Original Article

A Novel Murine Candidiasis Model with Severe Colonization in the Stomach Induced by N-acetylglucosamine-treatment and Its Scoring System Based on Local Characteristic Stomach Symptoms

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ABSTRACT

We developed a novel murine candidiasis model of the gastrointestinal tract using N-acetylglucosamine (GlcNAc) as a tool to aggravate symptoms. Forty-eight hours after intragastrically inoculating *Candida albicans* cells to immunosuppressed and GlcNAc-treated mice, vigorously accumulating patchy whitish plaques were observed on their inner stomach surface. *Candida* cells colonizing the plaques consisted of both yeast and mycelia, and were directly stained with Calcofluor White M2R. Aggravation of the candidiasis symptoms was dependent on GlcNAc concentration in drinking water, wherein administration of 50 mM GlcNAc not only severely worsened stomach symptoms, but also significantly increased *Candida* cell number in the stomach and small intestine. The aggravation effect of GlcNAc was enhanced by addition of sedative chemical chlorpromazine chloride after inoculation. In order to semi-quantitatively assess colonization by *Candida* in the stomach, we devised a new symptom scoring system that represents the extent of the patchy whitish plaques on the mucosal epithelium of the stomach. Histochemical analysis of *Candida*-infected tissues revealed not only a large amount of thick *Candida* mycelia invading mucosal epithelial stomach tissues but also infiltrating inflammatory cells. These results suggest that this murine gastrointestinal candidiasis model could serve as a useful tool for evaluating the protective activity of antifungal agents, probiotics, or functional foods against gastrointestinal candidiasis. Furthermore, from another point of view, this novel murine model could also be used to analyze the pathological mechanisms behind the translocation of *C. albicans* across intestinal barriers, which results in systemic *Candida* dissemination and infection.

Key words : *Candida albicans*, N-acetylglucosamine (GlcNAc), candidiasis, symptom score

Introduction

Systemic candidiasis is one of the most severe and life-threatening mycoses attacking advanced AIDS patients and immunocompromised, particularly neutropenic or elderly, patients treated with anti-inflammatory steroids or anticancer drugs^{1–4)}. Historically, catheters were believed to be the main infection route of *Candida*, but in the last ten years, the dissemination of *Candida* through gastrointestinal routes under various

abnormal conditions, such as gastrointestinal surgery, has become recognized as another possible route for infection. This route is particularly associated with intra-abdominal sepsis, leukemia, or lymphoma in patients that received chemotherapeutic regimens^{4, 5)}. Local translocation of *Candida* cells across intestinal layers, therefore, is a key step in the pathogenesis of candidaemia and systemic candidiasis^{1, 5)}.

Use of animal models of gastrointestinal candidiasis followed by systemic candidiasis enabled researchers to understand key steps in the

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mechanism of *Candida* translocation from local to systemic infection. Currently, however, there is no simple animal model for significant or severe symptoms of gastrointestinal candidiasis followed by systemic candidiasis^{6–9}. We therefore designed a murine gastrointestinal model that allows inducing severe inflammatory infections leading to systemic dissemination.

One major cause of candidiasis is *Candida albicans*. Its virulence is derived from its ability to undergo morphological change from yeast to hyphae, which is induced by various factors, such as CO₂, serum, temperature, and N-acetylglucosamine (GlcNAc). We reported in a previous study that, among these factors, GlcNAc *in vivo* led to severe *Candida* colonization and infectious symptoms in a murine oral candidiasis model¹⁰.

Here, we established a novel gastrointestinal candidiasis model of severe *Candida* infection that is coupled with the virulence-inducing effect of GlcNAc. In addition, we developed a scoring system to determine the degree to which colonization could exacerbate or alleviate symptoms of candidiasis in the stomach.

Materials and Methods

Yeast isolate

Candida albicans strain TIMM1768, recovered from a patient with candidiasis, was stored at –80°C in Sabouraud dextrose broth (Becton Dickinson, Sparks, MD, USA) containing 10% glycerol. For the preparation of the oral inoculate, each strain was grown on Sabouraud dextrose agar (Becton Dickinson) at 37°C for 20 h, harvested with a micro-spatula and resuspended in modified RPMI1640 (diluted to 1:3; Sigma Chemical Co. St. Louis, Mo, USA) containing 0.8% (v/v) fetal calf serum as inoculum medium.

