

Allergy to Flea Bites. III. The Experimental Induction of Flea Bite Sensitivity in Guinea Pigs by Exposure to Flea Bites and by Antigen Prepared from Whole Flea Extracts of *Ctenocephalides felis felis*¹

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(Submitted for publication, 6 June 1960)

Guinea pigs were sensitized to flea bites by previous exposure to *Ctenocephalides felis felis*. Delayed reactions appeared at from 5-7 days following daily exposures. Two to 5 days following the appearance of delayed reactions, the animals showed both delayed and immediate types of reactivity. The induction of sensitivity by flea bites was independent of the number of fleas biting. Sensitivity to flea bites induced either by intradermal injection of whole flea extract in saline or by flea bites was systemic and appeared to be an allergic phenomenon.

Sensitivity of guinea pigs to flea bites was induced by intradermal injections of whole flea extracts in saline. Guinea pigs injected intradermally with whole flea extract reacted to flea bites. Conversely, when sensitized by flea bites, they reacted to challenge with the extract. While sensitivity to flea bites induced by flea bites required a latent period of 5-7 days, sensitivity to flea bites induced by whole flea extract required 10-14 days.

Previous reports from our laboratory showed that guinea pigs could be sensitized to the cat flea *Ctenocephalides felis felis* (Bouché) by previous exposure to flea bites (Hudson *et al.*, 1960). It was demonstrated that hypersensitivity developed within 5-7 days after daily exposures to flea bites, during which time the animals manifested delayed skin reactions characterized by induration and erythema at the bite site. These

reactions appeared within 10-24 hours following the challenging bite.

The next step in our study of allergy to flea bites was to determine whether or not flea-bite sensitivity in guinea pigs could be induced by extracts of whole fleas, and if so, to determine the course of the reaction and to obtain preliminary information on the nature of the allergen. It is the purpose of this paper to report the results of these studies which demonstrate that whole flea extract contains the necessary substance or substances responsible for the bite reaction.

¹ This investigation was supported by Research Grant E-1389 from the National Institute of Allergy and Infectious Diseases of the National Institute of Health, Public Health Service.

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MATERIALS AND METHODS

Fleas

Ctenocephalides felis felis were reared in our insectary according to the method described by Hudson and Prince (1958). For ease of manipulation, the insects were anesthetized lightly with CO₂. Under such anesthesia complete recovery occurred after less than 1 minute.

Whole Flea Extract

This was prepared according to the method employed by Hudson (personal communication). Essentially, the method consists of saline extraction of lyophilized ground defatted fleas, dialysis of the extract, and finally lyophilization of the extract, which yields a pale-gray amorphous powder. Our experience has been that processing 1,000 fleas in the above manner yields 5 mg of the final saline soluble, non-dializable whole flea extract. A 1% solution of the whole flea extract in 0.87% saline contains 81.0 mg % nitrogen.

Spectrophotometric studies of the whole flea extract in saline, using the Beckman D.U. spectrophotometer revealed that the material absorbs at the ultraviolet region with a maximum absorption at 275 millimicrons. Consequently, standard absorption determinations for various concentrations of the extract were carried out, the results of which gave a straight line when plotted on logarithmic paper.

The electrophoresis of the whole flea extract, on the Karler-Misco continuous paper curtain electrophoresis apparatus, using 3 mm Whatman paper with 0.01 M barbital buffer pH 8.6 at 350 volts (0.2 ma), separated the preparation into several fractions. After drying at 95°C for 35–40 minutes, the developed electrochromatogram showed three fluorescing bands upon examination with ultraviolet light. Staining the paper with bromphenol blue revealed the presence of several proteinaceous fractions. This was confirmed by the spectrophotometric density determination of the collected fractions at a wavelength of 280 millimicrons, as shown in Fig. 1.

For injection, the whole flea extract was suspended in physiological saline containing 1:20,000 merthiolate, filtered either through a Seitz filter or through ultrafine ground glass filters, and finally cultured on Brewer's thioglycolate media. When results of culture for bacterial contamination were negative, the preparation was injected either in saline or in combination with the complete Freund adjuvant.⁵

⁵ Bacto adjuvant complete Freund. Difco Laboratory, Inc., Detroit, Mich.

