ble hybrids occurred after the 8th day of the insemination period and in Exp. 2, none occurred after the 18th day. Also, in eggs laid by hens not previously injected with semen, the incidence of development rose to a high level early in the insemination period but later subsided to the level encountered before inseminations began.

There is no way of knowing whether the suppression of embryonic development induced by semen injections and the decline in embryonic development in eggs of both semen injected and control hens after repeated artificial inseminations are the product of similar responses. Moreover, there is no direct evidence on the nature of the mechanism(s) involved. Nevertheless, one hypothesis which should be considered is that in both instances the results may derive from an immunological reaction of the hens to antigens associated with foreign spermatozoa. Certainly, if immunological reactivity were to play any role in infertility, it should do so in a heterologous situation such as this one where strong antigenic differences are expected.

The hypothesis is consistent with earlier findings that in chickens a) transient infertility occurred in hens following a single subcutaneous injection of testicular sperm suspension(4), b) duration of fertility was reduced by repeated intravenous injections of hens with spermatozoa(5) and by repeated artificial inseminations(6), and c) serum antispermatozoa titres increased in hens subjected to repeated artificial inseminations or extended periods of natural mating(6). Similar recent work with turkeys has shown that intramuscular injections of semen and semen nuclear extracts resulted in a drop of 26-53% fertility over a 7-week period. However, no reduction in fertility was detected in uninjected control hens artificially inseminated 5 times each week for 7 weeks(7).

Summary. Eight weekly artificial inseminations of Beltsville Small White turkey hens with Dark Cornish chicken semen were only partially successful in achieving cross-fertilization. Identifiable hybrid embryos were produced only in an 18-day period immediately following the first insemination. Intraperitoneal injections of turkey hens with chicken semen in Freund's adjuvant before artificial insemination by the same semen donors suppressed the ability of their subsequent eggs to undergo hybrid embryonic development. Similar injections of adjuvant alone had no suppressing effect.

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Sensitization Reaction to Carrageenan in the Guinea Pig.*† (31976)

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Carrageenan, a sulfated galactan derived from marine algae, was demonstrated to be a connective tissue growth stimulant in experiments carried out over a decade ago by Robertson and Schwartz(1). Since that time, it has been used extensively as a model of collagen synthesis. The chance observation that a second injection of carrageenan provoked

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TABLE I. Sensitization to Carrageenan SeaKem Type 21.

Sensitization period, wk	Pre-treatment	Animals, No.	Granuloma wt, g	Dry wt, mg/g	Collagen conc, mg/g	Collagen total, mg
3	Carrageenan	6	3.75 ± 1.37*	98.8 ± 6.2	14.5 ± 2.3	60.8 ± 8.8
	Saline	6	2.71 ± .60	109 ± 5.8	17.3 ± 1.2	46.5 ± 6.5
5	Carrageenan	8	2.32 ± .39*	85.9 ± 6.8	16.7 ± 1.8*	35.4 ± 4.9
	Saline	8	1.24 ± .20	86.9 ± 9.1	23.0 ± 1.7	27.8 ± 4.3
8	Carrageenan	8	5.91 ± 1.75*	91.9 ± .5	13.3 ± 1.9*	67.4 ±15.5
	Saline	8	2.71 ± .88	94.5 ± 5.5	19.7 ± .8	50.5 ± 8.0

* Significantly different from control value, $p < 0.02$.

Data presented as mean ± standard error. Granuloma weight is weight of tissue exclusive of serous fluid.

an exaggerated tissue response in pretreated guinea pigs suggested that the polysaccharide possessed immunological activity. The present communication describes the *in vivo* sensitization reaction to carrageenan in the guinea pig.

Materials and methods. Young guinea pigs of both sexes from our own colony were injected subcutaneously with 0.1 ml 0.9% NaCl or with 0.1 ml 1% carrageenan, SeaKem Type 21‡, prepared in 0.9% NaCl and adjusted to pH 7.0-7.4 before autoclaving, as described previously(2). After intervals of 3, 5, or 8 weeks, the animals were challenged with 5 ml 1% carrageenan, the usual dose for granuloma formation in this species(3), and were sacrificed 2 weeks later. The granulomas which developed as sheets or masses of tissue between abdominal muscle and skin were described grossly and dissected quantitatively, free from underlying muscle. Infected granulomas (incidence 4%) were discarded from the series. Samples of tissue were weighed for chemical analysis, and a sample taken for histological study. In a second series of experiments, lambda carrageenan, the active principle for connective tissue growth stimulation(2), was substituted for SeaKem Type 21, which is an unfractionated extractive containing both kappa and lambda carrageenans. Sensitization periods of 3 weeks were employed in both experiments.

