Stimulation of Interferon Production by Mannans (34902)

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Several interferon-stimulating agents such as bacteria and their endotoxins contain polysaccharides as constituents [for review see (1)]. Their role in the interferon-stimulating capacity of the preparation is unclear (2). The finding that a highly purified mannan from *Candida albicans* may function as a stimulus to interferon production in leukocytes lends support to the view that polysaccharides may by themselves play a triggering role in interferon release (3). However, only equivocal results have been achieved with this dose of compounds in other laboratories (4).

In this paper we present the results of further experiments aimed at a further understanding of the interferon-stimulating capacity of purified mannans obtained from *C. albicans* and/or from *Saccharomyces cerevisiae*, as well as of nonpurified polysaccharide–protein complexes from *C. albicans*, and of a lipoprotein fraction isolated from this microorganism.

These studies confirm that it is the polysaccharide itself, rather than impurities, which stimulates the release of interferon from leukocytes. Furthermore, they demonstrate that such factors as differences of species of microorganisms employed as a source of mannans and the molecular weight of preparation may influence the interferon-stimulating activity.

Materials and Methods. Substances tested as interferon stimulants. A. Purified mannans from *C. albicans* Berkhout strain 109: (a) cell-wall mannan with 1% N (code "CB"); (b) cell-surface mannan with 1% N (code "ED"); (c) cell-surface mannan with 0.4% N (code "El"); (d) mannan isolated from culture medium (code "ZP"). The molecular weight of purified mannans was estimated between 5500–20,000. The dried preparations were kept at room temperature in tightly stoppered tubes between tests.

B. Purified mannan from *S. cerevisiae* with indetectable N and a molecular weight of about 50,000.

C. Polysaccharide–protein complexes from *C. albicans* Berkhout strain 109: (a) polysaccharide–protein complex Nos. 4 and 9 from the cell wall with a molecular weight of 22,000 and 17,000, respectively; (b) polysaccharide–protein complex Nos. 1 and 3 from the cell wall after treatment with 2% NaOH (glucan–protein complexes) with a molecular weight of about 6500; (c) polysaccharide–protein complex Nos. 5 and 6 from cell surface with a molecular weight of 5800; (d) polysaccharide–protein complex Nos. 2 and 8 with a molecular weight of about 210,000.

The isolation procedure and the characterization of mannans and of polysaccharide–protein complexes was described by Masler *et al.* (5) and Sikl *et al.* (6, 7).

D. A lipid fraction from *C. albicans* was obtained by the method of Letters (8).

Stimulation and titration of interferon. (a) *In vitro.* Mouse peritoneal cells (2×10^6/ml of medium 199 with 5% calf serum) were treated with polysaccharide (10–100 μg) immediately after explantation. The cells were kept at 26° overnight. The interferon activity of the supernates was titrated on L-mouse fibroblasts against encephalomyocarditis virus (EMC) (9).

It has been found previously that mannans in the amounts employed neither neutralize EMC virus nor induce interferon in L-cells. (b) Mice of Decin strain (15–20 g) were injected with preparations tested into a tail

vein with a 0.3-ml volume (35–300 μg)/mouse. They were exsanguinated from the orbital plexus 2 hr after injection. Concurrently, the spleens of injected mice were assayed as supernates from a 25% emulsion in medium 199. (c) Cycloheximide (Upjohn Co., lot No. St. 713) was injected intraperitoneally in 2.5–5 mg amounts/mouse 1 hr before the mice were injected with the polysaccharide preparation intravenously (10).

Chemical analysis of polysaccharide preparations. The following methods were used for quantitative analysis of preparations: the methods of Dumas and of Kjeldahl as modified by Colson (11) and Markham (12), respectively, for determination of nitrogen; the method of Fiske and Subbarow (13) for the determination of phosphorus; the method of Dubois *et al.* (14) as well as paper chromatography were used for determination of sugars, and the method of Stimson and Reuter (15) was used for estimation of nucleic acids. The ratio of mannose to glucose was estimated by quantitative paper chromatography. The molecular weight of preparations was calculated from the sedimentation constant, diffusion coefficient, and the partial specific volume. The results of analysis are shown in Table I. Estimation of double-stranded ribonucleic acid in preparations was performed according to Lampson *et al.* (16) using a crystalline ribonuclease from bovine pancreas (Reanal, Budapest, lot 6202763). The effect of hydroxylamine hydrochloride (Lachema, Brno) on nucleic acids was estimated according to Skehel and Burke (17).

