Inhibition of the immune response by rapamycin, a new antifungal antibiotic

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Rapamycin, a new antifungal antibiotic, was found to inhibit the immune response in rats. It totally prevented the development of two experimental immunopathies (experimental allergic encephalomyelitis (EAE) and adjuvant arthritis (AA)) and the formation of humoral (IgE-like) antibody. It was about half as potent as cyclophosphamide in inhibiting EAE. In AA and on antibody formation, rapamycin and cyclophosphamide were about equipotent, whereas methotrexate was more potent. The immunosuppressant activity of rapamycin appears to be related to inhibition of the lymphatic system.

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La rapamycine, un nouvel antibiotique antifungique, inhibe la réponse immunitaire chez le rat. Il empêche totalement le développement de deux immunopathies expérimentales (encéphalomyélite expérimentale allergique (EEA) et arthrite adjuvante (AA)) et la formation d'anticorps humoraux semblables aux IgE. Sa puissance est la moitié de celle du cyclophosphamide dans l'inhibition de l'EEA. L'effet sur l'AA et sur la formation d'anticorps est approximativement le même pour la rapamycine et le cyclophosphamide alors que le méthotrexate est plus puissant. L'activité immunosuppressive de la rapamycine paraît liée à une inhibition du système lymphatique.

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Introduction

Rapamycin is a recently described (Vézina *et al.* 1975; Sehgal *et al.* 1975) antifungal antibiotic extracted from a streptomycete (*Streptomyces hygroscopicus*) isolated from an Easter Island soil sample. It is particularly effective against *Candida albicans* both in vitro and in vivo (Sidorowicz *et al.* 1975).

The inhibitory effects of this new antibiotic on two experimental immunopathies (EAE and AA) and on the formation of IgE-like antibody are described in this report.

Methods

EAE

EAE was induced in female, inbred Wister–Lewis rats (120–140 g). The rats were injected in the left hind foot pad with 0.05 ml of an emulsion consisting of guinea pig spinal cord (4.2 g) homogenized in a mixture of 5.8 ml of 0.5% aqueous phenol and an equal volume of complete Freund's adjuvant containing 4.4 mg/ml of heat-killed, dried *Mycobacterium butyricum* (Difco). The sensitized rats were treated orally with rapamycin or cyclophosphamide according to different schedules (see Table 1). The animals were observed for signs of hindleg paralysis from day 10 to day 16 (day of sensitization is day 0). The rats that did not show paralysis of the hindlegs during the observation period were considered protected.

AA

AA was induced in male inbred Wister–Lewis rats (180–200 g). The rats were injected intradermally in the foot pad of the left hindpaw (day 0) with 0.05 ml of a fine suspension of killed and dried *M. butyricum* (Difco) at a concentration of 5 mg/ml in liquid paraffin (Freund's adjuvant). For the prophylactic study, compounds were administered per os daily starting on the day of adjuvant injection (day 0) and until day 16. For the therapeutic study, treatment was started on day 14 and continued until day 22. Hindleg volume was determined by mercury displacement 2 h after the last treatment. Hindlegs were dipped in mercury up to the hair line. The mercury displaced represents the volume of the hindlegs (13.6 g of mercury = 1 ml).

IgE-like Antibody Formation

A modification of the method described by Mota (1964) was used to produce IgE-like antibody in the rat. The effect of rapamycin on this response was studied. Male Charles River rats (180–200 g) were

ABBREVIATIONS: EAE, experimental allergic enceph-

TABLE 1. Effect of rapamycin on EAE

Treatment, daily	Duration, d	Dose, mg/kg, po	Rats paralyzed[a] / Rats sensitized	% protected
Control			33/41	20
Rapamycin	0–13	10	0/12	100
	0–13	5	9/18	50
	0–13	2.5	11/17	35
	0– 6	10	2/12	83
	7–13	10	7/12	42
Cyclophosphamide	0–13	5	1/19	95
	0–13	2.5	9/19	53
	0–13	1	4/6	33