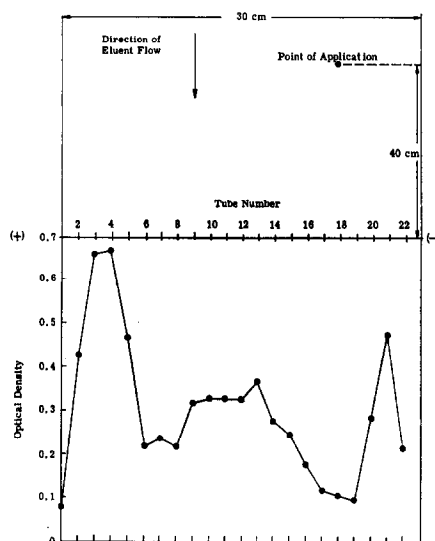


FIG. 1. The electrophoretic pattern resulting from fractionation of whole extract of *Ctenocephalides felis felis* by use of the Karler Misco continuous paper curtain electrophoresis apparatus.

Experimental Animals

Animals used were albino guinea pigs weighing between 300–400 grams.

Sensitization and Challenge Procedures

Technic of Exposure to Fleas. Twenty unfed fleas, 2 days old, were transferred under light CO₂ anesthesia into small plastic tubes, 2.5 cm in diameter and 2.0 cm in height, covered at both ends with nylon chiffon screens. These cages were placed on the abdomens of guinea pigs which were previously shaved with an electric razor and depilated with a commercial agent containing calcium thioglycollate. The cages were secured to the animal's abdomen by means of a 3-inch-wide strip of elastic bandage. The guinea pigs were exposed to flea bites for 15 minutes, at the end of which time reactions were observed and recorded. Readings also were taken at intervals of 20 minutes, one hour, 6 hours, 24 hours, and sometimes 48 hours following the exposure. The guinea pigs were exposed to flea bites daily until they showed skin reactions.

The inclination of fleas to feed was determined by anesthetizing the fed fleas with ether and then crushing each individual flea

on white paper; the appearance of a blood spot indicated feeding. Throughout the study feeding percentages of fleas in each of over 100 cages were found to average over 90 % per cage with a range of 80 % to 100 %. Accordingly, it was felt that in instances where no immediate or delayed reactions were observed, failure to react could not be attributed to failure of the fleas to feed.

Injection Procedures and Preparation of Inocula. Attempts to sensitize guinea pigs to flea bites by intramuscular or subcutaneous injections of whole flea extract in saline resulted in failure (Hudson, B. W., personal communication). Accordingly, the present experiments were carried out either by intramuscular injection of the extract in combination with the complete Freund's adjuvant; intramuscular injection of alum-precipitated extract, or intradermal injection of the extract in saline.

The preparation of the whole flea extract in combination with the complete Freund's adjuvant was as follows: to 20 ml of sterile Freund's adjuvant 6.66 ml of sterile Seitz-filtered 8 mg/ml suspension of whole flea extract in saline was added. The final concentration was adjusted with sterile saline to 2 mg of whole flea extract per ml saline. The preparation was preserved with 1:20,000 merthiolate and shaken well before injection.

The preparation of the alum-precipitated whole flea extract was as follows: seven (7) ml of a sterile suspension of 8 mg/ml whole flea extract in saline were adjusted to pH 6.5 with 1N NaOH. To this, 7 ml of 20 % w/v ammonium aluminum sulfate were added. The formed precipitate was collected following refrigerated centrifugation at 1000 g for 30 minutes and resuspended in 0.85 % saline to give a final concentration of 2 mg whole flea extract per ml. The preparation was preserved with 1:20,000 merthiolate. Generally, the volume injected into each animal was 1 ml/kg body weight. Occasionally, the injected volume was kept constant at 0.5 ml per animal.

Methods of Challenge. Sensitivity of guinea pigs to fleas was determined by exposure of the animals to flea bites in the manner described above. When animals were challenged with whole flea extract, the material

was injected intradermally in 0.05 ml physiological saline. In this manner, animals sensitized by flea bites were challenged with the extract, and animals sensitized by the extract were challenged with flea bites. In the latter case the animals were exposed to fleas daily until reactions appeared.

EXPERIMENTAL

Types of Sensitization Phenomena Elicited

Throughout this study over 100 animals were sensitized to flea bites by daily exposure to fleas (in most cases by exposure to 20 fleas). Twenty-four hour delayed skin reactions, characterized by induration and erythema, appeared within 5–7 days. Within 2–5 days following the appearance of these delayed reactions, immediate reactions appeared within 20 minutes to one hour following exposure. The immediate reactions lasted but a short time and usually faded completely within 4–6 hours. These reactions, characterized by erythema and edema, were significantly different from the minute hemorrhagic areas at the punctures sites caused by the flea feeding mechanism.

