INFECTION AND IMMUNITY, Mar. 1994, p. 1125–1127 Vol. 62, No. 3
0019-9567/94/$04.00+0

Ca Ions Stabilize the Binding of Complement Factor iC3b to the Pseudohyphal Form of *Candida albicans*

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Received 7 October 1993/Returned for modification 4 November 1993/Accepted 20 December 1993

The pseudohyphal form of *Candida albicans* is able to bind iC3b. This may play an important role in the pathogenesis of disseminated candidiasis and, in particular, in adherence to endothelium, protection against complement action, and iron acquisition from erythrocytes. Here we report that Ca^{2+} ions are required to maintain stable binding of iC3b to *C. albicans* pseudohyphae.

Candida albicans is a wide-spread saprophyte of the mucous membrane which may cause opportunistic infections in patients with immune disorders. T-cell deficiencies, e.g., human immunodeficiency virus infection, are often accompanied by a mucocutanous form of candidiasis. In contrast, the disseminated form is frequently associated with defects of the phagocytic system. Neutropenia caused by immunosuppressive or cytostatic therapies serves as an example. Adherence of *C. albicans* to the vascular endothelium is believed to be a critical step in the pathogenesis of disseminated candidiasis (15). Several mannoproteins of the cell wall have been identified as binding structures for fibronectin (17, 24), fibrinogen (4, 25), laminin (2, 25), type I and IV collagens (15), mucin and salivary proteins (22), and even acrylic (27) and plastic (26). *C. albicans* pseudohyphal forms, in contrast to yeast forms, express iC3b- and C3d-binding structures on their surfaces (3, 5–14, 18, 20, 23). Several proteins with different molecular weights have been proposed by different groups to be involved in the binding of iC3b to *C. albicans*. Recently, iC3b-binding proteins of 66, 55, and 42 kDa were purified by affinity chromatography in our laboratory (1). Since binding of iC3b to the human or murine complement receptor type 3 (CR3) requires Ca^{2+} and Mg^{2+}, we investigated in the present study whether the binding of iC3b to *C. albicans* is also dependent on the presence of divalent cations.

The buffer used throughout was physiological NaCl solution (0.9% = 154 mM; pH 7.3). Calcium, magnesium, iron, tin, and zinc chlorides were diluted with physiological NaCl solution to the required concentrations, and the pH was adjusted to 7.3.

C. albicans CBS 5982 (serotype A) was used throughout this study. In order to transfer *C. albicans* blastoconidia into the pseudohyphal growth form we used a two-stage cultivation procedure described previously (1). At least 90% of the cells obtained by the cultivation were in pseudohyphal form. To study iC3b binding to the *C. albicans* pseudohyphal form, we performed a rosetting assay with complement-coated sheep erythrocytes (EAiC3b) according to published methods (1, 7). For the rosetting assay, 25 μl of EAiC3b (5×10^8 erythrocytes per ml) was mixed with 25 μl of pseudohyphal particles (2×10^7/ml) and washed with 1 ml of buffer. The samples were resuspended in 50 μl of buffer and incubated for 30 min at 37°C. Then, the samples were put on ice for 10 min to immediately lower the incubation temperature and were stored at 4°C over night. On the next day the samples were washed once with 1 ml of buffer and transferred to glass slides. The percent rosettes (i.e., pseudohyphal forms with at least four adhering erythrocytes each) was evaluated by light microscopy (magnification, ×400). A minimum of 100 pseudohyphal forms having a length of 40 to 130 μm were counted in each incubation mixture.

As reported previously (7), no difference between the samples with and without calcium was observed when the percent rosettes immediately after incubation was evaluated, since in both cases about 90 to 100% rosettes were counted. In contrast, when we put the samples on ice for 10 min after incubation, stored them overnight at 4°C, and washed them once with the particular buffer, only the sample with calcium ions remained at nearly the same rosette index (81.0% ± 7.3%). Without calcium the adherence of EAiC3b to *C. albicans* was reduced to 46.8% ± 5.4%. When further washing steps were added, the rosette index of the sample with calcium decreased constantly by about 10% per step. In the sample without calcium, EAiC3b was completely removed at the second or third washing step. Thus, we could demonstrate that calcium is able to stabilize the binding of iC3b to *C. albicans* effectively at the physiological concentration of 2.5 mM. Without calcium this binding was not very stable and erythrocytes could be detached easily.

