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Protective Immunity Against Murine Candidiasis Elicited by *Candida albicans* Ribosomal Fractions

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Candida albicans ribosomes were prepared from mechanically disrupted cells through differential centrifugation and purification in a sucrose-ammonium sulfate solution. The ribosomes were analyzed chemically and physically and exhibited characteristics of eucaryotic ribosomes (78S). ICR female mice were immunized with two subcutaneous inoculations, 2 weeks apart, of 100 μg of ribosomes (expressed as ribosomal protein). Immunized mice were challenged either intraperitoneally or intravenously with a lethal dose of live *C. albicans* cells. The 31-day survival rate of immunized mice challenged intraperitoneally was 64% (mean value) versus 27% in controls; in intravenously challenged mice the survival rate of the immunized animals was about 60%, with no survivors among the controls. In intravenously challenged mice, incomplete Freund adjuvant enhanced the protection elicited by the ribosomes. Protection by ribosomal immunization was obtained against challenge doses causing chronic and acute infection.

It is well established that candidiasis is a prevalent mycotic opportunistic infection of the compromised host (3, 19). As such, it is a medical problem in patients with malignancies (8) or burns (15), after renal transplantation (21) or intravenous (i.v.) hyperalimentation (2), and in patients with other debilitating conditions. Besides the fact that candidiasis is difficult to diagnose in such patients, the chemotherapeutic treatment currently available can be toxic and is generally not recommended for prophylactic use. Thus, prophylaxis through immunization would appear to be a logical approach. Efforts to prevent experimental candidiasis through vaccination with live (6, 9, 18, 30) or killed (6, 18) *Candida albicans* cells did not lead in most instances to a significant protection against lethal challenge with live organisms; partial protection was reported for immunization with a *C. albicans* cell sonicate (18). When partial protection was obtained, it was expressed mainly in prolongation of life of the infected animals.

Vaccination with ribosomes has been shown to protect against experimental infections with numerous microorganisms, including various bacterial species (5, 12, 14, 22, 24, 34, 36, 37), certain parasites (11), and the dimorphic fungus *Histoplasma capsulatum* (4, 32, 33); vaccination with ribosomes has already reached the stage of clinical trials (17).

We recently reported the results of preliminary studies showing that *C. albicans* ribosomal preparations are immunogenic (26) and potentially protective in mice against an intraperitoneal (i.p.) challenge with live *C. albicans* cells (27). The present work continues these studies, and we describe a systematic investigation of the protective ability of *C. albicans* ribosomes in different models of experimental systemic murine candidiasis.

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MATERIALS AND METHODS

Microorganism and preparation of cultures. *C. albicans* CBS 562, obtained from Centralbureau voor Schimmel-Cultures, Delft, Holland, was maintained on yeast extract agar and subcultured monthly.

For i.p. challenge *C. albicans* cells were grown in M 199 medium for 18 h at 28°C with continuous shaking. Cells were collected by centrifugation, washed three times with sterile saline, counted in a hemacytometer, and suspended in saline or 5% gastric mucin (Sigma Chemical Co.) to the desired concentration.

For i.v. challenge *C. albicans* cells were grown in yeast extract broth for 18 h at 28°C with continuous shaking and prepared in a way similar to that used for i.p. challenge. The exact concentration was checked by viable count.

Preparation of crude ribosomes. *C. albicans* cells were grown in 6-liter batches for 18 h in yeast extract broth at 28°C in a gyratory shaker (New Brunswick Scientific Co.), with a shaking speed of 150 rpm (25). The ribosomes were prepared by a modification of the procedure of Rubin (23). Briefly, this consisted of harvesting the organisms by centrifugation at 4°C, washing three times with cold tris(hydroxymethyl)aminomethane-hydrochloride 0.01 M buffer containing 0.1 M NaCl and 0.03 M MgCl,

