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The Bioassay of Contact Allergens in the Guinea Pig*

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Synopsis—A technique for the prospective testing in the GUINEA PIG of POTENTIAL HUMAN SENSITIZERS is described. The method utilizes local inflammation of the sensitization site, Freund's complete adjuvant, and repeated applications of prospective sensitizer under occlusion to intensify the acquisition of hypersensitivity sufficiently so as to identify weak and moderately weak, as well as strong, sensitizing materials. The test materials need not be chemically defined.

Introduction

The guinea pig is the only small laboratory animal suitable for evaluating the allergenicity of prospective sensitizers of man. Thus, only weak and/or erratic contact sensitization can be induced in other species such as mouse, rat, rabbit, hamster, dog, chicken, etc. (1–3). It is known that, in general, contact allergens that sensitize the guinea pig also sensitize man; further, there is a parallelism of efficiency of sensitization to particular chemicals in the guinea pig and human such that strong and weak sensitizers of the guinea pig are, relatively, strong and weak sensitizers of man (4). For example, *p*-phenylenediamine (a chemical that is the basis for most hair dyes) behaves as a strong sensitizer in both guinea pig and man, whereas aluminum chlorohydroxide (an antiperspirant) does not sensitize individuals of either species (5, 6).

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Present methods for the detection of prospective allergens in the guinea pig, such as the Landsteiner-Draize test, are successful with strong sensitizers; however, sensitizers of weak or intermediate strength are often missed (6, 7). For instance, Marzulli and coworkers, in a study of allergic contact dermatitis in the guinea pig and in man, reported that they were unable to sensitize any of several hundred guinea pigs to the topical anaesthetic "benzocaine," whereas they *could* sensitize a substantial number of men in a human test population to this compound (5). Clinically, benzocaine is a fairly common cause of allergic contact dermatitis in man (8).

We here propose a new method for rating, in the guinea pig, the allergic potential of prospective sensitizers of man. Means are taken to render the guinea pig more agreeable to sensitization, so that weaker allergens are recognized. The method derives from the "split-adjuvant" technique, in which chemical allergen and Freund's complete adjuvant are administered separately to the skin, rather than as an emulsion (9, 10).

Materials and Methods

Animals

The subjects of these experiments are closed-colony, controlled randomly-bred, Hartley guinea pigs weighing 350–550 g. The animals are maintained on fresh Purina guinea pig chow supplemented by thrice weekly lettuce, and water *ad libitum*. Males or nonpregnant females are used. The colony is clinically free of infection with Streptococcus C; we exclude animals of questionable health (skinny, poor coat, etc.). The toe nails and distal portion of both rear feet are wrapped with water-proof adhesive tape so as to prevent skin damage from scratching or clambering by cage mates (Fig. 1). These "boots" are renewed as they are shed.

Sensitization

The test animal is clipped in an area of skin on the back just behind the right shoulder girdle. The area to be clipped is delimited by a cloth frame whose square center measures about 2 x 2 cm. We use an Oster animal clipper with a size "0000" head; the lower teeth of the head are adjusted so as to evenly, and only slightly, over-ride the upper teeth—this minimizes skin irritation. Long hairs at the edges of the clipped patch, which might protrude into the site, are cut away with a scissor. The clipped patch is then shaved so as to remove a good portion of the loose keratin; in practice, a single-edge adjustable safety razor was found to be the best. We shave just short of bleeding and, in place of shaving cream, saturate the site with water. The animal is now wrapped in a "window dressing" which consists of an outer layer of adhesive

