Demonstration of Shared Antigenic Determinants between *Streptococcus mutans* BHT Cell Membrane, Human Heart Tissue and Myosin Using Monoclonal Antibodies to *S. mutans*

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Monoclonal antibodies (MAb) raised to intact *Streptococcus mutans* P-4 cells (serotype *e*) were used to demonstrate the presence of shared antigenic determinant(s) between *S. mutans* BHT (serotype *b*) cell membranes and human heart tissue. MAb binding to both BHT membrane and human heart tissue was demonstrated by ELISA. Common antigens were identified by immunoblot analysis following separation of BHT membrane components and human heart antigens by SDS-PAGE. MAb 22C4 recognized three polypeptides from the BHT membrane preparation, having molecular masses of 42, 56 and 85 kDa. MAb 22C4 also recognized an 85 kDa component and a 200 kDa component from human heart tissue. MAb D159 was specific for a single 82 kDa polypeptide in BHT membrane, and also bound to two high molecular mass components in human heart (165 and 200 kDa). When both MAb D159 and 22C4 were first absorbed with *S. mutans* P-4 cells, subsequent reactivity to the aforementioned BHT membrane components was inhibited, indicating that these cross-reactive components are found in *S. mutans* P-4 as well as in *S. mutans* BHT micro-organisms. Competitive binding analysis showed that both MAb D159 and MAb 22C4 bound to myosin, indicating that *S. mutans* BHT membrane, human heart tissue and myosin share at least one immunodeterminant. This indicates that myosin could be the cross-reactive tissue component in human heart.

INTRODUCTION

Immunochemical studies of *Streptococcus mutans*, considered by some to be the major causative agent of dental caries, indicate that while immunization with whole *S. mutans* cells is protective in several model systems (Bowen *et al.*, 1975; Lehner *et al.*, 1975; McGhee *et al.*, 1975), the presence of components which are cross-reactive with mammalian tissue (van de Rijn *et al.*, 1976; Ferretti *et al.*, 1980; Hughes *et al.*, 1980) precludes using the intact bacterial cell for immunization. Clearly greater knowledge concerning the immunogenic structures of *S. mutans* is required in order to identify and purify non-cross-reactive components which could be used prophylactically.

A series of cell-wall-associated cross-reactive antigens have been described by various laboratories (Russell & Lehner, 1978; Russell *et al.*, 1980, 1982; Russell, 1979, 1980; Forester *et al.*, 1983). All these cross-reactive components have been found in the cell walls and culture supernatants of all *S. mutans* serotypes except *b*; much less is known concerning the presence of such cross-reactive components in this serotype. However, in the earliest series of experiments

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Abbreviation: MAb, monoclonal antibody/antibodies.

demonstrating cross-reactivity with *S. mutans*, van de Rijn *et al.* (1976) showed that *S. mutans* BHT (serotype *b*) does in fact contain one or more components which are cross-reactive. Ayakawa *et al.* (1985*a*) have studied the possible localization of cross-reactive components in the cytoplasmic membrane of *S. mutans* BHT. They showed that rabbit anti-human heart antiserum reacts with *S. mutans* BHT membrane, and, by Western blot analysis of the *S. mutans* BHT membrane preparation, that the anti-human heart antiserum recognizes four major polypeptide components with molecular masses of 42, 46, 62 and 82 kDa. They also showed that rabbit antiserum raised to *S. mutans* BHT membrane reacts with human heart tissue and recognizes a major polypeptide of 69 kDa. While these results do indicate that rabbits immunized with heart tissue develop an immune response to *S. mutans* BHT membrane antigens and vice versa, whether or not antigenic determinants are shared between human heart and *S. mutans* BHT membrane remains to be proven.

Cross-reactive components are also associated with *Streptococcus pyogenes* (group A streptococcus), which has been implicated in the pathogenesis of a variety of conditions, most notably rheumatic fever (Kaplan *et al.*, 1964; Zabriskie *et al.*, 1970). The presence of heart-reactive antibodies in serum from patients with acute rheumatic fever is well documented (Kaplan & Suchy, 1964; Zabriskie & Freimer, 1966) and as with *S. mutans*, immunization of animals with the micro-organism resulted in the production of antibodies with heart cross-reactivity (Cunningham & Russell, 1984). Cunningham *et al.* (1984) have produced a panel of heart-cross-reactive murine monoclonal antibodies (MAb) to *S. pyogenes* and recently have shown that one of these MAb also reacts with myosin derived from rabbit muscle (Krisher & Cunningham, 1985), suggesting that myosin shares an immunodeterminant with a component from *S. pyogenes*. They also reported that reactivity with heart tissue and *S. pyogenes* decreased when the MAb was absorbed with *S. mutans* GS5.

