The Use of Carrageenan as a Granuloma-Producing Agent in Freund's Adjuvant (34955)

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Enhanced production of circulating antibody and delayed hypersensitivity to protein antigens incorporated in Freund's complete mycobacteria adjuvant generally parallels the extent of reticuloendothelial cell hyperplasia (1, 3). Since processing of antigen by reticuloendothelial macrophages and binding with macrophage RNA is currently thought to play an important role in transfer of information to potential antibody-producing cells (4–7), it is likely that granuloma-producing agents such as *Mycobacterium tuberculosis* may serve as adjuvants by virtue of their macrophage-stimulating property. Conversely, substances that are toxic for macrophages might serve as immunosuppressive agents. Carrageenan, a high molecular weight sulfated galactan derived from marine algae, is a known granuloma-producing agent in the guinea pig (8). It is ingested by macrophages as demonstrated by its metachromatic reaction in secondary lysosomes with toluidine blue and is toxic for these cells (9).

In the current study carrageenan was substituted for mycobacteria in complete Freund's adjuvant and observations on antibody production and delayed hypersensitivity to crystalline bovine serum albumin (BSA) were made.

Materials and Methods. Purified powdered Sea-Kem 21 calcium carrageenan (Marine Colloids, Springfield, New Jersey) was substituted for mycobacteria in Freund's adjuvant (prepared by adding 8.5 parts of medium-weight mineral oil to 1.5 parts of mannide monooleate).

Random-bred 250-g female albino guinea pigs were injected via the foot pad route with 0.1 ml of an emulsion containing crystalline bovine serum albumin (BSA), 1 μg of N in saline solution, and an equal volume of a mineral oil—mannide monooleate mixture (8.5:1.5). Three types of emulsion were used: (a) incomplete adjuvant prepared with mineral oil; (b) complete adjuvant containing mineral oil plus a suspension of heat-killed *M. tuberculosis* H37Ra (5.0 mg/ml); (c) carrageenan adjuvant containing mineral oil and a suspension of powdered calcium carrageenan (5.0 mg/ml).

Animals were skin tested 10 days and 21 days after immunization with 1 μg N of BSA in 0.1 ml of saline and with saline controls. Reactions were read at 24 hr, and the extent of induration and erythema was recorded in millimeters. Bleedings were done by cardiac puncture 3 weeks after inoculation. Booster injections of 1 mg of BSA were given subcutaneously on the next day, and the animals were bled again 1 week later. Sera were heated at 56° for 30 min and assayed for guinea pig anti-BSA γ_2 antibody by passive hemolytic assay (10) and for γ_1 anti-BSA by passive cutaneous anaphylaxis (PCA) (11). All hemolytic assays were performed in duplicate with unsensitized cell controls for each serum, a negative serum control, and a known positive control.

A separate group of guinea pigs was divided into two subgroups, and inoculated via the foot pad route with carrageenan in either saline solution or in mineral oil adjuvant in concentrations of 2.5 mg, 0.25 mg, and 0.025 mg, respectively. These animals were sacrificed at various intervals for histologic study of foot pad injection sites and local draining lymph nodes (popliteal and axillary).

TABLE I. Delayed Hypersensitivity to BSA in Guinea Pigs Immunized with Adjuvant Containing Carrageenan or Mycobacteria.

No. of animals	Injection	Time (days)	Delayed hypersensitivity (24-hr reaction) Mean	Stand. dev.
10	1 μg N BSA in adjuvant with 0.25 mg *M. tuberculosis*	10	18.0[a]	5.7
		21	15.9	5.7
15	1 μg N BSA in adjuvant with 0.025 mg carrageenan	10	5.2	3.6
		21	9.0	6.1
14	1 μg N BSA in adjuvant with 0.25 mg carrageenan	10	2.6	3.2
		21	8.4	5.0
15	1 μg N BSA in adjuvant with 2.5 mg carrageenan	10	5.4	4.0
		21	10.2	5.4
10	1 μg N BSA in incomplete Freund's adjuvant	10	3.3	4.1
		21	3.8	3.6
10	Incomplete Freund's adjuvant only	10	0	—
		21	0	—

[a] Millimeters of induration and erythema.

Results. When carrageenan was substiuted for *M. tuberculosis* in Freund's complete adjuvant in several doses ranging from 0.025–2.5 mg, marked reduction in intensity of delayed hypersensitivity to incorporated protein antigen (BSA) was seen (Table I).

With *M. tuberculosis* adjuvant, reactions ranged from 10–27 mm of induration and erythema. With carrageenan–mineral oil adjuvant mean reactions were 4 mm at 10 days and 9 mm at 21 days with the three dose levels employed (Table I). In several of these animals the reaction was completely abolished or consisted of erythema alone, with only slight induration.

Carrageenan also failed to enhance γ_2 anti-BSA titers when compared with mycobacterial adjuvant. During the primary and secondary response, γ_2 anti-BSA titers ranging from 640–5120 were obtained when *M. tuberculosis* adjuvant was employed (Table II); whereas, titers ranged from 20–160 when carrageenan was substituted for *M. tuberculosis* over a selected dose range.

