Gastrointestinal Candidiasis in Rats Treated with Antibiotics, Cortisone, and Azathioprine

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Conventional albino rats treated with peroral chloramphenicol, gentamicin, and/or parenteral cortisone were challenged with Candida albicans. Antibiotics and cortisone were equally effective in predisposing the animals to colonization by the fungus. All animals treated with both antibiotics and cortisone developed defined, focal, superficial invasion of the cornified squamous epithelium of the stomach next to its junction with the glandular mucosa, as well as focal superficial invasion of the esophagus. Equivalent yeast cell and mycelial inocula of C. albicans were equally effective in producing colonization and invasion of the gut. Dissemination of the fungus from the gut was not found even after the addition of azathioprine to the treatment regimen; however, such addition did predispose to more extensive and severe lesions of the esophagus and stomach. Approximately 25% of infected cortisone- and antibiotic-treated rats developed agglutinins against C. albicans by 22 to 23 days after challenge, whereas 15% developed precipitins. The antibiotic-cortisone-treated rat may be a useful and consistent experimental model in the study of gastrointestinal candidiasis.

Infection by various Candida species is a serious complication of antibiotic, steroid, and immunosuppressive therapy and of altered host resistance in general (8, 11, 20, 29, 33, 34). A number of investigators have studied gastrointestinal candidiasis in germfree and conventional chicks and turkey pouls (1, 2, 35), and in germfree and conventional mice with antibiotic treatment and manipulation of gastrointestinal microflora (12, 13, 25). Experimental candidiasis in rats has also been studied (15, 16, 31, 32). We have investigated the course of Candida albicans colonization and infection of the alimentary tract of rats treated with cortisone, antibiotics, and azathioprine. The goal was to produce significant gastrointestinal populations of C. albicans and initiate gut invasion so that the conditions known to predispose to the disease in clinical situations could be examined. We have also studied the progression of gross and microscopic pathological lesions and the serological concomitants of experimental alimentary candidiasis by serial determinations of serum agglutinins and precipitins against C. albicans.

MATERIALS AND METHODS

Animals. Sixty-day-old female Holtzman rats, strain 4CD (Charles River Laboratories, Wilmington, Mass.), weighing 190 to 210 g, were used in all experiments. C. albicans and all drugs except cortisone were administered without anesthesia via peroral, gastric intubation with polyethylene catheters.

Inoculum. C. albicans, strain B311 (Hasenclever, National Institutes of Health), was maintained on modified Sabouraud dextrose agar with transfers every 2 to 3 weeks. Yeast-phase and mycelial growth were obtained using a synthetic medium (19). Stained smears of suspensions prepared from these cultures demonstrated a 9:1 predominance of the yeast phase or of mycelial elements. Organisms were harvested at 24 h, washed, and weighed. Dry weight equivalents of yeast cells or mycelium were suspended in 0.85% saline at 11.3 mg/ml (3 × 10^6 colony-forming units per ml) as determined by serial dilution and Sabouraud agar pour plates. Rats were given 1 ml of either the yeast cell or mycelial suspension.

Drugs. Rats received 5 mg of cortisone acetate suspension parenterally in the quadriceps femoris (Cortisone R acetate MSO) each day, commencing 6 days before inoculation with C. albicans and continuing through the course of the experiment. Twenty-five milligrams of chloramphenicol sodium succinate (chloromycetin sodium succinate for intravenous administration, Parke, Davis and Co., Detroit, Mich.) and approximately 20 mg of gentamicin sulfate (kindly provided by G. Evans, Scheiring Laboratories, Bloomfield, N.J.) were administered by gastric intubation on alternate days, beginning with a double dose of chloramphenicol 2 days before challenge with C. albicans. (Pilot studies had shown this combination to be the most effective of the antibiotics and combinations tried in suppress-
ing the normal bacterial flora without significant overgrowth of resistant bacteria.) The gentamicin dose was varied in response to increases in the growth of lactone-negative, gram-negative organisms in quantitative cultures of the fecal flora.