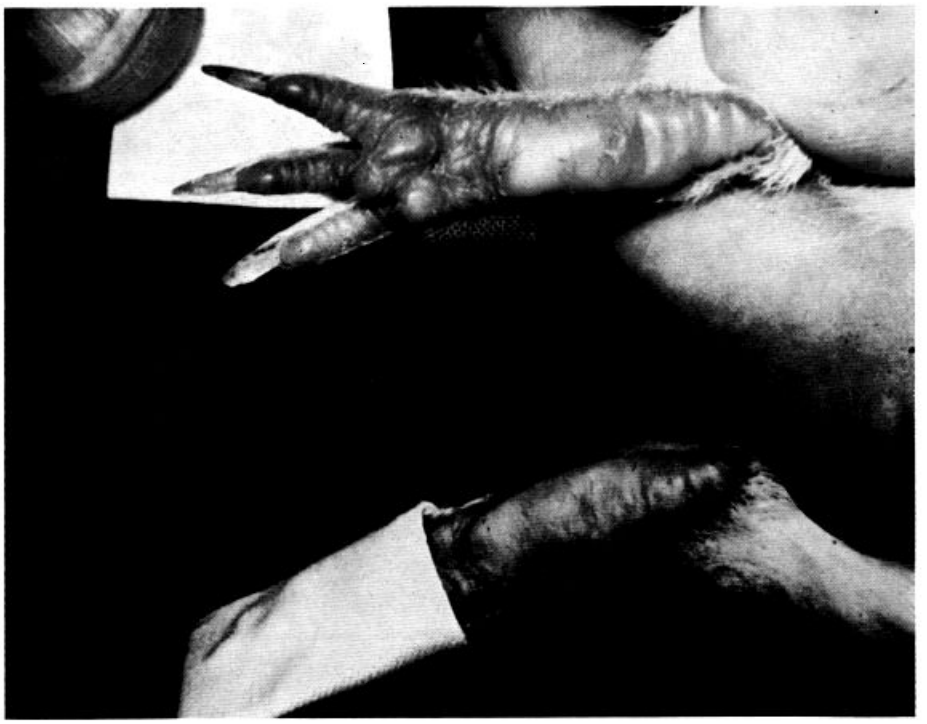

Figure 1. Adhesive tape wrapping protects the guinea pig's skin from scratches (necessary for hind feet only)

stretch fabric (Elastoplast®*) and an inner layer of loose mesh gauze (Kling®†). A 2 x 2-cm opening has previously been cut in the dressing; this opening is placed over the sensitization site, but overlapping slightly forward. The dressing is fixed with encircling adhesive tape fore and aft and is allowed to settle for a few hours; during this time it usually slides a ½ cm or so caudad of its initial placement. To the site, dry ice is applied for 5 sec with firm pressure. Then 0.2 ml of test ointment is applied, gently spread, and this is covered with a double thickness of Whatman #2 filter paper (Fig. 2). (In the case of liquids, 0.1 ml is usually used.) The filter paper has been cut so that its edges fit under the dressing. Then the area is covered with occlusive tape (Blenderm®‡) and this, in turn, fixed in place with adhesive tape. On Day 2, the dressing that covers the window is removed, 0.2 ml of test material is applied, and the window is reclosed with the same dressing. Day 4, the window is again opened and 0.1 ml of Freund's complete adjuvant (H37Ra, Difco§) is

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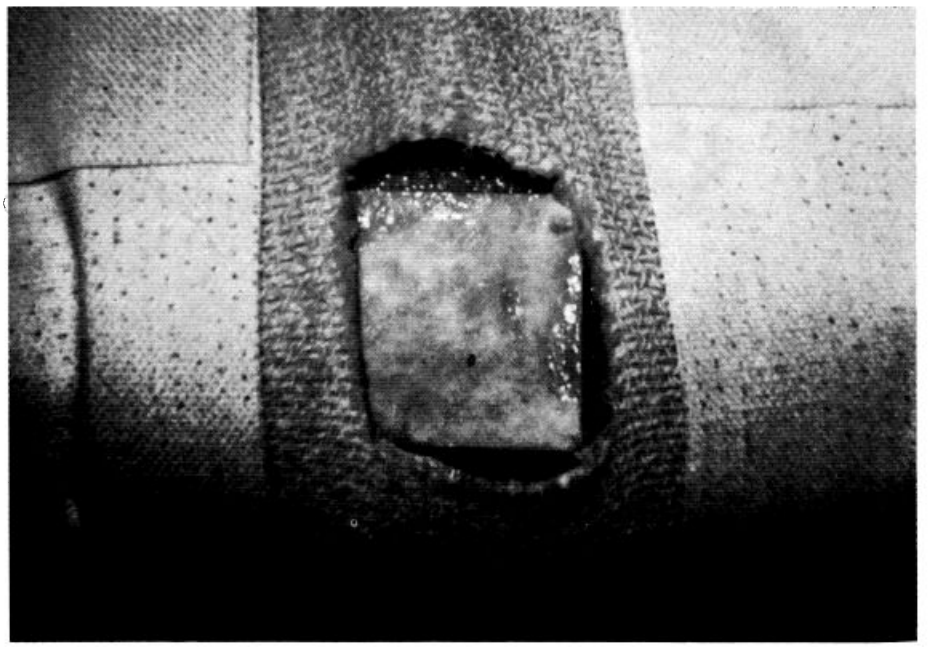

