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Defective handling of mannan by monocytes in patients with chronic mucocutaneous candidiasis resulting in a specific cellular unresponsiveness

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SUMMARY

Carbohydrate antigens from *Candida albicans*, essentially mannan, have previously been shown to persist in the serum of some patients with chronic mucocutaneous candidiasis, and to be able to inhibit specifically the candida antigen-induced proliferation of control lymphocytes. Lymphocytes from three out of six patients were shown to be hypersensitive to mannan inhibition. These data were explained by the demonstration of an apparently selective impairment of radiolabelled mannan handling by two patients' monocytes following a normal uptake. This defect was observed both in active and remission phases of the infection suggesting an intrinsic defect of patients' monocytes. In experiments performed with control lymphocytes, it was shown that mannan exerted its suppressive effect by interfering with candida antigen presentation by adherent cells to autologous T lymphocytes. Furthermore, mannan neither was cytotoxic nor induced suppressor T cells. Altogether, these data suggest that the *in vivo* persistance of mannan, in some patients, is secondary to a primary macrophage dysfunction leading to impairment of specific cellular immune responsiveness.

INTRODUCTION

The main immunological feature of chronic mucocutaneous candidiasis (CMCC) is a specific cellular immune unresponsiveness to candida antigens (CA). This has been shown by the absence of proliferation *in vitro* to CA, the inability of CA to induce migration inhibitor factor and the skin anergy (Edwards *et al.*, 1978). This defect of unknown origin, frequently disappears with the clearing of candida infections (Aronson & Soltani, 1976; Valdimarsson *et al.*, 1973). In some patients, a serum inhibitory activity (SIA) has been described which inhibits CA-induced lymphocyte proliferation (Valdimarsson *et al.*, 1973). We have previously shown in six patients that SIA is related to antigenic carbohydrate structures from *candida albicans*, especially mannan, a major constituent of yeast cell wall (Fischer, Ballet & Griscelli, 1978). Mannan itself, was shown to exert a specific suppressive activity on CA-induced lymphocyte proliferation. Two major points remained unclear. Firstly, why does mannan (or other candida carbohydrates) abnormally persist

Abbreviations: CA = Candida antigens; CMA = Candida metabolic antigen; CMCC = Chronic mucocutaneous candidiasis; E = Erythrocyte; MW = Molecular weight; PPD = Protein pure derivative; SIA = Serum inhibitory activity; ^3H = Tritium.

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in some patients? Secondly, what is the mechanism by which mannan inhibits specific T cell responses? We report here a comparison of the handling of radiolabelled mannan and S III pneumococcal polysaccharide by patients and control monocytes, together with studies of the interaction of mannan with control T lymphocytes and antigen presenting cells.

MATERIALS AND METHODS

Patients. Six patients with CMCC, already described (Fischer *et al.*, 1978), were studied in active and remission phases of the disease (Table 1). Three of them (patients 1, 2 and 3) were found to have a serum inhibitory activity (SIA) able to inhibit specifically candida antigen-induced lymphocyte proliferation *in vitro*. In these patients, *in vitro* and *in vivo* specific cellular immunity to candida antigens was undetectable in active phase of the disease while normal specific *in vitro* (lymphocyte proliferation) and *in vivo* (delayed skin reaction to candida) responses were found in the remission phase. The three other patients (patients 4, 5 and 6) had a specific cellular immune defect but no SIA could be found.

Candida antigens. Candida metabolic antigen (CMA) was purchased from the Institut Pasteur (Paris, France). Its characteristics have been described previously (Fischer *et al.*, 1978).

Carbohydrates. Mannan from *Candida albicans A* was a generous gift from Dr H. F. Hasenclever, NIH, Hamilton, Montana (Peat, Whelan & Edwards, 1961). S III pneumococcal polysaccharide (MW 350,000) was donated by Dr Jaton (Hopital Cantonal, Geneva, Switzerland).