A solution of 3 mg of azathioprine per ml (Imuran, Burroughs-Wellcome) was used, and its effect was monitored by leukocyte counts.

Fecal flora. The fecal flora of at least one-third of the animals in each experiment group was assessed before and after drug treatment and after challenge with C. albicans at 3- to 5-day intervals by the pour plate technique in Trypticase soy agar (BBL) with 5% whole human blood (incubated aerobically and anaerobically in a GasPak [BBL] apparatus), MacConkey agar, lactobacillus selection agar (BBL), Mycosel agar, and Sabouraud agar with 0.1% lactic acid. Cultures were incubated at 37°C for 48 h and counted, and the counts per gram of feces (wet weight) were calculated. Fungal cultures were also incubated at 37 and 25°C for 7 days.

Experimental groups. Animals were divided into groups as shown in Table 1.

Postmortem examinations. Animals were etherized and exsanguinated by cardiac puncture. Blood was cultured in Trypticase soy broth or on the surface of blood agar and in Trypticase soy broth with thioglycolate. Lung, liver, pancreas, and kidney were removed aseptically, and the cut surfaces were cultured on blood agar.

Viscera were removed and fixed in 10% buffered formalin. Paraffin sections of lung, liver, kidneys, spleen, tongue, duodenum, small intestine, cecum, colon, and rectum were prepared. The entire esophagus was sectioned longitudinally. The stomach was cut transversally. Sections were stained with hematoxylin-eosin and periodic acid-Schiff stain-hematoxylin.

Blood samples were obtained before and 11 or 14 days after challenge with C. albicans via the posterior orbital venous plexus under ether anesthesia and by cardiac puncture at sacrifice. The sera were examined for precipitins and agglutinins against C. albicans (23, 24).

Precipitins were determined in Ouchterlony plates prepared with 1% Ionagar (Oxoid). Sera were run against four concentrations of the antigen. Controls were run with serum from immunized sheep. The plates were stained with Coomassie blue.

RESULTS

General and bacteriological. Animals receiving cortisone and C. albicans failed to gain or lost between 5 to 30% of their initial weight within the first 16 days of treatment. Thereafter, weights tended to stabilize. Inoculated rats of groups not receiving cortisone consistently gained up to 30% in weight.

The average baseline leukocyte count in untreated animals was 11,200. In animals receiving only azathioprine the average count dropped to 6,100 by day 11 of treatment. In animals receiving concurrent cortisone and antifungals, the average count dropped to 3,600 by day 10 of treatment.

Bacterial colony counts at 24 h after gentamicin or chloramphenicol are compared in Table 2. No evidence of any acute or chronic bacterial infection was ever found.

There were no differences in prevalence, location, or degree of tissue involvement between those animals receiving the mycelial as opposed to the yeast cell inocula.

Colonization of the gastrointestinal tract by C. albicans. All inoculated animals receiving either cortisone or antibiotics or both showed significant and persistent colonization of the gastrointestinal tract as determined by fecal counts (Fig. 1). Rats receiving cortisone alone and those receiving antibiotics alone were colonized equally well with C. albicans. Animals receiving both regimens showed a consistently higher colonization than those getting

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals</th>
<th>Inoculum*</th>
<th>No. of animals</th>
<th>Days postinoculum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisone alone</td>
<td>3</td>
<td>Y</td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>M</td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>None</td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td>Cortisone + antibiotics</td>
<td>17</td>
<td>Y</td>
<td>12</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>M</td>
<td>5</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>None</td>
<td>3</td>
<td>22</td>
</tr>
<tr>
<td>Antibiotics alone</td>
<td>3</td>
<td>M</td>
<td>3</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Y</td>
<td>3</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>None</td>
<td>3</td>
<td>28</td>
</tr>
<tr>
<td>Cortisone + antibiotics + azathioprine</td>
<td>3</td>
<td>Y</td>
<td>3</td>
<td>23</td>
</tr>
<tr>
<td>(from day 10 after inoculation)</td>
<td>4</td>
<td>Y</td>
<td>4</td>
<td>22</td>
</tr>
<tr>
<td>Cortisone + antibiotics + alkaline saline (pH 8-9)</td>
<td>2</td>
<td>Y</td>
<td>2</td>
<td>23</td>
</tr>
<tr>
<td>from day 10 after inoculation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortisone + normal saline* via gastric intubation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azathioprine alone (3 mg/day) from 6 days before challenge and 23 days thereafter</td>
<td>3</td>
<td>Y</td>
<td>3</td>
<td>22</td>
</tr>
</tbody>
</table>

