Staphylococcus aureus protein A–antibody-mediated haemagglutination for the analysis of cross-reactivity between phenol-water extracts of *Bacteroides fragilis*

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Summary. Passive haemagglutination (PHA) and *Staphylococcus aureus* protein A–antibody-mediated haemagglutination (SAPA–AMHA) were used to analyse the cross-reactions of rabbit antisera against four stains of *Bacteroides fragilis*. There was cross-reactivity between all the strains tested but strain-specific reactions were obtained with three strains. Two to 32-fold higher antibody titres were obtained with SAPA–AMHA than with PHA. The antigen concentration required in inhibition assays was up to 32-fold higher for PHA than for SAPA–AMHA. The latter is a simple and superior alternative to PHA for such studies.

Introduction

The antigenic heterogeneity amongst strains of *Bacteroides fragilis* is similar to that seen in the genus *Salmonella* (Hofstad, 1977). Depending on the sensitivity of the procedure, various degrees of cross-reactivity have been demonstrated between strains of *Bacteroides* by agglutination (Elhag and Tabaqchali, 1978), passive haemagglutination (PHA) (Hofstad, 1977), indirect immunofluorescence (Schwan *et al.*, 1982) and enzyme linked immunosorbent assay (Poxton, 1979).

Staphylococcus aureus protein A–antibody-mediated haemagglutination (SAPA–AMHA) (Jagannath *et al.*, 1984) is a more sensitive assay for IgG antibody than PHA and was thus thought to be more suitable for the analysis of cross-reactivity between *B. fragilis* antigens with rabbit immune sera containing predominantly IgG antibodies.

Materials and methods

Bacteria

B. fragilis NCTC nos. 9343, 9344, 10581, 10584 and 8560 were kindly supplied by Dr A. Schwan, University of Uppsala, Uppsala, Sweden. Identification of these strains was confirmed by Gram's stain, aerobic and anaerobic

culture, biochemical tests and ability to grow on bile-aesculin agar (Sutter *et al.*, 1980).

Antigen preparation

Phenol-water extracts (PWE) were prepared as follows. Bacteria were grown anaerobically for 48 h on Brain Heart Infusion Agar supplemented with sheep blood 5%, vitamin K₁ 10 µg/ml, haemin 5 µg/ml and yeast extract 0.5% (Sutter *et al.*, 1980). Harvested bacteria were washed three times with cold sterile physiological saline and suspended to a concentration of 200 mg wet weight/ml. The suspensions were sonicated separately in ice baths for 10–15 min with a 150 watt MSE ultrasonic disintegrator at an amplitude of 12 µ. The sonicates were then extracted with aqueous phenol 90% w/v at 65°C (Sutherland, 1978) to yield PWE antigens. These were treated with RNAase (Sigma) and DNAase (Sigma) as described by Hofstad and Kristoffersen (1970) and adjusted to a concentration of 1 mg/ml. At the concentrations used in this study, the protein content estimated by the method of Lowry *et al.* (1951) was negligible in the PWE preparations.

Rabbit antisera

Antisera were raised separately for each strain in rabbits by injections of formalinised bacteria as described by Elhag *et al.* (1977).

Preparation of double-aldehyde-stabilised (DAS) red cells

Human group 'O' red blood cells were treated with glutaraldehyde, tannic acid and pyruvic aldehyde as described by Jagannath and Sengupta (1983).

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Sensitisation of DAS red cells

PWE antigens were treated with 0.25 N NaOH for 1 h at 56°C and neutralised with 0.25 N HCl. DAS red cells were washed three times with phosphate buffered saline, pH 7.2 (PBS) and 100- μ l volumes were suspended in various concentrations of PWE antigens and incubated for 2 h at 37°C and then overnight at 4°C. The sensitised red cells were then washed twice with PBS, once with PBS containing 0.1% bovine serum albumin (diluent) and resuspended to 1% in the latter. The optimal sensitising dose was defined as the lowest concentration of antigen that gave the maximum haemagglutination titre with homologous rabbit antiserum.

