



Original article

Anti-*Candida albicans* germ tube antibodies reduce *in vitro* growth and biofilm formation of *C. albicans*



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ABSTRACT

Background: Invasive candidiasis by *Candida albicans* is associated with high morbidity and mortality, due in part to the late implementation of an appropriate antifungal therapy hindered by the lack of an early diagnosis.

Aims: We aimed to evaluate the *in vitro* antifungal activity of the antibodies against *C. albicans* germ tubes (CAGTA) raised in a rabbit model of candidemia.

Methods: We measured the effect of CAGTA activity by colorimetric XTT and crystal violet assays, and colony forming units count, both on *C. albicans* planktonic cells and during the course of biofilm formation and maturation. Viability and cell morphology were assessed by optical, fluorescent or scanning electron microscopy.

Results: CAGTA ≥ 50 $\mu\text{g/ml}$ caused a strong inhibition of *C. albicans* blastospores growth, and DiBAC fluorescent staining evidenced a fungicidal activity. Moreover, electron microscopy images revealed that CAGTA induced morphological alterations of the surface of *C. albicans* germ tubes grown free as well as in biofilm. Interestingly, CAGTA ≥ 80 $\mu\text{g/ml}$ reduced the amount of *C. albicans* biofilm, and this effect started at the initial adhesion stage of the biofilm formation, during the first 90 min.

Conclusions: This is the first report showing that CAGTA reduce *C. albicans* growth, and impair its metabolic activity and ability to form biofilm *in vitro*. The antigens recognized by CAGTA could be the basis for the development of immunization protocols that might protect against *Candida* infections.

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Los anticuerpos dirigidos contra los tubos germinales de *Candida albicans* reducen el crecimiento *in vitro* y la formación de biopelículas de *C. albicans*

RESUMEN

Antecedentes: La infección invasora por *Candida albicans* está asociada a altas tasas de morbimortalidad, en parte debido al retraso en la instauración de una terapia antifúngica adecuada, dificultada a su vez por la falta de un diagnóstico precoz.

Objetivos: Evaluar la actividad antifúngica de los anticuerpos contra tubos germinales de *C. albicans* (CAGTA) obtenidos a partir de un modelo animal de candidemia en conejo.

Métodos: El efecto de los CAGTA se evaluó mediante los ensayos colorimétricos XTT y cristal violeta, así como mediante el recuento de unidades formadoras de colonias, tanto en células planctónicas de *C. albicans* como en distintos estadios de formación y maduración de biopelículas. La viabilidad y la morfología de las células tratadas con CAGTA se determinó mediante microscopía óptica, de fluorescencia o electrónica (SEM).

Palabras clave:

Candida albicans

CAGTA

Anticuerpos

Actividad fungicida

Biopelículas

Candidiasis invasora

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Resultados: Concentraciones de CAGTA ≥ 50 $\mu\text{g/ml}$ generaban una fuerte inhibición del crecimiento de *C. albicans*, y su actividad se mostró fungicida. Los CAGTA producían alteraciones en la superficie de los tubos germinales desarrollados tanto a partir de células en suspensión como de células en biopelículas. Además, concentraciones de CAGTA ≥ 80 $\mu\text{g/ml}$ redujeron la biomasa de biopelículas de *Candida*, y este efecto se desencadenaba en los primeros 90 min de su formación.

Conclusiones: Este es el primer estudio que demuestra la capacidad de los CAGTA para reducir el crecimiento de *C. albicans* y su actividad metabólica, así como para alterar la formación de biopelículas *in vitro*. Los antígenos reconocidos por los CAGTA podrían servir de base para el desarrollo de protocolos de inmunización protectores frente a infecciones por *Candida*.

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Invasive candidiasis (IC) concerns more than 250,000 people in the world and causes above 50,000 deaths every year.¹⁵ Despite the introduction of new auxiliary tests, the diagnosis of IC is difficult because there are no pathognomonic signs and it is hard to distinguish between colonization and invasion. Therefore, therapy starts late, and partly explains the high mortality associated with IC.^{25,28} In addition, the opportunistic fungal pathogen *Candida albicans* is able to organize into biofilm on both abiotic (i.e. medical devices) and biotic (i.e. oral mucosal) surfaces,¹³ what enhances its resistance to antifungal therapy and host immune mediated defenses.¹⁴

Our group of research developed an indirect immunofluorescence assay to detect antibodies (Abs) that react specifically with superficial antigens of the germ tubes of *C. albicans* (CAGTA) in patients with IC^{9,12,21} and such antibodies can also be found in a rabbit model of IC.³¹ Interestingly, the mortality rate of ICU patients receiving an antifungal treatment was significantly reduced in CAGTA-positive individuals.³⁴ Pitarch and colleagues²⁷ associated the best prognosis of IC patients with those showing the highest titer of specific Abs such as anti-Met6 (methionine synthase), anti-Hsp90 (heat-shock protein 90 kDa) and anti-Pgk1 (phosphoglycerate kinase 1). In a previous proteomic study of our group³¹ we identified some *C. albicans* antigens recognized by CAGTA: Met6, Ino1, Eno1, Pgk1, Adh1, 14-3-3 and Edg2. Moreover, Huertas et al. reported that, when infected with *C. albicans*, mice previously colonized with this yeast showed higher survival rates than non-colonized control counterpart.¹¹ The group of animals with good outcome presented high titers of IgG specific for Eno1 (enolase), Met6, Hsp70 (heat-shock protein 70 kDa), Pdc11 (pyruvate decarboxylase), Pgk1 and Cdc19 (pyruvate kinase).¹¹