* Y, Yeast cells; M, mycelium.
* One animal died from trauma incurred during intubation.
* Alkaline saline was substituted for the azathioprine solution in these animals to control for the effect of the pH of the azathioprine solution.
* Normal saline was substituted for the antibiotic solutions in these animals to control for the effect of the daily procedure of intubation and instillation.
TABLE 2. Fecal bacterial flora before and after various antibiotic doses administered via gastric intubation

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Blood agar (aerobic)</th>
<th>Blood agar (anaerobic)</th>
<th>MacConkey agar (lactase+)</th>
<th>Lactobacillus selection agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>8.50 (7.42–9.18)</td>
<td>9.15 (8.50–9.52)</td>
<td>7.62 (&lt;5.00–8.30)</td>
<td>9.56 (8.34–10.04)</td>
</tr>
<tr>
<td>Gentamicin (15 mg)</td>
<td>&lt;4.00–6.92</td>
<td>&gt;8.00</td>
<td>&lt;2.00–5.46</td>
<td>&lt;5.00–6.72</td>
</tr>
<tr>
<td>Gentamicin (20 mg)</td>
<td>&lt;5.00–7.72</td>
<td>&gt;8.00</td>
<td>&lt;3.00–5.56</td>
<td>&lt;6.00–7.65</td>
</tr>
<tr>
<td>Chloramphenicol (25 mg)</td>
<td>5.38–8.69</td>
<td>9.15</td>
<td>&lt;4.00–4.96</td>
<td>4.73–7.70</td>
</tr>
</tbody>
</table>

* Counts were made 24 h after last dose.

![Graph](http://iai.asm.org/)

**FIG. 1.** Colonization of gastrointestinal tract by *C. albicans* in rats treated with antibiotics and cortisone. (A) Animals receiving both cortisone and antibiotics (average from two to five animals) (●); (B) animal receiving antibiotics only (○); (C) animal receiving cortisone only (△).

either cortisone or antibiotics alone. Colonization in cortisone- and/or antibiotic-treated rats receiving a mycelial inoculum was essentially identical to the colonization over time seen in those inoculated with yeast cells.

Colonization after the addition of azathioprine to cortisone and antibiotics was not appreciably different from colonization in animals without azathioprine (Fig. 2). Animals receiving azathioprine alone showed a colonization pattern similar to those receiving no drugs. Both of the latter groups showed a rapid drop in fecal count within 2 days or, in one untreated rat, to nil after 21 days. Substitution of alkaline saline for azathioprine in cortisone- and antibiotic-treated rats did not produce a significant difference in colonization. No fungi were cultured from the rat feces before inoculation with *C. albicans*. After challenge, *C. albicans* was the only fungus cultured.

**Pathological examination.** Tissue invasion, when present, was limited to the esophagus, stomach, and oral cavity.

The frequencies of esophageal and stomach infection in the various groups are presented in Table 3. Almost 100% of animals treated with cortisone and antibiotics and *C. albicans* showed infection sharply limited to the squamous epithelium at the region of transition, where the forestomach joins the glandular epithelium. There were no pathological changes in other parts of the stomach. Lesions of the stomach in animals with azathioprine added to their regimen were also limited to this precise location, but were generally more severe. *C. albicans* invaded the squamous epithelium of the gastric mucosa but not the submucosa. As in the case of colonization, esophageal and gastric tissue invasion occurred only rarely in animals not receiving cortisone and/or antibiotics.