Figure 2. Dressing with window in place, test ointment applied and covered with filter paper

injected intradermally on either side of the sensitization site (total 0.2 ml per animal). This is followed by a further application of 0.2 ml of prospective allergen, and the window is closed. Day 7, the test material is again applied. Day 9, all wrappings are removed.

Challenge

Toxicity tests must be done on all materials prior to experimental challenge. In nonsensitized guinea pigs, the challenge material should not produce any substantial irritation. There are some materials such as soaps that are very difficult to test under occlusion since they are substantial primary irritants. However, most commercial materials that are intended for sustained topical application to human skin (e.g., cosmetics, topical antibiotics) can be used in challenge under an occlusive dressing. A negative control group of simultaneously tested toxicity guinea pigs is included wherever challenge is made of prospectively sensitized animals. A normal appearing area on the dorsal back is selected; it is best to test caudad of the shoulder muscles and cephelad of the muscles of the pelvic girdle since tests over muscles give more variable results. A site measuring 2 x 2 cm is atraumatically clipped and the overhanging long hairs from the edges are cut away with a scissor. One-tenth

milliliter of test compound is applied. This is covered with an occlusive dressing consisting of an outer layer of stretch adhesive (Elastoplast), a middle layer of wide-mesh gauze (Kling) and an inner layer of Blenderm tape, but with the sticky side away from the skin. The adhesive part of the Elastoplast overlaps the sides of the dressing. The dressing is fixed in place with adhesive tape (this may not be necessary). Twenty-four hours later, the dressing is removed and readings are made. The hair is clipped around the borders of the test site so as to enlarge it, precaution being taken to identify the test site by straddling marks made with a skin-marking pencil. Further readings are made at 48 hours, at 72 hours, and later, under special circumstances. Retestings at a different site for a second and third testing occasionally will bring out borderline earlier readings (5, 10).

The appearance of the challenge sites is recorded as word descriptions which are later scored (e.g., 0, trace, ±, +, ++ . . .) (9, 10). The scoring system rates the clinical intensity of the dermatitis induced by allergen as follows: 0, normal skin; ±, very faint pink, nonconfluent; +, faint pink; ++, pale pink, usually slightly elevated; +++, pale pink to pink, usually moderately elevated; ++++, pink and thickened; +++++, bright pink and markedly thickened. Intermediate scorings (trace, +±, etc.) are made as appropriate. Readings are best made by a senior person who has no knowledge of the test animals' prior history.

Prospective Sensitizers

Benzocaine, neomycin sulfate, hexachlorophene, Furacin, bithionol, and tribromosalicylanilide were obtained as powders.* Procaine crystals as Novacaine®†, 5% Xylocaine®‡ ointment, 5% mafenide cream (Sulfamylon®† cream), streptomycin sulfate, U.S.P.§, picric acid||, and petrolatum, U.S.P., were purchased in small quantity. When a powder was incorporated in petrolatum, this was done directly, i.e., without prior solubilization.

Results

In a typical early experiment we compared two topical anesthetics, *viz.*, benzocaine 5% and procaine 5%, each made up in U.S.P. grade petrolatum, as well as a commercial (lidocaine) topical anaesthetic, 5% Xylocaine ointment. Prospective sensitization was as outlined in Methods, except that the sensitization site was neither shaved nor treated with dry ice. The maximum

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|| Allied Chemical, Morristown, N.J.