In most cases, when the animals became reactive, all the sites of previously nonreacting bites flared up. The same phenomenon was also reported by Hudson *et al.* (1960).

Figure 2 shows the relationship between time and the development of delayed and immediate reactions. The shape of both curves indicates that there is a latent period for the development of hypersensitivity to the bites. Only very few animals reacted before 3–4 days of exposure; most animals showed delayed reactivity after 5–6 days of exposure, and immediate reactivity after 6–8 days of exposure. Some animals become hypersensitive more slowly than the majority, acquiring their hypersensitivity only after 10 daily exposures to flea bites. It is important to note that as many as 94.7 % of all animals showed delayed reactions, and 77.9 % of all animals showed immediate reactions. Except in a few isolated cases (less than 5 % of the total guinea pig population), the immediate reaction appeared after the onset of the delayed reaction.

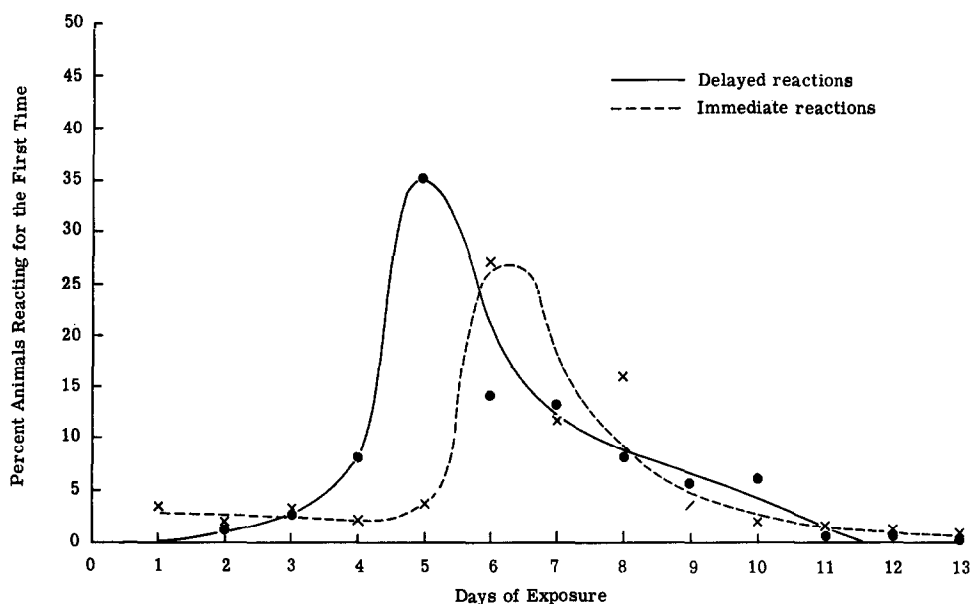


FIG. 2. The relationship between time and development of delayed and immediate reactions in guinea pigs induced through sensitization by bites of *Ctenocephalides felis felis*.

The Influence of the Number of Biting Fleas on the Development of Hypersensitivity

It was reported by Hudson *et al.* (1960) that guinea pigs could be sensitized to flea bites either by repeated daily exposure to small numbers of fleas (5–20), or by a single exposure to large numbers (several hundred), and that the animals showed hypersensitivity at the end of the usual period of from 5–7 days after the initial exposure. In order to establish whether or not the number of fleas, in repeated daily exposures, affects the development of hypersensitivity, an experiment was set up consisting of 5 groups of guinea pigs, 5 animals per group. The groups were exposed daily to variable numbers of fleas until the animals developed hypersensitivity. The data presented in Table I show that, except for a slight shortening of the latent period in development of delayed reactions in cases where animals were exposed to larger numbers of fleas, the course of development of the reactions was independent of the number of sensitizing fleas. Even in cases where 80 or 300 fleas were used for daily exposure, the slight reduction of the latent period in development of delayed reactions seems to be of little significance. However, when the animals were exposed to

TABLE I
Relationship of Number of Ctenocephalides felis felis Bites to Development of Hypersensitivity in Guinea Pigs

