SEROLOGICAL DIAGNOSIS

Detection of antibody to C-carbohydrate of group A streptococci with enzyme-treated whole bacterial cells as antigen for ELISA

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Antibody to specific cell-wall carbohydrate of group A streptococci in human sera was determined by enzyme-linked immunosorbent assay (ELISA) with enzyme-treated whole cells and purified group A carbohydrate (ACHO) as antigens. The optimal concentration of enzyme-treated whole bacterial cells to coat wells was 2×10^8 cells/ml and for purified ACHO antigen the optimal concentration was 1 µg/ml. Sera from patients with acute rheumatic fever and acute glomerulonephritis were screened for the presence of C-carbohydrate antibodies. Patients with acute post-streptococcal complications showed significantly higher titres of antibody when compared with normal healthy individuals. ELISA results were also compared with purified ACHO as an antigen; this showed a highly significant correlation ($r = 0.73$). Results showed that measurement of the anti-ACHO antibody by ELISA with enzyme-treated whole cells can be a useful, reliable and simple method for serological diagnosis of group A infection and sequelae.

Introduction

Several investigators have detected antibodies to streptococcal antigens in sera of patients with streptococcal infection and its sequelae [1–6]. Halpern and Goldstein [7] first utilised the radioimmune precipitin technique to quantify the levels of antibody in human serum. Subsequently, Smith and Lehner [8] developed a solid-phase immunoassay with whole bacterial cells, but this was not reliable because of the instability of bacterial adsorption to solid phase. Czerkinsky *et al.* [9] developed a solid-phase immunoassay with a polyaldehyde derivative (Methylglyoxal) to link bacteria directly to the solid phase. A method of measuring anti-CHO antibody by ELISA has been developed recently with purified group A streptococcal carbohydrate antigen-polyL-lysine [ACHO-PLL] complex [10]. Current serological methods for the diagnosis of streptococcal infection test for antibodies to extracellular products such as streptolysin O or DNAase B.

The present study investigated the potential advantage of ELISA for routine diagnosis of infection with group A streptococci (GAS) and sequelae with whole bacterial cells as antigen, and compared it with ELISA with purified ACHO antigen.

Materials and methods

Strain and culture

GAS strain J-17 was grown overnight at 37°C in Todd-Hewitt broth. The cells were harvested by centrifugation at 3000 rpm for 10 min and washed three times with sterile saline.

Preparation of enzyme-treated whole bacterial cells

GAS strain J-17 was suspended in saline 0.87% and heated at 60°C for 45 min, washed and suspended in 50 mM phosphate buffer (PB) at pH 7.4. Cells were then treated according to the method of Todome *et al.* [11]. Suspended cells were treated with DNAase 100 µg/ml and trypsin 100 µg/ml at 37°C for 4 h. Whole cells treated with trypsin were treated again with pronase E 100 µg/ml containing NaN₃ 0.1% at 37°C overnight. Enzyme-treated whole cells were washed thoroughly with 50 mM PB and suspended at a concentration of 2×10^8 cells/ml in methylglyoxal 0.3% at pH 8.0 by the method described by Czerkinsky *et al.* [9]. Treated whole cells were used as an antigen for ELISA.

Extraction of ACHO antigen

The cell walls of GAS were isolated by Mickle disintegration and differential centrifugation according to the method of Salton and Horne [12]. The cell walls

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were treated with DNAase 100 $\mu\text{g/ml}$, RNAase 100 $\mu\text{g/ml}$ and trypsin 100 $\mu\text{g/ml}$ at 37°C for 3 h. The enzyme-treated cell walls were washed three times with 50 mM PB. ACHO was then extracted from cell walls by the hot formamide method of Fuller [13] and assayed for rhamnose and glucosamine content [14, 15].

Coupling of group A carbohydrate to poly L-lysine

Coupling of ACHO with PLL was done by the method of Gray [16]; briefly, 2 mg of formamide-extracted ACHO was added to 1.0 ml of 10 mM NaOH, pH 12, and mixed gently for 10 s. This solution was then transferred to a tube containing cyanuric chloride 5 mg and mixed gently; pH was monitored continuously. At pH 8.4 the supernate was transferred quickly to a tube containing 0.2 ml of PLL, taking care to avoid transfer of cyanuric chloride crystals. The final pH was 8.0–8.2. The ACHO-PLL conjugate was stored at –20°C in small volumes until used.

