Contact Dermatitis, 1996, 34, 111–114
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CONTACT DERMATITIS
ISSN 0105-1873

Nonspecific hypersensitivity: false-positive responses with the use of Freund's complete adjuvant

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While conducting a guinea pig sensitization protocol, using the maximization test, it was discovered, at challenge, that the test animals were more responsive to the vehicle (acetone) than they were to the proprietary test material. During rechallenge, conducted to clarify the specific immune status of the test animals, it was determined that they were also hyperreactive to an alternate vehicle (diethyl phthalate), to which they were naïve. This bizarre set of data is presented and it is suggested that this type of response is the prototype for the presence of false-positive responses experienced by toxicologists using this test. The test conditions imposed on the immune system by the maximization test that could result in these anomalous results are discussed. These data suggest that investigators need cautiously to interpret data that are produced by the injection of Freund's complete adjuvant (FCA).

Key words: non-specific hypersensitivity; guinea pig maximization test; adjuvant; false-positive responses; carrier specificity; immunological basis. © Munksgaard, 1996.

Accepted for publication 12 June 1995

The guinea pig maximization test (1) is an accepted procedure as a preliminary screen, for the allergenic potential of newly introduced raw materials. The hallmark of the procedure is the multiple injections of Freund's complete adjuvant (FCA), both by itself and in conjunction with antigen. Although the mechanism of action of the adjuvant is not clearly understood, it must be recognized that it induces non-specific enhancement of the immune system, and that its efficacy seems to be directly related to its adverse side-effects (2). Although the maximization test is not intended for human risk assessment, it is used to classify potential allergens and is the favored approach by some regulatory agencies, particularly in the European Union. Although, it has the purported reputation of being the more sensitive of the accepted methodologies, it has been criticized for the multitude of adverse reactions that can occur (3), as well as for being an impractical approach for predicting allergenic potential (4).

For those who conduct this method, it should be recognized that the procedure can result in nonlinear dose responses, and that higher doses could lead to tolerance (5, 6). In contrast, the use of adjuvant alone has also been reported to result in immunosuppression (7). Additionally, the procedure can result in false-positives: this is often demonstrated when relatively innocuous vehicles produce increased reactivity (5, 8). These authors report 20–30% of the control groups as positive to the tested materials. From this standpoint, Andersen & Maibach (9) have suggested that a single injection of Freund's complete adjuvant (FCA) in the guinea pig can result in susceptibility to the excited skin syndrome (ESS) if combined with cumulative irritation. These discrepant and varying responses are probably related to the non-specific nature of the inflammatory cells induced, the resultant milieu produced and the subtle effects within this milieu that determine the qualitative and quantitative regulation of the immune system.

The present study demonstrates an exaggerated non-specific hyperreactivity that occurred during routine use of the maximization test.

Materials and Methods

Hartley/Dunkin strain albino guinea pigs (Harlan Sprague Dawley, Indianapolis, IN), of either sex,

weighing between 350 and 600 g were used throughout the experiment. The animals were housed singly, under environmentally controlled conditions, and were fed Teklad guinea pig diet and water, ad libitum.

The test material is a proprietary chemical currently being evaluated for safety under guidelines established by the US EPA and the European Union. Since its biologic activity is not a determinant factor in the results, it is not identified. Chemically, it is a relatively insoluble material that was soluble in either acetone or diethyl phthalate.

Screening studies were accomplished to determine the proper level of all materials for induction and challenge. The intent was to minimize the irritant effects of vehicle (acetone) and test material, when applied topically, even though adjuvant always produces necrosis when injected intradermally. All dosages were accomplished on shaved areas. In addition to a test group initiated topically by the proprietary chemical solubilized at 25% in acetone (20 animals), an appropriate vehicle control group (10 animals), and a naïve control group (10 animals) were included. Induction of the immune response was initiated by a series of intradermal injections at the nape of the neck (4 cm×8 cm) (0.1 ml) on day 1 and a single topical application (0.8 ml) on a double Webril® pad on day 8. The Webril® pad was placed directly over the injection site area and the animals were wrapped with Elastoplast® adhesive bandage (Beiersdorf Inc; Norwalk, CT 06856–5529). The topical dose was left in place for 48 h. The pattern of injection was as recommended by the authors of the maximization procedure (1). Duplicate injections were given in 2 rows of 3 on either side of the dorsal midline at the nape of the neck. The most anterior injections were of FCA (1:1 water emulsion) only. The 2nd injection sites injected caudally were the test material (10% in acetone) or acetone. The 3rd site was test material or acetone emulsified in FCA. As recommended, the 1st and 2nd injection sites were in closer proximity than the 3rd.

