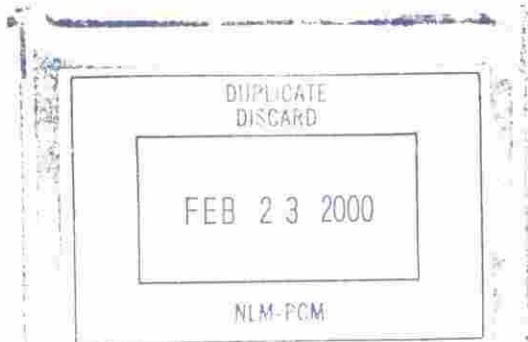




Review Article

The value and limitations of rechallenge in the guinea pig maximization test

S. FRANKILD, D. A. BASKETTER¹ AND K. E. ANDERSEN

Department of Dermatology, University Hospital, Odense, Denmark and

¹Unilever Environmental Safety Laboratory, Colworth House, Sharnbrook, Bedford, UK

The guinea pig maximization test (GPMT) has played a primary rôle in the evaluation of potential skin contact sensitizers for 25 years. In the OECD Guideline 406 from 1993, it is specifically suggested that equivocal results from the initial challenge in the GPMT should be evaluated further with a repeated challenge. However, there exist few published rechallenge data and the guideline does not describe how rechallenge data should be interpreted. In this paper, we have used examples from published results to illustrate both the positive value and the limitations of repeated challenges, including cross challenge. Testing with modified concentrations may also help to indicate whether or not the response is allergic in nature, particularly where there has been a low level of skin reaction observed in sham-treated controls, or where a low level of skin reaction is the dominant response in the test animals. In conclusion, the data presented demonstrate that, as a tool for the investigation of skin sensitizing potential, the GPMT can benefit from an experienced scientific evaluation of rechallenge data, but that this information should not be treated in a mechanistic fashion.

Key words: rechallenge; guinea pig maximization test; GPMT; dose-response; false-positive; cross-sensitization; contact allergy. © Munksgaard, 1996.

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Contact allergy is a delayed, immunologically mediated response to a sensitizing chemical. It is usually regarded as a long-lasting hypersensitivity. Even weak challenge reactions which are truly allergic can be reproduced, while non-specific irritant ones generally fade and are irreproducible. Prolonged and reproducible sensitization has been shown in man and in the guinea pig (1-3). However, in predictive testing methods, there is the potential risk of mistaking an irritant patch test reaction for an allergic one. Strong challenge reactions in the GPMT, showing sharp erythema and oedema, are readily recognized as allergic reactions, but weak reactions, showing only mild erythema, may be difficult to interpret. Unfortunately, there is no definitive way to distinguish the allergic from irritant reactions. One way to help avoid the problem is to determine the non-irritant patch test concentration in pilot studies on Freund's complete adjuvant (FCA) treated guinea pigs and at 1st challenge to treat with both

the test substance and the vehicle. Another way is to use FCA-treated control animals, blind reading of challenge and appropriate statistics. However, to clarify the nature of the challenge result further in the GPMT protocol, it is also possible to supplement the 1st challenge with a 2nd challenge, a rechallenge. Consequently, the OECD Guideline 406 suggests a repeated challenge 1 week after the initial challenge in the GPMT protocol (4). Unfortunately, additional challenge is rarely performed in the GPMT, though it provides a valuable tool for further evaluation of equivocal 1st challenge results or the investigation of cross challenge reactions. Repeated challenge may also provide data relevant to risk assessment, such as information on the threshold for the elicitation of sensitization.

In this review, the rationale for the conduct of a rechallenge is described, together with the considerations necessary for the interpretation of the results.

Irritancy and "False-positive" Reactions

The guinea pig maximization test (GPMT) requires the use of FCA to maximize the sensitivity of the test. It has been demonstrated that FCA-treated guinea pigs have a lower skin irritation threshold and that guinea pigs stressed by concomitant allergic or irritant inflammation remote from the site of skin testing can have a generalized state of hyperreactivity, also known as the "angry back" or excited skin syndrome (5-8). An altered state of reactivity in the skin may sometimes lead to questionable challenge results in test animals and, occasionally, non-specific irritant responses in control animals. Hyperirritable skin may account for 8% to 40% of weakly positive reactions in humans undergoing diagnostic patch testing, often caused by preexisting dermatitis. (9, 10). Either in the clinical setting or in predictive testing in animals, a positive patch test reaction observed in the absence of true skin sensitization is a "false-positive patch test reaction" (11, 12). Some important confounding factors for false-positive patch test reactions are listed in Table 1.

