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IN VITRO STUDIES ON THE ROLE OF COLLAGEN IN THE INDUCTION OF HYPERSENSITIVITY TO FLEA BITES[1]

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Previous reports have demonstrated that *in vitro* collected saliva of fleas contains a dialyzable, heat- and acid-stable hapten responsible for flea bite hypersensitivity (1, 2). Furthermore, activity related to flea bite hypersensitivity could be recovered from skin fractions of guinea pigs obtained from biopsies following flea bites (3). It was, therefore, postulated that in order to become a complete antigen, the flea hapten associates with components of the host's skin. Studies on the nature of the skin components associated with activity related to flea bite hypersensitivity revealed that both neutral salt soluble and acid soluble collagens, obtained from biopsies of skin previously exposed to flea bites, could induce hypersensitivity to flea bites upon their injection into recipient guinea pigs, whereas such activity could not be demonstrated in other skin fractions (4).

In view of the latter findings, *in vitro* experiments were performed in order to confirm the association of guinea pig collagen with saliva of fleas. For this purpose, *in vitro* collected saliva was used, and the immunologic activity, related to flea bite hypersensitivity, of the association product was tested. This article presents the results of these experiments.

MATERIALS AND METHODS

Preparation of collagen. Acid soluble guinea pig collagen was prepared according to the method of Piez *et al.* (5), with all extracting solutions and buffers containing a final concentration of 1:20,000 Merthiolate, so that when the final lyophilized pure collagen was made up to 1 mg/ml, the solution contained a final concentration of 1:20,000 Merthiolate. The amino acid composition of collagen was ascertained by amino acid analysis of a hydrolysate, using the Spinco model 120B amino acid analyzer. The percentages of glycine, proline and hydroxyproline residues from the total hydrolysate were 33, 14 and 9 respectively. These values are in agreement with those published for collagen (4, 5).

Collection of flea saliva. Cat fleas, *Ctenocephalides felis felis* were reared according to the method of Hudson and Prince (6). The fleas were then confined in enamel buckets and were allowed to probe on the enamel for several days. They were then removed and the buckets were washed in distilled water. The preparation was then passed through a 0.45-μ Millipore filter and lyophilized. The product was stored under nitrogen at −20°C until used. This preparation of *in vitro* collected saliva was chromatographically similar and immunologically identical to that collected according to the method of Young *et al.* (7).

Reaction of collagen with saliva of fleas. Twenty milligrams of acid soluble collagen were suspended in 20 ml water adjusted to either pH 10.6 or 7.2 with ammonia. Also, 20 mg collagen were dissolved in 20 ml of 0.1% acetic acid, pH 3.5. To each of these, 20-mg portions of lyophilized flea saliva were added and the reaction mixture was stirred at room temperature for 24 hr. This was followed by exhaustive dialysis of the reaction mixture in the cold against outer fluids of pH 10.6, 7.2 and 3.5 respectively. Controls, consisting of unreacted collagen and unreacted saliva, were similarly treated.

Preparation of inocula, sensitization and challenge. The dialyzed products were taken up in 20 ml of aqueous ammonia solution, pH 10.6 or pH 7.2, or in 20 ml of 0.1% acetic acid pH 3.5 to concentrations of 1.0, 0.1 and 0.01 mg collagen/ml. For injections, 2.5 ml of each sample was lyophilized, resuspended in 2.5 ml of 0.15 M saline, at a neutral pH, and was mixed with an equal volume of either Freund's complete adjuvant or 0.15 M saline. Guinea pigs in groups of five were each given 0.5 ml intradermal injection at a single site in the shaved abdomen; the

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following week each guinea pig was reinjected intradermally with another 0.5 ml at a different site. On the 3rd week each animal was challenged with bites of fleas according to the method of Benjamini *et al.* (8).

Skin reactions were evaluated 24 hr following challenge on the basis of the diameter and intensity of the erythematous area on a scale ranging from 0 to ++++ as follows: 0, no reaction; +, slight erythema approximately 1 mm in diameter; ++, moderate erythema approximately 2 mm in diameter; +++, intense erythema, 3 mm in diameter; ++++, very intense erythema, more than 3 mm diameter.

The sizes of reactions following challenges with bites were typical for flea bite hypersensitivity. The validity of the flea bite reactions was ascertained histopathologically (9).

Throughout this report the term "activity" denotes the capacity of a given preparation to induce hypersensitivity to flea bites as evaluated by skin reactions in response to challenges given with bites of fleas.