The aim of vaccination is to develop protective specific antibodies and immune memory against a particular pathogen. In recent years, studies in animal models are confirming the immunogenicity and efficacy of certain antigenic components of *C. albicans* as vaccines, including β -D-glucan and mannoproteins, or the recombinant proteins enolase (Eno1), hyphally regulated protein (Hyr1), hyphal wall protein (Hwp1), agglutinin-like sequence (Als3), secreted aspartyl proteinases (Sap) or heat-shock protein 90 (Hsp90).^{19,33} CAGTA recognize most of these components,³¹ consequently if these antibodies prove a protective role against *C. albicans*, other antigens reacting with them could add new candidates for a future *Candida* vaccine. Thus, the main objective of this study was to evaluate the antifungal activity of CAGTA on *C. albicans* growth and on its ability to form biofilm.

Material and methods

Strain and culture conditions

Candida albicans SC5314 (Stanford DNA Sequencing and Technology Center, Stanford, USA) was routinely grown on Sabouraud

dextrose agar (SDA, Difco, Sparks, MD, USA) at 24°C for 48 h. To obtain germ tubes, two or three colonies of *C. albicans* freshly grown on SDA at 24°C were inoculated into TC199 medium (Sigma-Aldrich, St. Louis, MO, USA) and incubated overnight at 24°C with shaking (120 rpm). The resulting blastospores were harvested and suspended in four volumes of TC199 medium pre-heated to 37°C. Germ tubes were collected after 4 h of incubation at 37°C and 120 rpm.³¹

Immune sera: fractionation, quantification and purification of CAGTA

Immune sera were obtained from White New Zealand female rabbits infected intravenously with *C. albicans* blastospores, as described previously,³¹ and were fractionated to run the different assays. Whole IgG fraction from immune serum (total-IgG) included Abs against the superficial antigens of *C. albicans* blastospores (anti-BI) and filamentous cells, including germ tubes, pseudo-hyphae and hyphae, together with non-specific Abs (CAGTA-enr fraction).

The CAGTA enriched fraction of serum (CAGTA-enr) was obtained after incubating the immune serum with an equal volume of heat-killed *C. albicans* blastospores (10^{10} cell/ml of PBS) for 2 h at room temperature, followed by centrifugation at 2500 rpm for 5 min to remove the blastospores. The anti-blastospore antibodies (anti-BI) adsorbed onto the surface of the yeast cells were eluted by gentle shaking in 2.5 M sodium iodide (Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 1 h, and then blastospores were eliminated by centrifugation. The eluted anti-BI antibodies were dialyzed (MWCO 12,000–14,000 Da; Medicell International, London, UK) against PBS and concentrated with polyethylene glycol 20,000 (Merck, Hohenbrunn, Germany).³¹

Purified CAGTA were obtained from *C. albicans* germ tubes that had been incubated with an equal volume of CAGTA-enr serum fraction at room temperature for 1 h with gentle agitation. The cell pellet was washed with PBS, and CAGTA were eluted, dialyzed and concentrated following the same protocol described for anti-BI antibodies.

The IgG class antibodies were purified with Melon™ Gel IgG Spin Purification Kit (ThermoFisher Scientific, St. Louis, MO, USA).

The protein concentration of serum fractions was estimated with Pierce™ Coomassie Plus (Bradford) Assay Kit (ThermoFisher Scientific, St. Louis, MO, USA) according to the manufacturer's instructions.

Evaluation of CAGTA activity against *Candida albicans* planktonic cells

The effect of the antibodies on *C. albicans* was evaluated by measuring the cell metabolic activity with the colorimetric XTT assay, and colony forming units (CFU) count.

Candida albicans blastospores grown overnight in Sabouraud broth (Difco, Sparks, MD, USA) at 30 °C in an orbital shaker (120 rpm) were suspended in fresh medium 10⁶ cell/ml, and distributed in a U-bottom 96-well plate (Costar, Corning Life Science, Lowell, MA, USA), 50 µl per well. Each well was supplemented with 50 µl of Sabouraud broth containing Abs to reach different final concentrations (50, 100 or 200 µg/ml). Plates were incubated at 37 °C for 2.5 h with gentle shaking. Then, for CFU estimation, each well content was homogenized and a 50 µl aliquot was inoculated onto SDA plates and incubated at 37 °C for 48 h. For the XTT assay, plates were centrifuged at 2500 rpm, the supernatant was discarded and the metabolic activity of cells was estimated with the 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)2H-tetrazolium-5-carboxanilide-inner salt (XTT; Sigma-Aldrich, St. Louis, MO, USA), according to the protocol of Henriques et al.¹⁰ Briefly, cell pellets in each well were supplemented with 90 µl of XTT 0.75 mg/ml in PBS and 10 µl of phenazine methasulfate (PMS; Sigma-Aldrich, St. Louis, MO, USA) 0.32 mg/ml in ultrapure water, and they were incubated at 37 °C for 2 h. Then each well content was homogenized and the absorbance at 492 nm (Abs_{492nm}) was registered using a spectrophotometer (Microplate Autoreader Elx808, Bio-Tek Instruments). Two different controls were included in every experiment, one without antibodies, and a second one with an equivalent concentration of an irrelevant non-specific rabbit IgG (irr-IgG; Sigma-Aldrich, St. Louis, MO, USA).