Labelling of carbohydrates. Mannan and SIII pneumococcal polysaccharide were subjected to the catalysed exchange in solution with tritium (^3H) as described by Evans *et al.* (1974). This procedure was reported to label reducing sugars and polysaccharides exclusively at C_1 in high yield and relatively high specific activity. Carbohydrates (5–15 mg) in 1 ml solution of 0·1 M sodium phosphate buffer pH 7 and 20–25 mg of $PdO/BaSO_4$ catalyst (Kuhn & Hass, 1965) were stirred under 97% pure tritium gas (10–15 Curies) for 2–5 hr. Following this exchange, the catalyst was filtered off. Labile tritium was removed *in vacuo* by two successive evaporations of a 50:50 mixture of methanol:water added to the reaction exchange mixture. ^3H-mannan was purified by size exclusion chromatography on a column of Ultrogel ACA (LKB, Sweden). ^3H-pneumococcal polysaccharide was purified on a column of Sephadex G-100 (Pharmacia Fine Chemicals Inc., Uppsala, Sweden). Elutions in both cases were carried out with 0·01 M sodium chloride solution. Solutions ($\neq$0·2 mCi/ml) were sterilized by filtration on millipore filter and stored at +4°C. After

Table 1. Cellular immune responsiveness to candida antigens and other antigens of 6 patients with CMCC

Patient	Serum inhibitory* activity	Proliferative responses‡ to CMA	Proliferative responses‡ to Other antigens†	Skin reactivity Candidin	Skin reactivity Other antigens†	Reversal of cellular§ immune defect by infection clearing
1	+	−	+	−	+	+
2	+	−	+	−	+	+
3	+	−	−	−	−	+
4	−	−	+	−	−	+/−
5	−	−	+	−	+	+
6	−	−	−	−	−	−

* Serum inhibitory activity on candida-induced lymphocyte proliferation was defined as previously described.

† PPD and tetanus toxoid.

‡ Proliferative responses of patient leucocytes tested in presence of homologous normal serum.

§ A complete reversal + means the disappearance of SIA together with a positive lymphocyte proliferative response to CMA (> 5,000 c.p.m.) and a positive delayed skin reaction to candida antigens.

about 1 year storage under these conditions mannan and S III were reanalysed by gel filtration on Ultrogel ACA 54. Radiochemical purity of mannan was in 81% and 96–98% for S III.

Uptake and release of labelled carbohydrates by monocytes. The method used is derived from that described by Weiner & Bandieri, 1974, in the mouse. Mononuculear cells were isolated from blood on a Ficoll–Hypaque gradient (Pharmacia) (Bishop, Blank & Gardner, 1960), washed three times in Hanks' BSS and resuspended in RPMI 1640 (GIBCO, U.K.) supplemented with antibiotics.

The monocyte concentration was adjusted to obtain 10^7 monocytes/ml. Monocytes were identified and counted by peroxidase staining and latex phagocytosis. Cell preparations were incubated for 1 hr at 37°C in a shaking waterbath with 10 μCi of ^{3}H-mannan or ^{3}H-S III. The cells were then washed five times, the final supernatant was checked for the absence of radioactivity ($<$ 100 c.p.m./ml). The cell suspension was divided into aliquots of 10^6 monocytes in 0·5 ml RPMI 1640 supplemented with 20% AB plus heat inactivated human serum and antibiotics, then incubated for between 0 and 72 hr at 37°C in a 5% CO_2 incubator. At the end of the incubation, supernatants were collected after centrifugation (400 *g* for 10 min) and stored at +4°C until counted. Cells were dissolved in 0·1 M NaOH (15 min at 4°C). Aliquots from supernatants and cell preparations were mixed with 5 ml Instagel (Packard III) and counted in a liquid scintillation counter (Intertechnique, France). In order to estimate the molecular weight of radioactive components released in cell supernatants, the latter were passed through an Ultrogel ACA 54 column equilibrated with 0·01 M NaCl. Radioactive components were shown to migrate with proteins. Dissociation could be achieved easily by pretreating the samples with trichloroacetic acid (10% v/v).

More than 90% of the radioactivity was recovered in the protein free supernatants. The uptake of ^{3}H-mannan and ^{3}H-S III by lymphocytes could be ignored since lymphocyte suspension depleted of monocytes by silica treatment (containing less than 1% monocytes) did not incorporate any radioactivity (5×10^6 monocytes + 2×10^7 lymphocytes = 4550 c.p.m. of ^{3}H-mannan, 2×10^7 lymphocytes alone = 200 c.p.m.). Furthermore, autoradiographic studies of suspensions containing both lymphocytes and monocytes, incubated with ^{3}H-mannan, did not show any labelling of lymphocytes. Therefore, in the rest of the study, we used unseparated mononuclear cells containing less than 2% granulocytes.

