J Gastroenterol 1996; 31:307–313

Journal of Gastroenterology

Esophageal *Candida* infection and adherence mechanisms in the nonimmunocompromised rabbit

KAZUNORI HOSHIKA,[1] MITSUO IIDA,[1] and HIROKO MINE[2]

[1] Division of Gastroenterology, Department of Medicine, Kawasaki Medical School, 577 Matsushima, Kurashiki 701-01, Japan
[2] Department of Clinical Nutrition, Faculty of Medical Professions, Kawasaki University of Medical Welfare, 288 Matsushima, Kurashiki 701-01, Japan

Abstract: *Candida* infection of the esophagus has been reported not only in immunocompromised hosts but also in healthy individuals. However, its mechanisms of action in healthy individuals have not been clarified. Our previous study suggested that physical contact was an important factor for the adherence of *Candida albicans*. The aim of the present study was to test our hypothesis and clarify the adherence mechanisms. Suspensions of *Candida albicans* cells were given to rabbits in drinking water without the use of immunosuppressive drugs and/or antibiotics, and the esophagus was examined. Candidial lesions were observed in 14 of 15 rabbits given the suspensions held in water with and without 30% sucrose for 13 days. The number of *Candida albicans* cells adhering to the esophagus per square millimeter by subepithelial cell insertion was significantly larger than that adhering by attachment. These results indicate that adherence of *Candida albicans* to the esophagus occurs by sustained physical contact alone under a nonimmunosuppressive state, and that subepithelial cell insertion results in greater attachment on adherence. Our findings provide a clue that may help clarify the mechanism of *Candida* infection in healthy individuals.

Key words: *Candida* infection, adherence, esophagus, nonimmunocompromised host

Introduction

It is well known that *Candida* infection occurs as an opportunistic infection in immunocompromised hosts such as patients with leukemia, organ transplant patients, or those with acquired immune deficiency syndrome.[1] The esophagus is a common site of *Candida* infection in the gastrointestinal tract.[2] In experimental studies, esophageal candidial lesions have been observed in immunocompromised hosts, such as animals treated with immunosuppressive drugs or genetically immunosuppressed animals, but not in nonimmunocompromised hosts.[3] Therefore, an immunodeficient state has been considered necessary for the formation of candidial lesions.

Esophageal candidiasis also occurs in patients experiencing long-term administration of antibiotics and in patients with diabetes mellitus, and the mechanisms of these *Candida* infections appear to involve overgrowth of *Candida albicans*, which leads to invasive infection under these conditions.[4]

Esophageal candidiasis has furthermore been reported in healthy individuals,[5,6] although the mechanisms of infection have not yet been clarified.

Our previous in vitro studies on the adherence of *Candida albicans* to the esophagus[7,8] as the first step of *Candida* infection showed four modes of adherence. In addition, our research suggested that physical contact of *Candida albicans* with the esophagus leads to its adherence, as well as that of inert particles, by these modes of attachment and/or subepithelial cell insertion. Subepithelial cell insertion is the characteristic adherence mode of the esophagus which we first reported and named.[7,8] This mechanism consists of the wedging of *Candida albicans* cells or other particles under the edges of epithelial cells. The present study was carried out to test our hypothesis that physical contact of *Candida albicans* with the esophageal epithelium can lead to the development

Offprint requests to: K. Hoshika
(Received Feb. 22, 1995; accepted Sept. 22, 1995)
Parts of this study were presented at the 78th General Meeting of the Japanese Society of Gastroenterology (Tokyo, 1992) and at the 79th General Meeting of the Japanese Society of Gastroenterology (Kyoto, 1993).

of candidial lesions in the esophagus in a nonimmunosuppressed state, and to clarify these adherence mechanisms.