Effect on guinea pigs	No. fleas used per animal for daily exposure				
	2	5	20	80	300
Average No. days to development of delayed reactions	6.3	5.2	5.0	4.8	4.8
No. reacting/No. used	4/5	5/5	4/4	4/4	5/5
Average No. days to development of immediate reactions	11.0	11.0	8.3	10.0	9.8
No. reacting/No. used	1/5	3/5	4/5	5/5	5/5

the bites of 2 fleas only, 2 out of 5 animals showed immediate reactions, and 4 out of 5 animals showed delayed reactions. The number of immediately reacting animals increased when 5 fleas fed, and increased more when the animals were exposed to 20, 80, or 300 fleas. These results suggested that a minimum of 20 cat fleas per cage should be

used in order to insure that a sufficient number of fleas actually feed.

Data presented in Table I also show that in this experiment the immediate reactions generally appeared within an average of about 5 days following the appearance of delayed reactions. In no case did the appearance of the immediate reactions precede that of the delayed.

Preliminary Experiments on Sensitization with Whole Flea Extract

Prior to detailed experimentation on the sensitization of guinea pigs to flea bites by whole flea extract, a preliminary experiment was performed in which animals were injected weekly, for 3 weeks, with whole flea extract in saline, whole flea extract in combination with the complete Freund's adjuvant, and alum-precipitated whole flea extract. Controls consisted of saline, Freund's adjuvant, a preparation of saline which underwent alum precipitation identical to that used in the preparation of alum-precipitated whole flea extract. Intramuscular injections were given with the antigen in combination with Freund's adjuvant, alum-precipitated whole flea extract, and the

corresponding controls. The other injections were given intradermally. Dosages used in these injections consisted of 1 mg whole flea extract per kg body weight. At the end of the sensitization period all animals were exposed daily to bites of 5 fleas each until delayed reactions appeared.

The data, which are presented in Fig. 3, show that both the intradermal injection of the extract in saline, and the intramuscular injection of the whole flea extract in combination with Freund's adjuvant sensitized the guinea pigs to flea bites. Most animals which were not treated with the extract developed delayed reactions within 6-8 days after daily exposure to fleas; none showed reactivity in less than 3 days after exposure. Most animals which were injected intradermally with the whole flea extract in saline developed delayed reactions within 3-4 days of daily exposure to flea bites, while some exhibited reactions as early as the first day of exposure. Similarly, most animals which were injected with the antigen in combination with Freund's adjuvant showed delayed hypersensitivity within 3-4 days of daily exposure to fleas, and some exhibited these reactions upon first exposure. No sensitivity

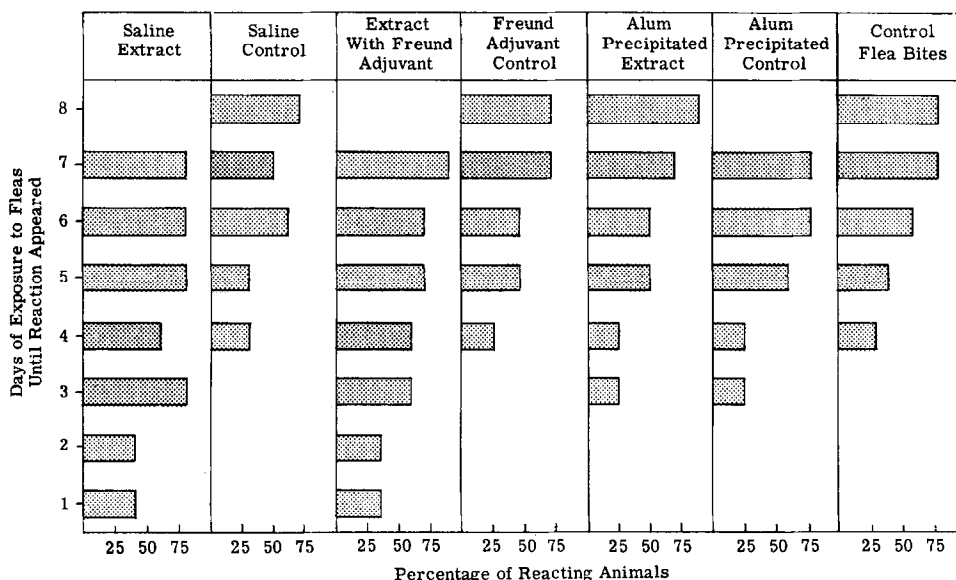


FIG. 3. Induction of flea bite sensitivity in guinea pigs by intradermal injection of whole cat flea extract in saline, intramuscular injection of the extract combined with Freund's adjuvant, and intramuscular injection of alum precipitated extract.