Table I
Comparative Trials: Benzocaine 5% *vs.* Procaine 5%

	Sensitization	T-22 Challenge	
		1% Intradermal	5% Topical
78	Benzocaine 5%	0[a]	tr
79	↓	0	tr
80		0	0
81		0	0
82		0	+ ±
83		0	+ ±
t. c.	...	**0**	0
t. c.	...	**0**	**0**
84	Procaine 5%	13.0 mm	+++ ±
85	↓	4.0 mm	++
86		9.0 mm	++++
87		16.5 mm	++++
88		12.5 mm	++++
89		13.0 mm	++++
t. c.	...	**0**	**0**
t. c.	...	**0**	**0**

[a]1% suspension.

reactions to intradermal and topical challenge tests made on Day 22 are shown in Table I. Clearly, procaine is the more efficient sensitizer; 5% Xylocaine did not sensitize any animals. In later experiments, the sensitization rate with 5% benzocaine was increased to 100% by shaving and freezing the sensitization site. Under the latter circumstances, in different experiments, simultaneous prospective testing with 5% Xylocaine ointment, with 5% bithionol in petrolatum (no ultraviolet light activation), with 5% Furacin in petrolatum, and with 5% tribromosalicylanilide in petrolatum gave no sensitization. This accords with clinical and experimental results in man (5, 6).

In another experiment, we tested the allergenicity of three different concentrations of picric acid in petrolatum. The sensitization method was as outlined in Methods. For challenge open patch tests were made on Day 16 with one drop of 1, ⅕, and 1/15% picric acid dissolved in dibutyl phthalate (10). The maximum readings and the arithmetic sum of the "pluses" of each group of testings are tabulated in Table II. As is generally, but not universally, true, the higher concentration of sensitizer elicited the stronger reaction and induced the higher incidence of sensitization.

In another experiment, we prospectively sensitized groups of guinea pigs with 5% mafenide cream (Sulfamylon cream), 5% streptomycin sulfate suspended in petrolatum, and 5% neomycin sulfate suspended in petrolatum. In the respective groups (plus toxicity control groups) challenge was made with 5% mafenide cream, 5% streptomycin sulfate ointment, and 5% neomycin sulfate ointment. Five per cent mafenide cream and 5% streptomycin sulfate

Table II
Sensitization with Different Concentrations of Picric Acid Ointment

Sensitization		Challenge[a]	
		1%	1/15%
58	1%	+++++	+++ ±
59		(± +)	(tr)
60		(++++ ±)	(++++)
61		(+++)	(++)
62		(++++)	(+ ±)
63		(+++)	(++)
		21	13
64	0.2%	+++	+
65		0	0
66		++++	((++))
67		++++ ±	++
68		((tr))	**0**
69		+++++	++++
		16-½	**9**
70	0.04%	+++	(+)
71		**0**	0
72		**(+ ±)**	((±))
73		**tr**	**0**
74		**0**	**0**
75		++	+ ±
		6-½	3

[a]24h, (48 h), ((72 h)) readings.

ointment each strongly sensitized ⅝ animals in their particular groups; whereas only ⅜ of the guinea pigs prospectively sensitized to 5% neomycin sulfate ointment in fact became sensitized. Further, the reactions to streptomycin and mafenide were considerably more intense and slower to resolve than those to neomycin. Sulfamylon cream is used in the therapy of extensive burns; however, its sensitizing potential precludes its use as a routine topical antibiotic. Streptomycin is a potent contact sensitizer, allergic contact eczema to streptomycin being an occupational hazard of nurses who frequently handle the medication (as in certain sanitariums). Neomycin is incorporated as an antibiotic into a wide variety of topical preparations, but at a concentration of 1% or less. Neomycin sensitivity occurs infrequently, but because of the large numbers at risk, cases of allergic contact dermatitis to neomycin are not uncommon in clinical medicine (8). Certainly neomycin sulfate is a weaker sensitizer than streptomycin sulfate.

We evaluated the sensitizing potential of a number of unlabelled topical preparations that had been purchased in the market place.* In a typical ex-

* Supplied as coded material by Dr. F. N. Marzulli.