[a]Rats which showed hindleg paralysis between day 10 and 16.

with 0.3 ml of EA (10 mg/ml). Oral administration of rapamycin and other compounds was started at the same time (day 0) and continued daily until day 11. On day 12, the animals were anesthetized with ether and bled from the abdominal aorta. The presence of EA antibody in the serums of control and treated groups (eight animals in each group) was determined by the PCA method. The pooled undiluted serum (0.1 ml) of each group was injected intradermally in six rats (three sites per rat). Forty-eight hours later the animals were challenged intravenously with 11.25 mg of EA per kilogram in 1% Evans blue dye in saline (0.9 ml per 100 g). After 30 min the rats were killed by CO_2 inhalation and the diameter of the wheals formed on the underside of the skin was measured.

The cyclophosphamide used in these studies was purchased from ICN Pharmaceutical, Inc., and the methotrexate was generously supplied by Lederle Laboratories.

Results

Effect on EAE

EAE is characterized by paralysis of the hind quarter. Rapamycin (10 mg/kg, po), administered daily for 14 d starting on the day of sensitization (day 0) completely prevented the development of hindleg paralysis (Table 1). None of these rats (10 mg/kg) showed late paralysis when observed until day 21. Rapamycin was more active when administered early (day 0 to 6) in the course of EAE than when administered from day 7 to 13 (Table 1). These data suggest that rapamycin exerts most of its effect during the induction of EAE.

The immunosuppressive drug cyclophosphamide, a potent inhibitor of EAE (Rosenthale *et al.* 1969), appeared to be about twice as potent as rapamycin in these experiments.

reaction of the hindlegs. When the adjuvant (mycobacteria in oil) is injected into a hindpaw (day 0) and treatment started on that day (prophylactic treatment), drugs can be evaluated for their effect on two distinct inflammatory phases of the disease: (*a*) and early phase in the injected paw which peaks around day 3 and is mainly dependent on an acute inflammatory reaction to the adjuvant, and (*b*) a late phase (starting around day 10) in the injected and the noninjected hindlegs, resulting from a delayed or cellular-type hypersensitivity reaction to some constituent of the mycobacteria (Rosenthale 1974). The immunosuppressive agents inhibit only the late or immune phase, whereas the anti-inflammatory drugs inhibit both phases (Walz *et al.* 1971; Rosenthale 1974).

Rapamycin (5 mg/kg), cyclophosphamide (5 mg/kg), and methotrexate (0.25 mg/kg), when administered orally, completely blocked the secondary immune response (day 16) in both hindlegs. However, they did not decrease significantly the primary nonimmune phase in the injected paw. The protective effect of the three compounds was still complete on day 22, 6 d after treatment was stopped (Table 2). The doses reported in the table are about the lowest that will inhibit AA completely.

In established arthritis (six controls and six treated), rapamycin (10 mg/kg) was inactive. It did not prevent further swelling of the hindpaws. However, the swelling was slightly less than in the untreated arthritic rats.

Effect of IgE-like Antibody Formation

TABLE 2. Effect of rapamycin on AA

Treatment[a]	Hindleg volume, ml ± SE				
	Injected			Noninjected	
	Day 3	Day 16	Day 22	Day 16	Day 22
Normal control	2.1 ± 0.02	2.3 ± 0.06	2.4 ± 0.06	2.3 ± 0.06	2.4 ± 0.06
Arthritic control	3.7 ± 0.08	4.9 ± 0.24	5.7 ± 0.22	3.3 ± 0.24	4.2 ± 0.23
Rapamycin, 5 mg/kg	3.7 ± 0.10	3.1 ± 0.02	2.9 ± 0.24	2.2 ± 0.12	2.3 ± 0.19
Cyclophosphamide, 5 mg/kg	3.4 ± 0.10	3.6 ± 0.26	3.3 ± 0.21	2.1 ± 0.02	2.2 ± 0.22
Methotrexate, 0.25 mg/kg	3.9 ± 0.20	3.3 ± 0.15	3.2 ± 0.12	2.2 ± 0.04	2.2 ± 0.04

[a]Orally from day 0 to day 16 (8–10 rats per group).