This effect exerted by calcium was concentration dependent (Fig. 1). As confirmed in five separate experiments, the percent rosette formation increased with rising concentrations of calcium in a linear way. For the control without calcium the mean percent rosette formation was 46.8% ± 5.4%; at a calcium concentration of 2.5 nM it was 63.2% ± 4.8%, at 2.5 μM it was 67.8% ± 7.7%, and at 2.5 mM it was 81.0% ± 7.3%. The statistical significance of the effect of calcium concentration on rosette formation was tested by linear regression analysis, with logarithmically transformed calcium concentration values as the independent variable and percent rosette formation as the dependent variable. Linear regression analysis of these data (calcium concentrations logarithmically transformed) demonstrated a highly significant effect of calcium concentration on percent rosette formation ($P < 0.0001$, t test of the regression coefficient; the correlation coefficient is 0.88). In these experiments the precursor intermediates of EAiC3b showed a background of about 10% rosettes. This nonspecific binding, however, was not influenced by Ca^{2+} ions.

To prove that the binding of iC3b to *C. albicans* is Ca^{2+} dependent we used EDTA (Sigma, St. Louis, Mo.). EDTA was prepared as 10 mM and 0.1 mM solutions in the particular buffer. The calcium specific effect was inhibited completely by

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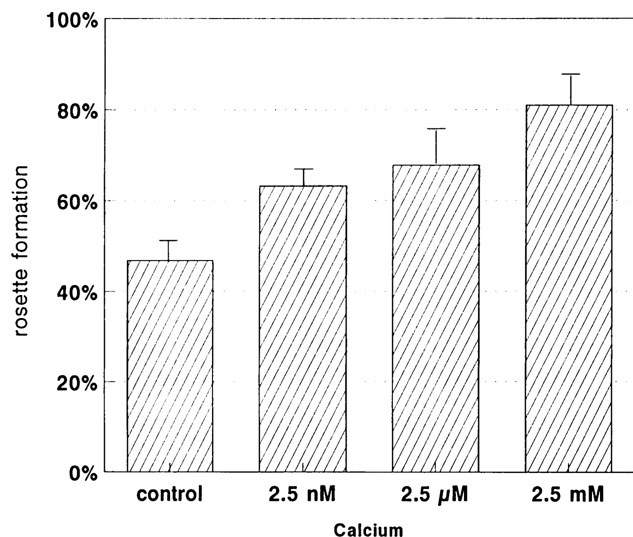

FIG. 1. Increase in adherence of EAiC3b to *C. albicans* by calcium in a dose-dependent manner. The effect of calcium concentration on rosette formation is highly significant ($P < 0.0001$, *t* test of regression coefficient; the correlation coefficient is 0.88).

EDTA (Fig. 2). A percent rosette formation of 81.0% ± 7.3% at a calcium concentration of 2.5 mM was reduced to 43.5% ± 5.5% by 10 mM EDTA. This was about the level that we observed with the control: the percent rosette formation with calcium-free buffer alone was 46.8% ± 5.4%, and with calcium-free buffer plus 10 mM EDTA it was 45.5% ± 3.5%. This control shows that EDTA did not alter the Ca^{2+}-independent rosette formation. At 0.1 mM, EDTA reduced the percent rosette formation to 65.0% ± 7.0%. Linear regression analysis

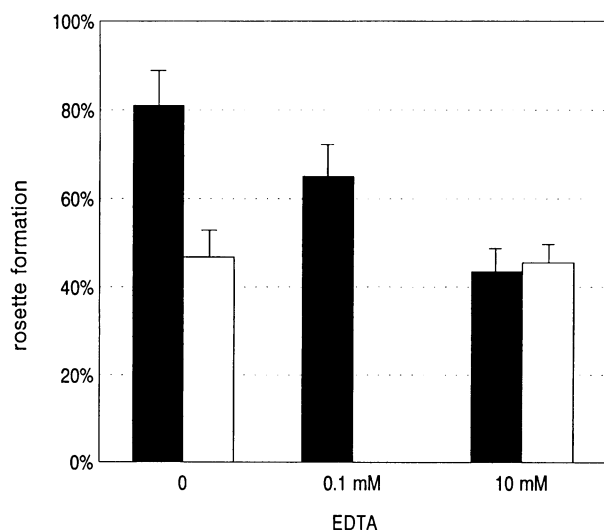

FIG. 2. Inhibition of EAiC3b adherence to *C. albicans* by addition of EDTA. Black bars show rosette formation with 2.5 mM calcium. The inhibition of calcium-dependent rosette formation by EDTA is highly significant ($P < 0.0005$, *t* test of regression coefficient; the correlation coefficient is 0.92). White bars show rosette formation without calcium. EDTA at 10 mM did not alter the calcium-independent rosette formation.

