Local Cellular Immune Reaction to Oral *Candida albicans* Inoculation in Normal and Immunodeficient Mice: Implications of Infectious Dose

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Purpose: To address two questions by means of an animal model: Firstly, does a dose/effect relationship exist between *C. albicans* load and the emergence of an oral *C. albicans* infection; secondly, which cellular immune response takes place following inoculation with defined pathogen loads.

Material and Methods: Immunocompetent inbred mice (Balb/c) (n=19) and mice with combined B- and T-cell defects (SCID) (n=21) were orally inoculated with pathogen loads between 10^4 and 10^8*C. albicans* cells/10 µl (strain ATCC 32032). One half of the tongue tissue was examined with the Periodic Acid Schiff (PAS) method for displaying the invasion of hyphae. The other half of the tissue was examined by immune peroxidase technique for analyzing the distribution of immunocompetent cells (CD4, CD54, CD74, CD103) in the epithelial layers and subepithelial connective tissue.

Results: One week following the inoculation, neither group's tissue showed clinical signs of oral candidiasis. Using PAS preparation hyphae were seen with the inoculation dose of 10^8*C. albicans* cells in the case of Balb/c mice and a load of 10^5 pathogens in SCID mice. The extent of the immunologic reaction depended both on the inoculation dose given to the animals and on their immune status.

Conclusion: Despite the lack of clinical signs of oral candidiasis in both group's tissue, infections of the tongue epithelium were evident histologically leading to immunologic reactions of the tongue mucosa.

Key words: Candida, candidiasis, immunologic dose response relationship, oral

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INTRODUCTION

The term candidiasis refers to a disease with clinical signs and symptoms which is most often caused by *C. albicans*, but also by *C. tropicalis*, *C. krusei*, and *C. glabrata* among others. Macroscopically visible mucosal changes are characteristic for the condition, as are the histological demonstration of hyphae and histopathological changes in the infected mucosa. These changes vary from atrophy to hyperparakeratosis, akanthosis, and microabscesses in the upper regions of the *stratum spinosum* and can include a band of mononuclear cells in the underlying connective tissue.

Data regarding the incidence of C*andida spp.* colonization without clinical signs or symptoms of mucosal disease vary between 32% and 60% (Fotos et al, 1992; Mooney et al, 1995) or 20% and 50% (Epstein et al, 1984; Odds, 1988; Samaranayake, 1989). The number of Candida cells per millilitre saliva among persons with no clinical symptoms of disease lies between 200-500 CFU/ml (Epstein et al, 1980). Using oral rinse techniques, it was possible to demonstrate 600 CFU/ml (McKendrick, 1968; McKendrick et al, 1967). It is significant that the pathogen load (measured by swab or oral rinse technique) is not indicative of a mucosal infection with *Candida spp.* There is no direct associ-

ation between microbiological demonstration of a high pathogen load and the occurrence of mucosal changes (Barone et al, 1990; Luangjamekorn et al, 1990; Schmidt-Westhausen et al, 1993; van Meter et al, 1994). Among persons with a healthy immune response who showed no clinical manifestations, swab techniques yielded up to 10^3 CFU/ml (Odds, 1988; Samaranayake, 1989), whereas clinical changes were seen in some HIV positive patients despite the fact that only 50 CFU/ml could be demonstrated (Barone et al, 1990).

C. albicans infection occurs when the microorganism penetrates the epithelial surface, for example as in the case of altered mucus production. Host defence against Candida infection depends on two types of immune reactions, unspecific cellular and acquired cell-mediated immunity. This occurs primarily through phagocytosis, cytokine secretion of phagocytes, and the subsequent activation of lymphocytes. In cases of Candida infection, a substantial part of the response comes from $CD4^+$-T-cells, which receive antigen peptides from antigen presenting cells (APC) through MHC class II molecules. Compared to the frequency and intensity of oral mucosa infections, the systemic complication of an invasive, disseminated Candida infection is rare in patients with HIV. The degree of the mucocutaneous lesions is limited, and in most cases the infection does not reach the subepithelial connective tissue. This can be explained by the fact that while the number of $CD4^+$-lymphocytes and T-cell involvement is diminished, the unspecific, non-adaptive, congenital immunity remains intact. The latter, which relies on neutrophil granulocytes, monocytes, macrophages as well as on Langerhans' cells (LHC), is decisive for the defence against fungal infection.

The aim of this study was to address two questions in a Candida infection model using immunocompetent and immunodeficient mice. Firstly, whether a correlation exists between the inoculated pathogen load and the emergence of a *C. albicans* infection (dose/effect relationship); and, secondly, which cellular immune response takes place in the oral mucosa following inoculation with defined pathogen loads.