The combination of antibiotics and cortisone was more effective in promoting stomach infec-
...in the inoculated rats than either drug alone (by a χ² Zuill-Yates correction; P < 0.002 in both cases); however, the difference in prevalence of esophageal involvement between these groups was not statistically significant. The results from the animals treated with cortisone and saline rather than with antibiotics suggests that intubation did not add significantly to the effectiveness of parenteral cortisone alone in promoting infection.

Although stomach invasion was found in only one of the animals treated with azathioprine alone, the severity of lesions at this location was significantly increased when azathioprine was added to cortisone and antibiotics.

Since coprophagy of feces with high counts of *C. albicans* could lead to oral lesions, the tongues of six animals treated with cortisone and antibiotics were examined histologically. Five of the six tongues examined did indeed show foci of *C. albicans* hyphae in the superficial layers of the lingual epithelium.

**Histology.** The lesions observed were either "superficial," i.e., small numbers of hyphae in superficial portions of squamous epithelium, or "transmucosal," i.e., numerous hyphae, total mucosal involvement, and inflammatory reaction. The latter were seen only in the stomach.

The lesions are tabulated in Table 3 and illustrated in Fig. 3 through 7.

Lesions of the lingual epithelum consisted of hyphal elements in the superficial layers without inflammation or other structural changes. Penetration of the fungus beyond the basement membrane was not observed in either the stomach, esophagus, or tongue.

**Serology.** None of the animals demonstrated serum agglutinins or precipitins before challenge with the organism. Agglutinins were developed in the serum of animals receiving cortisone only; however, some of the animals in the group receiving antibiotics and cortisone developed both agglutinating and precipitating antibodies (see Table 3). The animals which developed precipitating antibodies developed them to only cell wall components of the yeast.

**DISCUSSION**

The development of endogenous *C. albicans* infection probably occurs in three phases: gastrointestinal colonization, mucosal invasion, and ultimately, blood stream dissemination. However, it is not yet clear whether, or how, these phenomena succeed each other in natural or experimental infection.

Our rats were free of candida before the ex-
perimental inoculum, and the candida stool counts declined rather promptly in the unmodified controls. Chloramphenicol and gentamicin were used to suppress the gut flora and thus facilitate the establishment of \textit{C. albicans}, i.e., colonization and/or tissue invasion. Antibiotics are known to free fungi of competition by inhibiting the normal bacterial gut flora; they may also stimulate the fungus directly, or perhaps indirectly, by toxic effects on the host (33); however, removal of bacterial competition is probably the most significant factor. Growth inhibitors of \textit{C. albicans} have been described in a number of bacterial strains (2, 12, 38), and competitive removal of nutrients by bacteria has also been demonstrated (17). It has further been suggested that certain components of the normal flora suppress fungal growth by altering the pH and redox potential (13).

In our study, parenteral cortisone also significantly increased susceptibility of rats to stomach and esophageal candida infection and was equal to antibiotics in facilitating gut colonization. In fact, the two treatment modalities were virtually additive in these regards. Cortisone and antibiotics used together resulted in gastric infection of all of the experimental animals so treated.

The rat is considered "steroid sensitive" and shows lymphocytolysis and decreased effectiveness of remaining lymphocytes after cortisone treatment (6), as well as impairment of cell-