Passive haemagglutination (PHA)

Antibody assay. PHA titration was performed in U-bottom microtitration plates. After absorption with unsensitised DAS cells to remove heterophile antibodies, antisera were serially double diluted and 25 μ l of sensitised red cells were added to 25- μ l volumes of diluted antisera. The plate was shaken for 2 min and incubated at room temperature for 2 h. PHA patterns were recorded as described by Stavitsky (1954).

Inhibition of PHA (Hofstad, 1977). PWE antigens (25 μ l) were serially double diluted from wells 1 to 11 in microtitration plates. The first well contained 12.5 μ g and the eleventh well 0.012 μ g of the antigen. Antiserum (25 μ l), containing 8 haemagglutinating units, was then added to wells 2–12. Wells 1 and 12 were controls. The plate was shaken for 2 min and incubated at room temperature for 1 h before addition of 25 μ l of sensitised red-cells suspensions to each well. Haemagglutination patterns of 1+ or less were taken to indicate inhibition of PHA.

S. aureus protein A-antibody-mediated haemagglutination (SAPA-AMHA)

S. aureus cells bearing protein A (SAPA) were prepared from Cowan I cells grown in Casein hydrolysate-Cystine-Yeast extract broth (Arvidson *et al.*, 1971) and stabilised with formalin and heat as described by Kessler (1975). Before titration, antisera were absorbed with unsensitised DAS red cells and formalin-fixed protein A-negative *S. aureus* strain Wood 46 cells (Jagannath *et al.*, 1984).

Antibody assay. The microtitration protocol described by Jagannath *et al.* (1984) was followed. Briefly, to 25- μ l volumes of serially double diluted antisera in microtitration plates an equal volume of an optimal concentration (determined by a preliminary titration) of SAPA cells (usually 0.2% w/v) was added. The plates were then agitated gently on a horizontal shaker for 30 min before the addition of sensitised red cells as for PHA. Controls included sensitised red cells mixed with SAPA cells in the absence of serum. The co-haemagglutination (CHA) patterns, which are similar to PHA patterns, were scored as for PHA.

Inhibition of CHA (Jagannath *et al.*, 1984). Antibody-

coated SAPA cells were prepared by mixing equal volumes of SAPA cells at the optimal concentration (0.2% w/v) and 4 CHA units of antiserum (one CHA unit is the highest dilution of antiserum giving a 3+ reaction in SAPA-AMHA) which were then incubated at 37°C for 1 h. After washing three times in PBS, the cells were suspended to the original concentration in diluent.

The assay was conducted by adding 25 μ l of antibody-coated SAPA cells to 25- μ l volumes of serially double diluted PWE antigens in microtitration wells. The plates were shaken for 2 min, incubated at room temperature for 1 h and 25 μ l of sensitised red cells were added to each well. As controls, SAPA cells coated with normal rabbit serum were titrated against sensitised and unsensitised DAS red cells, and SAPA cells coated with immune rabbit serum were titrated against unsensitised DAS red cells. A CHA reaction of 1+ or less was taken to indicate inhibition of CHA.

Cross-absorption studies

Antisera were absorbed with equal volumes of appropriately sensitised packed DAS red cells before assay by PHA and SAPA-AMHA at starting dilutions of 1 in 20 and 1 in 40 respectively. For example (table III) antiserum to NCTC 9343 was absorbed with NCTC 10584 PWE antigen-sensitised DAS red cells and then titrated by PHA and SAPA-AMHA against PWE antigen-sensitized DAS red cells of the four stains of *B. fragilis*.

Results

Table I shows the results of cross-titration of the four PWE antigens against homologous and heterologous rabbit antisera by PHA and SAPA-AMHA. Strain NCTC 8560 was not included in the study because its PWE antigen bound poorly to DAS red cells.