MATERIALS AND METHODS

Mice

Immunocompetent inbred mice (Balb/c) (n=19) and mice with combined B- and T-cell defects (SCID) (n=21) were purchased from Charles River Breeding farms (Sulzfeld/Germany). Only male mice, 6 to 8 weeks of age, were used in the experiments. All the animals were housed and used in accordance with the federal guidelines of Germany for the protection of animals (Sign 0342/97). On arrival and throughout the experiments, SCID mice were kept in a sterile environment (filter-top caging system, laminar flow work and change stations, sterilization of feed, bedding and water). All the mice were given food and water ad libitum and kept, three per cage, at the Animal Care facilities of the Charité-University Medicine Berlin, according to established guidelines. Experiments started 7 days after arrival.

The drinking water contained tetracycline hydrochloride and was supplied according to the Russel and Jones technique (1973), i.e. 0.1% w/v aqueous tetracycline suspension for 5 days preceding the first inoculation, followed by 0.01% w/v aqueous tetracycline suspension for the remainder of the experiment. A fresh suspension was prepared twice weekly.

Inoculum

The *C. albicans* strain (ATCC 32032) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM, Braunschweig, Germany). The yeasts were cultured according to the recommendations of the vendor (Sabouraud-glucose agar with the addition of 5 mg of penicillin/streptomycin/500 ml Agar medium), incubated at room temperature for four days, and stored at 4°C until required. To obtain standardized cell counts for inoculation, the yeasts were suspended and the cell counts were quantified using a cytometer and diluted to a final concentration of 1×10^6 to 1×10^{10} per ml with sterile phosphate buffer (RPMI 1640).

Inoculation

In order to determine the dose/effect relations, the smallest inoculation dose leading to an infection of the epithelium was defined as the dose at which at least two of three animals were infected. As it was known from earlier studies that a single dose of 10^8 *C. albicans* cells leads to a colonization of murine mucosa within a few hours (Deslauriers et al, 1997; Lacasse et al, 1993; Lacasse et al, 1990), inoculation was begun with an initial dose of 10^6 *C. albicans* cells/10 µl, administered to the animals with an Eppendorf pipette.

Histochemistry

The animals were sacrificed one week following inoculation, and the tongue was excised. One half of the tongue tissue was fixed in 10% buffered formaldehyde and embedded in paraffin. Serial sections, 5 µm thick, were cut and stained with the Periodic Acid Schiff (PAS) method for displaying morphological structures of *C.*

Table 1 Number of mice per oral Candida albicans inoculation

Breed	*C. albicans* cells /10 µl	No. of mice
Balb/c	10^5	n=3
	10^6	n=3
	10^7	n=6
	10^8	n=6
	no (control)	n=1
		Total: n=19
SCID	10^4	n=2
	10^5	n=6
	10^6	n=3
	10^7	n=6
	no (control)	n=4
		Total: n=21

albicans. Serial sections (PAS stain) were evaluated according to the following criteria (per section): an infection was deemed present, if hyphae could be demonstrated in the tongue epithelium in at least one section using light microscopy at 400× magnification. In cases where 2/3 of the mice receiving the same dose were shown to be positive, the dose was reduced by one log level and administered to groups of three mice, until less than 2 were infected. This threshold, the pathogen load which was just capable of infecting the tongues of two of three mice, was then used to repeat a further inoculation experiment using the same pathogen load. Table 1 shows the number of animals per inoculation dose.

Immunohistochemistry

The remaining half of the tongue tissue was snap frozen, cryosectioned, air dried, fixed briefly in acetone and immunostained for antigens interacting with MHC-class-II molecules (CD4, CD74, CD103), an antigen thought to be involved in recruitment of subgroups of mucosal lymphocytes (CD103) and one antigen which promotes leukocyte adhesion to endothelium in inflammation (CD54).

The CD4 specific antibody (clone: RM4-5, isotype: Rat (DA) IgG_{2a}, κ Pharmingen, Hamburg Germany, dilution 1:45) labels murine helper T-cells; the antibody against CD54 (clone: 3E2, isotype: Armenian hamster IgG, group 1, κ Pharmingen, Hamburg Germany, dilution 1:200) labels endothelial and dendritic cells, lymphocytes and macrophages; the CD74 specific antibody (clone: In-1, isotype: rat (Wistar) IgG_{2b}, κ Pharmingen, Hamburg Germany, dilution 1:100) labels B-lymphocytes, monocytes, and Langerhans' cells; and the antibody against CD103 (clone: 2E7, isotype: Armenian hamster IgG, group 2, κ Pharmingen, Hamburg Germany, dilution 1:200) labels lamina propria $CD8^+$-cells.

