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Adherence of *Staphylococcus aureus, Klebsiella pneumoniae* from healthy persons and HIV carriers, under the influence of Broncho Vaxom *in vitro* and ascorbic acid *in vivo*

Fortis, A. A., Lianou, P. E. & Papavassilliou, J. T. Adherence of *Staphylococcus aureus, Klebsiella pneumoniae* and *Candida albicans* to human buccal epithelial cells, from healthy persons and HIV carriers, under the influence of Broncho Vaxom *in vitro* and ascorbic acid *in vivo*. APMIS *106:* 441–448, 1998.

We examined the *in vitro* effect of Broncho Vaxom (BV) (an immunobiotherapeutic preparation containing a lysate made from bacteria often involved in respiratory tract infections) on adherence of *Staphylococcus aureus, Klebsiella pneumoniae* and *Candida albicans* to human buccal epithelial cells (BEC) of healthy volunteers and HIV carriers. We also examined the *ex vivo* effect of ascorbic acid on the adherence of the same microorganisms to BEC of HIV carriers. The study reached the following conclusions: The presence of BV *in vitro* significantly reduces the adherence of the tested strains to BEC from healthy persons and HIV carriers. No significant difference was observed between healthy persons and HIV carriers regarding the adherence of the tested strains to BEC. Significant difference in the adherence of the tested strains to BEC was observed between HIV carriers who had been taking ascorbic acid over a 3-month period and those who had not. There was no further reduction in the adherence of the tested strains to BEC from HIV carriers who had been taking ascorbic acid in the presence of BV *in vitro*.

Key words: Adherence; Broncho Vaxom; ascorbic acid; HIV; AIDS.

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Respiratory infections are a frequent complication in patients with the acquired immune deficiency syndrome (AIDS). In most reports, *Staphylococcus aureus* is the third most common agent (*Bennett et al.* 1996). Carriage of staphylococcal organisms in HIV-infected patients is approximately twice that seen in the general population (*Isselbacher et al.* 1994). Gram-negative pneumonia is common in HIV-seropositive persons. The incidence of Klebsiella as an etiologic factor varies, reaching 26% in certain series, especially when other factors which influence immune status, such as malnutrition, coexist (*Ikeogu et al.* 1997). Candida infections are the most common fungal infections occurring in HIV-infected patients (*Isselbacher et al.* 1994). It has been demonstrated that, as HIV infection advances, the frequency of isolation of Candida increases (*Torrssander et al.* 1987), making it an important predictive sign of the subsequent development of AIDS (*Klein et al.* 1984).

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Bacterial adherence is required for the initia-

tion of infection and is positively correlated with virulence (*Beachey* 1981). In *S. aureus*, teichoic acid (*Aly & Levit* 1987), protein A, fibronectin-binding proteins, collagen-binding protein and fibrinogen-binding protein have important roles, acting as adhesins (*Foster & McDevitt* 1994; *Joh et al.* 1994). In Klebsiella, the type of fimbriae determines adherence. Adhesive capacities of Klebsiella might be affected by the bacterial growth phase, temperature, multiplicity of infection, and relative hydrophobicity of the cell wall (*Favre-Bonte et al.* 1995; *Jansen et al.* 1992). In *Candida albicans*, at least three recognition systems have been described. The systems are classified by the type of host cell, the growth form of the organism and the nature of the interaction at the molecular level (protein-protein or protein-oligosaccharide) (*Calderone* 1993). Fimbriae, in addition play, a role in *C. albicans* adherence (*Yu et al.* 1994).

Broncho Vaxom (BV) is an immunomodulating preparation containing extracts from common pathogenic bacteria involved in respiratory tract infections. It has been used as an oral adjuvant in the prevention of these infections (*Mauel* 1994). Various mechanisms of BV action are suggested. BV stimulates metabolic and functional activities of macrophages, induces interferon (*Mauel* 1992; *Emmerich et al.* 1992) and interleukin-1 production (*Bottex et al.* 1988), and enhances TNFa and interleukin-2 production (*Wybran et al.* 1989). In patients with chronic bronchitis, the bronchoalveolar lavage (BAL) CD4+/CD8+ ratio was increased after treatment with BV (*Emmerich et al.* 1992). Activation of natural killer cells has also been reported (*Wybran et al.* 1989). Finally, BV increases salivary levels of sIgA and serum levels of IgG and IgM (*Puigdollers et al.* 1980; *Spiropoulos et al.* 1993) and upregulates the expression of adhesion molecules on phagocytes (*Marchant et al.* 1992).

