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Oral Immunology

Inhibition of adherence of *Candida albicans* and *Candida dubliniensis* to a resin composite restorative dental material by salivary secretory IgA and monoclonal antibodies

N Elguezabal[1], JL Maza[2], J Pontón[1]

[1]*Departments of Inmunología, Microbiología y Parasitología;* [2]*Estomatología, Facultad de Medicina y Odontología, Universidad del País Vasco, Apartado, Bilbao, Vizcaya, Spain*

OBJECTIVE: The attachment of *Candida* to oral surfaces is a crucial step in the colonization of the oral cavity and the eventual development of oral diseases caused by this microorganism. Inhibition of adhesion is one of the strategies currently studied to prevent *Candida* infections. The main objective of this study was to investigate the inhibitory effect of the human salivary components on the adherence of *Candida albicans* and *C. dubliniensis* to Herculite, a widely used resin composite restorative dental material. We have also investigated the influence on the adherence of three monoclonal antibodies (mAbs) directed against *C. albicans* cell wall antigens.

DESIGN: The adhesion of three strains of *C. albicans* and one strain of *C. dubliniensis* was studied by a visual method after incubating the fungus and the resin in presence and in absence of human whole saliva, secretory immunoglobulin A (sIgA) and three mAbs directed against *C. albicans* cell wall surface antigens.

RESULTS: Adherence of *C. albicans* was inhibited by whole saliva (41.7%), salivary sIgA (55.7%) and the salivary components that bind to the cell wall (36.7%). Whole saliva significantly reduced the adhesion of *C. dubliniensis* to Herculite to 45.3% of the control level. Saliva previously adsorbed with fungal cells or sIgA depleted saliva had no effect on adherence. An inhibition in the adhesion of *C. albicans* and *C. dubliniensis* to Herculite similar to that shown by whole saliva was also observed when mAbs C7 and 26G7 were used. However, mAb 21E6 increased adhesion of all the strains to Herculite.

CONCLUSIONS: The results suggest that sIgA, as well as whole saliva, are important in blocking adherence of *C. albicans* and *C. dubliniensis* to Herculite and that this effect can be reproduced with mAbs directed against the cell wall surface of *C. albicans*.
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Keywords: *Candida albicans*; *C. dubliniensis*; adherence; Herculite; antibodies; secretory immunoglobulin A

Introduction

Candida albicans is a commensal fungus normally found in the oral cavity of 20–40% healthy individuals (Odds, 1988). Under host immunosuppression and other predisposing conditions *C. albicans* can cause a variety of oral infections, including pseudomembranous candidiasis, erythematous candidiasis and hyperplasic candidiasis, as well as *Candida*-associated denture stomatitis, *Candida*-associated angular cheilitis, rhomboid glossitis and chronic mucocutaneous candidiasis (Delgado and Aguirre, 1997). *Candida dubliniensis* is a recently described species which shows a worldwide distribution (Sullivan *et al*, 1997) and a close phenotypic and genotypic relationship with *C. albicans* (Sullivan *et al*, 1995). Although it seems to be primarily associated with recurrent oral infections in HIV-infected individuals, more recently it has also been isolated from healthy persons as well as from cases of superficial and systemic disease in non-HIV infected patients (Sullivan *et al*, 1999; Willis *et al*, 1999; Brandt *et al*, 2000; Salesa *et al*, 2001).

As the oral cavity has a number of defense mechanisms to control the growth of microorganisms (Marcotte and Lavoie, 1998), to remain in the oral cavity *Candida* species must adhere to the mucosal surface or to the hard surfaces which may be present in the mouth. Thus, adherence of *C. albicans* to plastic materials or to salivary macromolecules adsorbed on the surface of dental prosthesis is an important event in the ability of *C. albicans* to colonize dentures in the mouth (Vasilas *et al*, 1992). Although little work has been carried out