In this study, using two MAb raised to *S. mutans*, we attempted to demonstrate the presence of shared antigenic determinants between *S. mutans* BHT membranes and human heart tissue, and also examined the reactivity of these MAb with myosin.

METHODS

Bacterial strains. *Streptococcus mutans* P-4 (serotype *e*) was grown aerobically at 37 °C in 2% (w/v) yeast extract, 2% (w/v) dextrose broth (Linke & Chang, 1976) supplemented with 0.7% K_2HPO_4 and 0.3% KH_2PO_4 . Cells were harvested in the late-exponential phase and washed three times with PBS (0.15 M-NaCl in 0.05 M-potassium phosphate, pH 7.2) prior to their use for immunization of mice or for ELISA. *S. mutans* BHT (serotype *b*) was grown in the chemically defined medium of Terleckyj *et al.* (1975) as previously described (Ayakawa *et al.*, 1985*a*).

Production of MAb to *S. mutans* and polyclonal anti-BHT membrane antisera. The hybridoma procedure used was a modification of that of Kohler & Milstein (1975) as described by Doyle & Everhart (1985). Once cloned, the hybridomas were expanded and then injected either subcutaneously into athymic (*nu/nu*) BALB/c mice (Laboratory Supply Company, Indianapolis, Ind., USA), or intraperitoneally (i.p.) into BALB/c mice which had been 'primed' 2 weeks earlier by an i.p. inoculation of 0.5 ml pristane (2,6,11,14-tetramethylpentadecane, Sigma). Sera obtained from tumour-bearing mice containing high titres of MAb were used in ELISA and Western blot analysis of BHT membrane and human heart extracts. Ascites fluid was used as the source of MAb for inhibition experiments with myosin (see below). Polyclonal antiserum raised to *S. mutans* BHT membrane was prepared as described previously (Ayakawa *et al.*, 1985*a*).

Preparation of *S. mutans* BHT membrane and human heart tissue for ELISA and immunoblotting. Human heart tissue and the cell membrane of *S. mutans* BHT were prepared by our previously published methods (Bleiweis *et al.*, 1964; Ayakawa *et al.*, 1985*a*; Seigel *et al.*, 1981). Total protein contents of membranes and heart tissue preparations were determined by the Lowry method.

ELISA. MAb were screened, isotyped, titrated and characterized according to the method of Cobbald & Waldmann (1981) as described previously (Doyle & Everhart, 1985).

SDS-PAGE and Western blot analysis. The polypeptide components of *S. mutans* BHT membrane and human heart tissue were separated by SDS-PAGE as described by Doyle & Everhart (1985) and either silver stained (Merril *et al.*, 1981) or electrophoretically transferred onto nitrocellulose paper (Towbin *et al.*, 1979). The transferred polypeptides were then probed with biotinylated MAb according to the glucose oxidase method described by Porter & Porter (1984) with some modifications. Nitrocellulose paper was placed in blocking buffer consisting of 3% (w/v) BSA in 0.05 M-Tris/HCl (TBS, pH 7.4) overnight at room temperature. The nitrocellulose was then reacted for 1.5 h at room temperature with biotinylated MAb diluted either 1 in 20 or 1 in 200 in blocking

buffer. The nitrocellulose was then washed once for 10 min in TBS, twice for 10 min in the same buffer containing 0.5% (v/v) Nonidet P-40, and once more for 10 min in TBS. The paper was then reacted for 2 h at room temperature with glucose oxidase labelled avidin diluted 1 in 500 in blocking buffer, and then washed four times as in the previous step. The substrate consisted of 0.75% (w/v) D-glucose in 0.1 M-sodium phosphate buffer (pH 6.8) supplemented with 0.01% (w/v) phenazine methosulphate and 0.05% (w/v) paranitroblue tetrazolium chloride. The substrate solution was put on the paper and incubated for 1 h at 37 °C in the dark. The nitrocellulose was then rinsed once in PBS, soaked for 5 min in Lillie's neutral buffered formalin (100 ml 37%, v/v, formaldehyde, 4.0 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 12.2 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 900 ml H_2O) and rinsed in H_2O .