Materials and methods

Preparation of Candida albicans

Candida albicans IFO 1060 maintained on Sabouraud glucose agar at room temperature was used. Yeast cells were cultured in Sabouraud glucose broth for 24h at 37°C for experimental infections. The cells were washed three times in an arginine medium[7] and suspended in water or 30% sucrose at a concentration of 10^7 *Candida albicans* cells per milliliter. In the present study, sucrose was used to raise the specific gravity of the medium and to maintain a uniform distribution of *Candida albicans* cells, because these cells settle to the bottom of suspensions when they are left in water.

Experimental animals

Thirty-one New Zealand white rabbits (7-week-old males weighing 1.08–1.44kg) obtained from Japan SLC (Hamamatsu, Japan) were used, because the fine structure of the squamous epithelial surface of the rabbit esophagus is very similar to that of the human esophagus.[7] They were given ordinary feed (RC4, obtained from Oriental Yeast, Yokohama, Japan) in a Negativerack (Clea Japan, Tokyo, Japan) in which the temperature was kept at 25°C.

Experimental methods

Suspensions of *Candida albicans* yeast-type cells were given to the 29 rabbits per os for 1–13 days. Water without *Candida albicans* cells was given to two control rabbits. The feed, suspensions, and water were provided ad libitum. Following intravenous administration of anesthesia (pentobarbital sodium, 200mg/kg), the esophagus was resected and fixed in 2.5% glutaraldehyde at 4°C overnight. No immunosuppressive drugs such as corticosteroids or anticancer drugs, or antibiotics, which are well-known predisposing factors of *Candida* infection,[4] were used in this study.

Processing for light and electron microscopy

When lesions were recognized macroscopically as yellow or brown, and when they protruded into the lumen at killing, specimens including the lesions were taken from the esophagus for microscopic and electron microscopic examination. If no lesions were recognized macroscopically, specimens were taken from the lower esophagus, just above the esophagogastric junction.

For microscopic observation, specimens were stained with periodic acid-Schiff and hematoxylin-eosin.

For scanning electron microscopic observation, specimens were coated with platinum-palladium after additional fixation with 1% osmium tetroxide for 2h, dehydration, and critical-point drying. Specimens to be used with the freeze-cracking method were kept in 25% and 50% dimethyl sulfoxide (DMSO), respectively, for 30min each after double fixation, and then were frozen and cracked. They were then examined with a Hitachi S-570 scanning electron microscope (Hitachi, Tokyo, Japan). To clarify the adherence mechanisms, the surfaces of six specimens taken from rabbits given the 30% sucrose suspension for 13 days were scanned with a scanning electron microscope, and *Candida albicans* cells adhering to the esophagus were classified by attachment or subepithelial cell insertion into each of these modes. The area of each surface was calculated using an interactive image analysis system (IBAS 2000, Zeiss, Oberkochen, Germany). Then the numbers of *Candida albicans* cells adhering to the surfaces per square millimeter were calculated for each mode. The results were expressed as the mean and standard deviation of log (numbers). Statistical comparisons were made using the two-tailed unpaired *t*-test. Differences were considered significant if $P < 0.05$.

This study was approved by the Animal Research Committee of Kawasaki Medical School and conducted according to the "Guide for the Care and Use of Laboratory Animals" used by Kawasaki Medical School.

Results

All the rabbits used in the in vivo experiment ate enough feed and drank enough of the suspensions to increase their body weight. No diarrhea was noted, and their activity was maintained throughout the experiments. The results of this study are shown in Table 1.

Macroscopic findings

The candidial lesions were recognized in the esophagus as being yellow or brown, round and protruding, and 1–2mm in diameter. Some of the lesions, measuring 6mm in length macroscopically, were connected with each other (Fig. 1).