Clinically, irritant reactions are often distinguished from allergic reactions by evaluating the persistence of the patch test reaction. Irritant reactions generally decrease in intensity after removal of the patch but late maximal inflammation can be seen (13, 14). Reproducible reactions are generally allergic in nature, while irreproducible reactions are more likely to be irritant, as demonstrated by Kligman & Basketter (12). Histological or immunohistochemical evaluation of a patch test reactions does not distinguish allergic from irritant reactions (15, 16). However, proper rechallenge may provide information on the persistence and reproducibility of the reaction (17). It is important to confirm that a weak reaction is reproducible before it is considered as an allergic reaction.

In the original description of the GPMT by Magnusson & Kligman (18), rechallenge was suggested 3-4 days after the initial challenge. Non-specific irritant reactions may fade within such a short time, but in hyperreactive skin, irritant reactions may reappear after rechallenge. It is known that the hyperreactive state of the skin will diminish

with time and, consequently, a longer rest period between challenge and rechallenge will reduce the chances of getting false-positive reactions after rechallenge. In guinea pig tests, truly allergic reactions have been evoked to the same intensity 3 weeks after challenge (12). Animals that were strongly sensitized to potent allergens showed equally strong challenge reactions after 2-5 months, while weakly sensitized animals showed reduced challenge reactions in the same period (1, 12). Most rechallenge experiments have been conducted 1-3 weeks after the initial challenge, which seems reasonable.

Reproducibility of Patch Test Reactions from Challenge to Rechallenge

Reproducible results from challenge to rechallenge with different sensitizers in the GPMT have been reported by several authors (1, 5, 12, 19, 20). Others have observed a reduced sensitization frequency from challenge to rechallenge (19, 21). Table 2 shows a significant decrease in sensitization rate with chlorocresol from challenge (day 21) to rechallenge (day 35). The "down regulation" in sensitization rate was reversed only by treating the guinea pigs with cyclophosphamide (CY) on day 18, 3 days prior to the 1st challenge (Table 2). The suggested mechanism is an augmented effect of T-cell suppression compared to T-cell stimulation (22). It is important to note, however, that those animals that reacted at the 2nd challenge were among those that responded at the 1st challenge, confirming the allergic nature of the reaction. An "up regulation" of the sensitization rate from challenge to rechallenge has also been reported (6, 23, 24). Table 3 shows an example of an "up regulation" of sensitization with epoxy diacrylate and neopentyl methacrylate in the GPMT from closed challenge day 21 to open challenge day 35 and 49 (25, 26). The reason for the increased sensitization rate is unclear, but a booster effect caused by the challenge stimulus may enhance an already established immunological memory arising from the induction. Thus, sensitization from the challenge procedure must also be considered as a confounding factor for increased sensitization rate after rechallenge, especially if positive reactions are evoked in re-used control animals after rechallenge.

Hypersensitivity reactions represent a delicate balance between effector and suppressor mechanisms. This balance may be altered from challenge to rechallenge and may be dependent on the substance tested.

Table 1. Some important causes of "false-positive" skin test reactions (45)

allergen restricted	test concentration too high inappropriate vehicle local hyperreactivity due to previous allergic reaction at the test site
non-allergen restricted	excited skin syndrome due to inflammatory reaction elsewhere

Table 2. Sensitization frequency with chlorocresol after challenge and rechallenge in the GPMT; groups 5 and 6 were treated with cyclophosphamide on day 18 and 32 respectively

	Concentration			Challenge results (day)			ref.
	n	ic	ec	ch	21	35	
group 1	19	5%	10% (pet.)	1% (pet.)	13/19	4/19	(43)
group 2	20	1%	10%	1%	12/20	5/20	—
group 3	20	0.2%	10%	1%	12/20	6/20	—
group 4	20	1%	5% (pet.)	1% (pet.)	20/20	11/20	(44)
		1%	5%	0.5%	18/20	10/20	
		1%	5%	0.2%	19/20	10/20	
group 5	20/19 ^{a)}	1%	5%	1%	14/20	19/19	—
CY Day 18		1%	5%	0.5%	10/20	19/19	
		1%	5%	0.2%	9/20	18/19	
group 6	20	1%	5%	1%	17/20	6/20	—
CY Day 32		1%	5%	0.5%	18/20	9/20	
		1%	5%	0.2%	16/20	7/20	

n: number of animals tested. ic: intracutaneous induction concentration. ec: epicutaneous induction concentration. ch: challenge and rechallenge concentration. pg: propylene glycol. pet.: Petrolatum. CY: cyclophosphamide.