TABLE II. The Interferon-Stimulating Capacity of Purified Mannans (100 μg/2 × 10^6 cells).[a]

Sample (code)	Year of isolation	Source	Interferon titer (range)
CB	1967	*C. albicans*	8–32
ED	1967	*C. albicans*	16–64
El	1966	*C. albicans*	16–256
ZP	1966	*C. albicans*	8–64
S. cer.	1967	*S. cerevisiae*	<4

[a] The molecular weights of mannans from *C. albicans* were found between 5500 (El) to 20,000 (ZP); the molecular weight of mannan from *S. cerevisiae* was 50,000.

Results. The chemical composition of polysaccharide preparations from yeasts and their interferon-stimulating capacity. It follows from Table II that, while all mannans isolated from *C. albicans* possessed the capacity to stimulate interferon release from mouse peritoneal leukocytes, the mannan obtained from *S. cerevisiae* proved inactive repeatedly in this respect. The reason for this difference is unknown. The interferon-stimulating capacity of various mannans showed a fluctuation in repeated tests and, when compared with protocols from 1967, they showed a decreasing activity on storage at room tempera-

TABLE I. Characterization of Polysaccharide–Protein Complexes from *C. albicans* Berkhout.

Complex[a] (code no.)		Percentual content of: Ash	Nitrogen	Phosphorus	Nucleic acids	Sugar	Mannose–glucose ratio	Mol wt	Interferon titer (range)
ZP	2	21.5	3.6	1.64	2.9	77.4	2:1	210,000	<4–8±
	8	13.6	2.44	0.92	4.5	84.5	2:1	210,000	<4–8±
C	1	15.8	1.22	0.20	1.8	92.2	—:1	ND[b]	4–16±
	3	13.1	2.31	0.63	3.5	85.3	—:1	6500	<4–<4
	4	4.39	3.44	1.0	13.0	77.9	2:1	22,400	64–256
	9	2.96	2.69	0.63	6.1	82.7	2:1	17,000	128–512
E	5	ND	3.85	1.44	5.7	72.5	ND	5800	32–64
	6	6.96	3.48	ND	4.9	81.2	ND	ND	64–128

[a] The complexes were prepared in 1967.

[b] ND: not done.

ture. However, this observation may reflect a changed reactivity in the mouse strain used as the source of leukocytes.

The polysaccharide–protein complexes from *C. albicans* also differed in their interferon-stimulating activity. As shown in Table I, no or only a slight interferon-stimulating activity has been detected with preparation Nos. 2 and 8 isolated from the culture medium of *C. albicans*, as well as in glucan–protein complex Nos. 1 and 3 which were obtained after treatment of cell-wall polysaccharide–protein complexes with 2% NaOH. The glucan–protein complexes—especially the preparation No. 3—proved toxic when injected into mice.

The chemical analysis of polysaccharide–protein complexes showed no correlation of interferon-stimulating activity with any constituents which were studied. However, it seems probable that to be active, the mannan and/or mannan–protein complexes must possess a molecular weight of a certain range. This is suggested by the finding that both the mannan from *S. cerevisiae* (mol wt 50,000) and the polysaccharide–protein preparations with a molecular weight above 200,000 (obtained from culture medium) were inactive as interferon stimulants (Table I). The highest titers of interferon were observed when preparation Nos. 4 and 9, with molecular weights 22,400 and 17,000 respectively, were used for interferon stimulation. These preparations in contrast to glucan–protein complexes showed a high degree of solubility.

The possible role of nucleic acids and lipids in interferon-stimulating activity of polysaccharidic preparations. The possibility that nucleic acids present in the preparations might be responsible for the interferon-releasing capacity was tested by two methods.

Selected preparations of polysaccharide–protein complexes were treated with ribonuclease at 24 and 56° according to Lampson *et al.* (16). As shown in Fig. 1, the adsorbancy of ribonuclease-treated preparation Nos. 1 and 4 suggests the single-stranded character of nucleic acids present, while the 24° curve in preparation No. 9 suggests the presence of double-stranded fragments also. Nevertheless, the interferon-stimulating activity of preparations was not altered by ribonuclease treatment (Table III).