Correspondence: José Pontón. Departamento de Inmunología, Microbiología y Parasitología, Facultad de Medicina y Odontología, Universidad del País Vasco, Apartado 699, E-48080 Bilbao, Vizcaya, Spain. Tel: 34-94-6012855, Fax: 34-94-4649266,
E-mail: oipposaj@lg.ehu.es
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on the adhesion of oral microorganisms to restorative materials we are interested in the adherence of *Candida* to hybrid composites as we expect that once the fungus adheres to the restorative material, it can colonize other oral surfaces and cause oral infections in predisposed individuals. In an attempt to explore this area we have developed a model in order to study the adhesion of *C. albicans* to the resin composite Herculite (Maza *et al*, 2002). In the present work, we have studied the effect of the human salivary components on the adherence of *C. albicans* and *C. dubliniensis* to the composite restorative dental material Herculite, as we have recently demonstrated that whole saliva has an inhibitory effect on adherence of *C. albicans* to this material (Maza *et al*, 2002). We have also investigated the influence on the aforementioned adherence of three monoclonal antibodies (mAbs) produced in our laboratory against *C. albicans* cell wall antigens.

Materials and methods

Fungal strains and culture conditions

Candida albicans serotype A (NCPF 3153) and *C. dubliniensis* CD36 (NCPF 3949), two filamentous strains obtained from the National Collection of Pathogenic Fungi (NCPF, Bristol, UK), *C. ablicans* serotype B (ATCC 90028), a filamentous strain obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and *C. albicans* Ca2, a germ tube-deficient mutant of the parental strain serotype A NCPF 3153, kindly supplied by Dr A. Cassone (Istituto Superiore di Sanita, Rome, Italy), were used in these experiments. The strains were maintained at 4°C on slants containing 20 g of agar per liter.

Saliva and monoclonal antibodies

The experimental protocols were approved by the Institutional Review Board of the School of Medicine an Odontology at the University of the Basque Country, Leioa, Spain, and the subjects gave their informed consent. Unstimulated whole saliva samples were collected from a healthy female donor to eliminate sample variations. The donor had not taken any medication during the 3 months proceeding the study and had no active periodontal disease or active caries. Saliva was centrifuged at 6000 *g* for 30 min at 4°C and the supernatant was stored at 4°C to be used the same day or stored at −80°C until used.

To obtain the salivary components that bind to the surface of *C. albicans*, whole saliva was adsorbed with formalin killed germ tubes or yeast cells of *C. albicans* NCPF 3153 suspended at 10^{10} cells ml^{-1} of phosphate-buffered saline (PBS). Adsorption was performed by mixing equal volumes of the adsorbing suspension and saliva. After incubation in a rotary mixer for 2 h at room temperature, the organisms were removed by centrifugation. Supernatants were concentrated to the initial volume and stored at −80°C until used. The *C. albicans* cells used in the adsorption of saliva were washed with PBS (7 mM Na_2HPO_4, 2.7 mM NaH_2PO_4 and 145 mM NaCl; pH 7.2) and components attached to the cell wall surface of *C. albicans* were then released by treatment with 2.5 M NaI in PBS for 1 h at room temperature. Cells were removed by centrifugation, and the supernatant was dialysed against PBS for 48 h at 4°C. Eluted components were concentrated to the initial volume with polyethylene glycol 20 000 and stored at −80°C until used. The eluted components were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (Pontón *et al*, 1996). The total amount of protein loaded per lane was 5 μg.

To obtain saliva depleted of secretory immunoglobulin A (sIgA), saliva samples were applied to an Affi-Gel 10 Column (BioRad, Richmond, CA, USA) coupled to an anti-human IgA (Sigma, St Louis, MO, USA). The saliva depleted of sIgA was concentrated to the initial volume as described above and the depletion of sIgA was assessed by SDS-PAGE and Western blotting with both a goat anti-human IgA and a goat anti-human secretory component (Pontón *et al*, 1996). The purity of the sIgA eluted from the affinity chromatography column was also checked by SDS-PAGE and Western blotting where an irrelevant sIgA purified from human colostrum (Sigma) was used as a control.