Sections prepared as described above were immunolabelled with the antibodies as primary reagents followed by the peroxidase antiperoxidase technique. Briefly, frozen sections were thawed, air-dried and fixed in acetone. They were incubated with albumin or normal serum, washed in PBS, and incubated with optimally diluted primary antibodies for 1 h. Endogenous peroxidase activity was blocked with hydrogen peroxide. Sections were incubated with secondary antibodies (for CD4 and CD74: HRP-conjugated goat anti-rat immunoglobulin-specific polyclonal antibody; for CD54 and CD103: biotin-conjugated mouse anti-rat and hamster Ig as well as Streptavidin horseradish) for approx. 1 h and washed in PBS. Horseradish was shown with 3 aminocarbazole (AEC; Dianova, Hamburg, Germany). Nuclei were counterstained with Mayer's hemalaun. As negative controls, slides were incubated with secondary antibodies and the PAP-complex.

According to the study by Romagnoli et al (1997), labelled cells were counted in ten consecutive microscopic fields for quantifying evaluation using a light microscope at a magnification of 400× to quantify the number of labelled cells per 840 basal epithelial cells. The depth of the lamina propria analyzed for counts extended from immediately below to 170 µm from the basement membrane. The area of a microscopic field as designated above was 0.33 mm^2.

Positive reactions were semi-quantitatively analyzed as follows:

0 = no reaction
(+) = 1-2 positive cells
\+ = 3-15 positive cells
++ = 16-50 positive cells
+++= >50 positive cells

Activated endothelia were assessed as follows:

− = no expression
\+ = weak expression
++ = medium expression
+++= strong expression

RESULTS

Clinical Aspects

Macroscopically, neither the tongue of Balb/c mice nor SCID mice showed clinical signs of candidiasis, such as erythematous or pseudomembranous changes.

Table 2 Inoculated *C. albicans* doses and results of the PAS-evaluation of the serial sections for Balb/c mice

Animal No.[1]	Pathogens/10 µl[2]	Number of sections [3]	PAS findings[4]	Inflammatory cells[5]	Overall evaluation[6]
B0	no tetracycline no *C. albicans*	n=9	neg.	None	Negative
B1	10^7	n=20	neg.	None	Negative
B2	10^7	n=11	neg.	None	
B3	10^7	n=6	6/6 pos.	None	
B4	10^8	n=5	3/5 pos.	Infiltrates in 5/5	Positive
B5	10^8	n=5	4/5 pos.	None	
B6	10^8	n=5	5/5 pos.	Infiltrates in 5/5	
B7	10^6	n=36	neg.	None	Negative
B8	10^6	n=36	4/36 pos.	None	
B9	10^6	n=36	neg.	None	
B10	10^7	n=24	neg.	None	Negative
B11	10^7	n=24	12/24 pos.	Infiltrates in 3/12	
B12	10^7	n=24	neg.	None	
B13	10^5	n=24	neg.	None	Negative
B14	10^5	n=24	neg.	None	
B15	10^5	n=24	neg.	None	
B16	10^8	n=12	8/12 pos.	Infiltrates in 3/8	Positive
B17	10^8	n=12	neg.	None	
B18	10^8	n=12	6/12 pos.	Infiltrates in 3/6	

1) Specification of the inoculated animal
2) Number of inoculated Candida cells/10 µl
3) Number of sections per tongue half
4) Number of sections showing PAS positive structures (blastospores, hyphae) in the upper epithelium layers to the number of sections prepared
5) Sections demonstrating an inflammatory infiltrate among those PAS positive sections listed in column 4
6) The overall evaluation judges whether the inoculation dose infected at least 2/3 of the mice.

PAS Reaction

C. albicans is not part of the normal murine oral flora (Lacasse et al, 1990). Intraoral inoculation, however, resulted in colonization dependent on inoculation dose. Table 2 shows an overview of the inoculated pathogen load and the results of the evaluation of the serial sections for Balb/c mice. In addition to the invasion by hyphae, the reactive phagocyte infiltration was also documented.

No hyphae or blastospores could be demonstrated in 2/3 of the mice given a dose of 10^7 pathogens (B1, B2, B3). This result was confirmed in a further group which was inoculated with 10^7 *C. albicans* cells (B10, B11, B12). In total, 18/108 sections (16.6%) showed PAS positive structures at this pathogen load.

The pathogen load was raised by one log level. In the group inoculated with 10^8 *C. albicans* cells (B4, B5, B6), 3/3 of the mice showed PAS positive structures. In order to confirm these results, another group was inoculated with 10^8 (B16, B17, B18). In this case, 2/3 of the mice could be considered positive. At this pathogen load, a total of 26/51 sections (51%) showed PAS positive structures.

When the inoculation dose was reduced to 10^6/10 µl (B7, B8, B9), 2/3 of the tongues were PAS negative. In a further reduction to 10^5/10 µl (B13, B14, B15), no hyphae or blastospores could be demonstrated in the epithelium (0/72 sections). The Balb/c mouse no. B0 served as a control and showed no Candida structures. The minimal infective dose among Balb/c mice was 10^8 *C. albicans* cells/10 µl.

