Assessment of Germ Tube Dispersion Activity of Serum from Experimental Candidiasis: A New Procedure for Serodiagnosis

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Germ tubes of Candida albicans and C. stellatoidea clump in normal serum but disperse in serum from animals infected with either species of Candida. A new procedure for the assessment of grades of germ tube dispersion activity of serum is presented; this procedure is to count the number of freely dispersed germ tubes in test serum into which a definite number of yeast-type C. albicans has been inoculated. The relationship between the serum activity and macroscopic lesions caused by candidal infection is observed, indicating the possibility of applying the phenomenon to the serodiagnosis of deep-seated candidiasis. The specificity and sensitivity of the test are also examined.

Sera from healthy humans or normal animals clump the germ tubes of Candida albicans (4), but their clumping activity is known to be absent or decreased in sera from patients with some diseases, especially in systemic or chronic mucocutaneous candidiasis (4, 7).

Chilgren and his colleagues (4) obtained evidence that the decrease in germ tube clumping, which is observed as dispersion of germ tubes in serum, is caused by immunoglobulin G antibody to C. albicans. These reports urged us to study the possibility of applying germ tube dispersion to the serodiagnosis of deep-seated candidiasis, for which a reliable serological test has been urgently needed.

The present report deals with the procedure for the germ tube dispersion test and the specificity and sensitivity of the reaction.

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MATERIALS AND METHODS

Organisms used. Four strains of C. albicans, two each of types A (Y and J 1447) and B (W and J 1445), and one strain each of C. stellatoidea (IFO 1398), C. tropicalis (IFO 1070), Mycobacterium bovis BCG, and M. tuberculosis H37Rv were used.

J 1445 and J 1447 were given by Y. Fukuza (Meiji Pharmaceutical College), and Y was given by K. Yamashita (Kyoto National Hospital). W strain was isolated from the sputum of a pulmonary disease patient admitted to the hospital of our institute. Serotypes of C. albicans were determined by Candida check (10). IFO 1398 and 1070 were supplied by the Institute for Fermentation, Osaka. BCG and H37Rv were stock cultures of our institute. All Candida organisms were maintained on Sabouraud glucose agar slants and transferred to new media bimonthly. H37Rv and BCG were maintained on egg media and transferred bimonthly.

Animals used. Three strain of female mice, 10 to 12 weeks old, were used in most cases for infection experiments. In one case, a rabbit was also used.

Inoculum and infection. Candida organisms grown on Sabouraud glucose agar at 37 C for 48 h were harvested and washed three times in saline. The washed cells were resuspended in saline, and the number of cells in appropriate dilutions was counted in a Bürker-Türk hemocytometer. All cells in the suspension were yeast form, with a few paired, but no germ tubes or pseudohyphae were found. Intraperitoneal (ip) infection was made by inoculating 0.5 ml of the suspension containing desired number of cells per animal.

In one case, C. albicans Y strain suspended in saline was killed at 100 C for 1 h, and in another, a 1:1 mixture of the killed organism in saline and Freund incomplete adjuvant (Difco) was emulsified to a water-in-oil emulsion with a Waring blender. The former was injected into mice ip, and the latter was injected subcutaneously. BCG and H37Rv were grown on Sauton liquid medium for 1 month at 37 C. Pellicles were harvested, weighed, and suspended in saline after being ground thoroughly in a mortar. The suspension of each organism was injected into mice ip (BCG) or intravenously (H37Rv).

Procedures for the germ tube dispersion test. Yeast-phase C. albicans or C. stellatoidea was prepared from a 37-C overnight growth on a Sabouraud glucose agar slant. The cells were washed once in saline and twice in distilled water. One million yeast cells suspended in 0.1 ml of distilled water was inoculated into a 0.5-ml serum sample. The sample was shaken at 80 rpm in a 37-C incubator. The optimal incubation time of growing germ tubes to

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observe clumping varied from 3 to 12 h, depending on the strain used and animal species from which serum was taken. After incubation, the tubes containing samples were shaken by hand, and the number of freely dispersed germ tubes was counted in a hemocytometer (1 mm², 0.1 mm in depth; 10⁻⁴ ml in volume). The grades of germ tube dispersion activity of a given serum were expressed as in Table 1.