2 weeks after the topical application, all test and control animals were doubly challenged with 10% of the test material (acetone solvent) and acetone. Topical challenges were performed similarly to the topical induction procedure, except that they were placed on the flank. The wrappings were removed after 24 h. Test sites were graded 24 h and 48 h ours later after depilation (Neet hair remover cream; Reckitt & Colman Inc; Wayne, NJ 07474–0945) to facilitate scoring. The scoring system is as follows:

0=no perceptible reaction or only slight well defined erythema
1=slight but confluent or moderate patchy erythema
2=moderate erythema
3=severe erythema with or without edema.

Grade 1–3 responses at either time period at challenge were considered to be positive as an indication of hypersensitivity, provided that naïve control responses were grade 0.

Rechallenge of the test and vehicle control animals, as well as additional naïve controls, was performed 10 days after the primary challenge. Diethyl phthalate was used in place of acetone as the solvent of choice.

Results

The results of the primary challenge are summarized in Table 1, and the results of the rechallenge with the alternate vehicle in Table 2.

At primary challenge, the guinea pigs induced with the proprietary test material responded to acetone (4/20) as well as to the original test material (2/20) solubilized in acetone. Additionally, the animals induced with acetone alone were hyperresponsive to both the test material (2/10) and acetone (4/10). In all cases, the skin responses were limited to slight but confluent or moderate patchy erythema (grade 1).

It is instructive that, in both sets of animals, the incidence of responses was greater to the acetone challenge. The naïve control animals did not respond (grade 0) to either challenge material.

Table 1. The results of primary challenge with acetone as vehicle

Induction group	Challenge material	Incidence*
25% test material in acetone	10% test material	2/20
	acetone	4/20
acetone	10% test material	2/10
	acetone	4/10
naïve	10% test material	0/10
	acetone	0/10

*Number responders/number tested.

Table 2. The results of rechallenge with diethyl phthalate (DEP) as vehicle

Induction group	Rechallenge material	Incidence*
25% test material in acetone	10% test material in DEP	3/19
	DEP	4/19
acetone	10% test material DEP	0/10
		1/10
naïve	10% test material DEP	0/10
		0/10

*Number responders/number tested.

As is our practice when challenge results are ambiguous, we chose to rechallenge these same animals, and to substitute a vehicle to which the animals were naïve. A new set (10 guinea pigs) of naive controls was also included. When diethyl phthalate (DEP) was substituted for acetone, the animals displayed the same pattern of reactivity, except that the vehicle control animals appeared to be less responsive than was noted at the primary challenge. In this instance, the animals induced with the test material were responsive to the test material solubilized in DEP (3/19) as well as to DEP alone (4/19). Those animals originally induced only with acetone were responsive only to DEP at a low incidence (1/10). None of the naïve animals were responsive to the test material or DEP. Therefore, one is forced to conclude that both test and control animals are non-specifically hyperreactive as a result of the experimental conditions of the protocol. It is also apparent that the test material cannot be considered as being directly responsible for this bizarre set of results.

Discussion

The use of FCA as a stimulant to the guinea pig immune system, in prospective testing, to predict the sensitizing potential of test materials, has been a common practice, especially since the publication of the maximization test. The proponents of this approach have suggested that this procedure is more sensitive than others (6), particularly when compared to the approach that relies solely on occlusion (10). Nevertheless, the latter approach has been proposed as completely adequate to detect weak sensitizers and, furthermore, it was suggested that it is the more predictive procedure (11). The use of adjuvant has also been criticized because of its adverse effects and its impracticality (4, 11).