In cases where hyphae or blastospores could be demonstrated in epithelial layers 19/48 also showed an inflammatory infiltrate, showing that penetration by the microorganisms led to a reaction in 36.9% of these cases. Table 3 shows an overview of the inoculated

Table 3 Inoculation doses of *C. albicans* and results of the PAS studies of the serial sections for the SCID mice

Animal No.[1]	Pathogens/10 µl[2]	Number of sections[3]	PAS findings[4]	Inflammatory cells[5]	Overall evaluation[6]
S1	10^7	n=11	neg.	None	positive
S2	10^7	n=5	5/5 pos.	Infiltrate in 5/5	
S3	10^7	n=5	5/5 pos.	None	
S4	10^5	n=12	12/12 pos.	None	positive
S5	10^5	n=21	neg.	None	
S6	10^5	n=21	6/21 pos	None	
S7	10^6	n=42	17/42 pos.	Infiltrate in 3/17	positive
S8	10^6	n=21	19/21 pos.	Infiltrate in 19/21	
S9	10^6	n=28	neg.	None	
S10	10^7	n=42	11/42 pos.	None	positive
S11	10^7	n=21	13/21 pos.	None	
S12	10^7	n=42	21/42 pos.	Infiltrate in 21/21	
S13	no *C. albicans*	n=12	neg.	None	negative
S14	no *C. albicans*	n=9	neg.	None	
S15	no *C. albicans*	n=9	neg.	None	
S16	no tetracycline no *C. albicans*	n=9	neg.	None	
S17	10^4	n=12	neg.	None	negative
S18	10^4	n=12	neg.	None	
S22	10^5	n=21	neg.	None	positive
S23	10^5	n=12	12/12 pos.	None	
S24	10^5	n=21	3/21 pos.	None	

1) Specification of the inoculated animal
2) Number of inoculated Candida cells/10 µl
3) Number of sections per tongue half
4) Number of sections showing PAS positive structures (blastospores, hyphae) in the upper epithelium layers to the number of sections prepared
5) Sections demonstrating an inflammatory infiltrate among those PAS positive sections listed in column 4
6) The overall evaluation judges whether the inoculation dose infected at least 2/3 of the mice.

pathogen load and the results of the evaluation of the serial sections for SCID mice.

Hyphae were found in the epithelium (Fig. 1) in 2/3 of mice which received a dose of 10^7 pathogens (S1, S2, S3). This result was reproducible (S10, S11, S12). In total, PAS positive structures could be demonstrated in 55/126 sections (43.7%) at this pathogen load. When the pathogen load was reduced by one log level to a dose of 10^6/10 µl, PAS positive structures could be seen in 2/3 of the tongues and hyphae could be demonstrated in 36/91 (40%) of the sections in the stratum corneum of the papillae filiformes. In the group inoculated with 10^5 *C. albicans* cells (S4, S5, S6) 2/3 of the mice showed PAS positive structures. To confirm the results, a further group was inoculated with 10^5 (S22, S23, S24). Here, again, 2/3 of the mice could

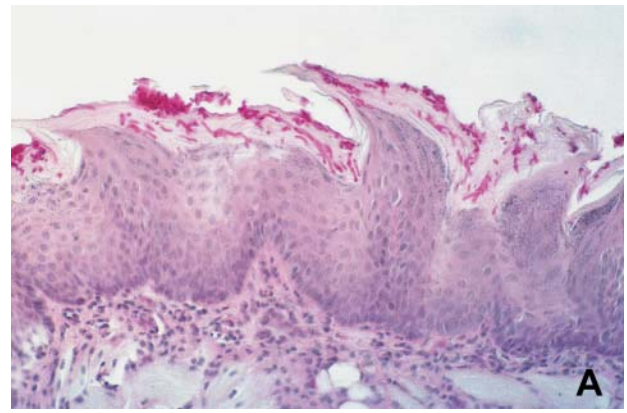

Fig. 1 SCID mouse: Hyphae invasion of the cornified layer of murine tongue epithelium after inoculation with *C. albicans* cells, moderate inflammatory infiltrate (PAS stain, ×80).

Table 4 Balb/c Mice: Semi-quantitative evaluation of the antigen markers within epithelium and connective tissue with respect to inoculation dose

Antibodies against	CD4	CD54	CD74	CD103
Inoculation dose 10^5				
Number	n=3	n=3	n=3	n=3
Str. spin. upper				
Str. spin. lower			+	
Basal cells	(+)			(+)
Subepithelial	(+)	+	+	(+)
Lamina propria				
Endothelium		+		
Inoculation dose 10^6				
Number	n=3	n=3	n=3	n=3
Str. spin. upper				
Str. spin. lower			+	
Basal cells	(+)			+
Subepithelial	+	+	+	(+)
Lamina propria				
Endothelium		+		
Inoculation dose 10^7				
Number	n=6	n=6	n=6	n=6
Str. spin. upper				
Str. spin. lower			+	(+)
Basal cells	+			+
Subepithelial	+	++	+	+
Lamina propria			(+)	
Endothelium		+		
Inoculation dose 10^8				
Number	n=4	n=4	n=3	n=3
Str. spin. upper				
Str. spin. lower	(+)		+++	+
Basal cells	(+)			+++
Subepithelial	++	+++	+++	+
Lamina propria			(+)	
Endothelium		+++		
No Inoculation				
Number	n=1	n=1	n=1	n=1
Str. spin. upper				
Str. spin. lower			+	
Basal cells	(+)			+
Subepithelial		+	+	(+)
Lamina propria				
Endothelium		(+)		

be considered positive. In total, PAS positive structures were present in 33/108 sections (30.6%).