TABLE II

*Correlation Between Delayed Reactivity due to Flea Bites and Delayed Reactivity due to Sensitization with Whole Flea Extract in Guinea Pigs**

	Flea sensitized	Whole flea extract sensitized
Challenge with flea bites	++++	++++
Challenge with whole flea extract	++	+++++

* The number of positive signs indicate the severity of the reaction.

to flea bites was induced by the alum-precipitated flea extract or by any of the control injections.

Results of the above experiment suggested that the routine sensitization of guinea pigs to flea bites should be carried out by intradermal injections of whole flea extract in saline.

The Relation Between Hypersensitivity to Flea Bites Induced by Exposure to Fleas, and that Induced by Injections of Whole Flea Extract

In order to ascertain whether or not a correlation exists between the antigenic effect of the flea bite and the antigenic effect

of the whole flea extract, guinea pigs, previously sensitized by exposure to fleas, were challenged with intradermal injections of 0.05 ml of 1 mg/ml whole flea extract. While slight immediate reactions resulted from the challenge, marked, delayed reactions appeared at the end of 24 hours. In a preliminary experiment of this kind, 10 flea-sensitized guinea pigs were used. However, later such challenges were given routinely to many animals, and they always resulted in positive reactions. Thus a correlation between reactivity due to flea bites and reactivity due to whole flea extract may be summarized as shown in Table II.

The Period Required for the Sensitization of Guinea Pigs to Flea Bites with Whole Flea Extract

In order to establish the period required for the sensitization of guinea pigs to flea bites with intradermal injections of whole flea extract, groups of 5 animals each were injected with 8 mg/ml/kg whole flea extract in saline. At intervals of 0, 2, 7, and 14 days following injection the animals were exposed daily to flea bites until delayed reactions appeared. Results in Fig. 4 indicate that all animals were hypersensitive, manifesting

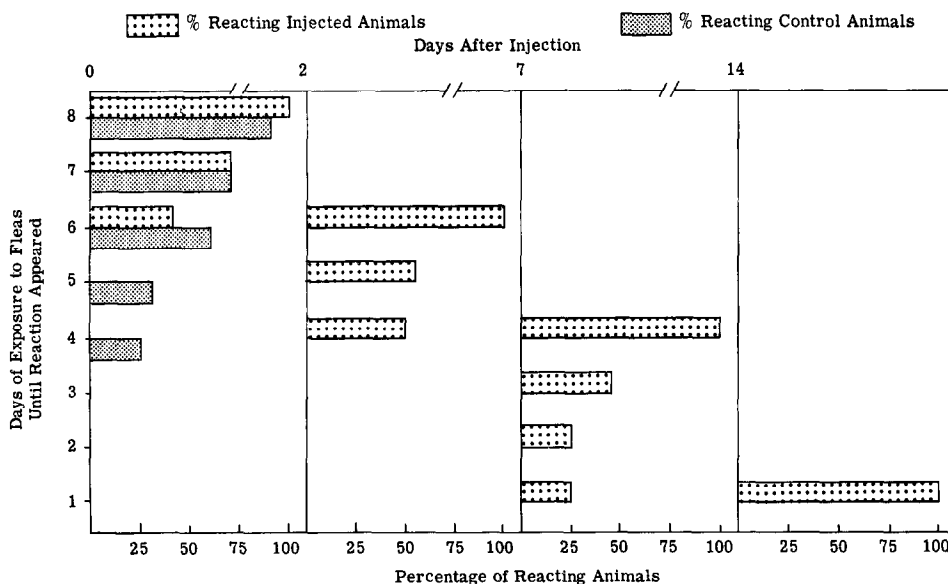


FIG. 4. The period required for sensitization of guinea pigs to flea bites by intradermal injection of whole cat flea extract in saline.

delayed skin reactions 14 days following the injection. At 7 days, all animals showed delayed type of reactivity, but only after 4 days of daily exposures to flea bites. In the case of animals which were challenged with flea bites 2 days after the injection, all became hypersensitive, but only after daily exposures to flea bites for 6 days. When the animals were challenged with flea bites immediately following the injection, few bite reactions appeared until 6 to 8 daily exposures to flea bites. This period corresponds to the latent period required for the sensitization of guinea pigs with flea bites. The data presented in Fig. 4 indicate that the latent period for sensitization with intradermal injections of whole flea extract in saline was approximately 14 days, with differences shown by individual animals.