Three IgM mAbs (C7, 26G7 and 21E6) were produced following standard methods with splenocytes from BALB/c mice immunized by subcutaneous injections of a 200 kD *C. albicans* cell wall stress mannoprotein recognized by human saliva (C7), *C. albicans* molecules attached to plastic surface after the elimination of the adhered germ tubes (21E6) (Pontón *et al*, 1993), and a 150 kD glycoprotein from the cell wall of *C. glabrata* (26G7). Monoclonal antibodies were purified from ascites fluid by affinity chromatography on an anti-mouse IgM column (Sigma). Purified antibodies were dialysed against PBS before use.

Immunofluorescence

Indirect immunofluorescence assay (IFA) was carried out as described previously (Pontón *et al*, 1993). Briefly, germ tubes produced in medium TC199 pH 6.7 for 4 h at 37°C were air-dried on IFA slides and incubated 30 min at 37°C with each mAb diluted 1 : 5 in PBS supplemented with Evans blue [0.05% (wt/vol)] and Tween 20 [0.05% (vol/vol) PBSTE]. After washing, the reacting antibodies were revealed by incubation with fluorescein isothiocyanate (FITC)-conjugated, goat anti-mouse IgM (Sigma) diluted 1 : 150 in PBSTE for 30 min at 37°C. Slides were mounted with carbonate-glycerol mounting fluid and examined with an epifluorescence microscope. The reactivity of mAb C7 and 21E6 was studied after mild periodate oxidation (20 min at room temperature with 10 mM sodium metaperiodate (Sigma) in 100 mM acetate buffer, pH 5.3).

Preparation of Herculite pieces

The acrylic mixture (Herculite XRV Restorative Resin; Kerr manufacturing Co., Romulus, MI, USA) was placed between two glass slides separated by spacers to leave a uniform distance of 1 mm between the glasses. After photopolymerization for 40 s by each side with a halogen curing light (Heliolux; Vivadent, Tonawanda,

NY, USA), the plates were cut into pieces of 10×10 mm, polished with Sof-Lex disks (3 M Co., St Paul, MN, USA), and washed with distilled water before being used in the adhesion experiments.

Adherence assay

The method described by Tronchin *et al* (1988) with the modifications described by Maza *et al* (2002) was used in all experiments. Briefly, yeast cells were inoculated in medium 199, pH 6.7, at a final concentration of 8×10^5 cells ml^{-1} and were incubated for 2 h at 37°C in 24-well tissue culture polysterene plates containing the Herculite pieces and 350 μL of the yeast cell suspension. After incubation, the pieces were removed from the wells, washed with saline solution, and stained with 0.1% calcofluor white in saline for 20 min at room temperature. Herculite pieces were washed three times with saline, and the fluorescence was read in a microscope equipped to detect fluorescence. Adhesion was quantified by counting the total number of cells in 12 fields (0.64 mm^2 each) per Herculite square by means of a graticule mounted in the focus of the ocular. Results were expressed as the number of cells per millimetre squared (mean values derived from four independent assays). To determine the effect on adherence of the whole saliva, adsorbed saliva, sIgA-depleted saliva or the salivary components eluted from the surface of *C. albicans*, the yeast cells were inoculated in medium 199 containing 280 μL of these fluids. In experiments with mAbs or purified sIgA, 20 μg of each antibody were added to the yeast cell suspension.

Statistics

The ANOVA test was used to assess the significance of differences between means in adherence assays. Data were considered significant at $P < 0.05$.

Results

Effect of the salivary components on the adherence of C. albicans *and* C. dubliniensis *to Herculite resin*

The ability of whole saliva to inhibit the adherence of *C. albicans* NCPF 3153 and *C. dubliniensis* NCPF 3949 to Herculite resin was initially assessed. In the absence of human saliva the adhesion levels of *C. albicans* NCPF 3153 and *C. dubliniensis* NCPF 3949 to Herculite resin at the end of the incubation period were 52.2 and 28.9 cells mm^{-2}, respectively (Figures 1 and 2). Whole saliva significantly reduced the adhesion of *C. albicans* NCPF 3153 and *C. dubliniensis* NCPF 3949 to Herculite to 41.7 and 45.3% of the control level, respectively ($P < 0.05$). When whole saliva was replaced by saliva adsorbed with *C. albicans* cells, the adhesion levels of *C. albicans* NCPF 3153 and *C. dubliniensis* NCPF 3949