Collagen was assayed by determination of hydroxyproline(4) in tissue extracted by a modification of the method of Neuman and Logan(4,5). After chloroform-methanol extraction, the residue of a second tissue sample was dried under vacuum to give an estimate of dry defatted weight of tissue. Total protein was estimated by the Folin-Ciocalteau procedure of Lowry(6), and iron determined by the method of Kennedy(7) as a measure of blood in tissue.

Results. The granulomatous reaction which occurs after subcutaneous administration of carrageenan in the guinea pig is characterized by an early leucocytic infiltration, a cellular phase in which fibroblasts and macrophages are prominent, accompanied and followed by synthesis of mucopolysaccharides and collagen, and a final phase in which the newly synthesized tissue undergoes total reabsorption(3). In all cases the present experiments were terminated 2 weeks after carrageenan injection, in the second phase of the reaction when collagen synthesis was approaching its maximal level. The control guinea pigs, which had received 0.9% NaCl as pretreatment, responded to carrageenan in this typical fashion, and the granulomas found in these animals were well vascularized, slightly edematous, highly cellular, with young collagen fibers in the matrix of the rapidly growing tissue.

Granulomas in the carrageenan preinjected guinea pigs appeared to be more highly vascularized, and in a number of instances were severely hemorrhagic. The time allowed for sensitization, 3, 5 or 8 weeks between first and second injections, did not appear to be critical. As the data in Table I indicate, at all time periods the carrageenan preinjected animals displayed significantly heavier granulomas, and in all but the first, significantly lower concentrations of collagen. This latter

‡ Marine Colloids, Inc., Rockland, Maine.

TABLE II. Sensitization to Lambda Carrageenan.

Pretreatment	Lambda carrageenan	Saline
A.		
Granuloma wt, g	7.01± 1.07*	2.22± .47
Dry defatted wt, mg/g	93.0 ±10.5	88.3 ± .2
Collagen conc, mg/g	10.6 ± 2.2*	21.7 ± 2.4
Collagen total, mg	71.9 ±14.3	46.6 ± 7.9
B.		
Granuloma wt, g	4.73± .48†	2.11± .31
Fe conc, μg/g	17.3 ± .9	21.7 ± 3.8
Protein conc, mg/g	92.1 ± 5.7	88.4 ± 5.1
Fe as Hb, mg	27.0 ± 3.2*	14.7 ± 2.6
Protein total, mg	447 ±65 †	180 ±16

* $p < .05$. † $p < .01$.

Data presented as mean ± standard error. Granuloma weight is weight of tissue exclusive of serous fluid.

Exp A: 3 and 4 animals pretreated with lambda carrageenan and saline respectively.

Exp B: 6 animals/group.

result was unexpected. The two differences almost balanced, *i.e.,* total collagen produced by 50 mg carrageenan in the sensitized animals was elevated only slightly. The difference never achieved stastistical significance and was certainly insufficient to explain the greater granuloma weights. The data suggested, in fact, that the collagen production stimulated by the challenging dose of carrageenan was diluted by production of some other material in excess. Dry weight determinations indicated this was not merely tissue fluid.

These observations were confirmed by two subsequent sensitization experiments using lambda carrageenan, the data from which are summarized in Table II. In the first of these, the same parameters were investigated as in the original experiments, with similar results. In the second, increases in total protein and total iron, with no change in their concentrations in the tissue, were demonstrated.