In some experiments, the biotinylated MAb were absorbed with washed *S. mutans* P-4 cells prior to use in Western blot analysis. In still other experiments, resolved BHT proteins were first incubated with unlabelled MAb followed by incubation with biotinylated rabbit polyclonal anti-BHT membrane antisera (diluted 1 in 100).

Inhibition experiments. Inhibition experiments were done to characterize MAb binding to myosin. Volumes (100 µl) of MAb 22C4 (1 in 100) and MAb D159 (1 in 50) were reacted with 100, 10 and 1 µg ml⁻¹ chicken myosin (Sigma) or with 5×10^{10} , 2.5×10^{10} , 1.25×10^{10} and 7.5×10^9 *S. mutans* P-4 cells. Each reaction was carried out at 37 °C for 1 h. Residual antibody activity was then assayed by reacting each sample with either myosin or *S. mutans* in an ELISA as described above. In these experiments, MAb was absorbed with BSA or *E. coli* at concentrations equivalent to those used with myosin or *S. mutans* to serve as a positive control. MAb replaced with PBS in each experiment served as a negative control.

Biotinylation of antibodies. Antibodies used in Western blot analysis were labelled with biotin (Kendall *et al.*, 1983). Biotinyl-*N*-hydroxysuccinimide (BNHS, Calbiochem) was used to covalently link biotin to MAb. MAb-containing sera from tumour-bearing athymic (*nu/nu*) BALB/c mice or rabbit polyclonal antisera were diluted 1 in 1 in PBS. BNHS (1.7 mg) was dissolved in 1 ml distilled dimethylformamide and added to MAb at a ratio of 1:50 (v/v). After incubation for 4 h at room temperature, the reaction mixture was dialysed for 24 h at 4 °C against several changes of PBS.

RESULTS

Two established hybridoma cell lines producing MAb to *S. mutans* P-4 were used in this study. Both hybridoma lines, D159 and 22C4, produced a MAb which was identified isotypically as IgMk. Titres of MAb to *S. mutans* P-4 in sera recovered from tumour-bearing athymic (*nu/nu*) BALB/c mice were 10^{-3} for MAb D159 and 10^{-2} for MAb 22C4 (Table 1). Normal sera from athymic mice showed no reactivity. Both MAb D159 (10^{-2}) and MAb 22C4 (10^{-1}) reacted with human heart tissue as well as BHT membrane antigens in the ELISA (Table 1).

The results obtained by Western blot analysis of BHT membranes and human heart tissue complemented those of ELISA and allowed individual specificities of MAb to be assigned to antigens of defined molecular mass. MAb 22C4 recognized three polypeptide bands in the BHT membrane preparations, having molecular masses of 42, 56 and 85 kDa (Fig. 1, lane A). In human heart tissue, two components, 85 and 200 kDa, were identified with this same antibody (Fig. 1, lane B). MAb D159 recognized a BHT membrane component of 82 kDa (Fig. 1, lane C) while in human heart, the same MAb recognized two polypeptide bands of 165 and 200 kDa (Fig. 1, lane D). Normal sera from nude mice showed no reactivity (data not shown). When

Table 1. *Characterization of monoclonal antibodies*

	Antibody class	Antibody titre*	Binding to BHT membranes†	Binding to heart†
22C4	IgMk	10^{-2}	0.110 ± 0.020 ‡	0.095 ± 0.005 ‡
D159	IgMk	10^{-3}	0.105 ± 0.005 §	0.050 ± 0.010 §
Normal sera	—	10^0	0.025 ± 0.005	0.010 ± 0.010
PBS	—	—	0.020 ± 0.010	0.005 ± 0.005

* Antibody titres to *S. mutans* P-4 cells were determined by ELISA with sera obtained from tumour-bearing athymic (*nu/nu*) BALB/c mice.

† Values represent the mean of total absorbance (490 nm) $\pm$ standard errors of the mean of duplicate determinations per sample.

‡ At 10^{-1} dilution.

§ At 10^{-2} dilution.

Fig. 1. SDS-PAGE and Western blot analysis of human heart tissue and BHT membrane antigens. Samples containing 20 µg protein were resolved by SDS-PAGE, electrophoretically transferred onto nitrocellulose and probed with MAb. BHT membrane antigens were probed with MAb 22C4 (lane A) and MAb D159 (lane C). Human heart antigens were also reacted with MAb 22C4 (lane B), and MAb D159 (lane D). MAb 22C4 was used at a final dilution of 1 in 20; MAb D159 at 1 in 200. As negative controls, both preparations were also reacted with normal nude mouse serum (not shown). The bands are labelled with their molecular masses (kDa).