Evaluation of CAGTA activity against *Candida albicans* biofilm

The day before each experiment, *C. albicans* was inoculated onto SDA plates that were incubated at 37 °C. Blastospores were harvested, washed, suspended at 10⁶ cell/ml in RPMI 1640 (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Defined Hyclone, Logan, UT, USA) and distributed (100 µl/well) into flat-bottom 96-well plates (Corning Incorporated, NY, USA). Plates were incubated at 37 °C and, according to the protocols shown below, CAGTA or irr-IgG were added at different times in order to test their effect at different stages of the biofilm formation process.

Protocol I: effect on biofilm formation. Irr-IgG or CAGTA (20–160 µg/ml) were added at time zero, and the effect on biofilm was checked after 24 h of incubation.

Protocol II: effect on biofilm maturation. Irr-IgG or CAGTA (20–160 µg/ml) were added to a 24 h-old biofilm and their effect was assessed after an additional 24 h incubation period at 37 °C.

Protocol III: effect on *C. albicans* adhesion. *C. albicans* cells were exposed to CAGTA 80 µg/ml for the first 90 min during the adhesion process, and then cells were washed and incubated without antibodies up to 24 h, to allow biofilm formation.

Protocol IV: effect on biofilm development. *C. albicans* cells were incubated for 90 min at 37 °C allowing the cells to adhere to the bottom of the plate, and then they were supplemented with CAGTA 80 µg/ml and incubated up to 24 h prior to biofilm assessment.

At the end of each experiment, non-adherent fungal cells were removed and total biofilm mass was assessed with crystal violet (CV, Abs_{540nm}), while the metabolic activity was estimated with the XTT assay (Abs_{492nm}), as described elsewhere.^{17,26} Two negative controls were included as explained for the planktonic cells experiments.

Optical and fluorescent microscopy analysis

Cell growth and morphology of *C. albicans* treated with CAGTA-enr serum fraction 6.25–200 µg/ml for 2.5 h at 37 °C were assessed by optical microscopy. Growth conditions of planktonic cells were the same as indicated for XTT and CFU assays. Two controls

were included in every experiment, one without antibodies, and a second one with an equivalent concentration of an Irr-IgG.

Cell viability of *C. albicans* incubated with purified CAGTA 20 µg/ml for 2.5 h at 37 °C was assessed with the fluorescent dyes 5,(6)-carboxyfluorescein diacetate (CFDA; Molecular Probes, Eugene, OR, USA) and bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC; Molecular Probes, Eugene, OR, USA), following the method described by Bowman et al.¹ Briefly, a stock solution of CFDA 5 mg/ml in dimethyl sulfoxide was diluted to 50 µg/ml with MOPS 3 (0.1 M MOPS–50 mM citric acid at pH 3.0), and cells were incubated with the dye in the dark at 37 °C for 45 min with gentle shaking and then stored on ice until analysis. DiBAC 2 µg/ml in MOPS 7 (0.1 M MOPS at pH 7.0) was prepared from a 1 mg/ml stock solution in ethanol, and cells were treated at room temperature for 1 h in the dark, washed twice with MOPS 7 and stored on ice until analysis.

Electron microscopy analysis

Candida albicans germ tubes and blastospores were grown in Sabouraud broth with CAGTA 40 µg/ml for 2.5 h at 37 °C, and biofilms were formed in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and CAGTA 80 µg/ml for 24 h at 37 °C. Negative controls were run without CAGTA. Then, cells were washed in Sorenson's buffer [0.133 M Na₂HPO₄, 0.133 M KH₂PO₄ (4:1, v/v), pH 7.2], treated with fixing solution (2% glutaraldehyde in Sorenson's buffer) at room temperature for 1 h, and washed three times with 6% sucrose in Sorenson's buffer. Cells were dehydrated using graded ethanol solutions (50%, 70% and 100%) for 5 min each. Then membranes were washed twice with 5 ml hexamethyldisilazane (Electron Microscopy Sciences, Hatfield, PA, USA) for 5 min each, and hexamethyldisilazane was removed by vacuum filtration. Membranes were left to dry in the air. Finally, the samples were processed for electron microscopy at the Service of Analytic and High Resolution Microscopy in Biomedicine (SGIker, University of the Basque Country).

Statistical analysis

The statistical analysis was performed using the GraphPad Prism software (version 6. GraphPad Software, Inc., La Jolla, CA, USA). For the analyses, the results of the different treatments were transformed into a percentage with reference to the mean value of the untreated control. Then variables were analyzed with the one-way ANOVA test or the Student's *t*-test for unrelated samples. *p* values <0.05 were considered statistically significant. All assays were performed at least three times and each assay was assessed in triplicate.