Autoradiography. After uptake of ^{3}H-mannan or ^{3}H-S III cell smears were made by cytocentrifugation. The slides were dipped in Ilford KS emulsion gel (Nuclear Research, Essex, U.K.) and were exposed for 3 weeks.

Cell fractionation and cultures. Monocytes were isolated from mononuclear cells by adherence to plastic (Microwells Falcon, Oxnard, California, USA) for 1 hr at 37°C. After repeated washings, adherent cells contained more than 90% monocytes as judged by latex phagocytosis. Depletion of monocytes was carried out by incubation of mononuclear cells with sonicated silica for 18 hr at 37°C. After removing dead cells on a Ficoll–Hypaque gradient, d = 1·09, lymphocyte suspensions contained less than 1% monocyte. E positive lymphocyte enriched preparations were obtained by rosetting mononuclear cells with neuraminidase-treated sheep red blood cells. E positive cells were separated by Ficoll sedimentation and recovered after lysis of the sheep red blood cells by an hypotonic shock. T cell suspensions contained between 90 and 95% E positive cells.

Cultures of various cell suspensions were carried out in microwells (Falcon). A number of 2×10^5 cells were layered in 200 μl RPMI 1640 supplemented with 20% heat inactivated human AB + serum and antibiotics and cultured for 6 days in the presence of 0·25 mg/ml CMA in a 5% CO_2 incubator. One microcurie of ^{3}H-thymidine (CEA, Saclay, France) was added per ml of culture fluid 18 hr before harvesting. Attempts to generate T suppressor cells were performed by incubating 5×10^6/ml control mononuclear cells or E positive cells for varying times (24 hr to 6 days) with different concentrations of mannan (0 to 1,000 μg/ml). Cells were then washed and added at various ratios (1:1, 1:3 or 1:5) to 1×10^5 autologous mononuclear cells in a 6 day CMA-induced proliferative assay. In these experiments, leucocytes were obtained from normal individuals who responded to CMA.

In order to study the presentation of candida antigens by adherent cells to T lymphocytes, adherent cells (2×10^4/well, counted through an inverted microscope) were incubated in 200 μl culture medium with 0·25 mg/ml CMA and/or mannan (250 mg/ml) for 2 hr at 37°C, then

repeatedly washed. To these CMA and/or mannan pulsed adherent cells, 2×10^5 autologous T lymphocytes (E+ preparation containing less than 1% monocyte) obtained from normal CMA responders were added and the culture was maintained for a further 6 days.

RESULTS

Increased sensitivity to inhibition by mannan of candida-induced lymphocyte proliferation

In three (patients 1, 2 and 4) out of five CMCC patients tested in the remission phase of the disease and who exhibited a positive lymphocyte proliferative response to candida antigens, it was found that mannan exerted a stronger inhibitory effect on candida antigen-induced lymphocyte proliferation than on control lymphocyte proliferation (Table 2). A similar effect was obtained when mannan was added to culture stimulated with suboptimal (0·01–0·25 mg/ml) concentrations of CMA (data not shown). This susceptibility to mannan was restricted to the response to candida antigen since in controls, as well as in CMCC patients, mannan inhibited only slightly the PPD-induced lymphocyte proliferation at high concentration (1,000 μg/ml). Although SIA was found in patient 3, no increased susceptibility to inhibition by mannan was observed.

Table 2. Increased sensitivity of candida-induced lymphocyte proliferation from three CMCC patients to inhibition by mannan

	Proliferative responses to CMA (c.p.m.)*			
Mannan concentration (μg/ml)	Control (mean of 10 subjects)	Patient 1	Patient 2	Patient 4
0	32,800 ± 11,800†	18,700	30,500	19,500
10	35,400 ± 13,200 (0)	7,400 (60·2)	20,000 (34·4)	10,500 (46·3)
100	15,400 ± 8,400 (53·1)	2,500 (86·7)	6,700 (78·2)	4,800 (75·6)
1,000	2,700 ± 1,100 (91·8)	500 (97·5)	900 (97·0)	1,300 (93·5)

* 2×10^5 leucocytes were cultured for 6 days in presence of 0·25 mg/ml CMA.