There is evidence that ascorbic acid, a well-known antioxidant, has immunoregulatory properties (*Anderson* 1984). There are studies, which indicate that ascorbic acid mediates an anti-HIV effect by diminishing viral protein production in infected cells and reverse transcriptase stability in extracellular virions (*Harakeh et al.* 1990; *Rawal et al.* 1995). In a previous work we have demonstrated the *in vivo* effect of ascorbic acid on anti-HIV antibody titers, T-cell subpopulations and serum immunoglobulins in human HIV carriers (*Lianou et al.* 1993).

The aim of this study was to establish: First, if BV can act *in vitro* as a competitive inhibitor reducing bacterial adherence of *S. aureus, K. pneumoniae* and *C. albicans* to human BEC from healthy volunteers and HIV carriers, and secondly, if ascorbic acid, which has been suggested as a possible alternative approach in the therapy of HIV infection (*Harakeh et al.* 1990; *Rawal et al.* 1995; *Lianou et al.* 1993; *Abrams* 1990), has a further role in the management of HIV-infected patients, by diminishing the adherence of the same strains *in vivo*.

MATERIALS AND METHODS

Patients

Epithelial cells were taken from the buccal mucosa of 25 healthy volunteers (23 men and 2 women; mean age 31 years, range 24–61) and 30 asymptomatic HIV carriers (28 men and 2 women; mean age 33.4 years, range 22–62) who visited our centre for a routine test and were diagnosed as HIV+. Laboratory diagnosis of HIV infection was based on EIA (Abbot Diagnostics) and was confirmed by Western blot (Diagnostics Pasteur). HIV-infected subjects had no AIDS-defining illnesses. According to the Walter Reed classification they were stage WR1. Neither healthy volunteers nor HIV carriers had any history of antibiotic therapy or other medication in the previous 6 months. Of the 30 HIV carriers, 25 (all men) were treated with 3 g of ascorbic acid daily for a period of 3 months. The adherence of the three strains and the effect of BV on the adherence *in vitro* were then re-examined.

Broncho Vaxom

Broncho Vaxom is a product of OM Laboratories Meyrin/Geneva (Switzerland). It is produced by exposing, under standardized conditions, bacteria that are frequently found in respiratory tract infections (*Haemophilus influenzae, Streptococcus pneumoniae, Klebsiella pneumoniae, Klebsiella ozaenae, Staphylococcus aureus, Streptococcus pyogenes, Streptococcus viridans* and *Neisseria catarrhalis*) to alkaline lysis. Subsequently, the hydrosoluble part is subjected to different extraction steps, further purified, and lyophilized. The germs are cultured separately, killed, and fractionated. The end product contains glycoproteins and ribonucleoproteins from these bacteria (*Delaval & Rey* 1986). The final dry preparation contains per net weight 35% protein, 8% free amino