\begin{table}
\centering
\caption{Prevalence of infection of esophagus and stomach and serology in rats variously treated with cortisone, antibiotics, and azathioprine$^a$}
\begin{tabular}{|l|l|l|l|l|l|l|l|l|l|}
\hline
Treatment, inoculum, and day of sacrifice & No. of animals & Esophag- & Stomach & Serology & & & & & \\
& & & & & & & & & \\
& & & & & & & & & \\
& & & & & & & & & \\
& & & & & & & & & \\
& & & & & & & & & \\
Cortisone, antibiotics; Y and M inocula; days 22 and 36 & 27 & 14/26 & 27/27 & Baseline & 0/27 & 0/27 & 11-14 & 0/27 & 2/27 & 22 & 1/18 & 3/18 & 0/1 & 36 & 4/9 & 2/9 & 2/4 \\
Antibiotics; Y and M inocula; day 28 & 5 & 0/5 & 3/5 & Baseline & 0/5 & 0/5 & 11 & 0/5 & 0/5 & 28 & 0/5 & 0/5 & & & & & \\
Cortisone; Y and M inocula; day 28 & 6 & 2/5 & 2/6 & Baseline & 0/6 & 0/6 & 11 & 0/6 & 0/6 & 28 & 0/6 & 0/6 & & & & & \\
Cortisone, antibiotics, azathioprine; Y inoculum; day 23 & 10 & 10/10 & 10/10 & Baseline & 0/10 & 0/10 & 14 & 0/10 & 0/10 & 23 & 1/10 & 3/10 & 1/1 & & & & & \\
Azathioprine; Y inoculum; day 23 & 2 & 0/2 & 1/2 & Baseline & 0/2 & 0/2 & 14 & 0/2 & 0/2 & 23 & 0/2 & 0/2 & & & & & \\
Cortisone, antibiotics, alkaline saline by tube from day 10; Y inoculum; day 23 & 3 & 3/3 & 2/3 & Baseline & 0/3 & 0/3 & 14 & 0/3 & 1/3 & 23 & 0/3 & 3/3 & & & & & \\
Cortisone, saline by tube; Y inoculum; day 22 & 4 & 3/4 & 2/4 & Baseline & 0/4 & 0/4 & 14 & 0/4 & 2/4 & 23 & 0/3 & 3/3 & & & & & \\
No drug; Y inoculum; day 22 & 3 & 0/3 & 0/3 & Baseline & 0/3 & 0/3 & 14 & 0/3 & 0/3 & 22 & 0/3 & 0/3 & & & & & \\
\hline
\end{tabular}
\end{table}

$^a$ Abbreviations: Ppt., Precipitins; Agg., agglutinins; Y, yeast-cell inoculum; M, mycelial inoculum.

$^b$ Azathioprine treatment (3 mg/day) begun 10 days after inoculation.

$^c$ Azathioprine (3 mg/day) begun 6 days before inoculation.

$^d$ Saline administered in the same manner as antibiotics.
Fig. 3. Periodic acid-Schiff stain-hematoxylin, x530; esophagus; 22 days. Treatment: Cortisone, and antibiotics. Shows typical focal superficial lesion; candida hyphae limited to and parallel with stratum corneum; minimal changes in deeper layers (note sparse neutrophils in submucosa).

Fig. 4. Periodic acid-Schiff stain-hematoxylin, x530; esophagus; 23 days. Treatment: Cortisone, antibiotics, azathioprine. Shows typical focal transmucosal lesion; top layer consists of rich fungal growth mixed with cell debris. Hyphae (arrow) invade through but not beyond epithelium. Stratum spinosum, partly destroyed by microabscesses. Marked intra- and intercellular edema, especially of basal layer; note pyknotic epithelial nuclei, migrating intramucosal neutrophils. Submucosa, edematous with dilated lymph channels, numerous polyps.
FIG. 5. Periodic acid-Schiff stain-hematoxylin, ×300; stomach; 22 days. Treatment: Cortisone and antibiotics. Moderately invasive lesion, limited to squamous epithelium. Note hyphae penetrating perpendicularly through stratum spinosum and leukocyte migration upward from submucosa, with superficial microabscesses. There is moderate acanthosis, hyperkeratosis, and parakeratosis.

