Rapid Clearance of Candida albicans Mannan Antigens by Liver and Spleen in Contrast to Prolonged Circulation of Cryptococcus neoformans Antigens

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Clearances of mannan antigen from Candida albicans and glucuronoxylomannan antigen from Cryptococcus neoformans were examined in nonimmune rabbits by using self-prepared latex agglutination tests. Injected intravenously, 20 mg of Candida mannan antigen was cleared from the serum with a half-life of approximately 2 h. In contrast, 20 μg of Cryptococcus glucuronoxylomannan antigen had a half-life in serum of approximately 24 h. At the latest, 9 h after injection, both antigens were no longer detectable without pretreatment of serum samples with protease and heating to 100°C, thus indicating rapid binding by serum proteins other than immunoglobulins. Candida mannan antigen clearance was also examined in nonimmune mice after intravenous injection of (i) 200 μg of Candida mannan antigen, which accumulated in the liver and spleen and persisted for 97 days; (ii) 2 × 10^6 ethanol-killed Candida blastospores, which was accompanied by rapid clearance of mannan from the blood but accumulation of mannan in the liver and spleen and slow clearance from these organs; (iii) 6 × 10^6 viable C. albicans cells (lethal infection), which resulted in a rapid decrease of Candida CFU in the blood, liver, and spleen during the first 8 h, after which blood cultures were negative on day 2 and viable Candida burdens in the liver and spleen persisted at 10^6 CFU/g, whereas Candida mannan antigen continued to circulate in the bloodstream and accumulated in the liver and spleen.

In deep-seated candidiasis, evidence points to the existence of mannan antigen in the bloodstream (2, 11). This antigen does not circulate freely but is bound by anti-Candida antibody and/or a recently characterized mannan-binding protein (9). Attempts to detect circulating mannan antigen regularly in early stages of deep-seated candidiasis have failed (11, 14, 20). For Aspergillus galactomannan antigen, rapid clearance from the bloodstream, mediated by urinary excretion and hepatic uptake of the antigen, has been shown in rabbits and rats (3, 7). In contrast, Cryptococcus glucuronoxylomannan (GluXM) antigen has been found to circulate in high quantities for prolonged periods in the bloodstream of patients with AIDS and cryptococcosis (8). Candida mannan antigenemia is fluctuating and transitory (11).

A general mechanism for clearing fungal mannans from the bloodstream may consist of mannosyl receptors of macrophages (2) and liver sinusoidal endothelial cells (22-24), which have a greater capacity to endocytose N-acetyl-glucosamine- and mannose-terminated glycoproteins than do Kupffer cells (21). Fibronectin has a role in antigen and immune complex clearance from and uptake by tissues (6). The clinical findings with mannan, galactomannan, and GluXM antigenemia in candidiosis, aspergillosis, and cryptococcosis, respectively (7, 8, 11), suggest differential clearance of the mannan antigens, depending on the side chains of the α(1-6)- or α(1-3)-linked mannose backbone of these polysaccharides. The side chains of Candida mannan antigen consist of α(1-2)- and α(1-3)-linked mannose units (12), those of Aspergillus galactomannan antigen consist of α(1-2)- and β(1-4)-linked galactose units (1), and those of Cryptococcus GluXM antigen (serotype D) consist of β(1-2)-linked gluconuronic acid and β(1-3)-linked xylose (4, 13). The capsular polysaccharides of Cryptococcus neoformans contain a mixture of at least three heteroglycans. However, it appears certain that most, if not all, of the serotype specificity resides in the major acidic polysaccharide GluXM (15). As a consequence, this study dealt with GluXM.

The fate of candidal and cryptococcal antigens in animal models has not been explored in detail. For Candida mannan antigen, this is due partly to the need for extraordinarily high doses to generate detectable antigen levels in serum. In this study, we examined the clearances of Candida mannan antigen and Cryptococcus GluXM antigen in nonimmune rabbits. Furthermore, we determined the fate of Candida mannan antigen in nonimmune mice after injection of ethanol-killed Candida blastospores and after lethal systemic infection with Candida albicans.