Results

Effect of CAGTA on *Candida albicans* planktonic cells

Candida albicans planktonic yeast cells incubated with whole IgG from rabbit immune sera showed a drastic reduction of their metabolic activity, as evaluated by the XTT assay, and the effect was concentration dependent (Fig. 1a). The reduction was statistically significant with respect to untreated cells and cells treated with an equivalent amount of a commercialized Irr-IgG. A parallel experiment with IgG from the CAGTA enriched serum fraction (CAGTA-enr) gave a similar result (Fig. 1b). However, anti-BI ≤200 µg/ml did not show any significant effect on the metabolic activity of the yeast cells and germ tubes which appeared after 2.5 h of incubation (Fig. 1c). The effect of the different serum antibody fractions on the metabolic activity of *C. albicans* planktonic cells was confirmed by the CFU assay, where whole IgG and CAGTA-enr fractions caused

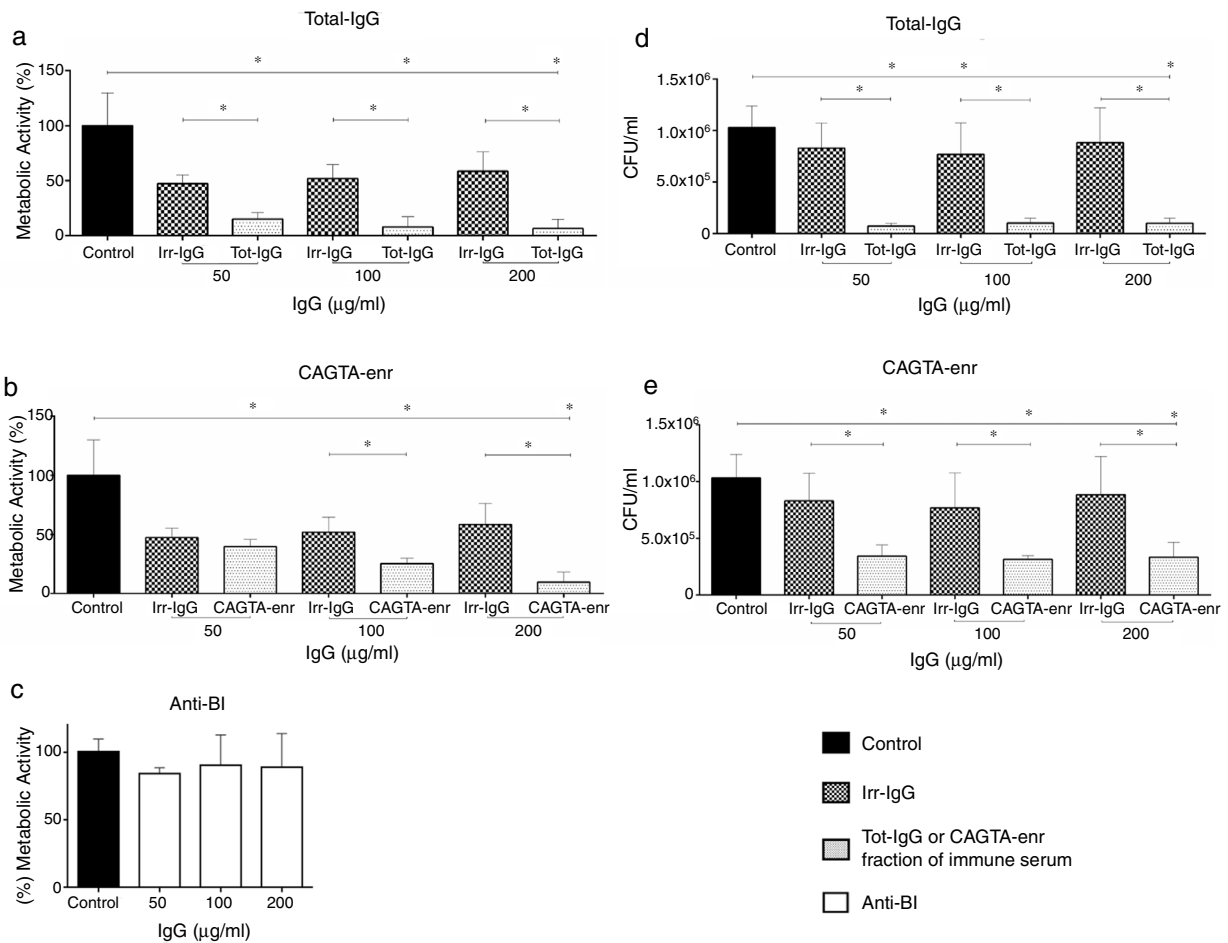


Fig. 1. Effect of antibodies on planktonic cells of *C. albicans* SC5314 grown at 37 °C for 2.5 h in Sabouraud broth supplemented with antibodies raised in a rabbit model of candidemia: total IgG (Tot-IgG), CAGTA enriched fraction (CAGTA-enr) and anti-blastospores antibodies (Anti-BI). Metabolic activity was measured with XTT assay (a, b and c). Viability is expressed as CFU (d and e). Bars represent mean value \pm SD from three independent experiments. Statistical significance * $p < 0.05$.