Among 14 rabbits given the 30% sucrose suspension for less than 7 days, candidial lesions were observed in only 3 rabbits. Lesions were observed in all 9 rabbits given the 30% sucrose suspension for 13 days. The

Table 1. Esophageal candidial lesions in this study

No.	Days[a]	Intake (ml)[b]	Macro.[c]	(Lower	Middle	Upper)	Micro.[d]
1	0		0	−	−	−	−
2	0		0	−	−	−	−
3	1	Sucrose	120	−	−	−	−
4	1	Sucrose	110	−	−	−	−
5	2	Sucrose	260	−	−	−	−
6	2	Sucrose	250	−	−	−	−
7	2	Sucrose	300	−	−	−	−
8	2	Sucrose	360	−	−	−	−
9	4	Sucrose	875	+	+	+	+
10	4	Sucrose	660	+	−	−	+
11	6	Sucrose	660	−	−	−	+
12	6	Sucrose	650	−	−	−	+
13	6	Sucrose	740	−	−	−	−
14	6	Sucrose	680	−	−	−	−
15	6	Sucrose	600	−	−	−	−
16	6	Sucrose	550	+	+	+	+
17	13	Sucrose	1700	+	+	−	+
18	13	Sucrose	1810	+	+	+	+
19	13	Sucrose	1480	+	+	+	+
20	13	Sucrose	1260	+	−	+	+
21	13	Sucrose	1690	+	+	+	+
22	13	Sucrose	1540	+	+	+	+
23	13	Sucrose	1430	+	+	+	+
24	13	Sucrose	1660	+	+	+	+
25	13	Sucrose	1600	+	+	+	+
26	13	Water	2485	+	−	−	+
27	13	Water	2730	−	−	−	−
28	13	Water	2930	+	−	−	+
29	13	Water	2960	+	−	−	+
30	13	Water	3220	+	−	−	+
31	13	Water	2940	+	+	+	+

[a] Number of days the suspensions of *Candida albicans* cells were given to the rabbits
[b] Kinds and volume of the suspensions given to the rabbits per os
[c] Macroscopic lesions
[d] Microscopic lesions

lesions were located in all areas of the resected esophagus.

Candidial lesions were also observed macroscopically in 5 of the 6 rabbits given a suspension of *Candida albicans* in water for 13 days. The lesions were located in the lower esophagus, near the esophagogastric junction. The amount of suspension consumed by the rabbits was about twice that of the suspension held in 30% sucrose.

Microscopic findings

The squamous epithelium of the candidial lesions had thickened because of widening of the intercellular space between the striated squamous epithelial cells invaded by mycelial elements of *Candida albicans*. Mycelial invasion was observed in the superficial part of the esophageal squamous epithelium (Fig. 2). The size of the candidial lesions varied, and tiny lesions, which were too small to detect macroscopically, were observed (Fig. 3) even in rabbits given suspensions for 13 days. In rabbits given the 30% sucrose suspension for 6 days, microscopic candidial lesions were recognized in two rabbits in which there had been no macroscopic detection of the lesions.

Adherence mechanisms

Attachment and subepithelial cell insertion were observed to occur in living rabbits by the modes seen in our previous in vitro study.[5,6] On scanning electron microscopic observation, *Candida albicans* cells adhering by the attachment mode were observed on the surface of the squamous epithelial cells (Fig. 4). *Candida albicans* cells adhering by the subepithelial cell insertion mode were observed through the superficial squamous epithelial cells around the candidial lesions (Fig. 5) and, by the freeze-cracking method, they were observed under the superficial squamous epithelial cells (Fig. 6).