With PHA and SAPA-AMHA, two-way cross-reactions were seen between strains NCTC 9343 and NCTC 10584 and between strains NCTC 9344 and NCTC 10581. However, by SAPA-AMHA alone, a significant degree of cross-reactivity with NCTC 9344 and NCTC 10581 could be demonstrated in rabbit anti-NCTC 9343 serum, and with NCTC 10581 in rabbit anti-NCTC 10584 serum.

In inhibition experiments (table II), the results of PHA and SAPA-AMHA appeared to be identical in that PWE antigens inhibited homologous antigen-antibody systems. No inhibition of heterologous systems was evident with PWE antigens of NCTC 9344 and NCTC 10581 at the concentrations used. PWE antigen from NCTC 9343 inhibited the reaction of PWE antigen from NCTC 10584-sensitized DAS red cells with rabbit anti-NCTC 10584, and similarly PWE antigen of NCTC 10584 was found to inhibit the reaction of PWE antigen of

Table I. Antibody titres to PWE antigens of *B. fragilis* in homologous and heterologous rabbit antisera tested by PHA and SAPA-AMHA

PWE antigen derived from strain		Titre with antisera to NCTC strains							
		9343		10581		9344		10584	
		P	S	P	S	P	S	P	S
NCTC 9343	9343	160	2560	<20	20	<20	40	80	5120
NCTC 10581	10581	20	160	320	5120	80	2560	20	160
NCTC 9344	9344	20	80	160	640	160	5120	20	20
NCTC 10584	10584	160	1280	20	<20	<20	<20	80	1280

P = PHA; S = SAPA-AMHA.

Table II. Degree of cross-reactivity between strains of *B. fragilis* evaluated by inhibition of PHA (IP) and inhibition of SAPA-AMHA (IS)*

PWE antigen used for inhibition		System		Minimum inhibitory concentration (μ g)	
		PWE antigen used for sensitisation	vs Rabbit anti-serum raised against strain	IP	IS
NCTC 9343	9343	NCTC 9343	NCTC 9343	1.56	0.39
NCTC 9343	9343	NCTC 10584	NCTC 10584	0.78	0.024
NCTC 10584	10584	NCTC 10584	NCTC 10584	0.39	0.048
NCTC 10584	10584	NCTC 9343	NCTC 9343	1.56	0.39
NCTC 9344	9344	NCTC 9344	NCTC 9344	6.35	0.78
NCTC 10581	10581	NCTC 10581	NCTC 10581	3.13	3.13

* Only combinations in which inhibition was observed are shown.

NCTC 9343-sensitised DAS red cells with rabbit anti-NCTC 9343.

Antigen concentrations in the range 0.024–0.78 μ g were sufficient to inhibit the reaction between antibody-coated SAPA cells and homologous antigen-sensitised red cells (inhibition of CHA), while antigen concentrations of 0.39–6.25 μ g were required for inhibition of PHA (table II). However, for PWE antigen of NCTC 10581 alone, inhibition of homologous system occurred at similar concentrations in both PHA and SAPA-AMHA.

After cross-absorption (table III), antisera generally retained the ability to react with homologous antigens except with rabbit anti-NCTC 10584, from which antibodies to homologous antigen were completely removed by absorption with DAS red cells sensitised with antigen from NCTC 9343.

Table III. Effect of cross-absorption of heterologous rabbit antisera on the antibody titres to PWE antigens of *B. fragilis* tested by PHA(P) and SAPA-AMHA (S)*

PWE antigen used for sensitisation		Titre with antisera to NCTC strains†							
		9343 ^a		10581 ^b		9344 ^c		10584 ^d	
		P	S	P	S	P	S	P	S
NCTC 9343	9343	—	40	nt	—	nt	—	—	—
NCTC 10581	10581	nt	—	80	320	—	—	nt	—
NCTC 9344	9344	nt	—	—	—	80	320	nt	—
NCTC 10584	10584	—	—	nt	—	nt	—	—	—

— = No reaction.

nt = Not tested.

* Starting dilution for P = 1 in 20 and for S = 1 in 40.