pH 7.4 (TMB), and resuspending in three volumes of buffer. The cells were then broken mechanically with glass beads in a Braun (MSK) cell homogenizer (two 1-min shaking periods) under liquid CO_2 cooling. The disrupted cell mass was centrifuged at 10,000 × *g* for 30 min to remove intact cells, glass beads, and cell debris, and the supernatant was recentrifuged twice at 30,000 × *g* for 30 min to remove any remaining cell debris. The final supernatant was collected and layered on 15% sucrose and 5% ammonium sulfate in TMB. The crude ribosomes were obtained by centrifugation at 27,000 rpm for 20 h in a Beckman ultracentrifuge (SW27 rotor, 4°C). The ribosomal pellet was suspended in TMB and, after an additional clearing (centrifugation at 30,000 × *g* for 30 min), maintained in small samples at −80°C until used.

Physical and chemical analysis of ribosomal preparations. Protein was determined by the method of Lowry et al. (13) with bovine serum albumin (fraction V; Sigma) as the standard. Ribonucleic acid was measured by the orcinol procedure (10) with yeast ribonucleic acid (Sigma) as the standard. Deoxyribonucleic acid was determined by the diphenylamine assay (1) with calf thymus deoxyribonucleic acid (Calbiochem) as the standard.

Sedimentation analysis was performed by using a Beckman model E analytical ultracentrifuge equipped with Schlieren optics (31). Sucrose density analysis was performed by layering 0.1 ml of the crude ribosomal suspension on a 15 to 30% sucrose gradient in TMB and centrifuging at 149,000 × *g* for 1 h at 4°C. Fractions of 0.1 ml were collected and read at 260 and 280 nm. Ribosomal subunits were obtained by centrifugation over a 10 to 25% sucrose gradient in low-Mg^{2+} (10^{-5} M) buffer at 22,000 rpm (SW27 rotor, 4°C) for 17 h (28, 35).

Immunization and challenge. Six-week-old female ICR mice were used in this study. The optimal immunization schedule was determined in preliminary experiments. This consisted of injecting mice subcutaneously with 100 μg of ribosomal suspension (expressed as ribosomal protein content), with or without incomplete Freund adjuvant (IFA), into two hind footpads and on both sides of the abdomen 2 weeks later. The immunized mice were challenged i.p. with live *C. albicans* cells at a concentration determined in preliminary experiments; the inoculum consisted of 4×10^7 or 8×10^6 organisms per mouse in 0.2 ml of saline or 5% mucin, respectively. This resulted in a chronic infection in which the mice showed signs of illness 1 week postchallenge and began dying about 7 to 10 days postchallenge, with death occurring up until 30 days after challenge. The i.v. challenge was carried out by injecting mice in the lateral tail vein with either 10^4 or 10^5 live *C. albicans* cells per mouse (in 0.2 ml of saline), yielding a chronic or acute infection, respectively. Acute infection was defined as a disease in which the animals began dying in the first week postchallenge.

RESULTS

Characteristics of ribosomes. Spectral analysis revealed that the ratio between absorbance at 260 nm and at 280 nm was 1.9, and the ratio between absorbance at 260 nm and at 235 nm was 1.5. Sedimentation analysis of the ribosomes showed one sedimenting particle with a sedimentation coefficient of 78S, which is comparable to values found for other yeasts (31).

As shown in Fig. 1, ultracentrifugation of the ribosomal preparation on a sucrose gradient at a high Mg^{2+} concentration yielded a single peak. Dissociation into two ribosomal units was obtained by centrifugation on a sucrose gradient at a low Mg^{2+} concentration. Chemical analysis of the ribosomal preparation indicated a protein/ribonucleic acid ratio of approximately 1:1; deoxyribonucleic acid was not detected.