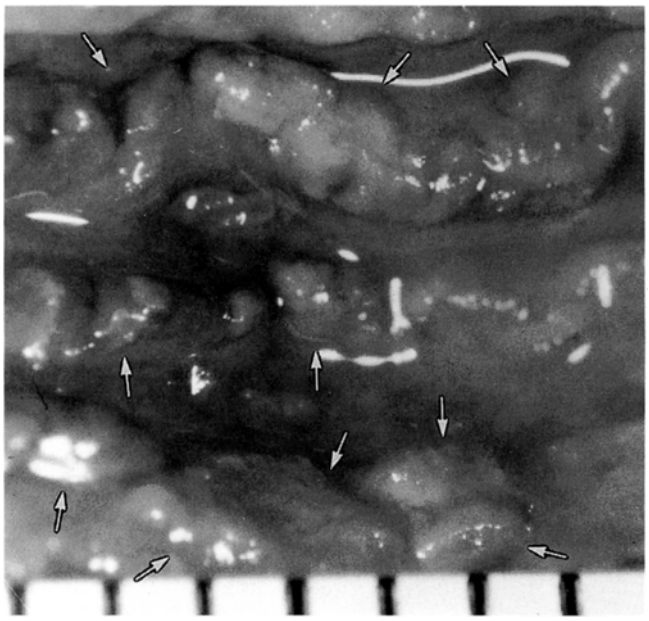

Fig. 1. Candidial lesions can be recognized as round protruding lesions, 1–2 mm in diameter. Some of the lesions are connected with each other (*arrows*). Scale is in millimeters

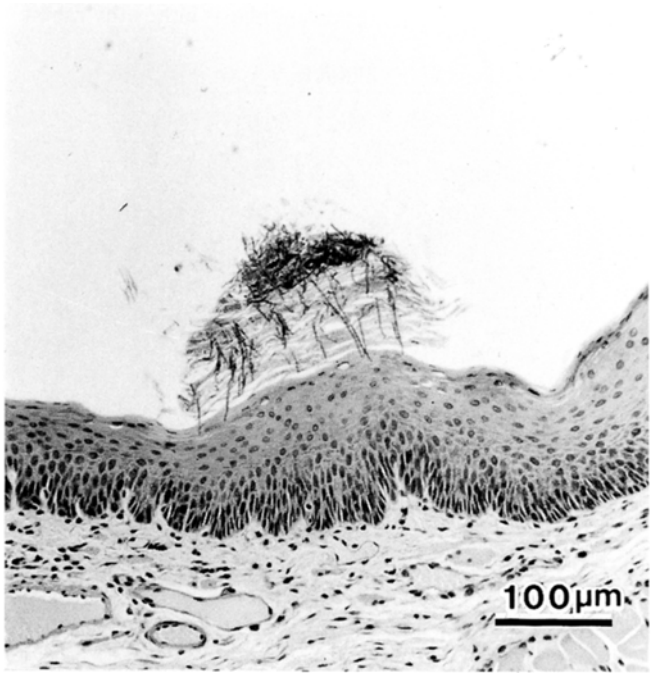

Fig. 3. Photomicrograph of a tiny candidial lesion. (Periodic acid-Schiff)