Murine model of gastrointestinal candidiasis

We performed all animal experiments in accordance with the guidelines for the care and use of animals approved by Teikyo University. All experiments involved six-week-old female ICR mice (Charles River Japan, Inc., Yokohama). Mice were randomized, kept in cages housing two to four individuals, and given food and water *ad libitum*. During the experimental period, photoperiods were adjusted to a daily 12–12 h light-dark cycle with environmental temperature maintained at 26 ± 0.5°C.

The experimental procedure to establish gastrointestinal candidiasis in mice was as follows. To induce an immunosuppressed condition, we injected mice with 100 mg/kg of prednisolone (Kyoritsu Seiyaku Co., Japan) subcutaneously 22 to 24 h before intragastric inoculation with *C. albicans*. Prior to prednisolone administration, we supplemented the drinking water with 4.2 mg/ml of tetracycline hydrochloride (CTC, SC Environmental Science Co. Ltd., Japan) throughout a 24-h period. Solid feed for mice was removed on the day of infection 3 h before inoculation. Mice were infected by intragastric inoculation using a syringe with a round-headed needle (39-mm length, Fuchigami, Ltd., Kyoto) at concentrations of approximately 4.0×10^5 , 4.0×10^6 , or 4.0×10^7 CFU of *C. albicans* TIMM1768 cells in 0.2 ml of modified RPMI1640 medium. Needle resistance at the stomach wall was used as an indication that *C. albicans* cells were properly injected. We sedated the animals by injecting 14.4 mg/kg of chlorpromazine chloride (3- (2-chloro-10*H*-phenothiazin-10-yl) -*N,N*-dimethylpropylamine monohydrochloride) in the femoral muscle at various times (0.5, 1, 2, or 4 h) after inoculation of *Candida*.

Assessment of stomach symptoms and fungal burden in the murine model of gastrointestinal candidiasis

At 48 h after *Candida* inoculation, mice were sacrificed by cervical dislocation, and their stomachs were removed. The stomachs were then cut open along the longitudinal axis. We removed stomach contents with tweezers and washed them twice with 5 ml of saline. We scored the severity of stomach lesions in terms of the degree of coverage of patchy whitish plaques coating the total inner stomach surface as follows: 0, normal; 1, patchy whitish plaques covering less than 20%; 2, patchy whitish plaques covering 20% or more, but less than 50%; 3, patchy whitish plaques covering 50% or more, but less than 90%, and resembling a squamous layer; 4, patchy whitish plaques covering 90% or more of the stomach and on the squamous layer; 5, thick, patchy whitish plaques covering 90% or more of the stomach and on the squamous layer. We weighed samples of complete stomach and small intestine (divided into three pieces) and the cecum, and placed them in 3 ml of sterile saline. Organs in the saline were homogenized, suspended by mixing on a vortex mixer, and diluted

with a series of 20-fold and 100-fold dilutions of sterile saline to verify the CFU of *Candida* cells. Next, 50 μ l of each dilution was incubated on a *Candida* GS agar plate (selection medium for *Candida*; Eiken Chemical Co. Ltd., Japan) for 20 h at 37°C. After counting *Candida* cells, the total cell number per gram of organ was calculated in terms of CFU.

Direct observation of Candida growth on the inner stomach surface by fluorescence microscopy

The fact that the upper part of the stomach fundus, including the muscle layers, was thinner than the middle and lower stomach body allowed us to easily identify the patchy whitish plaques on their inner surface using microscopy. We removed a small piece of fundus (3mm x 3mm) with patchy whitish plaque with a razor, washed it once with GH solution (2% glucose in 10 mM HEPES buffer, pH 7.2), then placed the sample in GH solution containing 5 μ M Calcofluor White M2R (Molecular Probes, Eugene, OR, USA) and incubated it for 10 min at room temperature. We washed a small piece of stained tissue once with GH solution, affixed it to a slide glass with a small amount of GH solution, put a cover slip on it, pressed the cover slip to flatten out the tissue, and observed the tissue directly using a fluorescence microscope (BH50, Olympus, Japan) equipped with an NB filter (narrow range of UV excitation) for Calcofluor white.