Not until an inoculation dose of 10^4 *C. albicans* cells was used, did two mouse tongues remain PAS negative (S17 and S18). None of the 24 sections showed an invasion by hyphae. The mice S13, S14, S15, S16 served as controls, no Candida structures were found in the tongue epithelium.

The minimal infective dose among SCID mice was 10^5 *C. albicans* cells/10 µl.

In cases where hyphae or blastospores were found in the layers of the epithelium, an inflammatory infiltrate was present in 48/124 PAS positive sections, showing that penetration by the organisms led to a reaction in 38.7% of cases.

Immunohistochemistry

Tables 4 and 5 show the distribution of the marked antigens in relation to the inoculation dose. A sum drawn from the semi-quantitative evaluation of the positive markings for individual steps was calculated and divided by the number of steps. The evaluation of the amounts of expressed antigen is portrayed for each individual layer.

Balb/c Mice (Table 4)

Inoculation Dose 10^5

The extent of the immune reaction to an inoculation with a pathogen load of 10^5 was minor and occurred primarily in subepithelial tissue. The activation of the endothelium was limited (CD54). A few $CD74^+$ cells could be seen in the stratum spinosum (lower layer). Individual $CD4^+$ and $CD103^+$ cells were found in the basal cell layer as well as in subepithelial tissue. Interestingly, it could be determined from the morphology of the $CD4^+$ cells that these were not T-lymphocytes, but dendritic cells.

Inoculation Dose 10^6

In cases involving inoculations one log level greater, no changes were seen in CD74 and CD54 compared with the lower dose. $CD4^+$ cells were demonstrated in the basal cell layer and below.

Inoculation Dose 10^7

In contrast to the previous inoculation dose, the expression of CD54 increased in subepithelial tissue. No increase of CD54 could be demonstrated in the endothelium. CD103 was expressed in three cell layers (lamina propria, basal cell layer and the lower part of the stratum spinosum). No changes were seen in comparison to the previous inoculation dose with respect to CD74. The number of $CD4^+$ cells in the basal cell layer and in subepithelial tissue increased minimally.

Table 5 SCID Mice: Semi-quantitative evaluation of antigen markers within epithelium and connective tissue with respect to inoculation dose

Antibodies against	CD4	CD54	CD74	CD103
Inoculation dose 10^4				
Number	n=2	n=2	n=2	n=2
Str. spin. upper				
Str. spin. lower			++	
Basal cells				+
Subepithelial	(+)	+	+++	+
Lamina propria			+	
Endothelium		+		
Inoculation dose 10^5				
Number	n=6	n=6	n=6	n=6
Str. spin. upper				
Str. spin. lower			+	(+)
Basal cells				++
Subepithelial	(+)	+++	+	+
Lamina propria			(+)	
Endothelium		+++		
Inoculation dose 10^6				
Number	n=5	n=3	n=3	n=4
Str. spin. upper				
Str. spin. lower			+	(+)
Basal cells				+
Subepithelial		+++	+++	(+)
Lamina propria			(+)	
Endothelium		+++		
Inoculation dose 10^7				
Number	n=9	n=6	n=6	n=6
Str. spin. upper				
Str. spin. lower			+	
Basal cells	(+)		+	+
Subepithelial	(+)	+++	+	+
Lamina propria			+	
Endothelium		+++		
No Inoculation				
Number	n=3	n=3	n=3	n=3
Str. spin. upper				
Str. spin. lower			+	
Basal cells			+	+
Subepithelial	(+)	+	+	(+)
Lamina propria			(+)	
Endothelium		++		

Inoculation Dose 10^8

A significant increase was seen in the expression of CD54 in the endothelium. Also, an increase in the expression of CD74 in the lower stratum spinosum and in subepithelial tissue was also measured (Fig. 2). $CD4^+$ cells could be demonstrated in the subepithelial layer, where they were evident in greater numbers compared to the lower parts of the stratum spinosum, where limited numbers were demonstrated. CD103 was limited to the subepithelial region, the basal cell layer and in the lower parts of the stratum spinosum, although the expression was markedly increased (Fig. 3).