As shown in Table III, hydroxylamine treatment (17) did not alter the interferon-stimulating capacity of preparations tested.

Taken together, these findings suggest that nucleic acids, single- or double-stranded, do not determine the interferon-releasing capacity of mannon-containing preparations.

Not more than 1% lipoprotein was found in the most active polysaccharide–protein preparation Nos. 4 and 9, and no lipid could be detected in purified mannans. This makes the role of lipid in the activity of these preparations highly improbable. Nevertheless, it seemed interesting to examine the interferon-stimulating capacity of a liproprotein with about 0.2% N obtained from *C.*

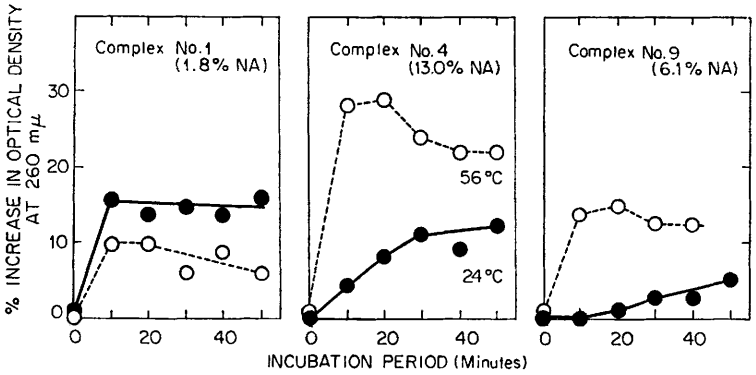

FIG. 1. Comparative rates of degradation of polysaccharide–protein complexes by ribonuclease at 24 and 56°, respectively.

TABLE III. Effect of Ribonuclease and/or Hydroxylamine Treatment on Activity of Mannans and Polysaccharide–Protein Complexes.

	Interferon titer after stimulation of leukocytes with preparations (100 μg/2 × 10^6 cells)			
	ED[a]	El[a]	No. 9[b]	Ribonuclease
Control (before treatment)	32	16	128	4±
After RNase treatment (100 μg):				
(24°, 30 min)	32	8	64	
(56°, 2 hr)	32	8	64±	
Hydroxylamine (0.2 *M*, pH 7)				
after 0 hr	—[c]	—	64	
1 hr	—	—	64	
1.5 hr	—	—	64	

[a] Purified mannans from *C. albicans*.
[b] Polysaccharide–protein complex from *C. albicans*.
[c] —, not done.

albicans. At a concentration of 1.64 mg/2.5 × 10^6 leukocytes, the lipid fraction showed a minimal interferon-stimulating activity in repeated tests (titers 1:4–1:16±/ml). However, it was inactive at lower concentrations and, on a quantitative base, the activity of this preparation fell far below the activity of mannans.

The interferon-stimulating activity of mannans and mannan–protein complexes in vivo. In contrast to the tests performed *in vitro*, the interferon-inducing capacity of purified manans *in vivo* proved to be a less regular phenomenon. The preparation El, which in 1967 stimulated interferon production when injected intravenously at 100–300 μg into mice, was ineffective in 1969 (higher amounts were not tested). As mentioned above, changes in preparation during storage as well as an altered reactivity of the mouse strain employed might be responsible for this observation.

Among polysaccharide–protein complexes tested at 500 μg/mouse level, the best results *in vivo* were obtained with preparation Nos. 5 and 9. Preparation Nos. 1, 3, and 4 were tested with negative results at a lower level (35–200 μg/mouse). In this respect, it is interesting that employing a technique introduced by Youngner and Feingold (10), a significant increase of serum interferon was observed after injection of polysaccharide–protein complex No. 9 into cycloheximide-treated mice. Interferon was also detected in cycloheximide-treated mice injected with purified mannan ED which gave negative results in normal mice (Table IV).

Discussion. The mechanism by which mannans stimulate interferon release from leukocytes resembles that of endotoxins (3). However, in contrast to the latter, mannans showed minimal, if any, toxicity and they are poor antigens (18). These properties are desirable from a practical point of view.