TABLE 3. Effect of rapamycin on IgE-like antibody formation

Treatment, daily	Dose, mg/kg, po	Diameter of skin wheal, mm ± SE
Control		28.1 ± 0.8
Rapamycin	10	0
	3	10.2 ± 0.2[a]
Cyclophosphamide	10	0
	3	12.0 ± 0.4[a]
Methotrexate	0.3	0
Phenylbutazone	25	27.0 ± 0.8

[a]$P < 0.01$.
NOTE: The skin was sensitized with 0.1 ml of the pooled serum of eight rats per group.

treated rats were assayed for the presence of EA antibodies by the 48-h PCA method. As shown in Table 3, the serum of the rats treated with 10 mg of rapamycin per kilogram and cyclophosphamide produced no skin wheals. A smaller dose of rapamycin and cyclophosphamide partially inhibited the response, while methotrexate was more potent. Rapamycin (100 mg/kg, po, administered to sensitized rats 1 h previous to challenge) or diluted anti-EA serum (one part with three parts of serum obtained from unsensitized rats treated with rapamycin, 50 mg/kg orally for 2 d) had no effect on the PCA response. From these results it was concluded that rapamycin, similar to the immunosuppressive agents cyclophosphamide and methotrexate, inhibited antibody formation. The nonsteroidal anti-inflammatory drug phenylbutazone had no effect. No drug-related adverse effects could be noted, apart from a depression of the growth curve with rapamycin

Discussion

Rapamycin, a new antifungal antibiotic, has been found to inhibit the immune response. Small, well-tolerated doses, 5–10 mg/kg, (Sidorowicz *et al.* 1975) of this antibiotic totally prevented the development of cellular immunity (EAE and AA), as well as the formation of IgE-like antibody. In EAE, rapamycin appeared to be half as potent as cyclophosphamide. In AA and antibody formation, the potency of rapamycin and cyclophosphamide appeared comparable, whereas methotrexate was more potent. Similarly, Walz *et al.* (1971) used 20-times less methotrexate than cyclophosphamide to block AA, and Rosenthale *et al.* (1969) showed methotrexate to be much more potent than cyclophosphamide in inhibiting paralysis in EAE.

All evidence indicates that the inhibitory effect of rapamycin in EAE and AA depends on suppression of the immune response. Rapamycin produced a complete and long-lasting inhibition of EAE. Prophylactic treatment with rapamycin in the AA model inhibited only the immune-mediated phase of inflammation and the inhibition lasted after treatment was discontinued. In established AA, 10 mg of rapamycin per kilogram, a dose which completely prevented the development of EAE and AA, was inactive. This profile was reported for immunosuppressive agents such as azathioprine, methotrexate, cycloleucine, and cyclophosphamide by Rosenthale (1974). Nonsteroidal anti-inflammatory drugs do not produce full protection in EAE (Komarek and Dietrich 1971). In AA, the steroidal and non-steroidal anti-inflammatory drugs inhibit both

phases of inflammation (Walz *et al.* 1971; Wax *et al.* 1974). They are about equipotent when assayed by the preventive and the therapeutic methods (Perper *et al.* 1971; Walz *et al.* 1971; Wax *et al.* 1974; Martel and Klicius 1976). Arthritis occurs readily after dosing is stopped (Perper *et al.* 1971). Furthermore, rapamycin inhibited antibody formation, whereas the non-steroidal anti-inflammatory drug phenylbutazone did not.

The mechanism of action of rapamycin on the immune system is unknown at the present time. However, long-term toxicity studies in dogs (Hemm, R. D., and Authier, L., personal communication) have demonstrated that rapamycin caused hypoplasia of lymphatic tissues (lymph nodes, spleen, thymus). Thus, it appears that the activity of this antibiotic on the immune response depends on an inhibition of the lympathic system.

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