MAb 22C4 and D159 were first absorbed with *S. mutans* P-4 cells, subsequent reactivity with resolved BHT membrane components was inhibited (data not shown).

With ascites fluid as the source of MAb, both 22C4 and D159 reacted with *S. mutans* P-4 cells at dilutions of 10^{-4} (data not shown). To characterize MAb reactivity with myosin, a series of inhibition experiments were done using ascites fluid as the source of MAb. MAb 22C4 was used at a dilution of 1 in 100 and MAb D159 at 1 in 50. Both MAb showed reactivity with myosin (Figs 2 and 3). When the MAb were absorbed with increasing concentrations of myosin (Fig. 2), subsequent reactivity to both *S. mutans* cells and myosin was increasingly inhibited. Absorption of these MAb with similar concentrations of bovine serum albumin did not inhibit ELISA reactivity (data not shown). Likewise, when MAb were absorbed with an increasing number of *S. mutans* cells (Fig. 3), subsequent reactivity was also reduced. Again, the degree of inhibition paralleled the concentration of bacteria used to inhibit.

DISCUSSION

In one of the first studies with *S. mutans* demonstrating the existence of antigens which cross-react with human cardiac tissue, van de Rijn *et al.* (1976) demonstrated cross-reactivity in strains from several serotypes, including serotype *b*. While subsequent studies have identified cross-reactive components associated with the cell wall and culture supernatants of *S. mutans*

Fig. 2.

Fig. 2. MAb 22C4 and D159 were absorbed with increasing concentrations of myosin and then reacted against either *S. mutans* P-4 cells or myosin in an ELISA. ●, Reactivity of D159 with *S. mutans* P-4 cells following absorption with myosin; ○, reactivity of D159 with myosin following absorption with myosin; ■, reactivity of 22C4 with P-4 following absorption with myosin; □, reactivity of 22C4 with myosin following absorption with myosin. Control values (▲) represent reactivity of PBS with myosin following absorption with myosin. Control values where PBS was absorbed with myosin and then reacted with *S. mutans* were comparable but are not shown. Each value represents the mean of duplicate determinations. MAb absorbed with BSA at concentrations equivalent to those used with myosin resulted in values approximately equal to those seen when MAb alone was incubated with antigen (data not shown).

Fig. 3.

Fig. 3. Reactivity of MAb following absorption with increasing concentrations of *S. mutans* P-4 cells. ●, Reactivity of D159 with P-4 following absorption with P-4; ○, reactivity of D159 with myosin following absorption with P-4; ■, reactivity of 22C4 with P-4 following absorption with P-4; □, reactivity of 22C4 with myosin following absorption with P-4; ▲, control values showing reactivity of PBS with P-4 following absorption with P-4. Control values where PBS was incubated with P-4 and then reacted with myosin were comparable but are not shown. Each value represents the mean of duplicate determinations. MAb absorbed with *E. coli* at concentrations equivalent to those used with *S. mutans* gave values corresponding to those seen when MAb alone was incubated with antigen (data not shown).

(Russell & Lehner, 1978; Russell, 1979, 1980; Hughes *et al.*, 1980; Russell *et al.*, 1982; Forester *et al.*, 1983), these components are apparently not found in serotype *b* micro-organisms. The cytoplasmic membrane has hence been focused upon as the possible source of cross-reactive antigens in *S. mutans* serotype *b*, specifically strain BHT. Ayakawa *et al.* (1985a) have shown that the cytoplasmic membrane of strain BHT contains cross-reactive antigens. Western blot analysis of BHT membranes showed that four polypeptides with molecular masses ranging from 42 to 82 kDa were reactive with rabbit anti-human heart sera. A mutually cross-reactive component of 69 kDa in human heart was identified with rabbit anti-BHT sera. These results clearly showed that rabbits immunized with *S. mutans* BHT developed an immune response to human heart tissue and vice versa. However, whether the same determinants were being recognized in human heart tissue and in BHT membranes could not be determined using polyclonal antisera.