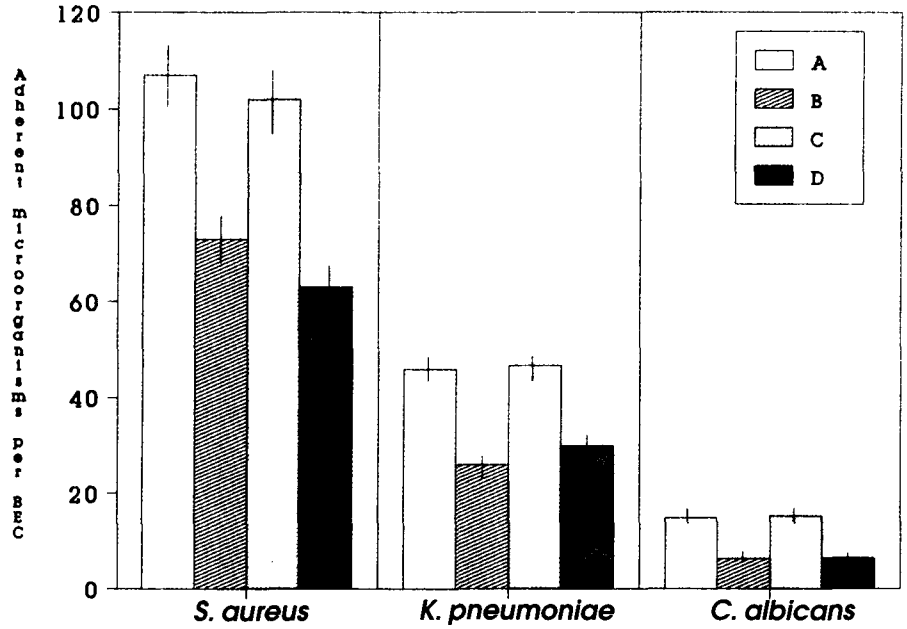

Fig. 1. In vitro adherence of the three microorganisms to BEC of healthy volunteers and HIV carriers, before and after addition of BV to the mixture (BEC-microorganisms). Thin vertical bars represent standard error (SE) of the mean. *A:* BEC from healthy volunteers. Adherence determination before addition of BV *in vitro*. *B:* BEC from healthy volunteers. Adherence determination after addition of BV *in vitro*. *C:* BEC from HIV+ individuals without ascorbic acid treatment. Adherence determination before addition of Broncho Vaxom *in vitro*. *D:* BEC taken from HIV+ individuals without ascorbic acid treatment. Adherence determination after addition of Broncho Vaxom *in vitro*.

acids, 10% lipids, 8% nucleotides, 2% carbohydrates and approximately 37% salt (*Mauel et al.* 1989). The BV concentrations employed were calculated in the BV preparation provided for us by Uni Pharma Pharmaceutical Laboratories, which represents OM laboratories in Greece. Pilot experiments using different BV concentrations were carried out. In our final experiments we used a BV concentration of 0.00875 mg/ml phosphate-buffered saline (PBS).

Preparation of epithelial cells

Epithelial cells, collected by scraping the buccal mucosa with a wooden applicator, were suspended in phosphate-buffered saline (0.02 M phosphate 0.15 M NaCl, pH 7.4). More than 95% of the epithelial cells appeared viable, as indicated by their ability to exclude 0.4% trypan blue. The epithelial cells were washed three times in PBS and resuspended at a concentration of 10^5 cells per ml.

Microorganisms

S. aureus was isolated from blood cultures. *K. pneumoniae* and *C. albicans* were isolated from urine cultures from patients with asymptomatic urinary tract infections. *S. aureus* and *K. pneumoniae* were cultured in nutrient broth at 37°C for 18 h. *C. albicans* was harvested after 3 days' culture in Sabouraud medium. The microorganisms were suspended in 10 ml PBS and washed three times with PBS by centrifugation, and a suspension of 10^8 microorganisms per ml for *S. aureus* and *K. pneumoniae*, and 10^6 microorganisms per ml for *C. albicans*, was prepared.

Bacterial adherence assay

This assay was performed according to *Ellen & Gibbons* (1972). The bacterial suspensions were mixed with an equal volume of epithelial cells (0.5 ml) and incubated at 37°C for 30 min. Following incubation, the cells were washed three times with PBS to remove unattached bacterial cells. Smears were prepared in duplicate and stained with crystal violet. The smears were examined by two observers using bright-field microscopy. Adherence was determined by counting the number of bacteria adhering per epithelial cell. For each case, at least 50 cells were examined. Control cells were incubated in PBS without the addition of microbes to determine the number of bacteria adhering to the cells before mixing the organism being tested. The background count was subtracted from the counts obtained following mixing of bacteria with epithelial cells. Assays were done in duplicate.