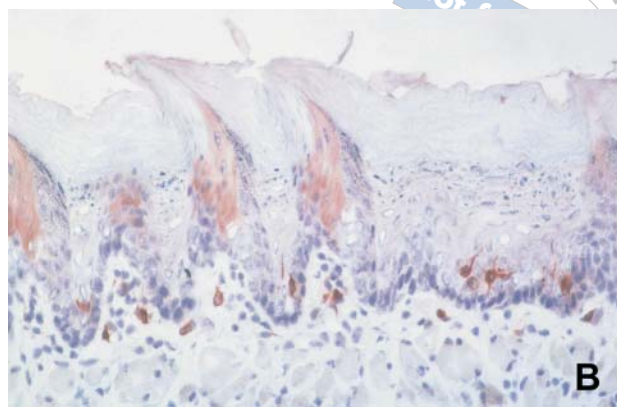

Fig. 2 Balb/c mouse: CD74 expressing dendritic cells in the stratum spinosum and in the lamina propria one week after inoculation with *C. albicans* cells (PAP stain, ×100).

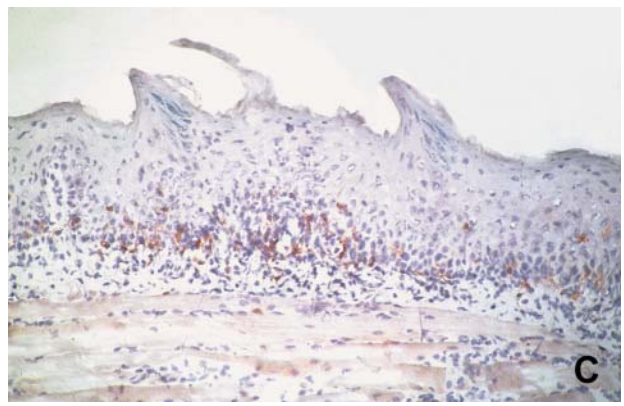

Fig. 3 Balb/c mouse: One week after inoculation with *C. albicans* cells CD103 antigen expressing cells were seen in the stratum spinosum, the basal cell layer and subepithelial connective tissue (PAP stain, ×65).

No Inoculation

In cases where no pathogens were inoculated, a distribution pattern similar to that of the inoculation dose 10^5 was found: $CD54^+$, $CD74^+$ and $CD103^+$ cells could be demonstrated in limited numbers in subepithelial tissue. Individual cases showed $CD4^+$ cells in the basal cell layer, and the endothelium showed minimal activation (CD54).

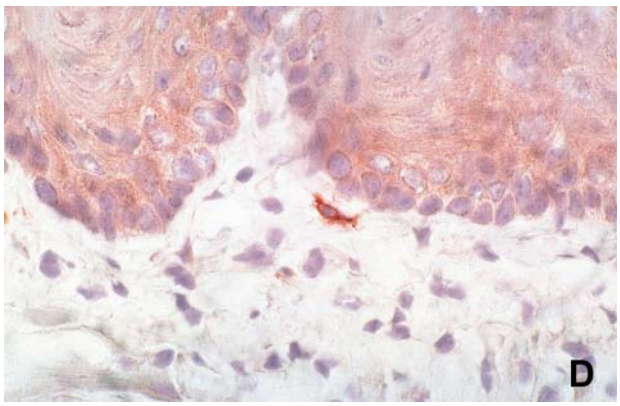

Fig. 4 SCID mouse: CD4 expressing cell in the subepithelial connective tissue one week after inoculation with *C. albicans* cells (PAP stain, ×250).

SCID Mice (Table 5)

Inoculation Dose 10^4

At this inoculation dose, positive immune reactions were primarily found in the subepithelial tissue. The expression of CD54 on activated endothelium and APC was weak, although demonstrable. Above the basal cell layer, no reaction could be seen, with the exception of CD74. In total, among all sections examined at this inoculation dose, only one or two cells expressing CD4-antigens could be demonstrated (Table 5). These were found in the subepithelial layer.

Inoculation Dose 10^5

At the inoculation dose of 10^5 pathogens, reactions were again primarily seen in the subepithelial region (CD54, CD74, CD103). A significant increase of marked endothelium cells expressing the functionally relevant adhesion molecule (CD54) was also seen here. CD54 was expressed in great quantity in subepithelial tissue, although these cells were gathered in nest configurations. In the basal cells, a strong expression of anti-CD103 was observed. Further markers were not present. As with the inoculation dose 10^4, only one or two cells expressing CD4 were demonstrated in the subepithelial region.

Inoculation Dose 10^6

In the evaluation of this inoculation dose, an increase in the expression of $CD54^+$ and $CD74^+$ cells in the subepithelial region was seen. Among the basal cells, minimal numbers of $CD103^+$ cells were found. In the higher cell layers, $CD74^+$ and $CD103^+$ cells could also be demonstrated in small numbers. $CD4^+$ cells were totally absent at this inoculation dose.