Histology

For the histological study, the stomach was resected and fixed with 4% paraformaldehyde (pH 7.4) at 4°C, dehydrated by ethanol series, and embedded in paraffin following standard procedures. We sectioned specimens at 8- μ m thickness across the longitudinal center line. Sections on the slide were deparaffinized with xylene and rehydrated by ethanol series, and fungal cell walls were stained with Periodic Acid-Schiff (PAS).

Statistical analysis

Values are shown as mean $\pm$ SD. We measured parameters using two-tailed unpaired Student's *t*-test, and compared them using one-way analysis of variance (ANOVA) with post-hoc testing and Bonferroni correction. Values of $p < 0.05$ were considered significantly different.

Results

Conditions necessary to establish severe infection of Candida in the gastrointestinal tracts

To develop severe infection in the murine candidiasis model, we used the sedative chlorpromazine chloride as an anesthetic to immobilize the gastrointestinal tract. We investigated the effects on *Candida* colonization of the injection of chlorpromazine chloride at 0.5, 1, 2, or 4 h after intragastric inoculation of *Candida* cells. The mice were sacrificed at 48 h after *Candida* inoculation. The gastrointestinal tract tissues removed from *Candida*-inoculated mice were homogenized to determine the average value of colony-forming *Candida* cells (CFU). Results of *Candida* colonization at 48 h after inoculation indicated that administration of chlorpromazine chloride between 1 h and 4 h after *Candida* inoculation significantly increased the number of viable *Candida* cells in stomach tissues (Figure 1A).

We also examined how the number of inoculated *Candida* cells affected *Candida* colonization. Figure 1B shows *Candida* colonization at 48 h after inoculation of different cell quantities of *C. albicans*. The maximal colonization of *Candida* occurred at 4.0×10^7 CFU/0.2 ml of inoculated *Candida* cells. These results confirm that adding chlorpromazine chloride 1 h after inoculation of 4×10^7 *Candida* cells in 0.2 ml solution was a suitable base condition to establish severe infection of *Candida* in the gastrointestinal tract.

GlcNAc aggravates the gastrointestinal colonization of Candida

We administered GlcNAc at various concentrations (0, 25, 50, and 100 mM) to each group of mice through their drinking water supplied *ad libitum* to examine the effect of GlcNAc administration on *Candida* infection in the gastrointestinal tract. We sacrificed the mice at 48 h after inoculation to obtain their stomach and intestinal tissues, and homogenized the tissues to estimate CFU. Figure 2 shows CFU per gram of stomach, small intestine (divided into three portions along the longitudinal axis; numbered 1–3 from the stomach-side), and cecum, at 48 h after inoculation of *Candida* to the stomach. The results show that the administration of 50 mM GlcNAc significantly increased viable cell numbers of *C. albicans*.

Figure 3 shows typical features of normal

Fig. 1. Effect of period between injection of chlorpromazine chloride and *Candida* inoculation on colonization of *Candida* (A) and inoculated cell number on colonization (B). * and ** denote significant differences of $P < 0.05$ and $P < 0.01$, respectively, with no GlcNAc control, as measured by one way ANOVA with post-hoc testing and Bonferroni correction. (A) Groups of immunosuppressed and 25 mM GlcNAc-treated mice were injected with 14.4 mg/kg of chlorpromazine chloride intramuscularly at different times (not added, $n = 5$; 0.5 h, $n = 6$; 1 h, $n = 6$; 2 h, $n = 5$; 4 h, $n = 4$) after inoculation of *C. albicans* TIMM1768 (4.0×10^6 CFU). (B) Groups of immunosuppressed and 25 mM GlcNAc-treated mice were injected with 14.4 mg/kg of chlorpromazine chloride intramuscularly at 1 h after inoculation with about 4.0×10^5 , 4.0×10^6 , or 4.0×10^7 CFU of *C. albicans* TIMM1768 ($n = 6, 6, 5$, respectively).