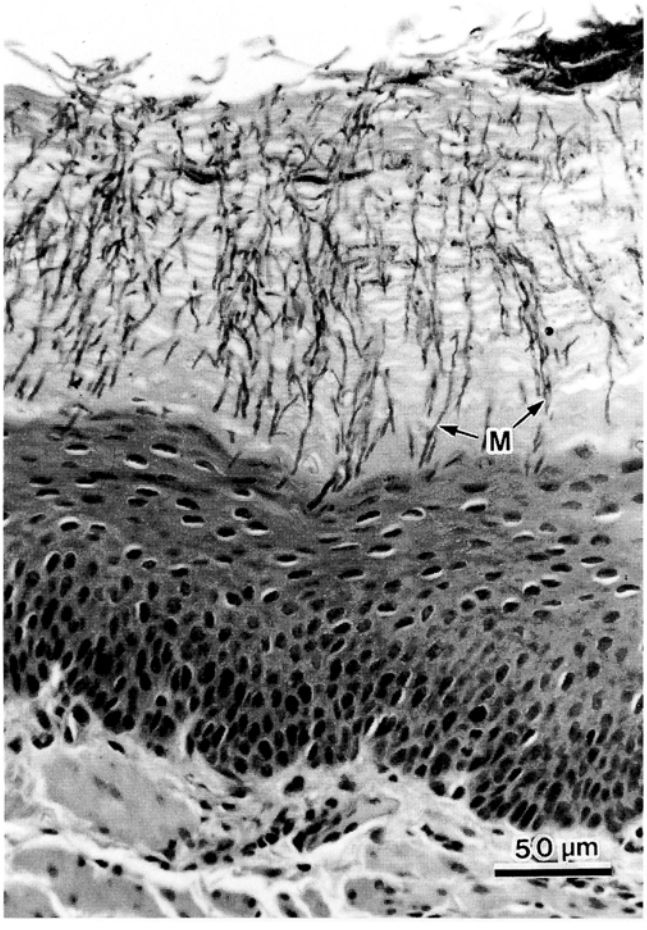

Fig. 2. Photomicrograph of the candidial lesions. Mycelial elements of *Candida albicans* (*M*) are infiltrating through the striated squamous epithelial cells. (Periodic acid-Schiff)

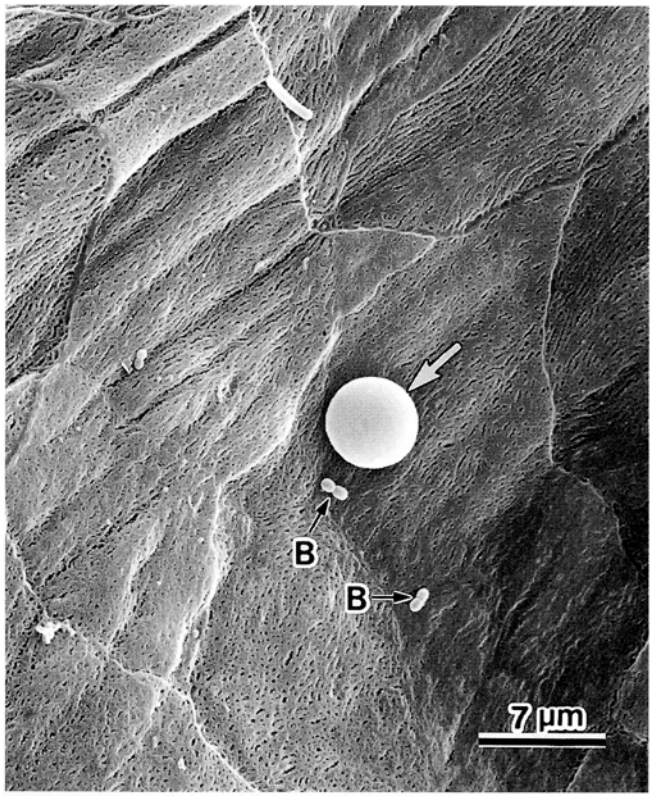

Fig. 4. *Candida albicans* cell (*large arrow*) and bacteria (*B*) are observed on the surface of the squamous epithelial cells