Inoculation Dose 10^7

A further increase in immunocompetent cell expression was not seen at this inoculation dose. It should be noted, however, that CD74 was expressed in four layers: the lamina propria, in subepithelial tissue, stratum basale, and in the lower regions of the stratum spinosum. $CD4^+$ cells were found in minimal numbers in the stratum basale as well as directly below the basal cells (Fig. 4).

No Inoculation

In the case of mice which were given no pathogens, only a minimal immune response could be round using the markers described here. With the exception of C74, this response was located in subepithelial tissue. A few $CD103^+$ cells were found in the basal cell layer. Anti-CD74 markers were found in the lamina propria, and, to a lesser degree, in the lower stratum spinosum. Anti-CD54 showed a significant reaction in vascular endothelium. Individual cells expressing CD4 were found in the subepithelial tissue.

DISCUSSION

Correlation between Inoculated Pathogen Load and Emergence of Candida Infection

It was possible to show from the data in this study that a significant difference exists between the number of Candida cells that are necessary to induce an infection in immunocompetent and immunodeficient mice. The minimal dose necessary for a hyphae invasion of Balb/c mice was 1,000 times greater than for SCID mice. Studies of humans have shown that no correlation exists between the number of pathogens measured and the occurrence of oral candidiasis, independent of the method used to determine the pathogen load (Barone et al, 1990; Luangjamekorn et al, 1990; Schmidt-Westhausen et al, 1993; van Meter et al, 1994). Among persons with a healthy immune response who showed no clinical manifestations, swab techniques yielded up to 10^3 CFU/ml (Odds, 1988; Samaranayake, 1989), whereas clinical changes were seen in some HIV positive patients despite the fact that only 50 CFU/ml could be demonstrated (Barone et al, 1990).

As is the case for all animal experiments, the results of a dose-effect relationship of an oral inoculation determined in this study should be discussed with respect to their validity for humans. However, because it has been exploited by various researches to elucidate clinical, therapeutic, and immunological features of oral

candidiasis (see review by Samaranayake and Samaranayake, 2001), the mouse model is especially suitable when studying short-term yet complex humoral and cellular immune responses associated with the disease.

Candida spp. is not a part of the murine oral flora, although the microorganism is present in 20% to 60% of the orogastrointestinal tract of healthy human individuals (Mooney et al, 1995; Odds, 1988; Samaranayake, 1989). In addition to the demonstration of hyphae in the keratin layer of the tongue mucosa this study also posed the question of an association between pathogen invasion and a reactive cell infiltrate. It was shown that inflammatory cells were present in only half of the cases where a pathogen invasion occurred. In this respect, no difference existed between immunodeficient and immunocompetent animals. A substantial explanation for this is that an isolate which causes no clinical changes can still lead to an infection. Studies of biopsies of HIV positive and HIV negative individuals with erythematous candidiasis have shown intense inflammatory reactions in many cases where neither hyphae nor blastospores were seen (Eversole et al, 1997; Scully et al, 1994). The conclusion was reached that the erythematous form could represent a hypersensitivity reaction to Candida antigens. On the other hand, in the case of the pseudomembranous form, even marked hyphae invasion of the keratin layer led to only a weak reaction.

Cellular Immune Response following Inoculation with Defined Pathogen Load

A further aim of this study was an examination of the cellular immune response in the epithelium as well as in the subepithelial connective tissue with respect to defined pathogen loads in relation to immune status. Previous immune histological and electron microscopic studies have shown that a constant population of immunocompetent cells is constantly present in the oral epithelium, serving as a local immunological barrier (Becker et al, 1985; Bos and Burkhardt, 1980; Reibel et al, 1985). With respect to the number, distribution and migration of immunocompetent cells, no significant difference between murine and human oral mucosa could be determined (Burkhardt, 1992).

This study shows that an immune reaction occurred in the tongue mucosa of the mice groups following inoculation with *C. albicans* despite the fact that clinical symptoms were not present. The extent of the reaction was dependent on the inoculation dose as well as on the immune status of the animals. A significant difference between immunocompetent and immunodeficient animals was seen both in the number and in the distribution of the $CD4^+$ cells within the cell layers. While the expression was reduced at low inoculation levels in SCID mice and occurred in subepithelial tissue (with the exception of the highest inoculation dose), the response in Balb/c mice involved the subepithelial region and the basal cell layer.

The intracellular adhesion molecule CD54 is expressed both on the (mouse) endothelium and on APCs (Scheynius et al, 1993) and murine/human LHCs (Tang and Udey, 1991; Teunissen et al, 1994). The expression of CD54 increases with stimulation by inflammatory mediators like lipopolysaccharides or cytokines. Furthermore, CD54 increases the antigen specific T-cell activation (Siu et al, 1989). In both animal groups CD54+ cells were found in the superficial lamina propria, and the endothelium was thoroughly activated. A significant difference was seen between the immunodeficient and immunocompetent mice with respect to the number of cells expressing the markers: while Balb/c mice did not show a strong expression in the subepithelial region or an activation of the endothelium until the highest inoculation dose was used, SCID mice showed a high degree of expression with an inoculation dose as low as 10^5, with the dose causing infection of the upper epithelial layers.