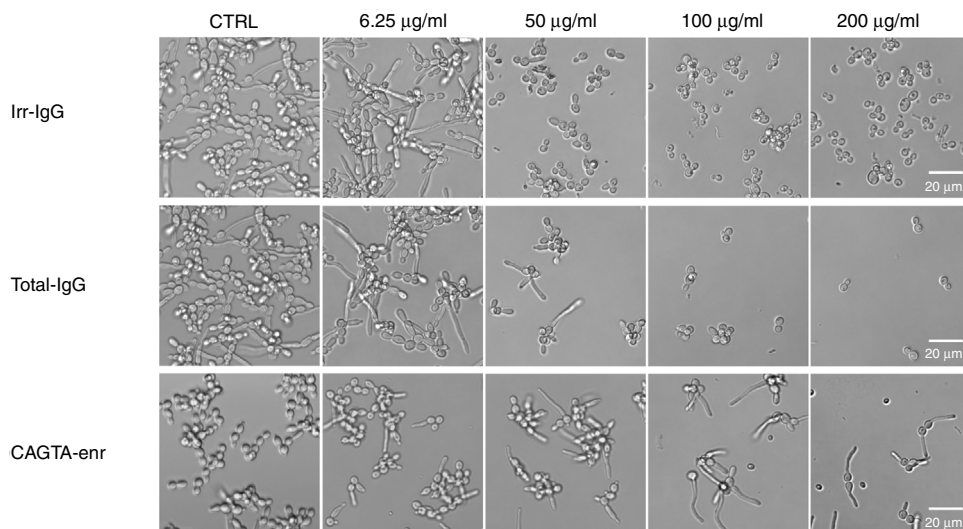


Fig. 2. Phase contrast microscopy images of *C. albicans* SC5314 grown at 37 °C for 2.5 h in Sabouraud broth supplemented with different concentrations of IgG antibodies (irrelevant IgG [irr-IgG], rabbit immune serum total IgG [Tot-IgG] or CAGTA enriched serum fraction [CAGTA-enr]). Control cells (CTRL) were incubated without antibodies.

a dramatic decrease in the number of colonies at IgG concentrations ≥ 50 $\mu\text{g/ml}$ (Fig. 1d and e). These data were also corroborated with the optical microscopy images of *C. albicans* planktonic cells incubated with increasing concentrations of different IgG fractions (Fig. 2). Whole IgG from rabbit immune serum reduced the growth

of cells in a concentration dependent manner. However, CAGTA-enr IgG induced a slight reduction of *C. albicans* growth and, even at the highest concentration (200 $\mu\text{g/ml}$), short germ tubes were observed. This paradoxical effect did not correlate the results of XTT and CFU assays. Nevertheless, electron microscopy images of

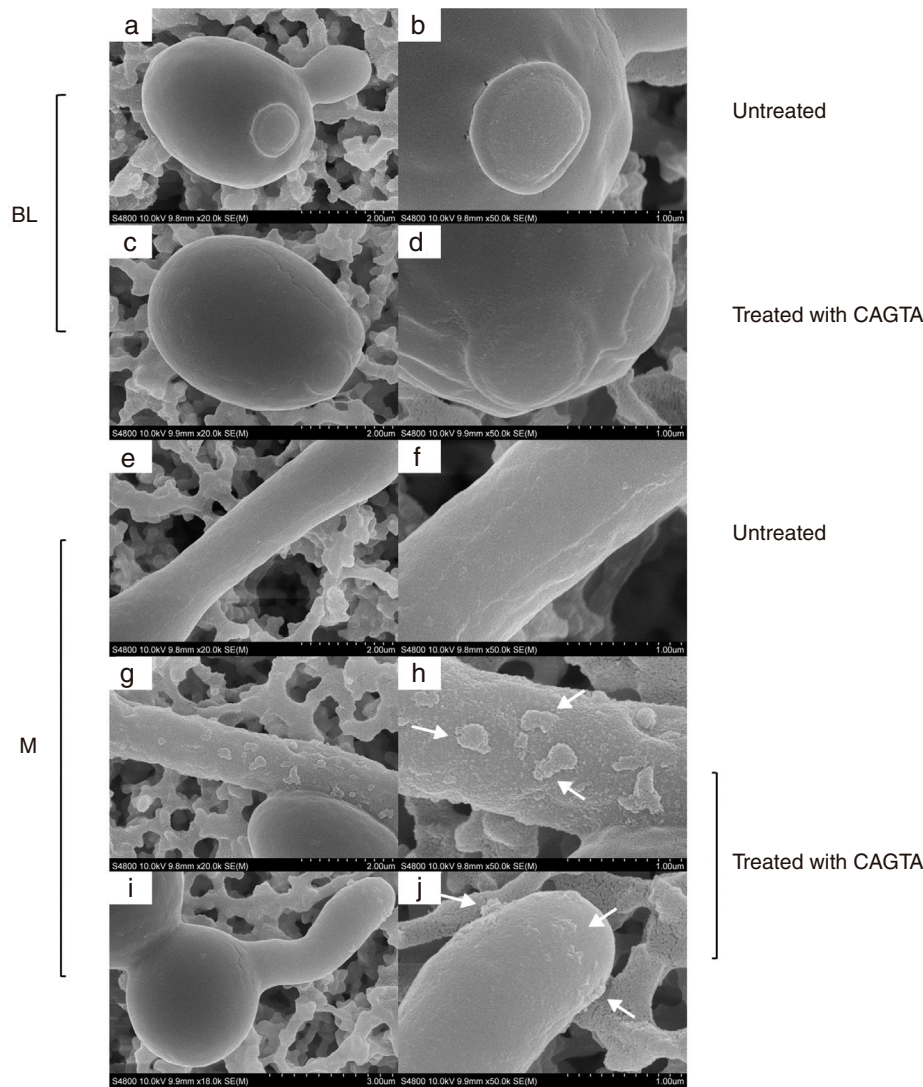


Fig. 3. SEM images of *C. albicans* SC5314 blastospores (BL) and mycelia (M) grown at 37 °C for 2.5 h in Sabouraud broth with CAGTA 40 µg/ml (c, d, g–j) or without antibodies (a, b, e and f). Magnification: ×20,000 (left column) and ×50,000 (right column) for details. Arrows highlight surface protuberances.