Antisera production

Antiserum was raised in rabbits against whole GAS cells and purified ACHO by the method described by McCarty and Lancefield [17]. The serum antibody was determined by gel immunodiffusion.

Sera

Serum was collected from 100 clinically and serologically diagnosed patients with post-streptococcal complications. Fifty were from cases of acute rheumatic fever (RF) and 50 from those with acute glomerulonephritis. Serum from 50 normal healthy subjects with no evidence of AGN or RF served as a control group.

ELISA with ACHO-PLL complex as antigen

ELISA was done according to the method described by Gray [16] and Barrett *et al.* [10] with slight modifications. Purified ACHO coupled to PLL was used as an antigen to coat the wells of the microtitration plate. Antigen diluted to 1 $\mu\text{g/ml}$ in 10 mM PBS, pH 7.4, was used to coat wells of an Immunol II 96-well microtitration plate. Incubation was for 1 h at 37°C followed by overnight incubation at 4°C. Plates were washed with phosphate-buffered saline (PBS) containing 0.05% Tween-20 and 500 mM NaCl (PBST-NaCl). PLL alone was coated as a control. Plates were blocked with BSA 1% in 10 mM PBS and incubated for 1 h at 37°C. At the end of incubation, plates were washed three times with PBST-NaCl, then standard or samples were added, incubated at 37°C for 1 h and washed as above; this was followed by application of anti-human IgG labelled to horseradish peroxidase (HRPO). Colour was produced with 2,2-azino-di-(3-ethylbenzthiazolin)

sulphonic acid substrate. The OD was measured at 490 nm.

ELISA with enzyme-treated whole cell antigen

For ELISA, wells of an Immunol II 96-well microtitration plate were coated with enzyme-treated whole cells suspended at a concentration of 2×10^8 cells/ml in methylglyoxal (Sigma) 0.3%, pH 8.0, adjusted with sodium bicarbonate 10%. Plates were incubated at 37°C for 3 h, washed three times with 50 mM PBST and blocked with BSA 1% in PBST. After incubation for 90 min at 37°C, wells were washed with PBST and human serum was added to each well and incubated at 37°C for 90 min. Wells were washed and HRPO-labelled anti-human IgG or biotinylated monoclonal mouse anti-human IgG1 (clone 8c/6–39), IgG2 (clone HP-14), IgG3 (clone HP-50) and IgG4 (clone HP-25) antibodies optimally diluted and added to wells, followed by incubation with HRPO-labelled Extra avidin. Plates were washed and 2,2-azino-di-(ethylbenzthiazolin) sulphonic acid was added as substrate to show colour and plates were read at 490 nm.

Results

Detection of anti-ACHO antibody in rabbit serum

Rabbit antiserum raised against whole cell vaccine, showed a line of precipitation with purified ACHO in gel immunodiffusion. A line of precipitation with purified ACHO was observed up to 1 in 6400 dilution, and, with group A extract, the line was up to a 1 in 12 800 dilution.

Detection of ACHO antibody with enzyme-treated whole cells as antigen

Microtitration plates were coated with various concentrations of trypsin-protease-treated (T-P) whole cells ranging from 2×10^5 to 2×10^{10} cells/ml (100 μl /well). Serum dilutions ranging from 1 in 10 to 1 in 12 800 were added to each dilution of antigen. The optimal concentration of antigen was 2×10^8 cells/ml and the serum dilution 1 in 100 (Figs. 1 and 2). Patients' and control sera were screened for ACHO antibodies. Patients' sera showed a significant difference ($p < 0.01$) compared with controls.

Distribution of IgG subclasses of antibody to ACHO

Serum diluted 1 in 50 was used to detect IgG subclasses of anti-ACHO antibody. The predominant subclass of antibody found against ACHO in sera of all normal and most RF and AGN sera was IgG2. Only five (2.5%) of 50 AGN and seven (3.5%) of 50 RF patients' sera showed high titres of IgG3 subclass antibody (Fig. 3).