A comparison between the latent period required for the appearance of delayed reactions by the sensitization of guinea pigs by flea bites, and that required for sensitization by intradermal injection of whole flea extract in saline, reveals a difference of approximately 6 days. This may indicate that the sensitizing mechanisms of the two methods differ somewhat, although both result in

the same type of sensitization and both show a high degree of cross reactivity. The latent period between the appearance of delayed reactions and the appearance of immediate reactions was approximately the same for both methods of sensitization. An illustration of the induction of delayed and immediate flea bite reactions in guinea pigs by the intradermal injection of whole flea extract in saline is presented in Fig. 5.

The Effect of Dosage on Hypersensitivity to Flea Bites When Induced by Intradermal Injection of Whole Flea Extract in Saline

In order to ascertain the effect of dosage on the induction of hypersensitivity by the intradermal injection of whole flea extract, six groups of 5 animals each were injected intradermally with 0., 0.05, 0.2, 1.0, 4.0, and 8.0 mg/ml/kg whole flea antigen in saline. Fourteen days following this single injection the animals were challenged with flea bites until delayed and immediate reactions appeared. Results summarized in Table III show that sensitization was independent of the injected dose.

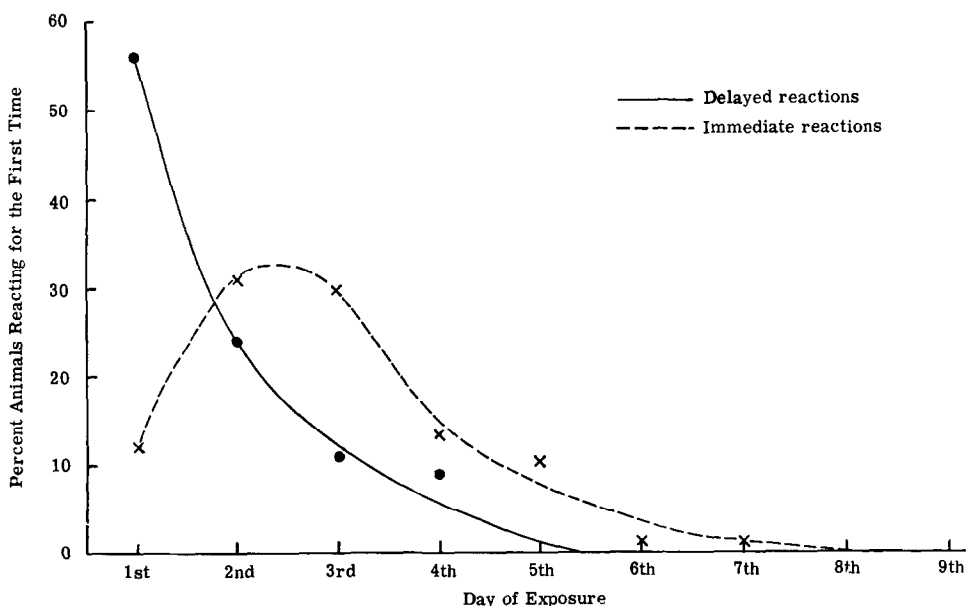


FIG. 5. Sensitivity of guinea pigs to flea bites induced by intradermal injection of cat flea extract in saline, showing development of delayed and immediate skin reactions.

*The Systemic Effect of Sensitization to
Flea Bites Induced by Intradermal
Injection of Whole Flea
Extract in Saline*

In order to establish whether sensitization is a local or systemic phenomenon, an experiment was carried out in which each of 10 guinea pigs was injected intradermally with whole flea extract; the injection was given in the right hind thigh. Fourteen days following this single injection each animal was challenged with bites of 20 fleas on the central portion of the abdomen. All animals responded to the challenge with delayed skin reactions upon first exposure to flea bites; the hypersensitive response taking its usual course. This suggested that the sensitization was systemic rather than localized.