The following experiments were done:

a) Determination of *in vitro* adherence of the three strains to BEC of 25 healthy volunteers before and after addition of a BV dilution of 0.00875 mg/ml to

TABLE 1. *Results of the various tests*

		Healthy volunteers		HIV carriers			
				Before taking ascorbic acid		After taking ascorbic acid	
		Without BV *in vitro* A	With BV *in vitro* B	Without BV *in vitro* C	With BV *in vitro* D	Without BV *in vitro* E	With BV *in vitro* F
Sta[b]	MEAN	107	73	102	63	55	54.5
	SE[a]	±5.6	±5.3	±4.4	±3.7	±1.9	±1.46
Klp[c]	MEAN	45.9	26.1	46.7	30	25	23
	SE	±1.64	±1.38	±1.5	±1.2	±1.08	±0.96
Caa[d]	MEAN	14.8	6.3	15.2	6.4	6.5	6.1
	SE	±0.78	±0.46	±0.6	±0.5	±0.38	±0.32

A, C, E=No addition of Broncho Vaxom (BV) *in vitro*, B, D, F=Addition of (BV) *in vitro*. BV=Broncho Vaxom, a: SE=Standard error of the mean, b: Sta=*S. aureus*, c: Klp=*K. pneumoniae*, d: Caa=*C. albicans*.

the mixture (BEC-microorganisms), and incubation at 37°C for 30 min.

b) Determination of adherence of the three strains to BEC of 30 HIV carriers who were not taking ascorbic acid, before and after addition of a BV dilution of 0.00875 mg/ml to the mixture (BEC-microorganisms), and incubation at 37°C for 30 min.

c) Determination of adherence of the three strains to BEC of 25 HIV carriers before and after they were treated with 3 g of ascorbic acid daily for a period of 3 months, each time before and after addition of a BV dilution of 0.00875 mg/ml to the mixture (BEC-microorganisms) and incubation at 37°C for 30 min.

Statistical evaluation. Mean values were compared using Student's *t* test.

RESULTS

a) *In vitro* effect of BV on bacterial adherence to BEC of healthy volunteers.

Adherence of all tested strains to BEC of healthy volunteers was significantly reduced after the addition of BV ($p<0.001$), as seen in

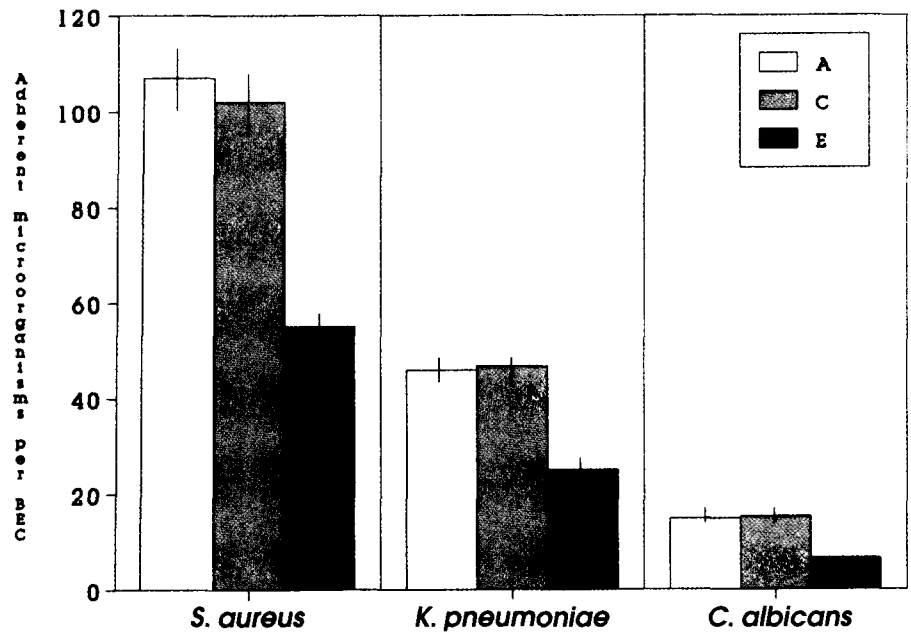

Fig. 2. Determination of adherence of the three microorganisms to BEC of healthy volunteers, HIV carriers not taking ascorbic acid, and HIV carriers after ascorbic acid treatment. Thin vertical bars represent standard error of the mean (SE) *A:* BEC from healthy volunteers. *C:* BEC from HIV+ not taking ascorbic acid. *E:* BEC from HIV+ after three months' treatment with ascorbic acid.