Fig. 1. Reference curve to determine optimal concentration of T-P whole cells to coat wells of ELISA plate. Serum dilutions: —■—, 1 in 50; —▲—, 1 in 100; —●—, 1 in 200.

Fig. 2. Determination of optimal dilution of human serum to detect GAS anti-ACHO antibody. Wells were coated with 2×10^8 T-P-whole cells/ml.

Screening of anti-CHO antibody in human serum with ACHO-PLL complex as antigen

Antigen was diluted to 1.5, 1.0, 0.5, 0.25 and 0.15 $\mu\text{g/ml}$ in 10 mM PBS, pH 7.4. Serum dilutions ranging from 1 in 10 to 1 in 250 were added to each dilution of antigen. The antigen diluted to 1.0 $\mu\text{g/ml}$ showed the highest OD and the serum dilution showed a plateau at a 1 in 100 dilution and thereafter a decrease with further dilution (Fig. 4). These results indicate that the optimal dilution for antigen was 1.0 $\mu\text{g/ml}$ and the serum dilution was 1 in 100; 100 sera from patients

with AGN and RF and from 25 normal healthy control subjects were screened for anti-ACHO antibodies. The titres in patients' sera were significantly higher than in the control group ($p < 0.01$) (Table 1).

Discussion

Antibodies to ACHO antigen have been detected in patients with GAS infection and in those with complications such as RF and AGN. Persistence of a

Fig. 3. Distribution of IgG subclass (IgG1, IgG2, IgG3 and IgG4) antibodies to ACHO in normal human sera (■) and patients with post-streptococcal complications (AGN, □; RF, ▨).

Fig. 4. Anti-ACHO antibody in human serum with ACHO-PLL complex as antigen. ELISA plates coated with ACHO-PLL antigen at concentrations of 1.5 µg/ml (■), 1 µg/ml (▲), 0.5 µg/ml (●), 0.25 µg/ml (□) and 0.15 µg/ml (○).

Table 1. Mean antibody titre in AGN and RF patients and controls assessed by ELISA with ACHO-PLL complex and enzyme-treated whole cells as antigen

Subjects	Mean antibody titre (SD) with	
	ACHO-PLL antigen	Whole cell antigen
AGN	0.71 (0.19)	0.85 (0.25)
RF	0.63 (0.12)	0.77 (0.27)
Controls	0.26 (0.17)	0.30 (0.18)

high level of antibodies to ACHO has been demonstrated in patients with rheumatic heart disease and has been utilised as a marker for persistence of rheumatic mitral valve disease [18–20].

This report describes ELISA to measure the anti-ACHO antibody with enzyme-treated whole cells as

antigen and compares it with ELISA with purified ACHO-PLL complex. The anti-ACHO antibody titre was significantly higher in patients than in the control group. Comparison of ELISA results with enzyme-treated whole cells as antigen with those of purified ACHO antigen showed significant correlation ($r = 0.73$).

T-P enzyme treatment of GAS strain J-17, produced complete digestion of protein antigens on the cell surface and exposure of ACHO on the surface. The mechanism of action between enzyme-treated whole cells and methylglyoxal is not clearly understood. It may cross-link amino groups exposed at the bacterial surface [21] or produce an electrostatic action with polystyrene. ACHO adsorbs poorly on to plastic surfaces because of a net negative charge conferred

by acidic groups. This was solved by conjugating ACHO to poly-L-Lysine which adsorbs strongly on to plastic surfaces.

In humans, antibodies to carbohydrate and dextran predominantly belong to the IgG2 subclass [22]. However, mice immunised with purified ACHO produce antibody of IgG3 subclass [23, 24]. Riesen *et al.* [22] also reported IgG3 subclass of antibody in the sera of persons with GAS infection. Todome and colleagues [11] reported IgG2 subclass antibody in the sera of normal persons and patients with RF and AGN. The present results also show that serum from normal subjects showed IgG2 subclass only. However, 3.5% of RF patients' sera and 2.5% of AGN patients' sera showed antibodies of subclass IgG3.

The ELISA technique with whole bacterial cells as antigen offers a promising alternative to other methods for the routine diagnosis of streptococcal infections and their complications because it is sensitive and easy to perform. Furthermore it avoids biohazards and the use of expensive equipment.

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