**Autopsy.** When serum was obtained from infected mice by decapitation, macroscopic findings were recorded. The grades of severity of lesions produced by ip injection with *Candida* were expressed as in Table 2.

In the case of infection with tubercle bacillus H₃Rv, the degree of tuberculous changes in mice was assessed by "specific lung weight" (SLW), proposed by Aoki (2), which is expressed as follows: SLW = [(lung weight (in milligrams)]/[body weight (in grams)] × 10. (According to Aoki, SLW of normal mice was 70.0 ± 2.9.)

### RESULTS

**Germ tubes of *Candida* in sera from normal animals and humans.** All *C. albicans* and *C. stellatoidea* strains tested produced germ tubes when inoculated into serum at 37 C. They clumped in normal serum after a certain incubation time. After 3 h of incubation, most germ tubes of W strain *C. albicans* clumped and produced masses in sera of normal mice, calves, and rabbits (Table 3 and Fig. 1). The number of freely dispersed germ tubes was less than 30. However, germ tube production in some human sera was slower than in the other sera, and short germ tubes which were sometimes indistinguishable from buds were observed after the same incubation period. This resulted in 31 to 50 free germ tubes in 2 sera and 51 to 100 in 6 sera among 25 sera examined (Table 3). However, after 6 h of incubation most cells produced germ tubes and clumped, and the number of free germ tubes became less than 30.

Two other strains of *C. albicans*, J 1445 and J 1447, and *C. stellatoidea* IFO 1398 behaved similarly to W strain in normal mouse sera. But germ tube production and clumping of Y strain *C. albicans* in normal serum were much slower than the other strains, and it took 12 h when the number of free germ tubes became less than 30.

These results indicate that the incubation time necessary to produce germ tubes long enough to clump varies with the strain of *Candida* and the animal species from which serum was taken.

**Germ tube dispersion activity of sera from infected animals.** In contrast to the behavior in normal serum, germ tubes did not clump but dispersed in sera of infected animals. Figure 2 shows the dispersion of term tubes of W strain after 3 h of incubation in serum of a mouse

### TABLE 1. Grades of germ tube dispersion activity

<table>
<thead>
<tr>
<th>Grade</th>
<th>No. of freely dispersed germ tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Less than 30</td>
</tr>
<tr>
<td>1</td>
<td>31 to 50</td>
</tr>
<tr>
<td>2</td>
<td>51 to 100</td>
</tr>
<tr>
<td>3</td>
<td>More than 101</td>
</tr>
</tbody>
</table>

- *Inoculum size is 10⁶ cells per 0.4 ml, which corresponds to 250 per 10⁻⁴ ml.*

### TABLE 2. Severity of lesions in grades of germ tube dispersion activity

<table>
<thead>
<tr>
<th>Grade</th>
<th>Macroscopic findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>None</td>
</tr>
<tr>
<td>±</td>
<td>A few submiliary to miliary nodules in visceral organs, mostly in liver or omentum.</td>
</tr>
<tr>
<td>+</td>
<td>Several nodules in visceral organs, some larger than 1 to 2 mm in diameter; kidneys frequently deformed.</td>
</tr>
<tr>
<td>++</td>
<td>Numerous nodules in visceral organs; kidneys enlarged or atrophied.</td>
</tr>
</tbody>
</table>

### TABLE 3. Number of freely dispersed germ tubes of *W* strain *C. albicans* in sera from normal animals and humans

<table>
<thead>
<tr>
<th>Serum from</th>
<th>No. of samples</th>
<th>No. of samples in the following no. of freely dispersed germ tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&lt;30</td>
</tr>
<tr>
<td>Mice</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Calves</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Rabbits</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Humans</td>
<td>25</td>
<td>17</td>
</tr>
</tbody>
</table>

**FIG. 1.** *W* strain of *C. albicans* incubated for 3 h in normal mouse serum. Large masses of germ tubes are seen.
mice with marked lesions macroscopically, and sera from mice without lesions were unable to disperse germ tubes.