MATERIALS AND METHODS

Microorganisms and antigens. C. albicans serotype A (Institute Pasteur Paris, strain 628) was maintained on 2% Sabouraud dextrose agar (Merck, Darmstadt, Germany) containing 0.5% yeast extract (Difco, Detroit, Mich.) at room temperature and transferred weekly. For mouse inoculation, C. albicans was grown on 2% Sabouraud dextrose agar containing 0.5% yeast extract for 48 h at 37°C, harvested in sterile phosphate-buffered saline, and adjusted to the desired concentration with a Neubauer chamber. The Candida suspensions contained >90% blastospores and had a viability of >90%, as determined by plate counts of serial dilutions. Candida mannan antigen was prepared by the method of Peat et al. (19). Briefly, C. albicans blastospores were extracted with 0.02 M citrate buffer (pH 7.0) for 2 h at 121°C; the mannan antigen was precipitated as a copper
complex with Fehling’s solution, redissolved in 3 N hydro-
chloric acid, and finally precipitated with methanol-acetic
acid (8:1, vol/vol). A 195-g (wet cell mass) sample of C. albi-
cans yielded 1.270 mg of mannan antigen. The Candida
manna antigen contained less than 5% protein and varied in
molecular size from less than 30 to 2,000 kDa, with the main
quantity around 200 kDa, as demonstrated with a Sephadex
column. Cryptococcus GluXM antigen (prepared from C.
neoformans serotype D strain 52) was a gift from H. J.
Scholer, Basel, Switzerland.

Animals. Outbred rabbits weighing 3.2 to 4.2 kg were used
in the study. Only rabbits lacking anti-Candida antibodies
(titers of <1:10 with both the indirect hemaggulination and
indirect immunofluorescence tests [both from Hoffmann-
LaRoche, Basel, Switzerland]) were included. Rabbits re-
ceived injections into and were bled from a lateral ear vein.
They were housed in steel cages with urine collectors and
given food and water ad libitum. Male and, for some experi-
ments, female BALB/c mice were purchased from
Charles River Wiga, Sulzfeld, Germany. At the beginning of
the experiments, they were 3 months old and weighed 25 g.
The mice checked for anti-Candida antibodies (one of each
batch) had titers of <1:10 with both the indirect hemaggulina-
tion and indirect immunofluorescence tests; noninfected
mice were negative for Candida antigen as tested by latex
agglutination test (LAT). The 50% lethal dose of C. albi-
cans serotype A (Institut Pasteur Paris, strain 628) for 2-month-
old male BALB/c mice was 106 bacteria injected intra-
venously. The mice received injections by a lateral tail vein
and were sacrificed by ether inhalation and cardiac puncture.
Groups of three to five mice were housed in boxes and given
food and water ad libitum.

Sample preparation. Organs of mice were weighed, and
complete organs were homogenized with 2 ml of 0.25% trypsin in phosphate-buffered saline in a sterile glass homoge-
izer (Braun, Melsungen, Germany). Spleens generally
weighed approximately 120 mg, and livers weighed approxi-
ately 1,000 mg, resulting in dilutions of 1:18 and 1:3,
respectively. All serum samples, urine samples, and organ
homogenates were kept at 4°C for less than 24 h before being
subjected to pretreatment as follows. Samples (300 μl) were
incubated with 50 μl of pronase (33 mg/ml of glycine-buffered
saline; Boehringer, Mannheim, Germany) for 15 min at 40°C
and then for 5 min at 100°C and centrifuged. Supernatants
were used for the antigen detection assays. In some experi-
ments, serum samples were tested without pretreatment.

Antigen detection assays. (i) Latex reagent for Candida
manna antigen detection. Polystyren beads (0.8 μm in
diameter; Serva Unisphere Lyphe Licates, Frankfurt, Ger-
many) were coated overnight at 4°C with polyclonal anti-C.
albicans antibodies, raised in a goat by repeated injections of
C. albicans serotype A (Institut Pasteur Paris, strain 628).
This antibody was a gift from E. Kuttin, Ness Ziona, Israel.
The ready-to-use latex reagent was 0.25% latex particles
with 50 μg of anti-C. albicans immunoglobulin per ml of
glycine-buffered saline.