† Results are the difference between ^{3}H-thymidine uptake in CMA-stimulated and unstimulated cultures (patient 1, 2, 4). Patient 1 and 2 displayed a serum inhibitory activity (SIA) in infected phase, but not patient 4. In brackets percentage of inhibition. Mannan alone did not induce any cell proliferation. Standard deviation of every individual culture performed in presence of mannan was below 15% of the mean ^{3}H-TdR uptake.

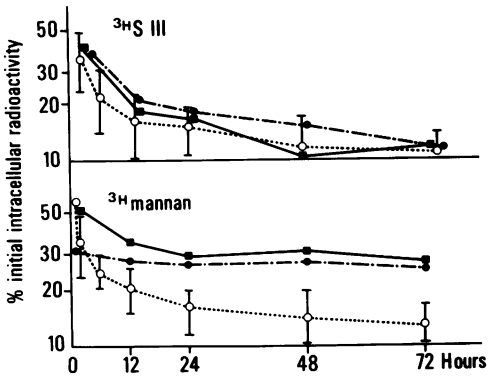

Fig. 1. Uptake and catabolism of labelled mannan or S III pneumococcal polysaccharide by control or patient monocytes. (- - - -) Control ± 2 s.d.; (- · - ·) Patient 1; (——) Patient 2.

Defective catabolism of mannan by patients' monocytes

Uptake of catabolism of two ^{3}H-labelled carbohydrates, mannan from *C. albicans* and S III pneumococcal polysaccharide, by monocytes from six CMCC patients (three in whom SIA was found and three in whom it was not) were studied. No difference was shown in the uptake of both carbohydrates by patients and 10 different control monocytes (Fig. 1). In controls, the release of radioactive material followed a biexponential decay. The first exponential component ($t_{1/2} = 2{\cdot}5$ hr) was related to the release of intact ^{3}H-carbohydrates from monocyte cell membranes. About two-thirds of the radioactivity initially associated to monocytes was membrane bound. In fact, autoradiography studies performed after a 2 hr incubation following the uptake phase, showed a diffuse labelling pattern on the whole cell area indicating that carbohydrates were mainly localized on the external membrane of monocytes. The second exponential ($t_{1/2} = 4{\cdot}5$ days) for mannan and 4·2 days for S III was related to a true release of split products (mol. wt between 1,000 and 8,000 for mannan). This material had been shown to be intracellular by autoradiography on the 12th hr of incubation when membrane bound intact carbohydrates have been almost completely released.

In all six patients the release of radioactive materials after S III uptake was identical with controls. In contrast, in two patients with SIA (patients 1 and 2), the release of mannan was abnormally low since the second slope of decay that referred to intracellular catabolism was characterized by a $t_{1/2} > 100$ days (Fig. 1). A precise analysis of the molecular weight of the radioactive products released by patients monocytes could not be achieved since insufficient material was collected in the supernatants. This abnormally slow mannan release was observed twice in both patients during active and infection-free phases of the disease. Catabolism of mannan by monocytes from the third SIA positive patients and the three SIA negative patients was not different from controls. One can notice that in the SIA + patient (3) a normal catabolism of mannan by monocytes was associated with an absence of hypersensitivity to mannan induction of candida-induced lymphocyte proliferation (see Table 3) in contrast to patient 1 and 2 (data not shown).

Failure of T suppressor lymphocyte activation by mannan and absence of cytotoxic effect

In order to understand how mannan could inhibit candida antigen-induced lymphocyte proliferation, we investigated a possible activation of T suppressor lymphocytes. As shown in Table 3, incubation of control T cell enriched suspensions (E +) with varying concentrations of mannan (up to 1,000 μg/ml, for 24 hr to 6 days) failed to generate any detectable suppressor cell activity. The same was true when total normal leucocytes incubated wih mannan were used. A similar negative result was obtained when leucocytes or E(+) lymphocytes from patients, in remission phase, were incubated with mannan and then added back to autologous lymphocytes (data not shown).