Fig. 2. Effects of GlcNAc on colonization of *Candida* in the gastrointestinal tract were detected by murine groups freely ingesting various doses of GlcNAc (0, 25, 50, and 100 mM each). The colony-forming cells per gram (CFU) of complete stomach, small intestine fragments (divided into three pieces along the longitudinal length, numbered 1–3 from the stomach-side), and cecum were detected from the homogenate at 48 h after inoculation of *Candida*. Groups of immunosuppressed mice (control, $n = 12$; GlcNAc 25 mM, $n = 10$; GlcNAc 50 mM, $n = 8$; GlcNAc 100 mM, $n = 8$) were inoculated with *C. albicans* TIMM1768, and GlcNAc was administered in drinking water as described in the Methods. * and ** denote significant differences of $P < 0.05$ and $P < 0.01$, respectively, from each GlcNAc negative control, as measured using one way ANOVA with post-hoc testing and Bonferroni correction.

tissues (a), *Candida*-infected (b-f) inner mucosa of mice stomachs without (b and c), and with (d-f) GlcNAc treatment. Coverage of the patchy whitish plaque on the inner stomach surface varied from minute, in treatments without GlcNAc (c), to

extensive and layered, in treatments with 50 mM GlcNAc (e and f), as indicated by arrows. Patchy whitish plaques were more visible at the upper stomach fundus due to the aforementioned muscle layer thinness relative to the other regions. A

Fig. 3. Typical inner surface of stomachs from mice 48 h after inoculation with or without *C. albicans* TIMM1768. (A) Control (normal, not infected), (B, C) Control (infected without GlcNAc) and (D, E, F) Experimental (infected with 25-50 mM GlcNAc). Images are typical for each semi-quantitative score. Stomach of *Candida*-infected mice were scored mainly according to degree of formation of patchy whitish plaques as follows: 0, normal; 1, patchy whitish plaques covering less than 20%; 2, patchy whitish plaques covering 20% or more, but less than 50%; 3, patchy whitish plaques covering 50% or more, but less than 90%, and resembling a squamous layer; 4, patchy whitish plaques covering 90% or more of the stomach and on the squamous layer; 5, thick, patchy whitish plaques covering 90% or more of the stomach and on the squamous layer.

Fig. 4. Typical inner surface of murine stomach from 48 h after *C. albicans* inoculation with 50mM GlcNAc stained with Calcofluor White M2R and observed by fluorescence microscopy. Cell wall chitin was stained pale-blue in a1, a2 (a2 is an enlarged portion of a1) and b. c is bright field image identical with b.

Fig. 5. Effect of GlcNAc on (A) symptom score; (B) colony-forming cell (CFU); and (C) correlation between the two parameters of score and CFU for individual mice at 72 h and 96 h after inoculation of *C. albicans* TIMM1768. * and ** denote significant differences of $P < 0.05$ and $P < 0.01$, respectively, each with GlcNAc-negative control measured by one way ANOVA with post-hoc testing and Bonferroni correction.

small piece of the fundus with patchy whitish plaque dissected by razor and stained with Calcofluor White M2R was directly observed using fluorescence microscopy after applying a small amount of pressure on the cover slip (Fig. 4). Figure 4a1-b shows clumps of colonizing yeast and hyphal *Candida* with chitin (cell wall) stained (pale-blue) with Calcofluor White M2R. These results show that there is a higher incidence of patchy whitish plaques in the gastrointestinal tracts as a result of GlcNAc administration.