The CD74 antigen plays a role in the intracellular transport of MHC class II molecules and in the presentation of antigens. Studies of mice lacking CD74 antigens have shown that their transport of MHC class II was insufficient, resulting in a reduction of the amount and function of class II molecules on the cell surface and a reduced activation of $CD4^+$ cells (Viville et al, 1993). A similar pattern emerged among the mouse groups in the distribution of the marked cells within the strata. Reactions occurred in the lower region of the *stratum spinosum*, in the subepithelial tissue, and in the *lamina propria*. With respect to the number of cells expressing the molecules, a heterogeneous pattern was seen among the SCID mice, and a relation to the inoculation dose could not be established. Balb/c mice reacted to an inoculation dose of 10^7 *C. albicans* cell with a limited expression and to 10^8 pathogens with strong expression.

The CD103 antigen, a mucosal lymphocyte integrin, plays a role in the development and increase in $CD8^+$ intraepithelial lymphocytes following microbial colonization (McFarland et al, 2000; Schoen et al, 1999). Both SCID and Balb/c mice expressed this antigen in the superficial lamina propria, in the basal cell layer and, at higher inoculation doses, in the lower stratum spinosum. Depending on the inoculation dose, the ex-

pression of these antigens in SCID mice increases, but is expressed only weakly at high inoculation doses. On the other hand, in Balb/c mice, the increase in the expression is almost linear in relation to the inoculation dose.

Immune Response following Invasion of C. albicans in Murine Epithelium

As shown in the results of the PAS studies, the inoculation of SCID mice with 10^5 pathogens led to a hyphae invasion in the stratum corneum, while Balb/c mice required a dose 1,000 times greater for an infection of the tongue mucosa.

When comparing the 'threshold' of 10^5 pathogens for SCID mice with that of 10^8 for the Balb/c mice, the following differences emerge with respect to their immune reaction: in SCID mice, $CD4^+$ cells could only be demonstrated in individual cases and were limited to the subepithelial region; in Balb/c mice, however, $CD4^+$ cells could be observed in the lower stratum spinosum. Similar results with respect to the restriction of $CD4^+$ cells to the subepithelial region were observed in HIV positive individuals with erythematous oral candidiasis (Romagnoli et al, 1997).

In addition, high numbers of $CD103^+$ cells were observed in both groups, which points to the development of $CD8^+$ lymphocytes. In studies of the distribution of immunocompetent cells in cases of oral candidiasis in HIV positive patients, $CD8^+$ lymphocytes were also seen in the stratum basale (Romagnoli et al, 1997). Due to the fact that no immunocompetent control group with oral candidiasis was used in the study, it could not be shown whether $CD8^+$ lymphocytes were primarily in the basal cell layer and in small numbers in the stratum spinosum and subepithelial tissue as the findings of this study indicate. Studies of the distribution and dynamics of murine and human intraepithelial lymphocytes in normal oral mucosa did show, however, that these cells do occasionally cross the basement membrane and that it is unclear whether these cells wander into the epithelium or across the basement membrane into the lamina propria (Bos and Burkhardt, 1980; Burkhardt, 1992).

With respect to these present results, the following conclusions could be drawn: the intraepithelial protective function in SCID mice is reduced by limitations in the reaction to foreign antigens despite a normal number of LHCs. As such, low inoculation doses can lead to mucosal infection. Further sources of infection with a low inoculation dose in immunodeficient animals could be due to changes in the mechanism preceding the adherence and invasion of the pathogen. A significant factor in this respect is the constitution of saliva and of the saliva mucins (Seemann et al, 2001). *In vitro* studies have shown that the saliva of patients undergoing chemoradiotherapy provides a reduced adherence inhibition against *C. albicans.* The cause of this was the reduction of the glycoprotein lactoferrin in the saliva caused by the therapy, whereby an increase in the adhesive characteristics of both *C. albicans* and the cells of the oral epithelium was observed (Umazume et al, 1995).

The present study shows that despite the lack of clinical signs of candidiasis, a Candida infestation of tissue including the accompanying immune response may be present. Moreover, the findings indicate that it is necessary to precisely distinguish between the term Candida infestation and candidiasis: The term Candida infestation simply means that the organism has established itself in the epithelium. In these cases, no macroscopic changes are apparent in the mucosa (subclinical infection), but histopathology can demonstrate PAS positive structures in the epithelium as well as mild inflammatory reactions. The presence of yeasts signifies neither (clinically manifest) candidiasis nor subclinical infection with *Candida spp.*, but rather is a sign of colonization (Meinhof and Spring, 1989). These aspects should also be taken into account in clinical studies on the efficacy of antimycotic drugs.

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