In the present study, the existence of shared antigenic determinants between BHT membranes and human heart tissue was demonstrated with monoclonal antibodies. In an ELISA, two monoclonal antibodies, MAb 22C4 and MAb D159, reacted with both BHT membranes and human heart tissue (Table 1). Since MAb are, by definition, specific for a single epitope, it is evident that BHT membranes and human heart tissue share at least one antigenic determinant. Results from Western blot analysis of BHT membranes and human heart tissue suggest that MAb 22C4 and MAb D159 recognize different antigenic determinants (Fig. 1). Even though both MAb recognized a 200 kDa component in human heart, MAb 22C4 also recognized an 85 kDa component in the same preparation whereas MAb D159 recognized another polypeptide of 165 kDa. (It is possible that the 200 kDa component contains two antigenic sites.) The two antibodies recognized polypeptides of different molecular mass in BHT membranes. Results from competitive binding experiments where resolved BHT membrane components were first incubated with MAb, then with labelled rabbit polyclonal anti-BHT membrane antisera, also suggest that the two MAb recognize different polypeptides. When resolved BHT membrane components were first incubated with D159 followed by polyclonal anti-BHT antisera, five major bands corresponding to molecular masses of 85, 65, 56, 42 and 39 kDa were recognized by the polyclonal antisera. When the same experiment was done using MAb 22C4, only the 65 and 39 kDa bands were seen (data not shown). This experiment also suggests that the 85, 56 and 42 kDa components recognized by MAb 22C4 are the same as the 82, 62 and 46 kDa cross-reactive components first identified by the rabbit polyclonal, anti-BHT membrane antisera reported by Ayakawa *et al.* (1985*a*), variations in the reported molecular masses being attributable to differences in the conditions under which SDS-PAGE was performed. Furthermore, it appears that MAb D159 recognizes a cross-reactive component not previously identified by polyclonal antisera (Ayakawa *et al.*, 1985*a*).

It is of interest that van de Rijn *et al.* (1977) isolated and partially purified a cross-reactive antigen from the cytoplasmic membrane of *S. pyogenes*, the Lancefield group A streptococcus which has been implicated in the pathogenesis of rheumatic fever (Unny & Middlebrooks, 1983). Both MAb 22C4 and MAb D159 reacted with *S. pyogenes* in an ELISA (Doyle & Everhart, 1985). This suggests that the cross-reactive antigenic determinants identified here are shared between these two streptococcal species. It is not known whether the cross-reactive antigens in *S. pyogenes* recognized by MAb D159 and MAb 22C4 are located in the cytoplasmic membrane. However, these findings support those of Krisher & Cunningham (1985), who demonstrated that the reactivity of a heart-cross-reactive monoclonal antibody raised to *S. pyogenes* could be inhibited by absorption with *S. mutans* GS5.

Krisher & Cunningham (1985) have also shown that this same monoclonal antibody recognizes the heavy chain of myosin, suggesting that myosin contains an antigenic site which is shared by *S. pyogenes*, and thereby identifying myosin as the possible cross-reactive tissue component in heart. From competitive binding experiments shown here, it is evident that both MAb 22C4 and D159 recognize myosin, demonstrating that *S. mutans*, *S. pyogenes* and myosin share at least one immunodeterminant. Furthermore, preliminary data (Ayakawa *et al.*, 1985*b*) indicate that the myosin-cross-reactive MAb described by Krisher & Cunningham (1985) reacts with membranes of *S. mutans* BHT, strengthening the evidence in favour of a relationship between myosin, *S. pyogenes* and *S. mutans*. It would be of interest to determine whether the monoclonal antibody produced by Krisher & Cunningham (1985) recognizes the same antigenic determinants as MAb 22C4 and D159.

Since MAb 22C4 and MAb D159 were both raised in response to *S. mutans* P-4 (serotype *e*) cells, the cross-reactive antigens identified in this study are not unique to *S. mutans* BHT (serotype *b*). When the MAb were absorbed with *S. mutans* P-4 cells prior to Western blot analysis, subsequent reactivity with resolved BHT membrane components was inhibited (data not shown). Previously, both MAb D159 and 22C4 have been shown to react with a 35 kDa component present in a partially purified membrane preparation derived from *S. mutans* P-4. It would appear therefore, that the 35 kDa component from *S. mutans* contains at least two cross-reactive epitopes, both of which are also present in the BHT cell membrane. In addition, both of these MAb react with representative strains of serotypes *a-h* (Doyle & Everhart, 1985). The fact

that both MAbs react with intact cells strongly suggests that the relevant epitopes are exposed at the cell surface of this species. It would be of interest to see whether these antigens are also associated with the cytoplasmic membrane in these serotypes and whether they are related to cell wall cross-reactive antigens previously described (Russell, 1979, 1980; Hughes *et al.*, 1980; Russell *et al.*, 1982; Forester *et al.*, 1983).

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