Scoring patchy whitish plaques: a parameter for aggravation of candidiasis after inoculation of Candida in the stomach

We followed the previously described procedure in oral candidiasis^{10, 11)} to score the severity of *Candida*-infected lesions of the stomach. Stomach tissues of *Candida*-infected mice were scored mainly in terms of the degree of formation of patchy whitish plaques as described in Materials and Methods. Typical images of each score are shown in Figure 3. We used this scoring system to categorize the extent of patchy whitish plaques on the inner stomach surface through time following inoculation of *Candida* while at the

same time determining the CFU of the stomach. On the basis of this scoring system, images of control (a), 0mM (c), and 50 mM GlcNAc (f) in Figure 3 represent scores of 0, 2, and 5, respectively. Figure 5 shows the results of *Candida* colonization score (A), CFU (B), and the relationship between the two parameters for individual mice at 72 h and 96 h post-inoculation (C). Patchy whitish plaque formation following inoculation of *Candida* gradually increased in both control and GlcNAc-treated mice groups, but symptom scores differed significantly between control and GlcNAc-treated mice groups from 24 h to 144 h after inoculation (24 h and 144 h, $P < 0.05$; 48 h–120 h, $P < 0.01$ each). Meanwhile, CFU estimates differed significantly only 48 h after inoculation ($P < 0.05$). Figure 5C shows that there is no correlation between the two parameters of lesion score and CFU of individual mice at 72 h and 96 h after inoculation. Symptom score changed more remarkably than CFU with respect to the administration of GlcNAc, suggesting that the former is a more reliable indicator of susceptibility to the induction of pathogenic activity by GlcNAc for *C. albicans*.

Fig. 6. Histology of cross-section of formalin-fixed-paraffin-embedded (FFPE) tissues of mouse stomach inoculated with *C. albicans* TIMM1768. (A) Form control mouse; (B) Form mouse given GlcNAc (50 mM). Stomach tissues were stained with Periodic Acid-Schiff (PAS). Arrowheads indicate PAS-stained *C. albicans* of yeast and mycelial form. Arrows indicate infiltrated polymorphonuclear leukocytes (PMNs).

Histological analysis of *Candida*-infected stomach mucosa

We used PAS staining to analyze mice stomachs at 48 h after *Candida* inoculation. Figure 6 shows typical histopathological features of stomach mucosa in *Candida*-infected mice treated with (B) or without (A) GlcNAc. Mucosal epithelium of the inner wall of the control stomach (indicated by the shallow curve in the upper part of image A) shows a small amount of the yeast form of *Candida* (indicated by arrowheads); image B shows *Candida* stained dark-red with intertwined fibrous and outstretched hyphae from the mucus to the epithelium (indicated by arrowheads) in a GlcNAc-treated stomach. Arrows indicate polymorphonuclear leukocytes (PMNs) infiltrating the submucosa and lamina propria in Figure 6B.

Discussion

Here we report a novel murine candidiasis model showing severe gastrointestinal tract infection by *C. albicans*. The infection was characterized by local symptoms in the stomach, such as significant patchy whitish plaques in which mycelia of *Candida* grew abundantly in the inner stomach surface, thickening of lamina propria mucosae, and infiltration of PMNs.

The experimental conditions to establish this mouse model were as follows. First, the role of GlcNAc was paramount. We developed severe *Candida* infection by inducing mycelial growth of *Candida* through free ingestion of large quantities of GlcNAc to enhance virulence of *C. albicans*^{12, 13}. We used concentrations of GlcNAc (25, 50, and 100 mM) based on the oral candidiasis

model¹⁰. Figure 2 shows significant deterioration due to *Candida* colonization in the stomach and part of the small intestine upon administration of 25 mM GlcNAc, and aggravation reaching to the bottom of the small intestine at 50 mM GlcNAc. Our results show a closely similar tendency as with the murine oral candidiasis model reported in our previous paper¹⁰, which showed increasing, GlcNAc-dose-dependent deterioration. We speculate that the deep mycelial invasion of *C. albicans* observed in the stomach of GlcNAc-treated mice could cause mucosal inflammation with PMNs infiltration, as shown in Figure 6.