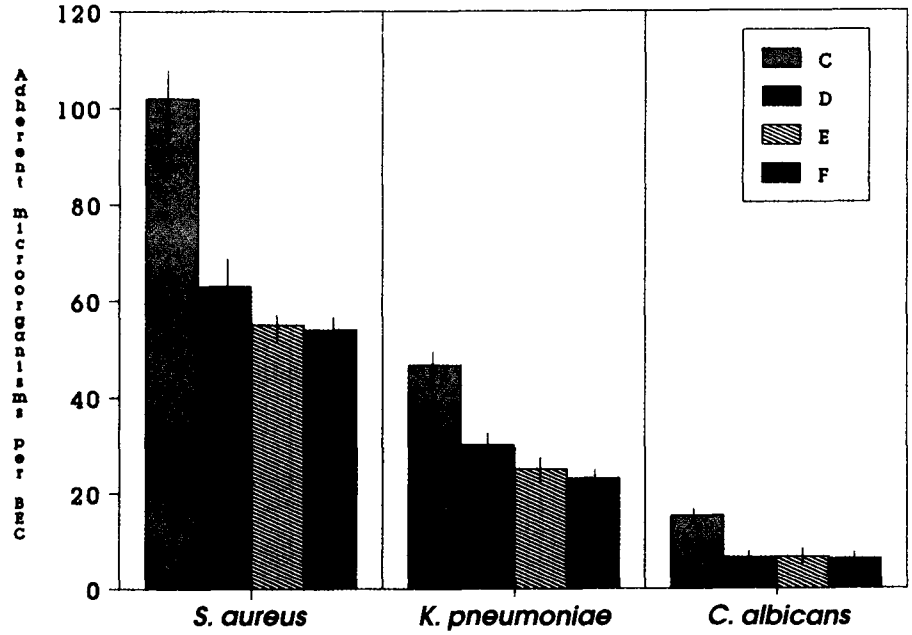

Fig. 3. Determination of *in vitro* adherence of the three strains to BEC of HIV carriers who had taken ascorbic acid for a period of 3 months before and after addition of BV. Vertical bars represent standard error of the mean. *C:* BEC from HIV+ individuals without ascorbic acid treatment. No addition of Broncho Vaxom *in vitro*. *D:* BEC from HIV+ individuals who had not been treated with ascorbic acid. Addition of Broncho Vaxom *in vitro*. *E:* BEC from HIV+ individuals after 3 months' treatment with Ascorbic acid. No addition of Broncho Vaxom *in vitro*. *F:* BEC from HIV+ individuals after 3 months' treatment with ascorbic acid. Addition of Broncho Vaxom *in vitro*.

Fig. 1 and Table 1. The addition of BV to the mixture inhibited the binding of *S. aureus* by 31.7%, of *K. pneumoniae* by 43%, and of *C. albicans* by 57%.

b) *In vitro* effect of BV on bacterial adherence to BEC of HIV carriers who were not taking ascorbic acid. Bacterial adherence was also significantly reduced ($p<0.001$) with all tested strains after the addition of BV *in vitro* (Fig. 1 and Table 1). The addition of BV to the mixture inhibited the binding of *S. aureus* by 38.3%, of *K. pneumoniae* by 36% and of *C. albicans* by 57%.

No significant difference was observed in the adherence of the tested strains to BEC of the 25 healthy volunteers and of the HIV carriers who were not taking ascorbic acid ($p>0.05$).

c) Adherence to BEC of HIV carriers before and after treatment with ascorbic acid.