(ii) Latex reagent for Cryptococcus antigen detection.
Polystyren beads (0.8 μm in diameter; Serva) were coated
overnight at 4°C with polyclonal anti-C. neoformans anti-
odies raised in a rabbit by repeated injections of C. neofo-
rmans serotype D (strain 52). This antibody was a gift from
H. J. Scholer. The ready-to-use latex reagent was 0.25%
lattice particles with 50 μg of anti-C. neoformans immuno-
globulin per ml of glycine-buffered saline. The LAT was
performed as described previously (14). Sensitivities were 8
ng/ml for Candida mannan antigen and 4 ng/ml for Crypto-
coccus GluXM antigen, as determined by serial dilutions of
the antigens in glycine-buffered saline.

Viable Candida counts. CFU of C. albicans in blood and
homogenized organs of mice were determined by plate
counts as described previously (17). Briefly, aliquots of
blood and serial dilutions of organ homogenates in phos-
phate-buffered saline were spread onto 2% Sabouraud dext-
rose agar plates containing 0.5% yeast extract, and colonies
were counted after 48 h at 37°C.

Statistical analysis. Results are presented in part as arith-
metic means with standard errors of antigen concentrations,
which were calculated from titers, LAT sensitivity (8 ng/ml),
and organ weights. Student’s t test for paired samples was
used to calculate the levels of significance.

RESULTS

Antigen clearance in rabbits. (i) Candida mannan antigen.
Three rabbits were each injected intravenously with 20 mg of
Candida mannan antigen. Blood samples drawn 5 min before
antigen injection were negative for Candida antigen. Freely
circulating antigen, as determined by LAT of untreated
serum, rapidly disappeared from the bloodstream during the
first 7 h. After dissociation of protein-antigen complexes
with protease, brief boiling, and subsequent removal of the
denatured proteins by centrifugation, Candida mannan anti-
gen was detectable for a few more hours (Fig. 1A). Filtration
of low-molecular-weight antigens in the urine paralleled
circulation in the bloodstream (Fig. 1B).

(ii) Cryptococcus GluXM antigen. One rabbit was injected
intravenously with 20 μg of Cryptococcus antigen (1,000
times less than Candida antigen). A blood sample drawn 5
min before antigen injection was negative for cryptococcal
antigen. Freely circulating antigen in untreated serum was
never detectable. Pretreatment of serum samples resulted in
prolonged demonstration of circulating protein-bound cryp-
tococcal antigen (Fig. 2). Urinary cryptococcal antigen was
not detected (data not shown).

Candida mannan antigen clearance in mice. (i) Injection of
soluble Candida mannan antigen. Twelve female BALB/c
mice were each injected intravenously with 200 μg of Can-
dida mannan antigen. At 5 and 18 h and days 2, 3, 4, 6, 8, 10,
15, 24, 56, and 97, one mouse each was sacrificed and blood
and organs were examined for Candida antigen. As with
rabbits, Candida mannan antigen was rapidly cleared from
the bloodstream. Antigen titers in serum were 1:16 and 1:1
at 5 and 18 h, respectively, and negative on days 2, 3, 4, and 6.
Antigen titers in kidneys, lungs, and heart paralleled those
in the serum. The titers in the right kidney, left kidney, right
lung, left lung, and heart were 1:4, 1:4, 1:2, 1:1, and 1:1 at 5
h and 1:1, 1:1, 1:2, 1:1, and 1:1 at 18 h and all were negative
on days 2 and 3. The antigen titers in liver and spleen were
approximately 10 times higher (1:256 in the liver and 1:32 in
the spleen at 5 h), they increased during the first 18 h
(reaching 1:500 in the liver and 1:48 in the spleen at 18 h),
and then they gradually dropped (with reciprocal liver titers
of 128, 500, 256, 500, 128, 128, 32, and 2 reciprocal
spleen titers of 16, 32, 64, 32, 8, 32, and 8 and 2 on days 2 to
56, respectively), persisting for as long as 97 days (liver titer,
1:8; spleen titer, 1:2). These data are not shown.