DISCUSSION

The hypothetical course of reactivity of humans to insect bites is composed of 5 main stages: 1) stage of no reactivity as a result of the lack of sensitization or exposure to bites; 2) after a certain latent period, starting from the initial sensitization of exposure to bites, the individual exhibits delayed reactions only; 3) upon continuous exposure to the sensitizing agent the individual exhibits both immediate and delayed reactions; 4) further exposure reduces the delayed reactions and the individual exhibits immediate reactions only; 5) continued exposure would finally cause no reactions at all as a result of complete desensitization of the individual.

This course of events in human reactions is partially supported by fragmentary experiments reported by various workers such as Theodor (1935), Hecht (1943), Mellanby (1946), Heilsen (1949), and summarized by McKiel (1955). Nevertheless, it would be premature to analogize human and animal reactions to insect bites. The small amount of work accomplished with laboratory animals has presented a rather confused picture in view of the contradictory reports by several authors. Thus it has been demonstrated that both guinea pigs and rabbits show only immediate reactions to mosquito bites, with no delayed reactions (McKiel, 1959). Hudson *et al.* (1960) showed that guinea pigs

TABLE III

The Effect of Dosage on the Induction of Hypersensitivity to Flea Bites in Guinea Pigs by the Intradermal Injection of the Whole Flea Extract in Saline

	Dosage in mg/kg					
	0	0.05	0.2	1.0	4.0	8.0
No. of daily exposures to fleas until delayed reactions appeared	6	1	1	1	1	1
% Reacting animals	80	60	100	100	100	100
No. of days from delayed to immediate reactions	2	3	3	3	3	3
% Reacting animals	60	80	60	80	100	100

exhibit delayed reactions to flea bites, with no immediate reactions, while the present report demonstrates that guinea pigs react to flea bites with delayed skin reactions which, after several days, are accompanied by immediate reactions. Experiments on the sensitization of rabbits to flea bites, by daily exposures to bites of 20 fleas, resulted in the induction of immediate reactivity only. The stage of delayed reactivity, which seems to be requisite for the appearance of immediate reactivity in guinea pigs, was completely missing in rabbits (Benjamini, unpublished).

This confusion may stem partly from the fact that different species of sensitized hosts may react differently to insect bites, as well as from the fact that various workers in this relatively new field are not using standardized techniques in the experimental procedures and in the evaluation of results. It is therefore felt that, at the present state of knowledge on hypersensitivity to insect bites, no general conclusions should be drawn. Accordingly the present discussion will be limited to the experimental results presented in this report.

It appears that the sequence of reactions of guinea pigs, in their development of hypersensitivity to flea bites by exposure to fleas, or by the intradermal injections of whole flea extract, is similar to the first three stages of the generalized reactions of humans to insect bites. The animals first show no reactions at all; after a certain latent period,

the animals exhibit delayed reactions, and finally, upon repeated exposure for only a few more days, the delayed reactions are accompanied by immediate reactions. We were unable to reach the stage of immediate reactions without delayed reactions, or the stage of complete desensitization even after repeated exposure for several months. It is possible that in time we shall reach these stages. It is also possible that while either of the last two stages may occur in the reactivity course of humans to insect bites, they do not occur in the reactions of guinea pigs to flea bites.

That the reaction to flea bites is allergic in nature, and that sensitization is systemic appears unquestionable; guinea pigs sensitized in one region of the body reacted to challenges given in other regions. Also, sensitivity either induced by flea bites or by intradermal injection of whole flea extract was independent of the sensitizing dosages used.

While it is realized that the whole flea extract contains a variety of compounds which may be antigenic, as revealed by electrophoresis, it undoubtedly contains the substance or substances which are related to flea bite hypersensitivity since a good correlation was noted between reactivity to the bite and reactivity to the extract. This correlation existed when guinea pigs reacted to flea bites after being sensitized by intradermal injections of the extract, and when animals reacted to the extract after being sensitized with flea bites. However, the latent period for the sensitization of guinea pigs to flea bites by injection of the extract extended from 10–14 days. This fact, and the fact that sensitization with the extract in saline was achieved only by intradermal injection, suggests that the sensitizing mechanism of the flea bite and that of the extract

may differ somewhat (perhaps in the injection method or injection site) although both result in identical reactivity.

Work is now in progress on the purification and isolation of the compound or compounds, found in whole flea extract, which are related to flea bite hypersensitivity in guinea pigs. Results of this work will be the subject of a future report.

ACKNOWLEDGMENTS

The authors wish to express their sincere thanks to Mr. R. D. Matarangas and Mr. R. S. Simpson for their technical assistance.

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