^{a)} 1 animal died between challenge and rechallenge.

Table 3. Sensitization rate after closed challenge day 21 and open rechallenge day 35 and 49

Substance	Concentration				Challenge results (day)			ref.
	<i>n</i>	ic	ec	ch	21	35	49	
epoxy diacrylate ^{a)}	15	5% (o.o.)	100%	10% (pet.)	11/15	15/15	—	(26)
epoxy diacrylate ^{b)}	15	5%	100%	10%	7/15	14/15	—	—
neopentyl methacrylate	10	0.17M (arac.)	3M (eth.)	3M (aram.)	0/10	8/10	8/10	(25)
—	10	0.5M	3M	3M	8/10	10/10	10/10	—
—	10	1.5M	3M	3M	2/10	7/10	7/10	—

n: number of animals tested. M: molar concentration. arac.: arachis oil. eth.: ethanol. aram.: mixture of arac and methylethylketone. pet.: petrolatum. o.o.: olive oil.

^{a)} Epicote DRH-370

^{b)} XD-8008.04

Rechallenge Concentration and Vehicle

The elicitation of an allergic patch test reaction is dose dependent (18, 27). Selection of an inappropriately low challenge concentration may result in failure to elicit an allergic reaction in a sensitized animal, causing a "false-negative" patch test reaction. In contrast, an application of too high a challenge concentration causes irritant reactions. If non-specific challenge reactions have been observed in sham treated control animals after challenge, it is reasonable to do a rechallenge with a lower concentration to avoid irritant reactions after rechallenge. On the other hand, if many low grade challenge reactions are observed in the test group, a higher rechallenge concentration may be used to evoke true positive sensitization reactions at rechallenge which were not elicited at challenge, provided that there is no skin irritation confounding the interpretation at the higher concentration.

If multiple concentrations are applied at challenge and rechallenge, it can provide information on the concentration needed to elicit an allergic

response. The dose-response relationship between the challenge concentration and patch test reaction may be relevant for risk assessment (27). However, there may be a limitation imposed by technical requirements of the application of multiple patches. In guinea pigs, positive patch test reactions at one test site have not been shown to evoke "false-positive" reactions at other test sites (7). When multiple patch test reactions are evaluated there is also a potential risk of biased reading, because one or more obvious positive patch test reactions may imply an overestimation of doubtful reactions in the same animal. Bruze et al. did not experience such a difficulty when they evaluated multiple patches in the GPMT (28), but random application of multiple patches combined with blind reading of the reactions may improve their evaluation.

It has been demonstrated that when the same vehicle is used for induction and challenge, ambiguous results may occur in the GPMT (29, 30). If equivocal results occur after challenge, the vehicle may be substituted for rechallenge to avoid non-specific hyperreactivity.

Table 4. Animals sensitized to either one of the active ingredients in Kathon CG[®] and related compounds; Challenge and rechallenge results after induction with either 5243-K-CG, 243-K-CG or 45243-K-CG

	n	45243-K-CG	5243-K-CG	243-K-CG	K-893	BIT	eth.	Ref.
5243-K-CG								(32)
challenge	24		19					
rechallenge	24	3	12	1	0	0	0	
243-K-CG								(32)
challenge	24			11				
rechallenge	24	2	3	8	0	1	2	
45243-K-CG								(36)
challenge	24	23						
rechallenge	24	17	19	0	1	1	1	

n: number of animals tested. 5243-K-CG: 5-chloro-2-methyl-4-isothiazolin-3-one. 243-K-CG: 2-methyl-4-isothiazolin-3-one. 45243-K-CG: 4,5-dichloro-2-methyl-4-isothiazolin-3-one. BIT: 1,2-benzisothiazolin-3-one. eth.: 99.5% ethanol, (vehicle control). K-893: 45% of 2-n-octyl-4-isothiazolin-3-one.