FIG. 6. Periodic acid-Schiff stain-hematoxylin, ×130; stomach; 23 days. Treatment: Cortisone, antibiotics, and azathioprine. Marked increase in severity and extent of lesion; note rich fungal growth and leukocyte accumulation. Marked hyper- and parakeratosis with eosinophilic degeneration of prickle cells (arrow).
mediated immunity, which is the main immunological defense against fungal infection (9, 10). Glucocorticosteroids also decrease host inflammatory responses through decreased lyso-osomal lability and containment of lytic enzymes (41). Although these steroid effects may have played a role in decreasing host resistance, they do not provide a completely satisfactory explanation for our results, especially in view of the uniform vigor of host cell responses of all gastric candida lesions, whether in steroid-treated rats or in animals treated otherwise. Steroids have a diabetogenic effect; Knight and Fletcher (17) found C. albicans overgrowth in human saliva with increased glucose levels regardless of whether this had been induced by diabetes mellitus or by steroid administration. Cortisone also decreases gastric mucus secretion and qualitatively changes carbohydrates in mucus (22). Yet another possibility is steroid-induced mucosal ulceration followed by superinfection with C. albicans. However, we have no pathological evidence that infection occurred at the site of any preexisting lesion.

Cortisone- and antibiotic-treated rats were additionally treated with azathioprine in the hope of stimulating dissemination from the alimentary foci of infection. Dissemination did not occur within the observation time of 12 days (which may have been too brief). On the other hand, mucosal lesions in the azathioprine-treated rats were more extensive and severe, and this could not be attributed to the alkalinity of the azathioprine solution. Azathioprine is known to depress lymphocyte function and inhibit inflammation (14). However, in spite of falling peripheral leukocyte counts, the gut lesions in our azathioprine-treated rats showed profuse inflammatory responses. Possibly, an effect of azathioprine on mucosal cell turnover could also have played a role in increasing susceptibility to local mucosal invasion.

A striking finding of the present work was the limitation of fungal invasion to the squamous epithelium of the rat alimentary tract and in particular the involvement of the epithelial fold ("Grenzfalte") immediately adjacent to the glandular stomach. Taschdjian et al. (40) found that vaginal candidiasis in mice could be induced only during that phase of the estrus cycle at which the mucosa is fully cornified. In the rat gut the most susceptible tissue is also a cornified epithelium. Localization of infection at the squamous-glandular junction may con-
cevably be related to the increased thickness of the cornified epithelium at this point or to the interruption of the smooth mucosal contour with pooling of juices that contain neutral cardiac gland secretions.

Candida serology is considered a potentially useful diagnostic tool in high-risk patients, e.g., in acute leukemia (26, 27, 30), but many normal humans have measurable agglutinating antibodies against C. albicans (5), and precipitins are usually correlated with high agglutination titers (26). High mean agglutination titers, precipitins, and increased prevalence of significant fluorescent antibody titers have been found in patients with vulvovaginitis, candidiasis of the skin, oral candidiasis, and chronic mucocutaneous candidiasis (20, 37, 39), but in superficial candidiasis serological tests were not helpful (4, 28, 36). Our results demonstrate that localized lesions of the gastrointestinal tract can be associated with production of antibodies measurable by routine serological tests in a proportion of rats and suggest that serology in such situations may be of value in early clinical diagnosis, especially in light of the fact that up to 25% of patients with significant esophageal candidiasis are asymptomatic (8).

Experimental studies, including the present work, suggest that the processes leading to gut colonization and local tissue invasion are not necessarily the same as those leading to dissemination by C. albicans. Evidence that C. albicans is capable of crossing the normal bowel wall was obtained by Krause et al. (18). They found fungemia and funguria within several hours of ingestion of large numbers of C. albicans by a healthy subject and ascribed this passage to "persorption." Stone et al. (38) have implicated such passive transfer in the development of funguria in surgical patients with gastrointestinal colonization after antibiotics. Other investigators have produced clear-cut fungemia in dogs after instillation of large numbers of yeasts through duodenal tubes (3). It is not clear whether true systemic candida lesions occurred in any of these fungemias, nor is it clear whether systemic candidiasis can occur in the absence of any gastrointestinal, mucosal invasion unless the inoculum takes place directly into the blood stream. In the present work, there was no evidence of metastatic candidiasis in spite of significant infestation of the gut, but there was some evidence of fungemia without visceral lesions (see pathological findings, above). Therefore, additional conditions must be met for candidiasis to proceed from gastrointestinal to systemic lesions.

The model presented in this paper could be used to elucidate some of these variables.

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LITERATURE CITED