untreated control cells (Fig. 3a, b, e, and f), as well as those of blastospores treated with CAGTA (Fig. 3c and d), appeared with regular and smooth surface, while filamentous phase forms of *C. albicans* treated with CAGTA-enr IgG 40 µg/ml showed an altered surface (Fig. 3g–j). In addition, fluorescent DiBAC and CFDA staining of *C. albicans* cells treated with CAGTA-enr IgG 20 µg/ml revealed that these antibodies exerted a fungicidal effect (Fig. 4).

Effect of CAGTA on *Candida albicans* biofilm development

The formation of biofilm by *C. albicans* can be divided in phases that were analyzed following different time-based protocols of treatment with CAGTA. Yeast cells of *C. albicans* exposed to CAGTA for the first 24 h of incubation (protocol I) experienced a statistically significant reduction of biofilm production at CAGTA concentrations ≥ 80 µg/ml (Fig. 5a), while no differences were observed at the lowest CAGTA concentrations or in the presence of the Irr-IgG (80 µg/ml). Similar profiles were obtained with the CV estimation of biofilm mass and metabolic activity according to the XTT assay. When CAGTA (20–160 µg/ml) were added onto the 24 h-old preformed biofilm, according to protocol II, the maturation of the biofilm was not affected after an additional 24-h incubation period (Fig. 5b).

To further characterize the effect of CAGTA on *C. albicans*, two additional experimental protocols were performed focusing on the early phases of the biofilm formation process. On the one hand, the presence of CAGTA only during the first 90 min of incubation (protocol III), corresponding to the *C. albicans* adhesion step, significantly reduced the biofilm biomass to 48% while the metabolic activity remained close to the control group values (Fig. 5c). On the other hand, when CAGTA were added after the first 90 min of incubation, no significant effect on the development of the 24 h-old biofilm was recorded (protocol IV; Fig. 5d).

In agreement with the observed effect of CAGTA on *C. albicans* biofilm formation (protocol I), electron micrographs of 24 h-old biofilms exhibited a reduction of the microbial structure density of treated cells (Fig. 6b) when compared to the untreated control (Fig. 6a). Moreover, micrographs revealed that CAGTA treatment had altered the cell wall surface of fungal filaments, that appeared rough and with protuberances (Fig. 6d and f), while the control group cells showed regular and smooth surfaces (Fig. 6c and e).

Discussion

The opportunistic pathogen *C. albicans* can cause invasive infections that are associated with its capacity to grow as hyphae. Several

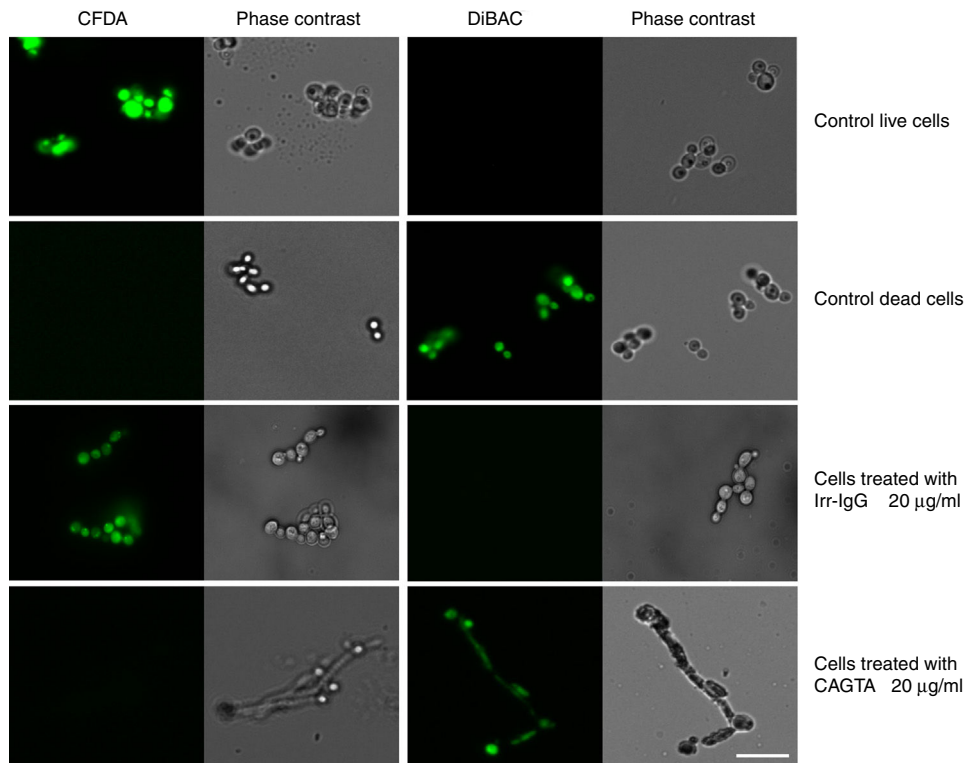


Fig. 4. Photomicrographs of *C. albicans* SC5314 grown at 37 °C for 2.5 h in Sabouraud broth supplemented with CAGTA 20 µg/ml and then stained with the fluorescent dyes CFDA and DiBAC. A rabbit irrelevant IgG (Irr-IgG) did not affect *C. albicans* viability. Staining control consisted of live and dead (heat-killed) yeast cells grown in Sabouraud broth without antibodies. Paired images depict epifluorescent and phase contrast microscopy of the same field. Bar 20 µm.