Cross-sensitization, Sensitizing Impurity and Formulations

Cross-challenge is performed when a sensitized guinea pig reacts to a 2nd allergen to which it has not been exposed previously (31). Rechallenge has been used as a tool for detecting cross-reacting substances in guinea pigs (32–35). Table 4 shows the results from rechallenge with the 2 active ingredients in Kathon CG[®], 2-methyl-4-isothiazolin-3-one (243-K-CG) and 5-chloro-2-methyl-4-isothiazolin-3-one (5243-K-CG), the contaminant of Kathon CG[®], 4,5-dichloro-2-methyl-4-isothiazolin-3-one (45243-K-CG), and other chemically related substances: Kathon 893 containing 45% of 2-n-octyl-4-isothiazolin-3-one (K-893) and 1,2-benzisothiazolin-3-one (BIT) (32, 36). When the animals were sensitized and rechallenged with 45243 K-CG it was observed to be a strong sensitizer and almost as strong as when rechallenged with 5243-K-CG. No cross-sensitization was observed with the other substances. However, when the animals were sensitized to 5243-K-CG, less than 1/2 the number of animals reacted to rechallenge with 45243-K-CG, compared to rechallenge with 5243-K-CG. Bruze et al. (36) demonstrated that the difference in the cross-sensitization pat-

tern was not due to contamination of 5243-K-CG in 45243-K-CG, but suggested that metabolism of 45243-K-CG to 5243-K-CG in the skin was the reason. This example demonstrates that important information can be gained from studying cross-sensitization with rechallenge, but also that results may be difficult to interpret.

Rechallenge in the Buehler test may be used to study the sensitization pattern of different ingredients in a formulated product. Table 5 shows the sensitization frequency of a formulated substance and the sensitization frequency of each ingredient in it. As in the example, one of the components was identified as the sensitizer (37). However, it is not recommended that finished products composed of several substances are tested in the GPMT. Usually 1 or 2 of these are potential allergens, while the remainder is water and minimally or non-allergenic substances, which may affect the choice of induction concentration. A locally irritating non-allergenic ingredient will decrease the test concentration and might prevent the detection of the allergen by giving "false-negative" results (38, 39).

Comments

The GPMT can be supplemented with a 2nd challenge, a rechallenge, which is considered to be a valuable tool for evaluating questionable reactions obtained after initial challenge, and may help to clarify cross-challenge patterns of chemically related substances or provide elicitation dose-response information (40, 41). Importantly, rechallenge after 1–3 weeks will improve the interpretation of test results if there is an altered irritation state of the skin.

It is important to remember that allergenicity is a delicate balance between effector and suppressor mechanisms, and that the substance tested may in-

Table 5. Identification of the sensitizing component in a mixture with the Buehler test (37)

Incidence	Severity ^(a)	
primary challenge	10/20	1.2–1.4
test substance A		
rechallenge ^(b)	0/10	0.1–0.2
component A-1		
component A-2	0/10	0.1–0.2
component A-3	7/10	0.9–1.1

^(a) Mean response grade.

^(b) Rechallenge of the animals judged positive after primary challenge.

fluence this balance by an up- or down regulation occurring as a consequence of the 1st challenge procedure.

A further option which can be taken by means of a rechallenge is to evaluate the effect on elicitation of a different vehicle. This proved to be an important action in a recently reported study, confirming the non-specific nature of the responses (30). However, it may also be used to evaluate the effect of various vehicles on the intensity of the sensitization response (42). The concentration chosen for rechallenge depends on the test reactions of the initial challenge and the concentration used to produce them. A 2-fold reduction in concentration may be indicated where a low level irritation is suspected of complicating the reading. On the other hand, a marginal level of reactivity in the absence of any indication of a reaction in the controls might be further investigated by means of rechallenge with a modestly increased concentration.

Ultimately, any rechallenge data must be interpreted with care in the context of the entire study. The interpretation has to be done on a case-by-case basis, using as a guide the best understanding of immunology integrated with the knowledge of those with substantial experience of conducting the test.

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Address:

S. Frankild
Department of Dermatology
Odense University Hospital
DK-5000 Odense
Denmark

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