† Antisera absorbed with red cells sensitised with PWE antigen of NCTC strains: a = 10584; b = 9344; c = 10581 and d = 9343.

Again, with SAPA-AMHA alone, a minor degree of reactivity to homologous antigen-sensitised red cells was demonstrable in rabbit anti-NCTC 9343 serum absorbed with red cells sensitised with antigen from NCTC 10584.

Discussion

Previous studies with agglutination (Elhag and Tabaqchali, 1978) and immunofluorescence (Schwan, 1981) suggested that a pool of *B. fragilis* strains consisting of NCTC nos. 9343, 9344, 10581, 10584 and 8560 would be suitable for serodiagnosis of infections caused by this organism. After appropriate modification with NaOH or heat, PWE antigens have been shown to bind to red blood cells (Neter, 1956). Thus, in this study, we have analysed the PWE antigens of these *B. fragilis* strains for cross-reactivity by PHA and SAPA-AMHA. Our results indicate that an antigen or antigenic determinant is common to strains which are thought to be representative of *B. fragilis* (Kasper *et al.*, 1983; Linko-Kettunen *et al.*, 1984). While strain-specific determinants have been demonstrated by cross-absorption experiments, PWE antigens of strains NCTC 9343 and NCTC 10584 appear almost identical.

These results are similar to those of Hofstad (1977), who provided evidence for cross-reacting and strain-specific determinants in PWE antigens of *B. fragilis* strains by PHA. While confirming the earlier observations (Elhag and Tabaqchali, 1978; Schwan *et al.*, 1982) that there is a significant degree of cross-reactivity between *B. fragilis* strains NCTC 10581 and NCTC 9344, it was possible in this study to demonstrate greater levels of cross-reactivity amongst the strains.

PWE antigens are likely to contain both lipopolysaccharide (LPS) and capsular polysaccharide (CP). A recent study with monoclonal antibodies (Linko-Kettunen *et al.*, 1984) confirmed that *B. fragilis* LPS is responsible for species specificity while CP appears to confer strain specificity (Kasper *et al.*, 1983). Because we did not attempt to increase the CP content by passage through mouse spleen (Kasper *et al.*, 1983), the greater level of cross-reactivity observed in this study could be due to

differences in the antigens or in techniques used for analysis or both.

A comparison of PHA and SAPA-AMHA (table I) confirmed the greater sensitivity of SAPA-AMHA. In agreement with the previous observations (Jagannath *et al.*, 1984) SAPA-AMHA gave titres two to 32-fold greater than PHA. This range of enhancement has been attributed to the presence of different levels of IgG class antibodies in the antisera and to the Fc binding activity of protein A-bearing *S. aureus* (Jagannath *et al.*, 1984). The increased sensitivity of SAPA-AMHA made possible the demonstration of minor cross-reactions between the various strains in this study.

Inhibition experiments with SAPA-AMHA (table II) also proved to be more sensitive than PHA. This was ascribed to the smaller number of antibody molecules involved in the CHA reaction and the correspondingly smaller quantities of antigen necessary for inhibition (Jagannath *et al.*, 1984). It is apparent that a decrease in the concentration of antibodies used to coat SAPA cells will enhance the sensitivity of the inhibition assay. However, the optimal dilution of antiserum for conducting the assay must be determined by a preliminary titration. Although a detailed study of antibody concentrations in the inhibition assay was not undertaken, a dilution of antiserum containing 4 CHA units appears to be suitable for most assays because at this dilution there was good correlation between inhibition and cross-absorption experiments (table III).

The results of this study do not indicate the combination of antigens necessary for serodiagnosis with PWE antigens. This will have to be determined with PWE antigens derived from a larger number of *B. fragilis* strains. Moreover, defined antigen preparations, such as purified LPS (devoid of CP), will have to be evaluated in this system.

However, SAPA-AMHA appears to offer some advantages for the study of cross-reactivities between antigen preparations suitable for sensitisation of red blood cells.

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