RESULTS

Reaction at pH 10.6 of collagen with flea saliva

Collagen was reacted at pH 10.6 with flea saliva as described. Results of the assays for the immunogenicity of the preparations and their appropriate controls are summarized in Table I. The results demonstrate that the immunologic activity of flea saliva at the concentrations tested resided in the dialyzable portion only; no significant activity could be demonstrated in the nondialyzable portion of the unreacted saliva, regardless of whether or not it was injected in saline or in combination with Freund's complete adjuvant. However, saliva which was reacted at pH 10.6 with collagen yielded a nondialyzable product which could sensitize guinea pigs to bites of fleas, whether injected in saline or in combination with Freund's complete adjuvant.

Reaction at pH 7.2 of collagen with flea saliva

Results of the assays for the immunogenicity of collagen reacted at pH 7.2 with saliva and their appropriate controls are shown in Table I. The results indicate that the activity of the saliva was dialyzable, and that the collagen which was reacted at pH 7.2 with saliva could not significantly sensitize the animals to bites of fleas, especially when injected in saline. The activity induced by the highest concentration (of collagen reacted with saliva) when injected in combination with Freund's complete adjuvant may indicate that either a very low degree of reaction took place between the collagen and saliva, or that sensitization was induced by residual amounts of unreacted saliva which were not completely removed by dialysis.

Reaction at pH 3.5 of collagen with flea saliva

Table I also shows the results of the assays for the immunogenicity of collagen reacted at pH 3.5 with saliva of fleas and of their appropriate controls. The results demonstrate the capacity of this preparation to induce hypersensitivity to flea bites when injected, especially in saline, into recipient guinea pigs. In contrast, whereas only the dialyzable portion of unreacted saliva was immunogenic, neither unreacted collagen nor the nondialyzable portion of saliva exhibited significant activity.

Reaction of various amounts of saliva with collagen

Since a high degree of reactivity was demonstrated by the product of the reaction, at pH 10.6, between 20 mg collagen and 20 mg saliva, it was of interest to ascertain the minimal amount of saliva necessary to render 20 mg collagen active in the induction of hypersensitivity to flea bites. For this purpose, 20-mg portions of collagen, thoroughly suspended in 20 ml of aqueous ammonia solution, pH 10.6, were reacted with various amounts of saliva ranging from 0.25 to 20 mg. Following exhaustive dialysis against outer fluid of pH 10.6, the preparations were made up with aqueous ammonia to various dilutions and were lyophilized. The preparations were then resuspended in physiologic saline and were injected into guinea pigs. Results are shown in Table II.

DISCUSSION

Previous observations showed that following bites of guinea pigs by fleas, the flea hapten(s) was found to be associated with collagen fractions of the bitten skin (4). Results given in the present communication on the *in vitro* interaction

TABLE I

Induction of flea bite hypersensitivity by collagen reacted in vitro at pH 10.6, 7.2 and 3.5 with saliva of fleas

Preparation	Dosage Injected (Mg Collagen or Saliva per Injection)	Reaction at pH 10.6		Reaction at pH 7.2		Reaction at pH 3.5	
		No. reacting animals/no. treated	Degree of response to challenge	No. reacting animals/no. treated	Degree of response to challenge	No. reacting animals/no. treated	Degree of response to challenge
Collagen reacted with saliva (injected with FCA[a])	0.25	4/5	++++	4/5	++	4/5	+
	0.025	5/5	+++	1/5	±	0/5	0
	0.0025	5/5	+++	0/5	0	1/5	±
Collagen reacted with saliva (injected in saline)	0.25	5/5	++++	1/5	±	5/5	++
	0.025	3/5	+++	1/5	±	5/5	+++
	0.0025	1/5	+	1/5	±	4/5	++
Collagen only (injected with FCA)	0.25	0/5	0	0/5	0	0/5	0
	0.025	0/5	0	N.D.[b]		0/5	0
	0.0025	0/5	0	N.D.		0/5	0
Collagen only (injected in saline)	0.25	0/5	0	0/5	0	0/5	0
	0.025	0/5	0	N.D.		0/5	0
	0.0025	0/5	0	N.D.		0/5	0
Dialyzable portion of saliva (injected with FCA)	0.25	4/5	+++	4/5	+++	4/5	+++
	0.025	5/5	++++	2/5	+++	3/5	++
	0.0025	N.D.		2/5	++	1/5	+
Dialyzable portion of saliva (injected in saline)	0.25	3/5	++	1/5	+	2/5	+
	0.025	3/5	+++	0/5	0	3/5	++
	0.0025	N.D.		N.D.		0/5	0
Nondialyzable portion of saliva (injected with FCA)	0.25	1/5	±	0/5	0	1/5	±
	0.025	2/5	±	0/5	0	1/5	+
	0.0025	1/5	±	1/5	±	1/5	±
Nondialyzable portion of saliva (injected in saline)	0.25	1/5	±	0/5	0	1/5	+
	0.025	0/5	0	0/5	0	1/5	+
	0.0025	0/5	0	1/5	+	1/5	±

[a] Freund's complete adjuvant.