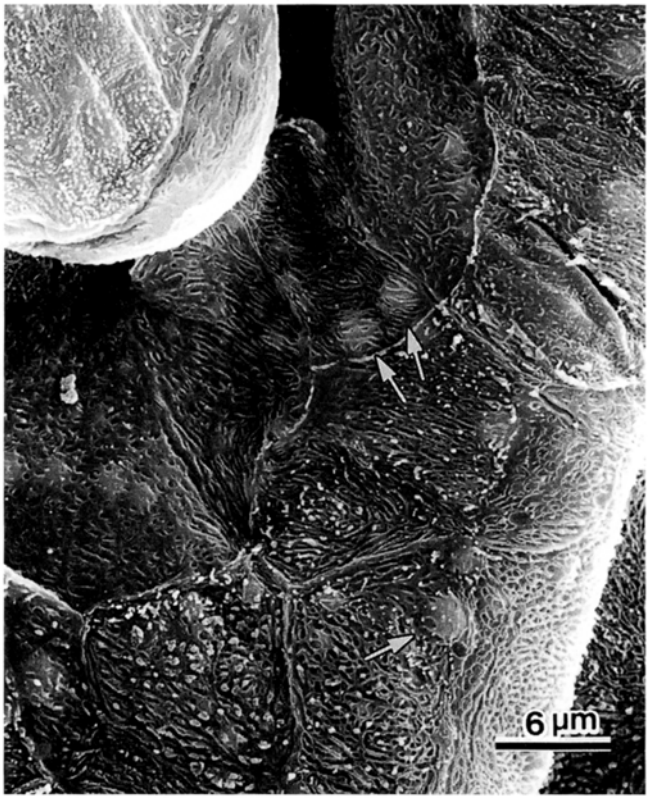

Fig. 5. *Candida albicans* cells (*arrows*) are observed through the superficial squamous epithelial cells

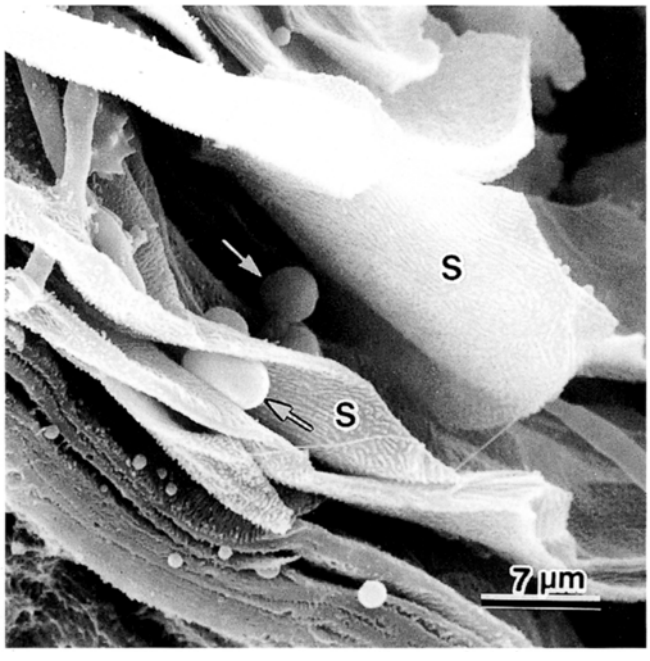

Fig. 6. *Candida albicans* cells (*arrows*) are observed under the superficial squamous cells (*S*)

Table 2. Adherence modes of *Candida albicans*

Modes of adherence	*Candida albicans* cells/mm^2 (n = 6)
Attachment	0.745 ± 0.273*
Subepithelial cell insertion	1.942 ± 0.780*

*$P < 0.01$
Values are mean ± SD of log(numbers)
Two-tailed unpaired t-test

Table 2 shows the number of *Candida albicans* cells adhering to the surfaces per square millimeter in each mode[7,8] in six specimens. The number of cells adhering by subepithelial cell insertion was significantly larger than that adhering by the attachment mode.

Discussion

Esophageal candidiasis in healthy individuals[5,6] cannot be fully explained by the predisposing conditions mentioned in the introduction, and there is no clearly defined basis which can explain the mechanisms of this infection. In previously reported experimental studies of *Candida* infection of the gastrointestinal tract, immunosuppressive drugs or genetically immunosuppressed animals were usually used to create an immunocompromised host model.[3,9–13] Therefore, these models were unsuitable as models for esophageal candidiasis in healthy individuals.

In an interesting study using suckling mice without immunosuppressive drugs, candidial invasion to the esophagus was reported.[14] This excellent study may provide a clue to clarification of the mechanisms of *Candida* infection in healthy individuals; however, we chose rabbits as the experimental animals for the following reasons: In previously reported studies, mice were commonly the experimental animals, but the structure of their esophagogastric regions and the fine structure of the squamous epithelial surface differ significantly from those of humans. In addition, the main site of invasive disease in studies in mice was usually the stomach, particularly the cardial-atrium ridge. In rabbits, the structure of the esophagogastric regions and the fine structure of the squamous epithelial surface of the esophagus are very similar to those of humans,[7] and the main site of invasive disease is the esophagus, as in humans. Therefore, we consider our model using rabbits to be more suitable as a model for esophageal candidiasis in humans than one using mice. In addition, our model is suitable as a model in healthy individuals, because immunosuppressive drugs and/or antibiotics are not used.