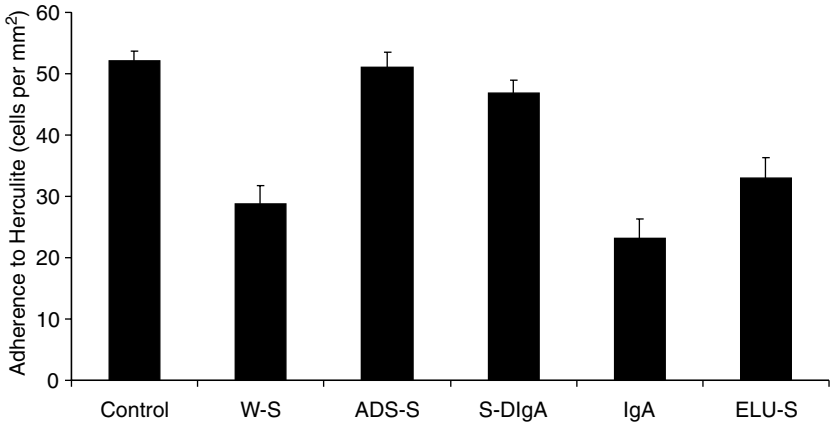

Figure 1 Adhesion of *Candida albicans* A NCPF 3153 to Herculite resin in absence of saliva (Control) or presence of whole saliva (W-S), adsorbed saliva (ADS-S), saliva depleted of IgA (S-DIgA), purified IgA (IgA) and eluted saliva (ELU-S). Results represent the means of quadruplicate determinations ± s.d.

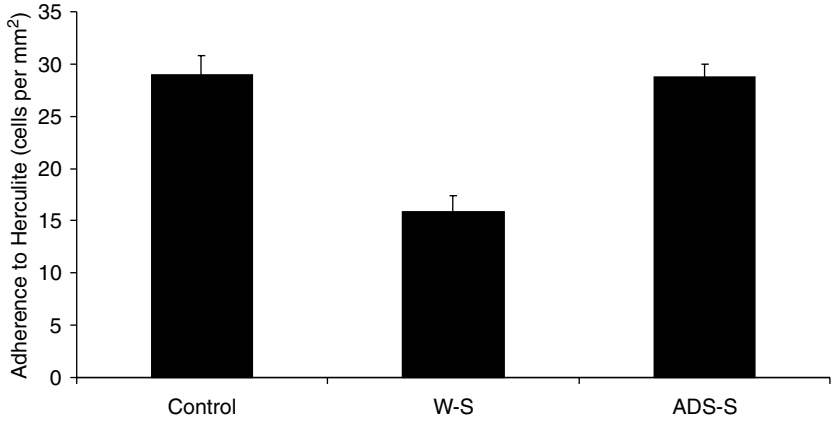

Figure 2 Adhesion of *Candida dubliniensis* NCPF 3949 to Herculite resin in absence of saliva (Control), or presence of whole saliva (W-S) and adsorbed saliva (ADS-S). Results represent the means of quadruplicate determinations ± s.d.

Table 1 Adherence of *Candida albicans* A NCPF 3153, *C. albicans* B ATCC 90028 *C. dubliniensis* NCPF 3949 and *C. albicans* A Ca2 to Herculite resin incubated with mAbs C7, 26G7 and 21E6 at 37°C for 120 min. Results represent the means of quadruplicate determinations ± s.d.