(ii) Injection of ethanolic杀死. Twenty-one female BALB/c mice were injected intravenously with 2 ×
106 ethanol-killed C. albicans blastospores. At 2 and 7 h and
1, 2, 4, 14, and 28 days, three mice each were sacrificed and
pretreated blood serum, livers, and spleens were examined
for Candida antigen. Circulating Candida mannan antigen
Candida mannan antigen in rabbits. Three rabbits were each injected intravenously with a single dose of 20 mg of soluble Candida mannan antigen. Serum (A) and urine (B) samples were tested for Candida antigen with a self-prepared LAT (sensitivity, 8 ng/ml). The data represent the median titers of three rabbits.

bloodstream throughout the study period, with titers ranging from 1:16 to 1:256 (corresponding to antigen concentrations of 0.1 to 2.0 μg/ml, as calculated from the antigen titers and the LAT sensitivity of 8 ng/ml). In livers and spleens, there was marked accumulation of Candida antigen: 52.8 ± 10.7 μg/g in liver and 102.4 ± 35.1 μg/g in spleen at 72 h versus 20.4 ± 5.4 μg/g in liver and 17.4 ± 10.8 μg/g in spleen at 48 h (P < 0.01; Fig. 4B). At 2 h, before generation of Candida foot in kidneys and other organs, the kidneys, lungs, testes, heart, brain, and bile did not contain measurable amounts of Candida antigen. One urine sample at 2 h was negative for Candida antigen, and a second one had a titer of 1:4. One surviving mouse was examined on day 6. The blood culture was negative, and the Candida antigen titer in serum was 1:64; in liver and spleen, the viable counts were 1.7 × 10^5 and 8 × 10^4 CFU/g and the Candida antigen concentrations were 48 and 64 μg/ml, respectively (data not shown).

DISCUSSION

Previous studies have shown that Candida mannan antigen detection in the diagnosis of deep-seated candidosis is inconsistent and unreliable because of the low sensitivities of various detection systems (11, 14, 20). Low sensitivity is due...
to (i) a lower detection limit (generally above 1 ng/ml with common immunoassays) and (ii) fluctuant and transient existence of Candida mannan antigen with different forms and stages of deep-seated candidosis (2, 11). The first point may be overcome by more sensitive methods, but the second point may be a handicap even for methods with a sensitivity well in the picogram-per-milliliter range.

To understand better the transitory character of mannan antigenemia in deep-seated candidosis, we explored first the clearance of soluble mannan antigen after a single intravenous injection. To secure serum samples at least 300 µl in volume in short sequence, rabbits were used in these experiments. Since preliminary experiments had shown that lower quantities of Candida antigen were cleared too rapidly to result in proper elimination data, we injected as much as 20 mg of Candida mannan antigen. In summary, clearance data of three rabbits allowed estimation of the serum half-life of Candida mannan antigen in rabbits as approximately 2 h (Fig. 1A). In contrast, with Cryptococcus GluXM antigen we injected 20 µg, i.e., 1,000 times less than with Candida mannan antigen. Nevertheless, we not only detected the antigen in the early phase but also showed its persistence for up to 48 h (Fig. 2), which indicates a serum half-life on the order of 24 h. These findings support the hypothesis that fungal mannan antigens are cleared by mannosyl receptors (2) since it predicts that Candida mannan antigen, which has low-molecular-mass units on the surface (12), is cleared more rapidly than Cryptococcus GluXM antigen, which has mainly glucuronic acid and xylose on the surface (4, 13, 15). Candida mannan antigen was shown to exist in urine samples, roughly reflecting the amount of circulating mannan antigen (Fig. 1B). Cryptococcal antigen was not detected in urine samples, probably because of the low injection dose of 20 µg with subsequent low titers in the serum. Candida mannan antigen does have low-molecular-mass portions below 30 kDa, the cutoff of the glomerular basement membrane. Our data suggest passive glomerular filtration of low-molecular-mass Candida mannan antigen rather than active renal excretion. The considerable amount of urinary antigen, as estimated from urine titer and volume, compared with the small low-molecular-mass portion of the original mannan antigen (approximately 5%), suggests that Candida mannan antigen is subject to partial in vivo breakdown.

