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INVESTIGATIVE REPORT

Comparison of the Sensitivities of the Buehler Test and the Guinea Pig Maximization Test for Predictive Testing of Contact Allergy

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International test guidelines, such as the Organisation for Economic Cooperation and Development (OECD) guideline #406, recommend 2 guinea pig methods for testing of the contact allergenic potential of chemicals: the Guinea Pig Maximization Test (GPMT) and the Buehler test. Previous comparisons between the methods suggested that the Buehler test was less sensitive than the GPMT although modified Buehler test protocols were used. Parallel GPMT and Buehler tests were conducted according to OECD guideline #406 using a multiple-dose design and test results were analysed using a standard logistic dose–response model. To compare the sensitivity of the 2 test procedures the test conditions were kept identical and the following chemicals with a range of sensitization potentials were tested: chloraniline, chlorhexidine, eugenol, formaldehyde, mercaptobenzothiazole and neomycin sulphate. Formaldehyde and neomycin sulphate were strong sensitizers in both tests. Mercaptobenzothiazole, eugenol and chloraniline were all strong sensitizers in the GPMT, eugenol and mercaptobenzothiazole were negative in the Buehler test and equivocal results were obtained with chloraniline. Chlorhexidine was negative in the GPMT and equivocal responses were obtained with the Buehler test. Higher induction concentrations were needed to show allergenicity in the Buehler test and for some allergens the Buehler test protocol was not sensitive enough to demonstrate allergenic potential. *Key words: dose–response; guinea pig; OECD guideline; allergic contact dermatitis; contact allergy*.

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Two guinea pig methods—the guinea pig maximization test (GPMT) (1) and the Buehler test (2)—are recommended for predictive allergenicity testing of chemicals according to the Organisation for Economic Cooperation and Development (OECD) guideline #406 (3, 4). For induction the GPMT combines the use of intradermal administration with and without Freund's complete adjuvant (FCA) and occluded topical application of the test substance. Two weeks after induction the animals are challenged by closed-patch tests to the flanks. The Buehler test employs 3 6-h duration topical induction patches (1 patch per week). Two weeks after induction the animals are challenged by closed-patch tests to the flanks for 6 h. All tests in the Buehler assay are performed on restrained animals.

The GPMT is the preferred test method in the EU and >90% of the tests on "new" and "existing" chemicals submitted to the EU regulatory authorities are carried out using this method (5). The Buehler test is the preferred test procedure in the USA. However, the regulatory authorities in the USA and EU accept results from both test protocols. According to the EU Commission Directive any chemical inducing sensitization in $\geq$ 30% animals in the GPMT and $\geq$15% animals in the Buehler test should be labelled R43. R43 labelling indicates potential allergenicity (type IV allergy) (6). Some comparisons on the sensitivities of the 2 test procedures have been published.

The GPMT is considered to be one of the most sensitive assays for detecting contact allergens. Marzulli & Maguire (7) found a good correlation between the GPMT and modified human Draize tests; however, only 10 out of 30 chemicals positive in the human tests were detected as sensitizers in the Buehler test. Furthermore, Klaschka & Vossmann (8) reported that guinea pig tests that do not use FCA underestimate the sensitization potential of relevance to humans. Two experimental studies compared the Buehler test with the GPMT by testing strong sensitizers and found the Buehler test to be less sensitive than the GPMT (7, 9). Similar findings were reported by Basketter et al. (10), who tested the 3 OECD positive control compounds mercaptobenzothiazole, hexyl cinnamic aldehyde and benzocaine. However, all these studies used a modified Buehler test procedure and it has been argued that the sensitivity of the Buehler test is reduced if the original protocol is not strictly adhered to (11–13). Therefore we decided to perform a systematic comparison of the sensitivity of the 2 methods using well-known chemicals with varying sensitizing potentials and a multiple-dose design (14) enabling us to evaluate the effect of the different test concentrations, which vary between the 2 methods.