Table III
Comparative Trials of 3 Unknowns

Sensitization		T-16	T-28
94	H[a]	+++ ±	...
95		++	...
96		++++	...
97		+++	...
98		++	...
99		+++	...
100		++	...
101		+++	...
t. c. x 4	...	0, 0, 0, tr	0
102	F[b]	0	0
103		0	0
104		0	tr
105		0	0
106		f. tr	0
107		0	0
108		±	0
109		0	0
t. c. x 4	...	0, 0, 0, 0	0, 0, f. tr, 0
110	G[c]	0	0
111		(tr)	0
112		0	0
113		0	0
114		0	0
115		0	0
116		tr	0
117		0	0
t. c. x 4	...	0, 0, 0, 0	tr, tr, 0, 0

[a]Hair dye containing 2.8% *p*-phenylenediamine derivatives.
[b]Spray deodorant containing 0.03% hexachlorophene.
[c]Green soap containing 0.75% tribromosalicylanilide.

periment, we compared a hair dye (H), a spray deodorant (F), and a bar of antibacterial soap (G). Saturating amounts of deodorant were sprayed onto the skin site for sensitization and challenge. The bar of soap was solubilized as chips in distilled water at a concentration of 1% (w/v) and 0.1 ml was used for sensitization; challenge was made by open patch test since the soap solution proved to be somewhat irritating under occlusion. The results of this experiment are outlined in Table III. The hair dye was a strong sensitizer, the antibacterial soap and deodorant spray were not. This accorded with clinical experience; the hair dye contained one or more synthetic dyes (*p*-phenylenediamine derivatives) for which skin testing prior to use is mandatory. In another experiment we tested three marketed hair coloring agents: two permanent dyes and one vegetable rinse. In parallel with human experience, the dyes sensitized % and the rinse did not.

Discussion

The success of experiments dealing with the acquisition and expression of allergic contact dermatitis in the guinea pig requires the use of healthy animals. Vitamin C deficiency, to which the guinea pig is uniquely susceptible, and bacterial infections (particularly with Streptococcus C) must be carefully avoided. The albino Hartley guinea pig is widely available and is well suited for ordinary experiments in allergic contact dermatitis. If, for special experiments, histocompatible animals are required, the inbred Family II or Family XIII can be used. However, these inbred strains are less fertile than the Hartleys and on the average are somewhat more difficult to sensitize. Which sex is to be preferred? We have not been impressed with significant differences of skin reactivity between males and nonpregnant females. However, the female has the advantage of being more placid, and thereby easier to handle and less apt to have damaged skin from fighting. Pregnant guinea pigs give poorer and less reliable skin reactions and should not be used. Guinea pigs do establish a social order and should not be rehoused after the start of an experiment. Very young guinea pigs, less than 2 weeks of age, sensitized poorly; the defect appears to be peripheral in that their skin does not express the reaction of allergic contact dermatitis even when the animals are passive sensitized (with viable cells from adult sensitized guinea pigs).

According to Magnusson and Kligman, guinea pigs older than 1 year do not sensitize well (6). The cause for this is not known. In practice, we select young adult animals weighing close to 400–500 g. The weight and sex of the guinea pig should be controlled between groups where comparisons are to be made. Warm temperatures in the animal room, as during the summer in the absence of air conditioning, tend to impair the delayed-type immunological responsiveness of the animal. The requirement for a healthy, well-cared for, suitably chosen guinea pig in experiments dealing with allergic contact dermatitis cannot be overstressed.