[b] Not done.

between guinea pig collagen and *in vitro* collected saliva of fleas support the above findings. This is indicated by the fact that while the allergenic activity of flea saliva is dialyzable, the active product resulting from the incubation of saliva with collagen was nondialyzable.

Results shown in Table I indicate that following incubation of saliva with collagen at pH 10.6 the dialyzable activity of saliva was now present in the nondialyzable portion of the reaction mixture. This is probably due to the association of the dialyzable hapten with collagen, since no significant activity was exhibited by the nondialyzable portion of saliva. A similar phenomenon occurred when the reaction between saliva and collagen was performed at pH 3.5. Here again, while the nondialyzable portion of saliva was inactive, the nondialyzable portion of the reaction product between saliva and collagen exhibited immunogenicity. It is interesting to note that activity of this nondialyzable product could be demonstrated best when the product was injected in saline, rather than in combination with Freund's complete adjuvant. A similar observation on the hindrance of immunogenicity

TABLE II

Induction of flea bite hypersensitivity by injections of collagen reacted in vitro at pH 10.6 with various amounts of flea saliva

Mg Saliva used for Reaction with 20 Mg Collagen	Dosage Injected (Mg Treated Collagen per Injection)	No. Reacting Animals/No. Used	Degree of Response to Challenge with Flea Bites
20.0	0.25	4/5	+++
	0.025	4/5	+++
	0.0025	4/5	+++
10.0	0.25	5/5	+++
	0.025	4/5	++
	0.0025	2/5	+
4.0	0.25	4/5	+++
	0.025	2/5	+
1.5	0.25	2/5	+
	0.025	5/5	+
	0.0025	2/5	±
0.25	0.25	2/5	+
	0.025	3/5	±
	0.0025	2/5	+
0.00	0.25	0/5	0
	0.025	0/5	0
	0.0025	0/5	0

due to Freund's complete adjuvant has previously been reported (4).

No significant activity was exhibited by the nondialyzable fraction after mixing flea's saliva and collagen at a neutral pH, although the activity of the dialyzable portion of saliva was apparent when it was injected in combination with Freund's complete adjuvant. These results indicate the lack of association between the flea hapten and collagen. Furthermore, although the products of the reactions at basic and acidic pH were dialyzed against the respective pH at which the reaction was carried out, and not against neutral pH, these final lyophilized products were taken up at neutral pH prior to injection. The fact that these preparations were immunogenic when injected in saline indicates that at neutral pH no dissociation of the reaction products occurred. Had such dissociation occurred, the product would not have been immunogenic when injected in saline, since the hapten injected in neutral saline was not immunogenic.

Data presented in Table I on the activity of the dialyzable portion of saliva indicate that the prolonged exposure to the basic or acidic conditions resulted in some change affecting its immunogenicity. It has been our experience that the injection of *in vitro* collected saliva into guinea pigs results in sensitization only if injected in combination with Freund's complete adjuvant (7, 8). However, results presented in Table I indicate that following prolonged exposure to basic or acidic conditions, sensitization could be achieved not only when injected with Freund's complete adjuvant, but even when injected in saline, and at a neutral pH. At present we do not have an explanation for this phenomenon.

The foregoing data indicate that the interaction between the flea hapten and guinea pig collagen, as measured by the immunogenicity of the reaction product to challenges given with bites of fleas, occurs at either basic or acidic, but not at a neutral pH. The type of reaction which takes place between the hapten and collagen is yet unknown; the finding that the reactions at basic pH and at acidic pH resulted in immunogenic nondialyzable products may indicate either the formation of the same product through different reaction mechanisms or the formation of chemically different but antigenically similar products. Studies on possible reaction mechanisms, utilizing synthetic polyamino acids as immunologic carriers for the flea hapten, are currently in progress.

Results presented in Table II shed some light on the quantitative aspects of the *in vitro* reaction, at pH 10.6, between collagen and the flea hapten. These data demonstrate that the immunogenicity of the reaction product between collagen and saliva depended on the ratio between the reactants. When reacted with 20 mg of collagen, both 20 mg and 10 mg of saliva yielded an immunologically active product, while 4 mg of saliva reacted with the same amount of collagen yielded a much weaker immunogenic material; smaller quantities of saliva yielded products of negligible or no immunogenicity.

SUMMARY

Acid soluble collagen and *in vitro* collected saliva of fleas were allowed to react at pH 3.5, 7.2 and 10.6. Whereas all the activity of saliva is dialyzable, the nondialyzable products of the reactions at both the acidic and the basic pHs had the capacity to induce hypersensitivity to flea bites in recipient guinea pigs; the product of the reaction at neutral pH was not immunogenic.

The ratio between the amounts of saliva and of collagen was shown to be important in the production of immunogenic products.

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