It was surprising that the concentration of iron, presumably present as hemoglobin, was not increased in the sensitized animals. Histologically both saline and carrageenan preinjected animals showed frequent hemorrhage in the tissue samples examined, but the gross appearance of granulomas in lambda carrageenan preinjected animals was definitely more hemorrhagic. These granulomas occasionally appeared as sac-like structures filled with a blood tinged serous fluid. More blood was present in the total granuloma, of course, just as more protein and more water were present, but one could not say that the increased weight of granuloma was due to the inclusion of blood products, or of water, as the concentration of these substances was essentially unchanged. It would appear from these results and from histological studies that the excess tissue in the sensitized animals consisted of the usual connective tissue cells but somewhat less of the extracellular connective tissue protein collagen.

Discussion. The present demonstration that the connective tissue site pretreated with a small dose of carrageenan retains a memory of that event and responds with a greater tissue reaction to the challenging dose of carrageenan suggests that this polysaccharide may possess immunological activity. For purposes of analysis, it might be instructive to compare the carrageenan sensitization reaction with two well-investigated immunological sensitization phenomena, the local Shwartzman and Arthus reactions. Despite a superficial similarity, in that all involve hemorrhagic tissue reactions, these show distinct differences.

Both local Shwartzman and Arthus reactions are shortlived, with duration measured in hours, and are immediate responses to a second dose of endotoxin and foreign antigen, respectively. Both are marked by the presence of polymorphonuclear leucocytes(8,9) and both are hemorrhagic in character. In the Shwartzman reaction, the initial dose of endotoxin is believed to block the reticuloendothelial mechanisms for removal of thrombin (10), which sets the stage for massive coagulation and hemorrhagic necrosis when the second, challenging dose of endotoxin is given.

The carrageenan reaction reported here, on the other hand, involves a continuing sensitized state over a period of several weeks. Leucocytes were not prominent at the time of the second injection and its sequel, the sensitization reaction. No unusual aggregation of leucocytes, or of other types of cells, was found at the site of injection in animals sacrificed after the initial dose only, at the time other guinea pigs were receiving the

challenging dose. The hemorrhagic appearance of the carrageenan granulomas was probably due not to coagulation but to the anticoagulant effect of this sulfated polysaccharide, recently investigated in detail by Anderson and Duncan(11).

The carrageenan sensitization reaction, thus, is of different character from these classical phenomena, but as it represents an overreaction to this material based on the animals' prior experience it may be termed a sensitization. Whether this is immunological in nature cannot be stated on the basis of the present experiments, but will depend upon demonstration of antibodies to the polysaccharide. The usual response to subcutaneous carrageenan administration is the establishment of a colony of connective tissue cells, predominantly fibroblasts and macrophages, which produce mucopolysaccharide and collagen and which in some manner dispose of the injected material. The same cells were prominent in granulomas at the two week stage of development in both sensitized and control animals. The sensitized guinea pigs responded to the challenging dose of carrageenan with a granuloma twice the size of that in controls. One can rather imagine that the increased total amount of blood, measured as the iron of hemoglobin, resulted from the physical presence of an increased mass of tissue. On the other hand, it is obvious that the total synthesis of protein was increased with the increased cellular population, despite the fact that protein concentration remained constant. Although total collagen appeared to be slightly higher in the sensitized animals, its insignificant increase never approached the level of the increase in total protein.

In an earlier study(12) we have reported that collagen production stimulated by various preparations of carrageenan bore an essentially straight line relationship to the amount of lambda carrageenan administered. In the present experiments, despite the presence of roughly twice as many of the appropriate cells, it would appear that the capacity for collagen production under the influence of the challenging dose of 50 mg carrageenan was not altered substantially by the establishment of a sensitized state in the guinea pig.

Experiments are in progress to determine whether anti-carrageenan antibodies can be demonstrated in sensitized animals.

Summary. Guinea pigs pretreated with unfractionated carrageenan subcutaneously exhibited a sensitization reaction when challenged 3, 5 or 8 weeks later with the same polysaccharide. The same phenomenon occurred in guinea pigs pretreated and challenged with lambda carrageenan, the active fraction for stimulation of connective tissue growth. Granulomas from sensitized animals were consistently larger, and had significantly lower collagen concentrations than granulomas from animals pretreated with saline. Total protein, dry defatted solids, and iron concentrations were not significantly altered, but a two-fold increase in granuloma weight indicated a large increase in these elements, and especially in non-collagenous protein synthesis.

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