The problem set in the predictive testing of possible contact allergens is to identify those materials that will cause significant difficulty in man. In the Landsteiner-Draize assay, the test material is dissolved, or suspended, in saline, and injected intradermally into the clipped dorsal skin of the guinea pig (7). The sensitizing schedule consists of a total of 10 injections given thrice weekly; the volume of the first injection is 0.05 ml, that of the others 0.1 ml. Two weeks after the last sensitizing injection, the animal is challenged with an intradermal injection of 0.05 ml delivered to normal skin. A significant difference in the reactions to the first sensitizing injection and the challenge injection is interpreted as sensitivity. Strong and moderately strong sensitizers can be identified by this technique. Magnusson and Kligman developed a maximization method in the guinea pig for identifying contact allergens (6, 11). Their sensitizing protocol involves the following: simultaneous but separate paired intradermal injections of (a) complete Freund's adjuvant (CFA),

(b) allergen, and (c) allergen emulsified in CFA. A week later, allergen is applied under a closed dressing to the area, and the dressing left in place for 48 hours. Challenge is made after a further 2 weeks. The maximization method of Magnusson and Kligman virtually eliminates the false negative results of the Landsteiner-Draize technique (Ref. 6, Table 8:6). However, there are several drawbacks to the maximization test. Materials for testing are administered to the skin both topically *and* parenterally. Thereby, the variable of the efficiency of penetration into the skin of prospective allergen is eliminated. Passage through the barrier layer (stratum corneum) is a prerequisite for sensitization and for challenge. Materials, such as nickel, that penetrate poorly are poorer sensitizers than they would be otherwise. It seems wise to avoid the artifice of an unnatural route of administration of a contact allergen. Materials designed for topical use are best tested for by the topical route alone. Conversely, materials to be assayed for parenteral allergenicity should be given by the particular parenteral route alone, at least in final testing. The Magnusson-Kligman maximization test is designed to optimize the conditions for inducing sensitivity with a particular substance. Accordingly, very high concentrations of prospective sensitizer are used and there is a standard solvent, usually petrolatum. It is the purpose of our test to assay the allergenicity of final materials, e.g., topical antibiotics, perfumes, etc., in their ultimate concentrations and vehicles. Clearly, the interaction of vehicle, active material(s) and preservatives as well as the particular concentrations of each will significantly affect the sensitization potential of a given preparation. It is the likelihood for inducing sensitization with proposed final products that our test is designed to measure. Of course, preliminary formulations can be assayed as well.

What is the mechanism of action of Freund's complete adjuvant in its intensification of acquisition of delayed hypersensitivity in the guinea pig? On the basis of studies utilizing a variety of cellular and soluble allergens, we have proposed the following scheme (12). Allergen administered to the skin interacts with circulating immunocompetent lymphocytes ("T" cells) thereby becoming immunocommitted. These lymphocytes pass to regional lymph nodes and clonalize. Freund's complete adjuvant acts in the regional node by making more efficient this clonalization. An important practical question is whether intensification by Freund's complete adjuvant changes the relative *ordering* of the sensitization potential of different materials or instead more or less regularly magnifies a given level of sensitivity. Our experience, and that of Magnusson and Kligman, and, additionally, a large body of information utilizing CFA to intensify the acquisition of sensitivity to macromolecular antigens suggests that there is no reordering of allergens.

It is to experiments from Landsteiner's laboratory that we owe observations that (a) Freund's complete adjuvant enhances the acquisition of allergic contact dermatitis in the guinea pig, (b) multiple exposures to a simple chemical

allergen increase the sensitization rate, and (c) previous irritation makes a skin site more agreeable for the induction of sensitivity (13–15). The technique of administering allergen under occlusion to the guinea pig was introduced by Buehler (16). Numerous studies in man and experimental animals have demonstrated that occlusion renders the barrier layer of the skin more permeable.

Clearly, the test method for the evaluation of putative contact allergens that is proposed here requires extensive evaluation to determine its usefulness. In a limited experience we have found it reliable. It should be emphasized that it is particularly suited for rating the allergic potential of unknown against known materials, especially where all are designed for the same final use. For example, a proposed topical antibiotic for a given purpose would be compared with two or more topical antibiotic preparations that are currently used for that purpose. Of course, all preparations would be studied, in different guinea pigs, at the same time. The results of such studies would then not give absolute ratings, but would imply a greater or lesser risk of sensitization by the unknown as compared to the known. With experience, certain likely extrapolations could be made between results of individual experiments.

Experience in other laboratories will doubtless improve our method. For instance, it may be that extensive shaving of the sensitization site is unnecessary and, in fact, that only clipping of the site is necessary. We have no experiments directly to that point.

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