In the present study we have compared the

TABLE IV. Effect of Cycloheximide Pretreatment on Resulting Interferon Titers in Mice Injected with Mannan or Mannan–Protein Complex.[a]

	After injection (hr)	Interferon titer in serum
Cycloheximide alone (2.5 mg/mouse)	2	16
	5	64
Polysaccharide–protein complex No. 9 (300 μg/mouse)	2	<16–32±
Cycloheximide + No. 9	2	<16–16
	5	256
Mannan ED (300 μg/mouse)	2	16
Cycloheximide + ED	2	32
	5	64–128±

[a] Cycloheximide was injected intraperitoneally 1 hr before intravenous injection of mannan and/or mannan–protein complex.

interferon-stimulating activity of several batches of purified mannans isolated from *C. albicans* and from *S. cerevisiae,* as well as that of mannan–glucan–protein complexes containing various amounts of N, P, nucleic acids, and ash.

While all preparations of purified mannans from *C. albicans* were, to a varying degree, active interferon-stimulating agents in mouse peritoneal leukocytes *in vitro,* the mannan isolated from *S. cerevisiae* showed no activity in repeated tests. This finding suggests that species differences most probably due to the structural relationships of mannose residues in the polymer might play a significant role in determining the interferon-stimulating capacity of mannans. A similar conclusion has been reached by Suzuki *et al.* (19) when studying the serological activity of mannans.

Since the titers of interferon obtained with stored preparations were lower than those found in 1966–67 when they were first prepared, these polymers may not be stable under our conditions of storage. A similar phenomenon has been observed by Came *et al.* (20) in studies with polyvinyl sulfate. However, it seems equally possible that during the long study period, a change occurred in the interferon responsiveness of the mouse colony.

In this study no correlation between interferon-stimulating activity and the presence of nonsaccharide constituents in the polysaccharide–protein complexes could be observed. In view of the fact that both nucleic acids (16) and glycolipids (2) were reported as interferon stimulants, the role of these potential contaminants of mannan preparations were tested. The attempts to inactivate the nucleic acids present in the preparations by ribonuclease or hydroxylamine did not alter the interferon-stimulating activity of our mannan preparations.

A lipid fraction from *C. albicans* at 10–100-fold greater concentration than the effective dose of purified mannans showed a low but consistent interferon-stimulating activity. However, the role of lipids in determining the activity of mannans seems to be unlikely as they could not be detected in active preparations of purified mannans, and in polysaccharide–protein complexes they did not exceed 1% in amount.

The experiment supports the view that it is the mannan itself which triggers by an unknown mechanism the production of interferon from leukocytes. The most active preparations were found in a molecular range between 5500 to 20,000. Samples with a higher molecular weight such as the mannan from *S. cerevisiae* and the complex Nos. 2 and 8 were devoid of any interferon-stimulating activity. This suggests that in addition to the primary structure differences of mannans isolated from *C. albicans* and/or from *S. cerevisiae,* the secondary structure of the molecule may play a determining role in its activity. The possibility of alteration of the secondary structure with increasing molecular weight is often discussed in the polymer literature (21). Recently, it has been suggested by De Clercq *et al.* (22) that the enhancement of interferon-inducing ability might be related to a better binding of inducer to cell receptors. It might be that the effective binding of higher molecular weight preparations was hindered in our experiments.

Summary. Four preparations of purified mannans from *C. albicans* Berkhout, one purified mannan from *S. cerevisiae,* a lipid fraction from *C. albicans* Berkhout, and eight polysaccharide–protein complexes from the same microorganism were tested for interferon-stimulating activity in explanted mouse peritoneal leukocytes. Whereas the mannan from *S. cerevisiae* was inactive, the purified mannans as well as the polysaccharide–protein complexes from *C. albicans* with a molecular weight around 20,000 regularly demonstrated an ability to stimulate interferon release. Glucan–protein complexes were inactive. No correlation between N, P, and nucleic acid content of preparation and its interferon-stimulating activity was found. Ribonuclease or hydroxylamine treatment did not alter the activity of these preparations. A lipoprotein fraction from *C. albicans* examined as a potential contaminant showed a low degree of activity. It was concluded that it is the mannan itself which stimulated the interferon production from leukocytes.

Possible explanations from different activity of various mannans are considered.

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