Although the mode of action of adjuvants is not completely understood, it must be recognized that, even though FCA has been demonstrated to enhance the cell mediated immune (CMI) response, it actually produces a non-specific enhancement (3). In fact, it has been suggested that the outstanding effect of FCA is to produce a high, long-lasting cell-mediated immunity to protein antigens, even though proteins are not generally considered to be contact allergens (12). Furthermore, the variables that affect the inflammatory cascade, to promote specific enhancement of one aspect of the immune response (i.e., immunoglobulin versus CMI) over another, are incompletely understood. It has been proposed that water in oil (w/o) emulsions enhance the hydrophobicity of antigens, target antigen-presenting cells and present antigens over a large surface area. (2). The addition of bacterial cell walls to this mixture may promote the synthesis not only of interferon (INF), which supports CMI, but also of lgG2a (13). Although these responses are not necessarily exclusive, they do represent divergent pathways that probably modify and regulate the resulting inflammatory lesions. Thus, FCA produces a complex response and its efficacy and side-effects are largely dependent on all the circumstances (route, antigen, species, dose etc.) of their use (3). The macrophage obviously plays a key rôle in this regulation, and can direct the inflammatory cascade in several directions by the production of a multiplicity of inflammatory cytokines. Many of these cytokines are pleiotropic and express multiple biological activities on differing target cells, both locally and systemically, and are also able to generate and modulate inflammation in several directions, depending on very many extenuating factors (14). Furthermore, the primary macrophage responding to intradermal injection differs morphologically and biologically, from the Langerhans cell of the epidermis, and would be expected to have a different effect on the qualitative and quantitative aspects of the immune response. Intradermal injection also alters the inter-relationships of the macrophage and the various lymphocytes with the keratinocyte, that also has a dominant influence on regulatory aspects within the skin associated lymphoid system (15).

FCA side-effects (generalized inflammation, necrosis, granuloma, autoimmunity) are well documented (3), but it is less well advertised that FCA can produce conflicting results and can result in a diminution of CMI, as reported by Asherson & Allwood (16) and Jancovic (6). It has also been suggested that a single injection of FCA can produce an excited skin syndrome in guinea pigs (9). From these standpoints, it is this author's experience that FCA, when used as recommended in the maximization test, will often result in hypersensitivity responses to common vehicles (e.g., water, ethanol, acetone), which are generally of low incidence (10–20%) and of only moderate intensity (grade 1). Although this aspect has been reported (5,8), it is not generally acknowledged in the literature, though one can assume that this phenomenon is not unique to just a few laboratories. As practiced in our laboratory, appropriate rechallenges are then necessary to properly interpret the biologic response to the test material particularly the false-positive responses to innocuous vehicles. A recent critical commentary on the maximization technique has discussed the potential for false-positivity, and suggests the inclusion of an irritant sham control as well as rechallenging responsive animals. Their experience suggested that at least some false-positive responses did not persist beyond 3 weeks (17).

The bizarre set of data recorded in this experiment, however, are unusual and require consideration by practicing toxicologists. To bring these circumstances into perspective, it is necessary to understand that allergic contact dermatitis typically occurs only after topical epicutaneous exposure during the induction and elicitation phases. The allergen must then penetrate the epidermis, combine with homologous protein and interact with Langerhans cells, to effect the inflammatory reaction that results in delayed contact hypersensitivity. Furthermore, the kinetics of antigen presentation and processing by Langerhans cells are specifically conducive to the development of delayed contact hypersensitivity rather than that of protective or homocytotropic antibody. In contrast, the maximization test utilizes artificial stimulation of the immune system, by using intradermal injections of antigen and FCA. The latter produces necrosis and a non-specific inflammatory cascade. Damage to dermal protein, as well as possible denaturation by the solvent action of vehicles, could result in these homologous proteins becoming antigenic, comparable to the "carrier specificity" discussed by Gell & Benacerraf (18). They demonstrated that delayed hypersensitivity occurred to homologous protein, in the presence of hapten or when denatured, and they speculated that this extension of specificity to homologous protein could also occur in vivo. One could then expect responses to slightly altered normal protein at challenge.

Additionally, the excited skin syndrome in individuals with contact dermatitis (19) and conditioned hyperirritability in atopics (20) has been reported, and the latter authors hypothesize that, in atopics, this phenomenon is due to abnormal intrinsic hyperreactivity of the inflammatory cells. In the presence of non-specific enhancement by FCA and the hyper-inflammatory nature of the resulting milieu, these factors could combine to produce an idiopathic response that has no obvious practical significance and results in false-positive responses to otherwise innocuous materials.

Regardless of the mechanism responsible for this bizarre phenomenon, it is necessary to understand that false-positives can and do occur when FCA is used to non-specifically enhance the immune system. If these should occur to a newly introduced test material, it could result in the improper classification of a useful compound.

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