM EndoCab rises to above the adult range by five years, peaks around the age of 12, and descends towards the upper adult range by 16 years.[55]

Anticore glycolipid antibodies are commonly detected in healthy individuals who appear to retain their antibody levels indefinitely. Systemic endotoxaemia results in perturbations of ACGA concentrations in patients who develop sepsis. In general both IgG and IgM are depleted by the initial endotoxaemia; however the humoral amanestic EndoCab response may be triggered and EndoCab levels can rise rapidly. Hence the assay can be used to provide information on recent endotoxin exposure or antiendotoxin immunocompetence in clinical studies. Antiendotoxin core antibody assays have been used in a variety of experimental and clinical settings where endotoxaemia is felt to fuel the inflammatory response.[56–63] Immunoglobulin is physically a more stable molecule than endotoxin and the assay is not affected by contamination. Antibody responses are sustained and are more likely to reflect previous endotoxin exposure, overcoming the problem associated with single sampling and transient endotoxaemia.

Significant falls in IgG and IgM EndoCab have been recorded following a variety of clinical interventions as evidence of systemic endotoxin release, namely lithotripsy for ureteric calculi, surgery for obstructive jaundice, cardio-

pulmonary bypass, major surgery, and abdominal aortic aneurysm repair.[55 60 63] It is postulated that systemic endotoxaemia may arise directly from the site of manipulation as in the first two examples or indirectly from the gastrointestinal tract through translocation following gut ischaemia as is seen in the latter examples. Evidence from tonometry indicates that failure to maintain gastrointestinal mucosal pH in protracted surgical intervention is associated with falling EndoCab concentrations and development of the multiple organ dysfunction syndrome postoperatively.[55 63]

Systemic endotoxin binds with endogenous ACGA resulting in consumption of antibodies, a feature described in other septic states.[56 58 59 61 62] Protracted depression in IgG EndoCab concentrations in acute pancreatitis has been shown to correlate with mortality in one clinical study.[56] In a recent clinical study carried out in a large cohort of septic patients in the intensive therapy unit, significant IgG EndoCab depletion occurred in 17% of patients, 75% of whom died, resulting in a positive predictive value of 69%. Patients with IgG depletion on entry to the study had significantly higher levels of endotoxin than those where the concentration of IgG EndoCab was not depleted.[58] The EndoCab concentrations may recover within hours as 20% of EndoCab IgG is present in the peripheral circulation and the remainder is available as interstitial antibody reserves which quickly reconstitute circulating levels. More profound falls in IgM are seen as virtually all IgM is present in circulating blood.

In this experimental study exposure to endotoxin is reflected in a rise in IgG EndoCab concentrations which reflects chronicity of endotoxin exposure over a 21 day period and hyperproduction of antibody by sensitised plasma cells. This is also seen in other clinical situations such as Crohn's disease where the endotoxin release is chronic.[57] It would appear from the data available to date that perturbations in EndoCab concentrations are dependent on whether the clinical situation results in rapid release of high concentrations of systemic endotoxin or more indolent chronic endotoxaemia.

Although the main thrust of this study was to show conclusively that endotoxaemia occurrs in obstructive jaundice, we have also shown that there is evidence of immune dysfunction which confirms the immunological abrogation of the normal immune response to be a contributory factor in the development of complications associated with obstructive jaundice.[13 16 30 31]

Summary and conclusions

In this experiment using an exogenous challenge with tetanus toxoid we have shown how the humoral response is specific for endogenous endotoxin and does not represent a generalised rise in circulating immunoglobulin. A depressed humoral response to TT probably reflects immunological deficiencies in mononuclear phagocytosis, antigen presentation, and subsequent interaction with T helper lymphocytes, all prerequisites for normal B cell differentiation, clonal expansion, plasma cell formation, and normal antibody production.

These data conclusively support the hypothesis that obstructive jaundice results in systemic endotoxaemia and depression of the normal humoral antibody response. The EndoCab ELISA overcomes many of the problems encountered with the LAL endotoxin assay and provides a powerful research tool in the field of endotoxin research. A wider application in research may elucidate the role of endotoxin in a variety of clinical situations and ultimately promote the development of beneficial therapeutic strategies in conditions where endotoxaemia is an integral factor.

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