Table 3. Failure of T suppressor lymphocyte activation by mannan

Treatment of control T (E+) lymphocytes	Proliferative responses to CMA (c.p.m.)* Ratio treated T lymphocytes/target cells 1:5	1:3	1:1
0	26,700 ± 11,200	28,400 ± 8,700	28,300 ± 11,700
mannan 100 μg/ml	30,500 ± 9,600 (0)	27,800 ± 11,400 (2)	26,600 ± 9,600 (6)
mannan 500 μg/ml	27,300 ± 7,400 (0)	26,500 ± 9,500 (0)	29,400 ± 10,900 (0)
mannan 1,000 μg/ml	25,400 ± 8,400 (5)	33,100 ± 11,800 (6)	30,600 ± 3,500 (0)
Con A 40 μg/ml	19,200 ± 5,100 (28)	14,100 ± 3,300 (50)	6,500 ± 2,000 (77)

* Results (c.p.m.) are given for 2×10^5 cells (for the ratio 1:5 × (2/1·2), 1:3 × (2/1.3) Con A-activated lymphocytes used as control exerted a suppressor activity which was dependent on number of cells added to culture.

Table 4. Suppression by mannan of adherent cell (AC) dependent T lymphocyte proliferation to candida antigens (CMA)

Cultured cells obtained from normal CMA-responders	Expt. 1 (c.p.m.)	Expt. 2 (c.p.m.)
Adherent cells (AC)	255±36	387±82
CMA-pulsed AC	344±41	311±50
T lymphocytes	172±24	143±14
T lymphocytes+CMA	423±28	277±31
AC+T lymphocytes	417±32	515±28
CMA-pulsed AC+T lymphocytes	26,536±743	12,730±603
Mannan-pulsed AC+T lymphocytes	463±12	398±27
CMA and mannan-pulsed AC+T lymphocytes	10,852±447	3,445±208
CMA and mannan-pulsed AC+T lymphocytes+CMA pulsed monocytes	24,260±926	8,235±511

The results of two representative experiments are given as the mean of triplicate cultures ± 1 s.d.

Mannan did not exert any cytotoxic activity since lymphocytes preincubated with mannan up to 1,000 μg/ml for 24 or 48 hr at 37°C, then washed and cultured in presence of candida antigens, proliferated as well as control cells that were preincubated in absence of mannan (data not shown). Moreover, the viability, as measured by trypan blue exclusion was similar after a 48 hr incubation with 250 μg/ml mannan (77±5 versus 79±6% viable cells).

Interference of mannan with antigen presentation by adherent cells to T lymphocytes

In two representative experiments out of five, normal adherent cells pulsed for 2 hr with CMA, but not with mannan, were further able to induce the proliferation of autologous T lymphocytes, whereas the same T cells alone, were unable to proliferate in the presence of candida antigen (Table 4). Addition of mannan (50–250 μg/ml) to adherent cells at the time of incubation with CMA resulted in a significant inhibition ($P<0{\cdot}0001$) of the T cell proliferation. Finally, addition of autologous monocytes contained in E+ depleted suspensions, pulsed with CMA, to adherent cells previously pulsed with CMA and mannan, restored significant ($P<0{\cdot}001$) proliferation of T lymphocytes, indicating that mannan does not act through the activation of suppressor monocytes.

DISCUSSION

We have previously demonstrated the presence in serum of carbohydrate antigenic materials from *C. albicans* in CMCC patients with serum inhibitory activity (SIA) and that the persistent candida carbohydrates exert a specific inhibitory activity on candida antigen-induced lymphocyte proliferation. Mannan which is the main component of the candida cell wall (Bishop *et al.*, 1960; Yu *et al.*, 1967) appeared to be the most likely candidate since it was shown to exert a specific immunosuppressive effect *in vitro* on candida-induced T cell proliferation (Fischer *et al.*, 1978). We show here, firstly, there is an abnormally low catabolism of mannan by monocytes in two patients with SIA. Secondly, as observed in experiments performed with control cells, mannan exerts its suppressive effect through interaction with antigen presenting cells although induction of suppressor T cells is not excluded. Together these data explain the susceptibility of the patient lymphocyte proliferation to inhibition by mannan. To study the first point, we took advantage of the availability of radiolabelled, unmodifed carbohydrates obtained by the catalytic exchange control. The specific activity (1–2 mCi/mg) was sufficient to perform catabolism kinetic studies but low enough to avoid an important radiolysis. Uptake kinetics of both carbohydrates studied, mannan and S III, showed no difference between control and patient cells in the presence as well as the absence of normal human serum. Two different phenomena occur resulting in a normal