Sera from mice injected with heat-killed C. albicans. When heat-killed Y strain was suspended in saline and injected into mice ip, an inoculum three times larger (3 x 10⁸) than that used in live cells (10⁷; see Table 4) did not elicit the germ tube dispersion activity (Table 6). When they were emulsified in Freund incom-

![Fig. 2. W strain of C. albicans incubated for 3 h in infected mouse serum. Most germ tubes are present separately.](http://iai.asm.org/)

infected with the same strain. Most germ tubes did not adhere to each other and were present separately.

To investigate the relationship between the germ tube dispersion activity and the dilution of the serum, serial fourfold dilutions of infected rabbit serum were made with normal serum and inoculated with yeast-form W strain. After 3 h of incubation at 37 C, the germ tubes which did not adhere to each other were counted in a hemocytometer. With the increase of dilution of test serum, the number of free germ tubes decreased (Fig. 3). This indicates that the antibody activity of test serum can be expressed by the number of freely dispersed germ tubes in the serum.

Relationship between infection and serum activity of dispersing germ tubes. When 10⁴ or 10⁷ cells of Y strain were inoculated into mice ip, slight germ tube dispersion activity (grade 1) was observed in some sera at day 20 or later, but none of the sera tested had activity of more than grade 2. However, the inoculation of 10⁴ cells gave mice the dispersion activity of sera as shown in Table 4. None of the sera examined had obvious activity at day 10 in spite of the presence of macroscopic lesions. At day 20, half of the sera examined showed the activity and mice had marked lesions. At day 31, the activity became more pronounced, and all sera had strong activity (grades 2 or 3) at day 39. This activity was observed as late as 102 days in more than half of the sera examined. Similar results were obtained when mice were infected with other strains of C. albicans.

In one instance when mice were injected ip with 3 x 10⁷ cells of W strain C. albicans and the activity of their sera was examined at day 50, close relationship between the activity and macroscopic findings was found (Table 5): sera that had strong activity were obtained from

![Fig. 3. Relationship between the number of freely dispersed germ tubes of W strain C. albicans and the dilution of serum from an infected rabbit. Numbers in parentheses indicate the number of trials. Means are calculated arithmetically and vertical lines indicate the maximal and minimal numbers of freely dispersed germ tubes in each dilution.](http://iai.asm.org/)

<table>
<thead>
<tr>
<th>Days after infection</th>
<th>No. of mice</th>
<th>Grades of germ tube dispersion activitya</th>
<th>Macroscopic findings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>10</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>31</td>
<td>10</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>39</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>102</td>
<td>9</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

a Examined by using Y strain C. albicans.
between their activity stellatoidea. Sera C. volume same or test serum performed Since the volume of dose same adjuvant (grade activity difference was a mouse C. stellatoidea IFO 1398, between two 7). Subcutaneous with saline 50 \text{ mg}
\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{Injection} & \textbf{Inoculum size} & \textbf{Days after injection} & \textbf{Germ tube dispersion activity}\textsuperscript{a}
\hline
Intraperitoneal in saline & 3 \times 10^7 & 30 & 0 1 2 3 \\
& 3 \times 10^4 & 30 & 0 1 2 3 \\
& 3 \times 10^7 & 50 & 0 1 2 3 \\
& 3 \times 10^4 & 50 & 0 1 2 3 \\
Subcutaneous with Freund incomplete adjuvant & 3 \times 10^7 & 30 & 0 1 2 3 \\
& 3 \times 10^4 & 50 & 0 1 2 3 \\
\hline
\end{tabular}
\end{table}

\textsuperscript{a} Suspension of cells was heated at 100 C for 1 h.