As in the case of delayed hypersensitivity, there was no evidence of active suppression of γ_2 anti-BSA production by carrageenan in the dose range employed since mineral oil adjuvant alone resulted in γ_2 titers comparable to those obtained with carrageenan (Table II). No clear-cut enhancing or suppressive effect of *M. tuberculosis* or carrageenan on γ_1 anti-BSA titers was evident, and titers were widely variable within each group (Table II).

Morphologic studies of local foot pad inoculation in animals receiving carrageenan in saline solution revealed an early edematous reaction in which polymorphonucleated neutrophils and large histiocytes were present. By the seventh day numerous activity dividing fibroblasts and histiocytes predominated in the granulomatous lesions. Between the second and third weeks heavy collagen fibers were prominent and these were predominantly resorbed by the fourth week as has been previously noted (8). Draining lymph nodes in these animals were small with little evidence of hypertrophy or hyperplasia.

Animals receiving carrageenan in mineral oil developed small draining nodes in striking constrast to the large hypertrophied and hyperplastic nodes reported after foot pad inoculation of *M. tuberculosis*-mineral oil adjuvant in guinea pigs (2).

Discussion. Our findings indicate that carrageenan, a known granuloma-producing

TABLE II. Antibody to BSA in Sera of Guinea Pigs Immunized with Adjuvants Containing Carrageenan or Mycobacteria.

No. of animals	Immunization	Time (days)	γ_2 anti-BSA titers[a]		PCA	
			Mean	SD	Mean	SD
10	1 μg N BSA in adjuvant with	21	3749	1826	71	66
	0.25 mg *M. tuberculosis*	7pb[b]	5120	0	263	199
5	1 μg N BSA in adjuvant with	21	100	60	64	49
	0.025 mg carrageenan	7pb	440	560	622	518
5	1 μg N BSA in adjuvant with	21	132	62	28	40
	0.25 mg carrageenan	7pb	140	40	280	481
8	1 μg N BSA in adjuvant with	21	62	57	162	370
	2.5 mg carrageenan	7pb	297	444	304	443
10	1 μg N BSA in incomplete	21	145	134	78	185
	adjuvant	7pb	1015	1600	167	339

[a] Reciprocal of highest serum dilution giving complete hemolysis (γ_2) or bluing of injected skin site (PCA).

[b] pb = postbooster.

agent with macrophage-toxic properties, did not enhance delayed hypersensitivity and γ_2 anti-BSA production in the guinea pig when substituted for *M. tuberculosis*, a macrophage stimulant, in Freund's complete adjuvant.

Since macrophages are a prominent cell type in delayed hypersensitivity reactions (12) and antibody synthesis is thought to involve macrophage antigen processing with transfer of immunogenic information to antibody synthesizing cells (13, 4–7), it is likely that our observed differences in immunologic response between carrageenan and *M. tuberculosis* adjuvants are due to their opposing effects on macrophages. This possibility is further supported by histological findings of scant draining lymph node hyperplasia in our animals receiving carrageenan adjuvant. These nodes were small and often difficult to locate in contrast to those in animals receiving complete Freund's adjuvant which were markedly hypertrophic and contained massive epithelioid cell infiltrates. Local injection sites of carrageenan–mineral oil adjuvant were also less inflammed, and indurated, than those inoculated with complete Freund's adjuvant.

Carrageenan has also been reported to inactivate the C'1 component of complement (14, 15) and to be highly antigenic in the rabbit (16). Since the complement system is thought to play a role in delayed hypersensitivity (16) and antigen competition is known to decrease antibody formation (17) either of these factors could also explain the less intense delayed hypersensitivity and lower γ_2 anti-BSA titers observed when carrageenan was substituted for mycobacteria in Freund's adjuvant. However, recent data indicate that carrageenan suppresses ongoing delayed hypersensitivity reactions without significant alterations in serum complement levels (18) which makes one of these possibilities unlikely.

Our results shed no light on the important question of the ability of carrageenan to suppress active immunity since its overall effect on delayed hypersensitivity and circulating antibody to BSA was not less than that observed when this antigen was administered in mineral oil adjuvant alone. Larger doses and different methods of administration would likely be necessary to demonstrate such *in vivo* suppressive activity since 10–50 mg of carrageenan given intraperitoneally have been necessary to demonstrate suppression of ongoing delayed hypersensitivity in the guinea pig (18). Such large doses have, in our hands, been technically impossible to incorporate into mineral oil adjuvants be-

cause of the physical properties of carrageenan.

Summary. Carrageenan, a known granuloma-producing agent with macrophage-toxic properties, was substituted for *M. tuberculosis*, a macrophage stimulant, in complete Freund's adjuvant. Carrageenan failed to enhance delayed hypersensitivity or γ_2 anti-BSA production when compared with *M. tuberculosis* over a wide dose range. Histologically, draining nodes from carrageenan-inoculated animals revealed little hyperplasia when compared with extensive epithelioid cell infiltrates in nodes from animals receiving complete Freund's adjuvant.

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