	Control		*MAb C7*		*MAb 26G7*		*MAb 21E6*	
Strain	*Adherence ± s.d.*	*% Inhibition*	*Adherence ± s.d.*	*% Inhibition*	*Adherence ± s.d.*	*% Inhibition*	*Adherence ± s.d.*	*% Enhancement*
NCPF 3153	37.4 ± 0.6	0	23.6 ± 2.9	36.7 ± 7.9	19.0 ± 2.4	49.0 ± 6.3	47.4 ± 3.3	26.7 ± 8.8
ATCC 90028	47.3 ± 1.0	0	17.6 ± 1.5	62.8 ± 3.2	45.3 ± 0.8	4.2 ± 1.7	53.8 ± 2.3	13.8 ± 4.9
NCPF 3949	27.2 ± 1.7	0	16.3 ± 1.3	40.2 ± 4.6	16.7 ± 0.9	38.3 ± 3.5	32.1 ± 2.1	18.0 ± 7.6
Ca2	9.5 ± 1.0	0	5.0 ± 0.6	47.2 ± 6.6	3.2 ± 0.8	65.9 ± 8.3	14.2 ± 2.4	49.4 ± 2.5

to Herculite resin were similar to that of the controls (Figures 1 and 2).

In an attempt to identify the salivary components involved in the inhibition of the adhesion of *C. albicans* NCPF 3153 to Herculite resin, sIgA purified from human saliva and salivary components eluted from the surface of *C. albicans* were also tested. Both salivary components significantly reduced the adhesion of *C. albicans* NCPF 3153 to Herculite resin to 55.7 and 36.7% of the control level, respectively ($P < 0.001$). However, sIgA-depleted saliva did not cause an inhibitory effect on adherence, showing an adhesion level similar to that of the control (Figure 1).

Effect of mAbs on the adherence of C. albicans *and* C. dubliniensis *to Herculite resin*

In a different experiment, the ability of three mAbs directed against epitopes expressed on the cell wall of *Candida* to inhibit the adherence of *C. albicans* NCPF 3153, ATCC 90028, Ca2 and *C. dubliniensis* NCPF 3949 was studied. MAbs C7 and 21E6 reacted by indirect fluorescence with the cell wall surface of all strains studied while mAb 26G7 reacted with *C. albicans* NCPF 3153, Ca2 and *C. dubliniensis* NCPF 3949 but not with *C. albicans* serotype B strain ATCC 90028.

Two of the MAbs studied inhibited the adhesion of *C. albicans* and *C. dubliniensis* to Herculite resin to levels similar to those observed with whole saliva and sIgA (Table 1). MAb C7 produced an inhibition of adhesion ranging from 36.7 to 62.8%. However, mAb 26G7 only inhibited the adherence of *C. albicans* serotype A strains (NCPF 3153 and Ca2) and *C. dubliniensis* NCPF 3949 but showed no effect on the *C. albicans* serotype B strain ATCC 90028. Interestingly, mAb 26G7 seemed to inhibit the adherence of the serotype A strains (NCPF 3153, Ca2 and NCPF 3949) to a greater extent than mAb C7. Conversely, mAb C7 showed an important inhibitory effect on serotype B strain ATCC 90028 (62.8%). In the presence of mAb 21E6 the adhesion levels of all strains to Herculite resin were increased, being higher than the controls in absence of mAb.

Discussion

The human mouth presents a variety of surfaces to which microorganisms of the oral microbiota can adhere. These surfaces are constantly bathed by the saliva, which provides water, nutrients, adherence and antimicrobial factors (Marcotte and Lavoie, 1998). A number of studies have shown that the adhesion of *C. albicans* to oral epithelial cells and plastic materials used to make dental prosthesis is modulated by human saliva (Samaranayake and MacFarlane, 1980; McCourtie, MacFarlane and Samaranayake, 1986; Umazume, Ueta and Osaki, 1995; San Millán *et al*, 2000) and we have previously reported that whole saliva caused a statistically significant decrease in the adhesion of *C. albicans* to Herculite resin (Maza *et al*, 2002). In this study, we have confirmed the inhibitory effect showed by whole saliva on the adhesion of *C. albicans* to the composite resin and we have identified the sIgA as the salivary component responsible for this effect, as sIgA purified from human saliva caused a reduction in the adherence of *C. albicans* to Herculite, and the removal of sIgA from the saliva abolished the inhibitory effect presented by whole saliva on the adhesion of *C. albicans* to the resin composite. These results extend the idea that salivary sIgA acts as a barrier for oral colonization by *Candida* by interfering the adhesion of the fungus with oral surfaces. In fact, sIgA and possibly other salivary components, are likely to block the adhesion of *C. albicans* to Herculite resin, by binding the cell wall surface of the fungus and masking the fungal adhesins, as the adsorption of saliva with *C. albicans* yeast cells abolished the ability of whole saliva to inhibit the adhesion of *C. albicans* to the resin and the salivary components eluted from the cell wall surface of the yeasts used in the adsorption inhibited the adhesion to levels similar to those found with whole saliva.