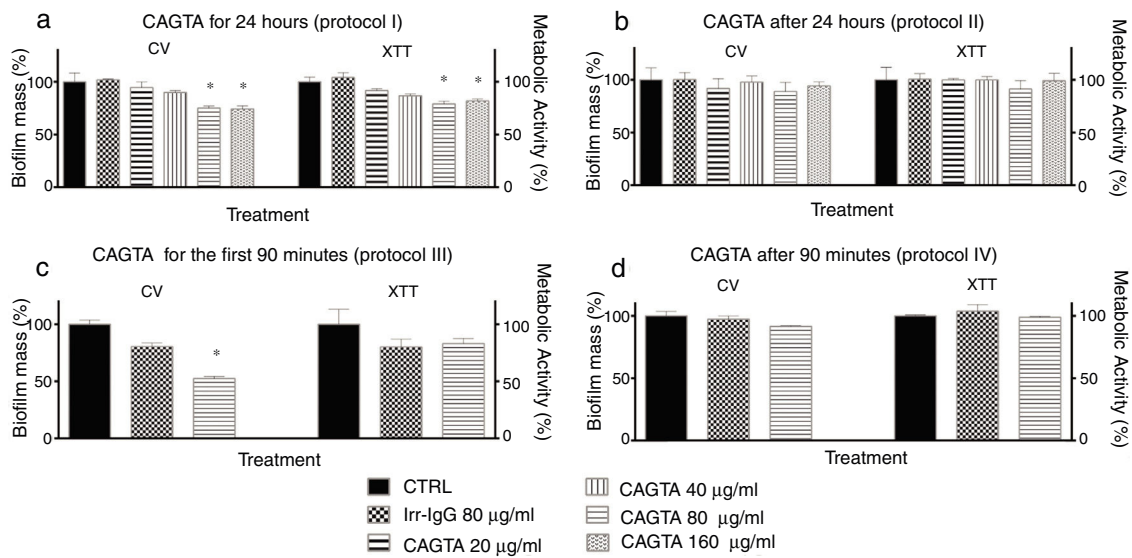


Fig. 5. Effect of CAGTA on metabolic activity (XTT assay) and biofilm mass (CV assay) of *C. albicans* SC5314 at different stages of biofilm formation. Experimental protocols are described in Material and Methods section: (a) Protocol I, (b) Protocol II, (c) Protocol III, and (d) Protocol IV. Bars represent mean value \pm SD from three independent experiments. Statistical significance * $p < 0.05$.

virulence factors related to the cell wall are known to interact with host epithelial cells, which in turn begin and develop an immune response. Hence, an efficient anti-*Candida* response requires the cooperation of different mechanisms of the immune system that include specific antibodies.^{4,22,30}

The development of an indirect immunofluorescence technique to detect specific antibodies against superficial components of the cell wall of the mycelial phase of *C. albicans* (CAGTA) has facilitated the diagnosis of IC in patients at risk, since it differentiates

colonization from invasion.^{9,21} Moreover, increasing titers of CAGTA have been related with a better prognosis for patients with IC admitted at the ICU.³⁴ Based on this premise, the present study was designed to investigate the antifungal activity of CAGTA against *C. albicans*.

In the animal model of IC, *C. albicans* blastospores were inoculated intravenously and developed germ tubes in the body environment, where they encountered predisposing factors such as temperature $>35^{\circ}\text{C}$, neutral pH and serum components.

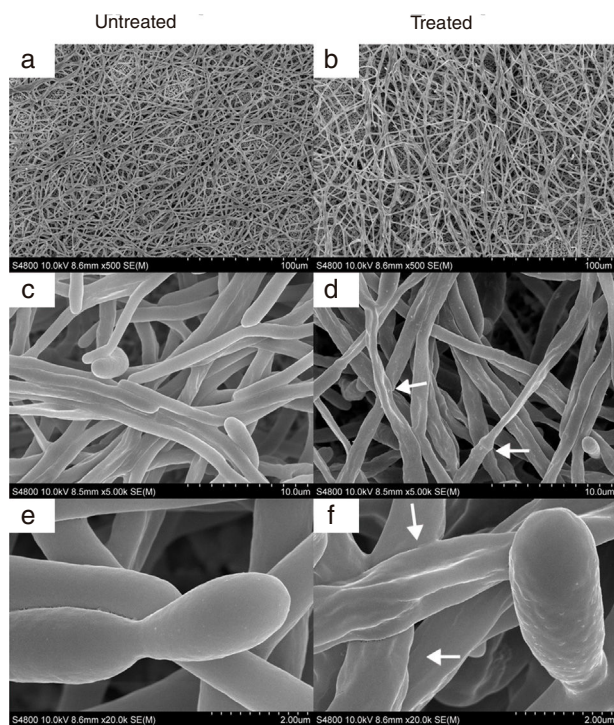


Fig. 6. SEM images of 24-h biofilm of *C. albicans* SC5314 developed at 37 °C in RPMI + FBS10% with CAGTA 80 µg/ml (right panel) or without antibodies (left panel). Magnification: ×500 (a, b), ×5000 (c, d) and ×20,000 (e, f). Arrows highlight the altered surface and protuberances of CAGTA treated cells.