biexponential decay of cellular bound radioactivity. (1) Carbohydrates that had bound to external membranes as observed on autoradiography were released within a few hours. (2) Intracellular radioactive material as shown by autoradiography, was metabolised into small m.w. residues which were released slowly indicating a true catabolism of the carbohydrates mannan and SIII by monocytes. The release of mannan breakdown products but not those of S III was found to be reduced in the monocytes of two out of three SIA positive patients and normal in the three SIA negative patients. The defect was observed in the active as well as in the remission phases of the disease. This last observation would make mannan overload an unlikely explanation of the low release of mannan metabolites by patient monocytes.

The hypersensitivity of SIA patient's lymphocytes to mannan inhibition should result from another unknown mechanism underlying again the heterogeneity of CMCC. The apparently primary defect of mannan catabolism by monocytes could well play a role in patient's susceptibility to candida infection. We can however only speculate about the precise mechanism. Particularly, we do not know whether carbohydrates of similar physical properties to mannan, unlike S III (linear polymer of glucose and glycuronic acid, m.w. 350,000) are handled ineffectively by patient monocytes. We do not know either if the catabolism of other candida carbohydrates like glucan, which is particularly resistant to enzymatic digestion (Meister *et al.*, 1977) is normal or not. One should also mention that patients affected with mannosidosis, a genetic defect of α-D-mannosidose activity (Canoll *et al.*, 1972) are not especially susceptible to candida infections although often infected (Desnick, Sharp & Grabowski, 1976). This observation however does not implicate directly an α-D-mannosidase deficiency in the monocyte pathology of some CMCC patients. The second question envisaged in this work concerned the mechanism by which mannan slowly degraded in some CMCC patients can suppress candida-induced cellular responses *in vitro*. We used normal mononuclear cells in the experiments studying the mannan-mediated immunosuppression in order to avoid interference with immunological dysfunctions of CMCC patients' cells. The suppressive effect of mannan, largely restricted to candida-induced lymphocyte proliferation (Fischer *et al.*, 1978) appeared not to be secondary to a cytotoxic effect. Although presence of T suppressor lymphocytes have been demonstrated in chronic fungal infections (Stobo, 1977), we could find no evidence for the induction of T suppressor lymphocytes by mannan in 6 days. However, one could not exclude that T suppressor cells could be activated in another environment *in vivo* or by longer incubations as Picollela has recently shown that T suppressor lymphocytes are induced by a 6-day incubation with a polysaccharide from *C. albicans*. However, experiments in which mannan was added to adherent cells being pulsed by candida antigens clearly showed that mannan acts by interfering with the presentation of the candida antigens to T lymphocytes. The direct activation of suppressor macrophages by mannan would seem unlikely, since T lymphocyte proliferation was restored by adding back candida antigen-pulsed monocytes. These data are similar to those obtained by Ellner & Daniel (1979) studying the non-specific immunosuppressive role of *Mycobacterium arabinomannan*.

Mannan is well known to induce a very strong antibody response both in normal subjects and more particularly in CMCC patients (Axelsen, 1976; Axelsen, Kirkpatrick & Buckley, 1974). Preliminary data indicate that mannan does not inhibit *in vitro* antibody production by normal lymphocytes to mannan and other candida antigens. Thus, the effect of mannan on candida-induced immune responses appears to affect T rather than B cell functions. This effect is also reminiscent of the inhibition of antigen presentation to T cells by free antigen whereas B cell responses are increased as observed in a murine model (Oppenheim & Seeger, 1976; Rosenthal *et al.*, 1976). Although *in vitro* high concentrations of mannan were required to induce an impairment of antigen presentation to T cells, such concentrations are not unlikely in CMCC patients where monocytes are defective in the handling of mannan. Moreover, indirect evidence of mannan accumulation in infected tissues has been presented (Sohnle, Frank & Kirkpatrick, 1976; Ray *et al.*, 1979). What we observed in a rather specific way with mannan could belong to a more general inhibitory mechanism of T cell functions by certain polysaccharides and oligosaccharides as described by Muchmore, Decker & Blaese, 1980.

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