Although *C. dubliniensis* presents a close phenotypic and genotypic relationship with *C. albicans* (Sullivan *et al*, 1995), it showed a statistically significant reduction ($P < 0.001$) in the adhesion to Herculite resin when compared with that shown by *C. albicans*. However, whole saliva reduced the adhesion of *C. dubliniensis* NCPF 3949 to Herculite in a way similar to that observed for *C. albicans* NCPF 3153.

Health disorders such as Sjogren's syndrome and medication may produce salivary gland dysfunction and xerostomia and cause an increase in the oral carriage of *Candida* (Tapper-Jones, Aldred and Walker, 1980; Baum, Ship and Wu, 1992). Oral candidiasis can, therefore, be increased in patients with salivary hypofunction. The administration of mAbs mimicking the anti-*Candida* activities of sIgA could be one of the ways to prevent the development of oral candididiasis in those patients. In the last years, there has been controversy

over the importance of antibody- and cell-mediated protection in candidiasis (Cassone *et al*, 1997; Casadevall *et al*, 1998) but considerable evidence is being accumulated on the role of antibodies in the protection of candididasis (Matthews and Burnie, 2001) and cryptococcosis (Casadevall *et al*, 1992). In order to explore a future role of antibodies in the protection of oral candidiasis the ability of three mAbs directed against cell wall antigens was assessed in this study. As mAb C7 was produced against a 200 kD cell wall stress mannoprotein which is a major target of salivary sIgA (Pontón *et al*, 1996), it was expected to act like salivary sIgA preventing adherence. Surprisingly, our results show that the activity of mAb C7 may be strain dependent as it was effective in blocking adherence of *Candida* to Herculite in all strains but the highest inhibitory activity was observed with the *C. albicans* serotype B strain. Conversely, mAb 26G7 only decreased adherence of the *C. albicans* serotype A strains and the *C. dubliniensis* strain, which also expresses the serotype A antigens (Sullivan *et al*, 1995). These results are in agreement with data obtained by indirect immunofluorescence showing that mAb 26G7 does not react with the cell wall of the serotype B strain but it strongly reacts with serotype A strains and that mAb C7 reacts with the protein moiety of a glycoprotein which is partially masked by the sugar moiety (Moragues *et al*, 2003).

Although whole saliva has been shown to decrease adhesion of *C. albicans* to denture acrylic *in vitro* (Samaranayake and MacFarlane, 1980; McCourtie *et al*, 1986) a saliva-mediated enhancement of the adhesion of *Candida* to denture acrylic has been also described (Vasilas *et al*, 1992). Interestingly, one of the mAbs used in this study, 21E6, enhanced adherence of all the strains studied to Herculite resin. These results are in agreement with data reported by our group about this mAb in adherence assays of *Candida* to polysterene (San Millán *et al*, 1996) and suggest that some antibodies present in the saliva may enhance the adhesion of *Candida* to oral surfaces. If this is the case, it would confirm the hypothesis of Casadevall and Scharff (1995) that the humoural immune response mounted against a pathogen includes protective antibodies, non-protective antibodies and disease enhancing antibodies.

The data provided in this article support the notion that sIgA can modify the course of infections caused by *C. albicans* associated with plastic devices by blocking the adherence of the fungus to these materials. This inhibitory effect can be mimicked by mAbs directed against antigens expressed on the cell wall surface of the fungus. However, taken into consideration the different effects observed by these antibodies on adherence, a careful selection and a combination of different mAbs should be considered in order to obtain an efficient blockage of *Candida* adhesion. Further studies should be performed to verify if saliva from other individuals also shows the effect described in this study and if the antibodies can also inhibit the adherence of *Candia* to buccal epithelial cells.

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