Cross-reaction in germ tube dispersion activity between C. albicans types A and B, and C. stellatoidea. Sera from mice infected with type A (J 1447) or B (J 1445) of C. albicans, or C. stellatoidea IFO 1398, were examined for their activity in dispersing germ tubes of the same or different strains of C. albicans or C. stellatoidea. Since the volume of serum taken from a mouse was not enough for the three tests performed in this experiment (Table 7), an equal volume of normal serum was added to the test serum before use. Although some strain difference was observed, cross-reactions between C. albicans and C. stellatoidea as well as between two types of C. albicans were observed (Table 7).

Serum from a rabbit infected with both types of C. albicans also dispersed germ tubes of not only both types of C. albicans but also of C. stellatoidea.

**Serum activity of mice infected with organisms other than C. albicans or C. stellatoidea.** Different doses of C. tropicalis IFO 1070 and a single dose of M. bovis BCG or M. tuberculosis H\textsubscript{3}Rv were injected into mice ip or intravenously. Germ tube dispersion activity was examined at different times after infection by using C. albicans Y strain as a test organism (Table 8). Although one serum of a mouse infected with C. tropicalis had activity, the grade was low. BCG (5 mg, ip) did not elicit activity in mice. Six out of 24 mice infected with tubercle bacilli as evidenced by marked SLW values showed only a slight germ tube dispersion activity (grade 1).

**DISCUSSION**

Since serological tests are still not yet generally accepted as reliable tools for the diagnosis of deep-seated candidiasis, any diagnostic measure that demonstrates promise is worthy of investigation (11).

Our results show that germ tubes of C. albicans or C. stellatoidea clump in sera from normal humans, rabbits, calves, and mice. But

\begin{table}[h]
\centering
\begin{tabular}{|c|c||c|c|c|}
\hline
\textbf{Infecting organism} & \textbf{No. of animals} & \textbf{Germ tube dispersion activity tested with}\textbf{Mice} & \textbf{Rab-} & \textbf{Type} & \textbf{Type} & \textbf{C. stel-} \\
 & & & \textbf{bit}\textsuperscript{a} & \textbf{A} & \textbf{B} & \textbf{latoidea} \\
\hline
C. albicans & 1 & 3 & 3 & 2 \\
Type A (J1447) & 2 & 3 & 3 & 3 \\
& 4 & 3 & 3 & 3 \\
Type B (J1445) & 1 & 1 & 2 & 2 \\
& 2 & 3 & 2 & 2 \\
Types A and B & 3 & 3 & 3 & 3 \\
C. stellatoidea & 1 & 1 & 1 & 2 \\
IFO 1398 & 2 & 2 & 2 & 3 \\
& 3 & 1 & 1 & 2 \\
& 4 & 2 & 2 & 3 \\
& 5 & 1 & 1 & 2 \\
& 6 & 1 & 0 & 1 \\
\hline
\end{tabular}
\end{table}

\textsuperscript{a} Mice were injected ip with C. albicans or C. stellatoidea in the dose of 10\textsuperscript{6} or 5 \times 10\textsuperscript{6} yeast-type cells, respectively. Serum was taken 30 days after infection.

\textsuperscript{b} Rabbit was given five biweekly ip injections with 10\textsuperscript{6} cells of C. albicans types A and B. Serum was taken 1 week after the last injection.
they disperse in sera from animals infected with either species of *Candida*. Grades of dispersion activity of a serum can be expressed by counting the number of freely dispersed germ tubes in the serum into which a definite number of yeast-type cells of *Candida* has been inoculated.

One of the problems to be solved before practical use, however, is the sensitivity and specificity of the reaction. As for the sensitivity of serological reactions of candidiasis, no test now used adequately indicates the possible extent and severity of the disease in a given serum (12). In our results, when Y strain *C. albicans* was used as an infecting (ip) and test organism, the activity did not appear until day 10, but at day 20 and afterwards the activity became stronger, and at day 39 all sera tested had the marked activity (grades 2 and 3) with macroscopic lesions. Similar results were obtained with other strains of *C. albicans*, and sometimes a close relationship between the activity and macroscopic findings was found (Table 5). As for the specificity of our procedure, cross-reactivity between *C. albicans* and *C. stellatoidea* as well as between types A and B of *C. albicans* was evident. However, most sera from mice infected with *C. tropicalis* had no or week cross-reactivity with *C. albicans*.