Protection against i.p. challenge. The first stage of our study consisted of determining the eventual protection of mice immunized with ribosomes versus nonimmunized mice challenged i.p. with live *C. albicans* cells. Preliminary results of immunization of mice with ribosomes and i.p. challenge showed that optimal results were obtained by immunization with ribosomes without adjuvant and by the administration of challenge 2 weeks after booster immunization. Table 1 summarizes the results obtained from seven independent experiments involving 140 animals. At day 30 postchallenge the survival rate of the immunized mice ranged from 46 to 90% as compared with 14 to 38% in controls, indicating statistically significant ($P < 0.01$) protection in the immunized mice. No difference in protection was observed between mice challenged with *C. albicans* cells suspended in saline or in mucin, although the number of organisms was different, indicating that immunization was effective even at this challenge dose (4×10^7 *C. albicans* cells per mouse).

Protection against i.v. challenge. The next stage in our study was to investigate the efficacy of ribosomal vaccination against an i.v. challenge with live *C. albicans*. Table 2 presents the 30-day survival rate of mice immunized with ribosomes or ribosomes emulsified in IFA and challenged with various concentrations of live *C. albicans* cells. Whereas control mice (inoculated with buffer only) did not survive the challenges, the survival rate of mice inoculated with ribosomes or ribosomes in IFA ranged from 22 to 67%. The data show that significant protection ($P < 0.01$) was obtained by immunization with ribosomes with and without IFA when the animals were challenged with 10^4 organisms per mouse (causing chronic infection), whereas at a challenge dose of 6×10^4 to 1×10^5 organisms per mouse (causing acute infection), significant protection was obtained only by ribosomes in IFA. In view of the finding that addition of IFA increased the efficacy of the vaccination, the

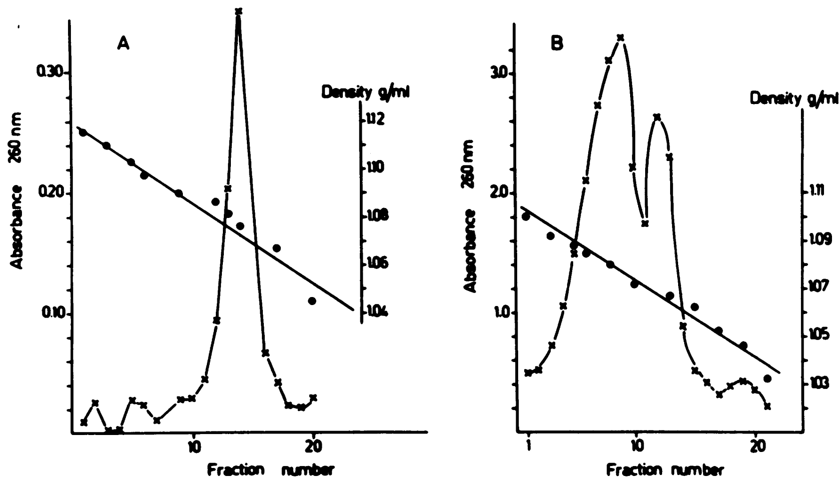

FIG. 1. *Sucrose gradient sedimentation of C. albicans ribosomal preparation. A, Single ribosomal peak sedimented by centrifugation in tris(hydroxymethyl)aminomethane buffer with 0.03 M Mg^{2+}. B, Separation of the ribosomal subunits by sucrose density gradient in tris(hydroxmethyl)aminomethane buffer with 10^{-5} M Mg^{2+}.*

TABLE 1. *Protection of mice immunized with ribosomes against i.p. challenge with live C. albicans cells*[a]

Expt. no.	No. of organisms injected/mouse	Cells suspended in:	30-Day survival of mice: Immunized Survivors/total	Immunized %	Control Survivors/total	Control %
1	4×10^7	Saline	4/7	57	1/7	14
2	4×10^7	Saline	5/7	71	2/7	29
3	8×10^6	Mucin	9/10	90	2/9	22
4	8×10^6	Mucin	7/10	70	3/10	30
5	8×10^6	Mucin	7/10	70	3/10	30
6	8×10^6	Mucin	8/15	53	2/10	20
7	8×10^6	Mucin	7/15	46	5/13	38
Total			47/74	64	18/66	27

[a] Statistical analysis was performed by the Mantel and Haenszel test (16), and the protection of immunized mice as compared with controls (mice inoculated with buffer) was significant ($P < 0.01$).