TABLE 1. Effect of divalent cations (at 25 μM) on the adherence of EAiC3b

Cation	% Rosette formation (mean ± SD)
Ca	66.6 ± 5.8
Mg	32.3 ± 6.8
Fe	40.3 ± 22.7
Sn	39.0 ± 2.6
Zn	39.3 ± 11.0
None (control)[a]	46.8 ± 5.5

[a] Physiological NaCl solution without any divalent cations.

of these data (EDTA concentrations logarithmically transformed) demonstrated a highly significant effect of EDTA concentration on percent rosette formation ($P < 0.0005$, *t* test of regression coefficient; the correlation coefficient is 0.92). Thus we demonstrated that the inhibition of the Ca^{2+} effect was directly dependent on the concentration of EDTA.

We also studied whether other divalent cations, like magnesium, iron, tin, and zinc, would have any influence on iC3b binding to *C. albicans*. This was done in three different experimental approaches. In the first experiment we tested all cations at a concentration of 25 μM. Calcium was the only cation that was able to enhance the rosette formation. Samples containing Mg, Fe, Sn, and Zn remained at the control level (Table 1). In the second experiment we compared the different cations at their specific physiological concentrations in human serum, i.e., Ca at 2.5 mM, Mg at 750 μM, and Fe at 25 μM. Sn and Zn were not tested because they are only trace elements in human serum. Calcium showed a distinct effect (percent rosette formation, 81%), whereas the samples with magnesium (44%) and iron (41%) remained at the control level (46.8%). In one particular experiment magnesium showed a small augmentation, but this effect could not be confirmed by repeated experiments. Finally, we tested all cations at 2.5 mM. Calcium (81%) could alter the iC3b-binding, but Mg (40%) and Zn (35%) could not (control, 46.8%; Fe and Sn were not soluble in a 0.9% NaCl solution at pH 7.3 at this concentration).

In conclusion, we were able to demonstrate that the binding of iC3b to the cell wall of *C. albicans* is influenced by Ca^{2+} ions. These results seem to be in contrast to a previous publication from our laboratory (7). Eigentler et al. reported no effect of divalent cations on the adherence of EAiC3b to *C. albicans*. These adherence assays were performed with Ca^{2+}- and Mg^{2+}-free buffers containing 20 mM EDTA. Under these conditions, the binding of EAiC3b was not influenced by the presence of divalent cations. Because binding to the human CR3 is dependent on Ca^{2+} and Mg^{2+} ions, we reinvestigated the effect of calcium ions on the iC3b-binding moieties in more detail. To achieve a higher sensitivity we modified the original method as follows: we used a lower ratio of EAiC3b to pseudohyphae, we put the samples on ice for 10 min immediately after incubation and stored them at 4°C overnight, and we washed the samples once before the evaluation of the rosette index. Introduction of these modifications revealed the influence of Ca^{2+} ions.

Klotz et al. recently reported that Ca^{2+} ions have an effect on a binding structure of the cell wall of *C. albicans* (16). They observed that adherence of *C. albicans* to the extracellular matrix proteins type I collagen and fibronectin was in particular dependent upon the presence of calcium (16). This observation is similar to our results.

The ability of *C. albicans* to bind to iC3b via a specific

receptor may play an important role in the adherence of this fungus to the vascular endothelium. Neutrophils, for example, are able to adhere to endothelial cells which are coated with C3 fragments by means of their CR3 (19). C3 fragments thus function as a bridge between neutrophils and endothelium. In the case of iC3b-mediated adherence of *C. albicans* to endothelium, this binding has to be stable in order to avoid the detachment of the fungus, e.g., by the flow of blood. We could indeed demonstrate that the physiological calcium concentration in blood is high enough to stabilize the adherence in vitro. Thus, calcium could be an important factor in the pathogenesis of disseminated candidiasis.

Binding of C3 fragments on the cell wall of *C. albicans* can have relevance in another respect: Gilmore et al. discussed a protective mechanism exhibited by the iC3b-binding structure of *C. albicans* (10). In their model, iC3b is noncovalently attached to the candidal receptor, thus masking the recognition site on iC3b for neutrophil CR3.

Finally, it is thought that *C. albicans* has evolved a strategy for acquiring iron from erythrocytes by means of complement (21), which would represent a third function of the iC3b-binding moieties. *C. albicans* is dependent upon iron for growth. By rosetting complement-coated erythrocytes, *C. albicans* might be capable of obtaining iron from them. Rosetting can be inhibited by monoclonal antibodies to the human CR3. In vivo, erythrocytes can be opsonized during a *Candida* infection because the activation of the alternative pathway of complement by *Candida* organisms leads to "bystander" deposition of C3 fragments on the surfaces of autologous erythrocytes.

Presumably, the iC3b-binding structures of *C. albicans* pseudohyphae carry out some important functions in the pathogenesis of disseminated candidiasis. Ca^{2+} ions are required to accomplish this function effectively.

We thank Maggie Rossmann and Reinhard Würzner for critical reading of the manuscript and Gilbert Reibenegger for help with the statistical analysis.

This work was supported by the Austrian FWF (P 8288).

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