Cross-reactive antigens between *Candida* and *Mycobacterium* have been documented by precipitin (1, 8, 9), passive hemagglutination (3, 8), allergic skin reaction (9), and complement fixation reactions (5). Recently developed "S" antigen of *C. albicans* is said to be the only one that does not cross-react with *Mycobacterium* (12).

One problem in this connection is the relationship between the tuberculous involvement of mice and their germ tube dispersion activity. In 1966, Aoki (2) proposed to express the grades of tuberculous involvement of mice by specific lung weight. According to him, SLW of normal mice was 70.0 ± 2.9 and never reached 100. But SLW of mice infected with tubercle bacilli for more than 20 days was more than 100. Lee (6) confirmed this finding when he examined the virulence of tubercle bacilli isolated in Korea.

Our results show that SLW of all mice infected with *H. Rv* was more than 100 and increased with days after infection. Sera taken from these mice had no or slight (grade 1) germ tube dispersion activity, none having more than grade 2 activity (Table 8).

From these results, one may reasonably conclude that the present test procedure can be used as a tool for the diagnosis of the disease if we take the activity of grade 1 as doubtful reaction and grades 2 and 3 as positive reactions, since there is no difference in the treatment of deep-seated candidiasis whether the causative agent is *C. albicans* or other species of *Candida*.

Louria et al. (7) have recently suggested that the use of the assay of clumping activity and interfering antibody in patients with overt or suspected candidiasis might have some diagnostic value. Their assay procedure was to measure the *Candida* population in serum by colony count, which is a little more time-consuming than our procedure. A difference in observations between our procedure and theirs is that in their experiment, normal murine sera were much less active in clumping germ tubes than were human sera, whereas strong clumping activity of normal mouse sera was observed in our experiment. The reason for the discrepancy is not clear, but it seems to be caused by the

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**Table 8.** Germ tube dispersion activity of sera from mice infected with organisms other than *C. albicans* or *C. stellatoidea*

<table>
<thead>
<tr>
<th>Infecting organism</th>
<th>Inoculum ip</th>
<th>Days after infection</th>
<th>No. of mice</th>
<th>No. with grades of germ tube dispersion*</th>
<th>No. with macroscopic findings</th>
<th>Mean SLW</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. tropicalis</em></td>
<td>3 x 10⁷</td>
<td>31</td>
<td>9</td>
<td>8 1 0 0</td>
<td>5 2 1</td>
<td></td>
</tr>
<tr>
<td>IFO 1070</td>
<td>10*</td>
<td>49</td>
<td>9</td>
<td>9 0 0 0</td>
<td>0 5 2</td>
<td></td>
</tr>
<tr>
<td><em>M. bovis BCG</em></td>
<td>5 mg</td>
<td>56</td>
<td>10</td>
<td>10 0 0 0</td>
<td>1 4</td>
<td></td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>0.4 mg (iv)</td>
<td>39</td>
<td>8</td>
<td>5 3 0 0</td>
<td>0 0</td>
<td>111</td>
</tr>
<tr>
<td>H₄, Rv</td>
<td></td>
<td>60</td>
<td>8</td>
<td>6 2 0 0</td>
<td>0 0</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80</td>
<td>8</td>
<td>7 1 0 0</td>
<td>0 0</td>
<td>176</td>
</tr>
</tbody>
</table>

* Y strain *C. albicans* was used as the test organism.

* About half of the mice infected with BCG produced lesions comparable to (+) to (++) in *Candida* infection.
difference in strains of *C. albicans* and mice employed.

Finally, the fact that an inoculum of heat-killed *C. albicans* cells three times larger than that of live cells elicited only slight activity (grade 1) compared with the strong activity of live cells (see Tables 2 and 3) seems to indicate a difference in the ability to elicit the germ tube dispersion activity between tissues having candidal infection and those in which *Candida* cells are merely present.

**ACKNOWLEDGMENT**

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**LITERATURE CITED**