Consequently, immune sera contained specific antibodies raised against *C. albicans* germ tube surface antigens (CAGTA). Whole IgG antibodies of rabbit immune serum reduced the metabolic activity and viability of *Candida* planktonic cells *in vitro*, and the effect was concentration dependent. However, when serum IgG content was fractionated, the anti-BI fraction did not show any effect on planktonic cells, while the CAGTA-enr fraction appeared as the main responsible for the observed inhibitory effect. These results are in agreement with those of Fujibayashi et al.⁸; these authors registered a reduction in the growth and metabolic activity of *C. albicans* SC5314 when treated with polyclonal Abs produced in a chicken immunized with that same strain of *Candida*. Furthermore, Brena et al. observed that the monoclonal antibody C7 (MAB C7), obtained from BALB/c mice immunized with *C. albicans*,²⁰ reduced the metabolic activity and the CFU count of *C. albicans*, demonstrating the fungicidal activity of such monoclonal antibody.³

Microphotographs of *C. albicans* cells incubated with the CAGTA-enr serum fraction evidenced the reduction of cell growth, although germ tubes emerged to some extent. This paradoxical behavior could be explained by the lack of anti-BI Abs in the CAGTA-enr fraction of serum, which would allow the growth of blastospores to some extent and the emergence of germ tubes. However, purified CAGTA eventually killed *C. albicans*, as evidenced by DiBAC staining. Moreover, when treated with CAGTA-enr IgG, the surface of germ tubes appeared altered with protuberances while blastospores retained their original smooth surface. Similar results were obtained using the MAB C7 that affected the surface of *Candida* blastospores,⁵ and altered the iron uptake pathway.² Although CAGTA seemed to affect only the surface of *C. albicans* mycelia, cell wall alteration is relevant for the fungus viability since it may disturb the traffic of nutrients, the osmotic stability and eventually the cell wall organization.⁵ Along these lines, Ribeiro et al. also observed that *C. albicans* exposed to a peptide from chilli pepper

seeds caused severe alterations in bud formation, the cell wall and the cytoplasmic membrane of the yeast.²⁹

At this point, we would like to explain the unexpected effect of the Irr-IgG from rabbit included in our experiments as a control antibody. Although the metabolic activity of *Candida* yeast cells was significantly reduced in the presence of this antibody, it never reached the extent of the effect of total-IgG or CAGTA-enr serum fractions, and it was not a concentration dependent effect. Moreover, the CFU counting experiment revealed that cell viability was not affected by the irr-IgG when compared to the untreated cells. A similar inhibitory effect with another Irr-IgG also from rabbit has been reported by Rodier et al.³⁰

While there is evidence of the inhibitory activity of CAGTA against *Candida* planktonic cells, no data are still available on the ability of such Abs to prevent biofilm formation, one of the major virulence factors of *C. albicans*. To the best of our knowledge, this is the first report showing that CAGTA reduce *Candida* biofilm formation. In particular, our data indicate that CAGTA inhibit the adherence of the cells to the surface and, consequently, impair biofilm formation, mainly at an early stage, within the first 90 min after *C. albicans* inoculation; in contrast, CAGTA showed no activity against an already structured 24 h-old biofilm. This observation is in accordance with the fact that fungal cells embedded in biofilms are less susceptible to conventional antifungal drugs as well as to antibodies.^{6,7} Nevertheless, the biofilm formation was significantly impaired by the continuous presence of CAGTA, and the surface of the hyphal cell walls appeared altered. Other studies have reported comparable changes of *Candida* cells morphology when exposed to peptide P-113Du and P-113Tri¹⁶ or marine polyunsaturated fatty acids.³²

Our results agree with previous evidence: other monoclonal and polyclonal antibodies, MAb 7D7,¹⁸ anti-C3-RP antibody and MAB OKM1,⁴ also altered the development of *C. albicans* biofilm. Interestingly, recent data demonstrate that antibody-derived peptides, such as the Killer Peptide (KP), and molecules contained in commercial mouthwashes, are able to significantly impair *C. albicans* biofilm formation and maturation in terms of both biomass and cell metabolic activity.^{23,24} Taken together, these findings open new ways for alternative strategies to counteract fungal biofilm formation.

In conclusion, CAGTA inhibit the growth of *C. albicans* planktonic cells as well as the formation of biofilm, alter the surface of the cell wall of hyphae developed in their presence, and eventually induce planktonic cells to die. The results presented in this work warrant further studies to evaluate the potential protective role of CAGTA, alone or combined with antifungal compounds, in an animal model of invasive candidiasis. Moreover, future research on the antigens recognized by CAGTA could help to find candidates for the development of immunization protocols that might protect against *Candida* infections.

Transparency declarations

None to declare.

Conflict of interest

The authors declare that they have no conflict of interest.

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