Until recently, the mechanisms of adherence of *Candida albicans* as the first step of *Candida* infection had not been clarified, despite the fact that esophageal candidiasis is a frequently occurring *Candida* infection in the gastrointestinal tract.[2] Therefore, we initially studied the modes of adherence of *Candida albicans* to the rabbit esophagus in vitro using scanning electron microscopy of resected ligated esophageal segments incubated with *Candida albicans* suspensions, and found four modes of adherence: (1) attachment, (2) subepithelial cell insertion, (3) cavitation, and (4) invasion.[7] Our second in vitro study of the significance of these adherence mechanisms suggested that physical contact of *Candida albicans* with the esophagus led to its adherence by attachment and/or subepithelial cell insertion.[8]

In the present study, we tested this hypothesis. At first, we used a 30% sucrose suspension to maintain continuous contact of *Candida albicans* cells with the esophagus. In rabbits drinking the 30% sucrose suspension, the formation of candidial lesions in the esophagus seemed to depend on the cumulative amount of the suspension consumed. We succeeded in inducing candidial lesions in all rabbits given a 30% sucrose suspension for 13 days. However, this result was not sufficient to support our hypothesis, because in addition to maintenance of uniform distribution, other effects of sucrose on adherence are also possible. Then, we gave a suspension of *Candida albicans* in water, in which there were no effects of sucrose, to rabbits for 13 days and succeeded in inducing candidial lesions in 5 of the 6 rabbits. Among rabbits developing lesions within 13 days, a smaller amount of suspension was consumed by rabbits drinking the sucrose suspension than by those drinking the suspension in water. In rabbits drinking 30% sucrose suspension, the formation of candidial lesions was facilitated, possibly due to the effects of sucrose, including maintenance of uniform distribution. In the present study, we were therefore able to create candidial lesions by establishing repeated physical contact of *Candida albicans* with the esophageal epithelium without the use of immunosuppressive drugs and/or antibiotics. This condition may be presumed to be that of healthy individuals.

The successful induction of *Candida* infection without the use of immunosuppressive drugs in the mouth of the mouse[15] supports our hypothesis. We believe that larger amounts of the suspensions than those used to induce oral candidiasis may be required to create lesions in esophageal candidiasis, because of esophageal clearance by peristalsis. In our opinion, the reason candidial lesions did not develop in the control rabbits in previously reported studies,[3,11] in which 5×10^6 viable units or 3×10^3 colony-forming units of *Candida albicans* per host were used, is that the suspension of *Candida albicans* cells was not large enough to establish repeated physical contact for the creation of lesions. A larger amount of the suspension provided continuously may create such lesions in control rabbits, possibly by increasing the statistical probability that some *Candida albicans* cells will come into sufficient proximity to esophageal cells to adhere.

With regard to the adherence mechanisms of esophageal *Candida* infection in healthy individuals, the success of *Candida* infection depends on the organism gaining a foothold that prevents its being washed away by food or water and/or by peristalsis of the esophagus at the initial stage. In the present study, minute candidial lesions, which were too small to detect macroscopically, were observed in rabbits given suspensions of *Candida albicans* for various periods. The results suggest that cumulative *Candida* infections occur when *Candida albicans* cells are provided continuously, because these minute lesions are presumed to occur following adherence of *Candida albicans* to the esophagus, at the initial stage of *Candida* infection. We scanned the specimens taken from rabbits given the suspension for 13 days to analyze adherence mechanisms, because lesions that occurred following adherence were easily observed in those specimens. While the number of cells adhering by attachment was larger than that adhering by subepithelial cell insertion in our in vitro study,[8] the number of cells adhering by subepithelial cell insertion was significantly larger than that adhering by attachment in this in vivo study. This result suggested that (a) the characteristic structure of the esophagus provides *Candida albicans* with a foothold for adherence, i.e., subepithelial cell insertion, whereby cells are physically protected from clearance by peristalsis,[8] and that (b) the mode of subepithelial cell insertion results in greater attachment on adherence.