Second, the administration of chlorpromazine chloride played an important role by facilitating pathogenesis in our gastrointestinal candidiasis model. Chlorpromazine-treated groups showed significantly increased CFU estimates compared with controls as shown in Figure 1. It is well known that saliva flow and peristaltic motion mechanically inhibit fungal adhesion to the epithelial tissues, an integral step for the successful colonization of the oral cavity by *Candida*¹⁴. Chlorpromazine was effective in the murine oral candidiasis model because of its sedative, partially anesthetic effect, as well as because it reduces the amount of secreted saliva as a medicinal function. Takakura *et al.* reported that chlorpromazine induced dose-dependent effects during the sedation period in a mouse model¹¹. Our results indicate that the condition under which chlorpromazine was administered affected the degree of colonization by *Candida* in the stomach. Injection of chlorpromazine at 1–2 h after *Candida* inoculation resulted in significant colonization. We suggest that it may take 1–2 h for *Candida* to become

widespread in the stomach and invade depressed areas of mucosa for colonization under a sedated condition accompanied by reduced peristalsis.

Third, it should be noted that the other experimental procedures used in the study also played roles in the development of this gastrointestinal candidiasis model. One is the use of a broad-spectrum antibiotic, namely, tetracycline hydrochloride, in this study. The administration of such broad-spectrum antibiotics to mice can effectively induce substituted microbism and subsequent host vulnerability to *Candida* colonization^{11, 15, 16}. Another experimental procedure that should be mentioned is the use of steroids, for which we used prednisolone in this study. The administration of steroids can induce an immunosuppressed condition in mice, making them more vulnerable to colonization by *Candida*^{4, 11, 16}. The validity of these procedures has also been confirmed by previous reports^{10, 11}.

The severe *Candida* colonization in the gastrointestinal tract, especially in the stomach, of this mouse model allowed for a novel scoring system based on local characteristic symptoms. Patchy whitish plaques, similar to symptoms of the oral candidiasis model described in our previous papers^{10, 11}, were observed clearly in the stomach (Figure 3). As noted in Materials and Methods, this symptom score reflected mainly two pathological conditions: width of the surface colonized by *Candida*, and mass of the patchy whitish plaques based on their thickness. Using this scoring system allowed us to clearly show the aggravating effect of GlcNAc in drinking water from 48 h to 120 h after infection (Figure 5). This simple scoring system appears to be valuable for semi-quantitative determination of the severity of gastrointestinal candidiasis at the macroscopic level.

It should be noted that the score values did not always correlate with the number of viable *Candida* cells (CFU), e.g., there is no correlation in the symptom score and CFU between 72 h and 96 h, as shown in Figure 5C. Score values, however, reflected the width and mass of patchy whitish plaques, which consisted primarily of the mycelial form of *Candida*. This was confirmed through direct observation of cell wall staining with Calcofluor White M2R (Figure 4), which showed that the patchy whitish plaques indeed mainly consisted of *Candida* hyphae. We speculate that one reason for this discrepancy could be

the tendency for yeast cells to be more represented quantitatively than mycelial cells in the CFU method. Determining the actual relationship between fungal burden and lesion score would require the estimation of total fungal volume or mycelial volume by measurement of hyphae-specific components¹⁷.

Animal models are useful tools for studying gastrointestinal candidiasis^{5–9, 18}. Rats and mice are commonly used as model organisms because of the similarities in anatomical structure between rodent and human digestive tracts. Previous gastrointestinal candidiasis models succeeded in inducing an increase in the number of *Candida* cells in feces or milled tissues, but they could not be used to determine in detail the histological colonization in intact body tissues^{6–9, 18}. Furthermore, colonization could not be assessed at all by macroscopic observation because the severity of infection was lower than in models of oral or esophageal infection^{8, 9}.

The murine model in this report gives us some clues for studying the pathogenesis of systemic candidiasis caused by translocation of *Candida* from gastrointestinal mucosal tissues that are inflamed or damaged under conditions of severe infection^{4, 5}. Our histological analysis showed an association between symptoms of inflammation, such as significant patchy whitish plaques, thickening of lamina propria mucosae, and infiltration of PMNs in the stomach, with severe *Candida* colonization. In future experiments, we intend to analyze the inflammation process and the local invasion of *Candida* hyphae in the stomach and small intestine.

Finally, we present the utility of our scoring system and gastrointestinal candidiasis model for *Candida*-related studies in two areas: 1) interpreting the translocation mechanism of *Candida* cells during the spread from local to systemic infection; and 2) evaluating the preventative functions of antifungal agents, probiotics, or functional foods against *Candida* gastrointestinal infection.

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