Significant reduction ($p<0.001$) in the adherence of the tested strains to BEC after treatment with ascorbic acid for a mean period of 3 months was observed (Fig. 2 and Table 1). The treatment with ascorbic acid decreased adherence by 42% in *S. aureus*, by 34.8% in *K. pneumoniae*, and by 57% in *C. albicans*.

d) *In vitro* adherence to BEC of HIV carriers taking ascorbic acid after addition of BV.

No further reduction was observed in the adherence of the tested strains to BEC from 25 HIV carriers who were taking ascorbic acid in the presence of BV *in vitro* (Fig. 3 and Table 1).

DISCUSSION

Since the discovery of HIV as a new pathogen, interest in substances with immunoregulatory action has increased. The main question regarding bacterial adherence is whether bacterial infection can be prevented by blocking adherence to the mucosal surface, especially in immunocompromised persons (AIDS patients).

Even though Broncho Vaxom's mode of action has been widely studied, little is known re-

garding its role in bacterial adherence. Polyantigenic immunomodulator adjuvants have been used in the management of patients infected with HIV, either alone (*Colon et al.* 1990) or in formulations of experimental HIV vaccines amplifying and directing immune responses to highly purified antigens (*Vogel* 1995).

In general, three approaches have been proposed with respect to the prevention of bacterial adherence. First, purified bacterial adhesin might be applied as a competitive inhibitor of bacterial adherence. Second drugs might be administered to suppress adherence (e.g. subMIC concentrations of antibiotics, enzymes or chemicals) and third anti-adherence vaccines which induce the formation of local antibodies might be developed (*Beachey* 1981). In our experiments, there was evidence that BV interacted with the BEC of healthy volunteers *in vitro* and decreased the adherence of all the microorganisms tested. It is logical to assume that the first of the above mechanisms was responsible for the diminished adherence seen in both healthy volunteers and HIV-infected patients, with BV probably operating as a competitive inhibitor of bacterial adherence. Further studies must be conducted in order to establish if BV has a similar effect on adherence *in vivo*.

Ascorbic acid has been used as a potential immunoregulatory agent in *in vitro* and *in vivo* experiments relating to the treatment of HIV infection. Exposure to high concentrations of vitamin C decreased the proliferation and survival of the HIV-infected cells and caused decreased viral production (*Rivas et al.* 1997). Whether ascorbic acid's action is virus specific, or whether it has a more general effect on cellular metabolism, remains controversial (*Harakeh et al.* 1990; *Rawal et al.* 1995; *Lianou et al.* 1993). In the present study we observed a significant decrease in the adherence of the three strains in HIV carriers after treatment with 3 g of ascorbic acid daily for a mean period of 3 months. Mucosal IgA has generally been viewed as an immune barrier which prevents the adherence and absorption of antigens (*Mazanec et al.* 1993; *Ravdin & Kelsall* 1994). We have already seen that administration of ascorbic acid may lead to an increase of immunoglobulin levels, including IgA (*Lianou et al.* 1993). These results suggest a mechanism of action which decreases adherence of the three strains in HIV carriers after such treatment.

The bacterial adherence assays in our study indicate no difference between healthy volunteers and HIV carriers when the latter were not receiving treatment with ascorbic acid. *Sweet et al.* (1995), found an increased adherence of *C. albicans* strains which were isolated from HIV carriers and AIDS patients. This suggests that HIV infection and AIDS are associated with the selection of strains of *C. albicans* with enhanced ability to adhere to BEC. We are not aware of data regarding the adherence in HIV patients' BEC of the other two microbes examined here, and the previous studies have all been done on *C. albicans*. However, it is logical to assume a similar activity. Further studies need to be conducted in order to establish whether different strains of the same microorganism differ regarding adherence under the influence of BV or ascorbate.

A final observation in our study was that, in HIV-infected patients who were receiving ascorbic acid therapy, no further reduction of adherence could be observed after BV addition *in vitro*, a fact that leads us to speculate that ascorbic acid has a maximum effect in preventing microbial adherence. The selected ascorbic acid dose was moderately high compared to in similar studies and no side effects were noted. Treatment of HIV carriers with ascorbic acid thus seems promising, since its beneficial effect is combined with extremely low toxicity and it can be taken as a nutritional supplement (*Pauling* 1970).

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