TABLE 2. *Protection of mice immunized with ribosomes against i.v. challenge with live C. albicans cells*[a]

Inoculum	No. of organisms injected/mouse	30-Day survival of mice: Survivors/total	%	Significance (P)
Ribosomes plus IFA	1×10^4	13/21	62	<0.01
Buffer plus IFA	1×10^4	4/19	21	
Ribosomes plus IFA	1×10^5	8/12	67	<0.01
Buffer plus IFA	1×10^5	0/11	0	
Ribosomes	1×10^4	10/22	45	<0.01
Buffer	1×10^4	0/22	0	
Ribosomes	6×10^4	2/9	22	0.26[b]
Buffer	6×10^4	0/8	0	

[a] Statistical analysis was performed by Fisher's exact test (29).
[b] Not significant.

subsequent immunizations were performed with ribosomes suspended in IFA.

Based on the results of i.p. challenge experiments, the i.v. challenge was administered at the same time, 2 weeks after booster immunization. We tested whether this schedule was indeed appropriate also for i.v. challenge, and the results are presented in Table 3. No significant difference in the survival rate of the immunized mice was observed when challenge was administered at days 4, 14, and 19 after booster immunizations. Thus, the pattern of a 2-week interval between immunization and challenge was continued.

DISCUSSION

The results of our experiments indicate that *C. albicans* ribosomes have the ability to protect mice against a lethal challenge administered either i.p. or i.v. This protection is only partial, as expressed by a survival rate of 64% in immu-

TABLE 3. *Protection of mice immunized with ribosomes against i.v. challenge administered at various days postimmunization*[a]

Day postimmunization	30-Day survival of mice					
	Immunized		Control 1[b]		Control 2[c]	
	Survivors/total	%	Survivors/total	%	Survivors/total	%
4	12/20	60	4/14	29	0/14	0
14	5/9	56	2/7	29	0/7	0
19	8/12	67	2/12	17	0/10	0

[a] Mice were challenged with 10^4 live *C. albicans* cells per mouse.

[b] Mice inoculated with buffer plus IFA.

[c] Mice inoculated with buffer.

nized animals challenged i.p. versus 27% in controls and a survival rate of above 60% in immunized mice challenged i.v. versus zero survival in controls; it is, however, significant.

Our data also demonstrated that IFA increased the protection in the i.v. challenge model. Moreover, addition of IFA increased the efficacy of the ribosomal vaccination so that protection against challenge causing even an acute infection was obtained. These data are consistent with the observations of others that Freund adjuvant may or may not enhance the protection elicited by ribosomes (12, 22, 24, 33, 34), a phenomenon probably dependent on the organism or experimental system used (or both). Since in our system Freund adjuvant increased the efficacy of ribosomal vaccination, we plan in the future to try other adjuvants more suitable for human use.

The protection rate obtained in our experiments was lower than that reported for ribosomal vaccine in experimental histoplasmosis (32, 33). It must be noted, however, that whereas protection against histoplasmosis can be induced by immunization with live organisms, which is of the same degree as that induced with ribosomal vaccination (33), the outcome of candidiasis immunization with killed or live organisms is lower or nil as compared with that provided by ribosomes in our system. Moreover, the experimental system of Giger et al. (7), in which protection was induced by live organisms, has limited applicability for clinical use.

In the course of the ribosomal vaccination study, the humoral and cellular immune responses of the immunized animals were analyzed. The results of the humoral immune responses were briefly reported previously (26), and the results of the cellular immune responses are being prepared for publication.

Further studies will investigate ways to elevate the protection, to possibly transfer the protective immunity, and to determine the component in the ribosomal fractions responsible for protection.

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