Minute candidial lesions observed in this study may occur following adherence at the initial stage of *Candida* infection. The existence of these minute lesions may be possible to determine clinically. However, these lesions may not be recognized during routine clinical examinations, i.e., by esophagogram or esophagoscopy, because these lesions are too small to detect. Further analysis of these minute lesions is certainly required to clarify the mechanisms of invasive infection following adherence. Such studies may lead to a new explanation of invasive infection in cases of esophageal candidasis not associated with an immunosuppressive state. The mechanisms of adherence and invasive infection in esophageal candidiasis are the subject of ongoing study in our laboratory.

Acknowledgments. This study was supported by funds from the Sanyo Broadcasting Foundation and a Research Project

Grant (nos. 5-501, 6-501) from Kawasaki Medical School. The authors thank Dr. Robert L. Owen for his helpful comments and suggestions.

References

1. Odds FC, Schmid J, Soll DR. Epidemiology of *Candida* infections in AIDS. In: Bossche HV, Mackenzie DWR, Cauwenberg G, Cutsem JV, Drouhet E, Dupont B (eds) Mycosis in AIDS patients. Plenum, New York, 1990;67–74.
2. Eras P, Goldstein MJ, Sherlock P. *Candida* infection of the gastrointestinal tract. Medicine 1972;51:367–369.
3. DeMaria A, Buckley H, von Lichtenberg F. Gastrointestinal candidiasis in rats treated with antibiotics, cortisone and azathioprine. Infect Immun 1976;13:1761–1770.
4. Odds FC. *Candida* and candidosis. A review and bibliography. Bailliere Tindall, London, 1988;93–114.
5. Phaosawasdi K, Rice P, Lee B. Primary and secondary *Candida* esophagitis in normal, healthy subjects. Illinois Med J 1986;169:361–365.
6. Naito Y, Yoshikawa T, Oyamada H, et al. Esophageal candidiasis. Gastroenterol Japon 1988;23:363–370.
7. Hoshika K, Kihara T, Mine H. Adherence modes of *Candida albicans* to rabbit esophagus. J Clin Electr Microsc 1992;25:261–267.
8. Hoshika K, Mine H. Significance of modes of adherence in esophageal *Candida albicans*. J Gastroenterol 1994;29:1–5.
9. Kennedy MJ, Volz PA, Edwards CA, et al. Mechanisms of association of *Candida albicans* with intestinal mucosa. J Med Microbiol 1987;24:333–341.
10. Kennedy MJ, Volz PA. Ecology of *Candida albicans* gut colonization: Inhibition of *Candida* adhesion, colonization, and dissemination from the gastrointestinal tract by bacterial antagonism. Infect Immun 1985;49:654–663.
11. Helstrom PB, Balish E. Effect of oral tetracycline, the microbial flora, and the athymic state on gastrointestinal colonization and infection of BALB/c mice with *Candida albicans*. Infect Immun 1979;23:764–774.
12. Cole GT, Lynn KT, Seshan KR, et al. Gastrointestinal and systemic candidosis in immunocompromised mice. J Med Vet Mycol 1989;27:363–380.
13. Cantorna MT, Balish E. Role of $CD4^+$ lymphocytes in resistance to mucosal candidiasis. Infect Immun 1991;59:2447–2455.
14. Cole GT, Lynn KT, Seshan KR. An animal model for oropharyngeal, esophageal and gastric candidosis. Mycosis 1990;33:7–19.
15. Lacasse M, Fortier C, Trudel L, et al. Experimental oral candidosis in the mouse: microbiologic and histologic aspects. J Oral Path Med 1990;19:136–141.