To study the distribution of cleared Candida mannan antigen in organs and clearance from the organs further, we injected mice intravenously with a single dose of 200 µg of soluble Candida mannan antigen. As already shown with rabbits, Candida mannan antigen was rapidly cleared from the bloodstream and not detected in serum samples thereafter. At 5 and 18 h, low antigen titers in the kidneys, lungs, and heart reflected contamination of these organs with serum. In contrast, antigen titers approximately 10 times higher in the liver and spleen increased during the first 18 h. Thus, Candida mannan antigen is cleared from the circulation mainly by the liver and spleen and to a lesser extent by renal filtration. Clearance from the liver and spleen was delayed, as indicated by persisting antigen titers in these organs on day 97 (data not shown). Thus, in vivo breakdown of Candida mannan antigen in the livers and spleens of mice appears to be very limited.

To check these results under conditions closer to the infectious situation, in which mannan is released from intact Candida cells, we injected mice intravenously with 2 × 10⁷ ethanol-killed C. albicans blastospores. By using dead Candida organisms, we were able to inject a dose high enough to allow detailed studies of titers in serum and a prolonged follow-up, undisturbed by Candida multiplication, additional mannan turnover, or premature death of mice. Circulating Candida mannan antigen dropped dramatically during the first 24 h, rose to a second peak on day 4, and thereafter gradually declined until day 28 (Fig. 3). The second peak of circulating Candida mannan antigen on day 4, which did not appear after injection of soluble antigen alone, is probably due to release of mannan antigen from Candida cells already cleared from the circulation by the liver and spleen. As we took no measurements between days 4 and 14, the true peak of circulating antigen may have occurred at any time between days 2 and 14. Accumulation of Candida antigen in liver and spleen, followed by a delayed breakdown, confirmed the previous results obtained with soluble antigen.

To approach the infectious situation as closely as possible, we finally chose a model of acute lethal systemic Candida infection in mice. This model is characterized by a complex array of processes, which all influence the antigen levels in
the bloodstream and the organs: (i) antigen clearance, (ii) multiplication of Candida organisms with establishment of Candida foci in organs, (iii) antigen release, and (iv) death of mice. We avoided a model of subacute, nonlethal candidosis, because in preliminary experiments antigen levels had been very low to undetectable, and we accepted the limitation of the study period due to early death of the mice. Negative blood cultures as early as 48 h after infection confirm the unsuitability of blood cultures for the diagnosis of deep-seated candidosis. Viable Candida burdens of the liver and spleen also dropped during the first 24 h. However, there was no complete elimination of viable Candida organisms, thus leaving a source of continuous antigen release and leading to persisting antigenemia. By day 3, mannan concentrations in the liver and spleen had increased significantly (Fig. 4B), probably because of trapped antigen released (Fig. 4B), probably because of trapped antigen released (Fig. 4B).

We did not examine fungal antigen clearances in immune animals. Most patients at risk for deep-seated candidosis do have circulating antifungal immunoglobulins G, M, and A. Circulating immune complexes have been demonstrated in association with human candidosis (5, 14), and release of specific mannann antigen-antibody complexes has been shown by immune electron microscopy in chronic human vaginal candidosis (16). Thus, Fc receptors of liver sinusoidal endothelium (18) will play a considerable additional part in the clearance of Candida immune complexes from the circulation of patients.

Our model of lethal infection provides further evidence for the existence of circulating mannann antigen in disseminated candidosis. However, persistently measurable antigen levels could be generated only by an infective dose far beyond the 50% lethal dose. This corresponds to the clinical observation of regular antigenemia only in patients with terminal overwhelming disseminated candidosis (11). With many clinical cases of deep-seated candidosis, especially early stages and diseases confined to certain organs, mannann clearance will be more effective than release of mannann antigen from Candida foci into the circulation. Therefore, some patients suffering from deep-seated candidosis may never enter a mannann turnover stage, which could result in persistent circulating antigen levels.

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REFERENCES

11. Fujita, S., F. Matusbara, and T. Matsuoka. 1986. Enzyme-linked immunosorbent assay of mannan in serum, and antigenuria neither preceded nor outlasted antigenemia. Our data do not confirm any advantage of urinary antigen testing over serum antigen testing, as has been suggested for several disseminated fungal diseases, including candidosis (10), aspergillosis (7), and histoplasmosis (25).