MATERIALS AND METHODS

Chemicals

Chemicals with a range of known sensitizing potentials were selected. All chemicals used were of analytical grade: 4-chloraniline (Merck); chlorhexidine digluconate (Degussa AG); eugenol (Daniel, Royal Tunbridge Wells, UK); formaldehyde pro analysis 37–38% (Merck); mercaptobenzothiazole (Aldrich); and neomycin sulphate (Pharmacia & Upjohn). FCA was purchased from Statens Serums Institute, Copenhagen, Denmark. Propylene glycol (Merck), petrolatum or water were used as vehicles.

Animals

Outbred albino female guinea pigs (Dunkin–Hartley, Sahlins, Malmø, Sweden) weighing 300–350 g at receipt were housed in

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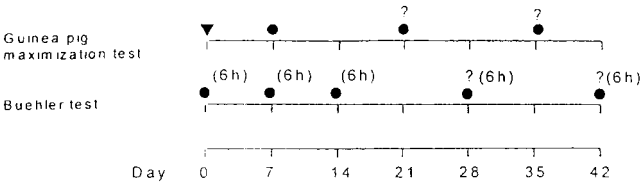

Fig. 1. Schematic presentation of the GPMT and Buehler test procedures: (●), patch test; (▼), intradermal injection; ($\overset{?}{\bullet}$), challenge patch test.

groups of 2 or 3 in plastic cages at the Biomedical Laboratory, Odense University, Denmark. The animals were kept on a 12-h photoperiod at temperature of 21±3°C, a relative humidity of 55% (±15%) and with food and water available *ad libitum* (standard guinea pig pellets, Altromin®, 3123, Chr. Petersen A/S, Ringsted, Denmark). Beech wood chips were used as bedding (Glamsbjerg Træindustri A/S, Glamsbjerg, Denmark). The animals were randomly assigned to control and test groups, ear-marked with baby simplex ear tags (Chevillot®, Paris, France) and allowed to adapt for 1 week before the study began. The skin areas used for treatment were clipped and shaved with an electric razor prior to each treatment. All animals were weighed before each test procedure.

Guinea Pig Maximization Test (GPMT)

The procedure described by Magnusson & Kligman (1) was followed, comprising intradermal induction with FCA on day 0; topical induction on day 7; a subsequent challenge on day 21; and rechallenge on day 35 by closed-patch tests to the flanks of the animal (Fig. 1). The GPMT procedure was modified with a multiple-dose design (14): 30 animals were assigned to 1 control group of 5 animals and 5 test groups containing 5 animals each. Simultaneous increases in both intradermal and topical induction doses were used. In the multiple-dose GPMT procedure pretreatment with sodium lauryl sulphate was omitted.

Dose finding. The concentration ranges for induction and challenge were determined from a pilot study utilizing 12 FCA-treated guinea pigs for each test chemical. Two weeks after FCA injections the animals were treated intradermally or topically (closed patch to the flank of the animal) with a range of test concentrations. Readings were performed after 3 and 24 h. The highest concentrations tolerated systemically and not producing skin necrosis were selected for induction. The highest non-irritating concentration and 1 or 2 lower concentrations were chosen for closed challenge.

Induction. For intradermal induction 3 pairs of 0.1 ml injections were given in 2 rows in the nuchal area of each guinea pig. Glass syringes with Luer–Lock G23 needles were used for intradermal injections.

For topical induction a 2×4 cm^2 filter paper (Whatman no. 3MM) saturated with 150 µl of test solution (Finn pipette) or petrolatum preparation was applied. Leucoflex® (Beiersdorf AG, Hamburg, Germany) was used to ensure occlusion and was secured by Acrylastic® (Beiersdorf AG).

Challenge. Patches were placed on the flank using small (8 mm) Finn chambers (Epitest Ltd., Helsinki, Finland) on Scanpor® (Norgesplaster A/S, Oslo, Norway) and secured with Acrylastic®. For the liquid preparations 2 filter papers (Epitest Ltd.) were placed in the Finn chamber and saturated with 50 µl of test solution. Petrolatum preparations were applied directly to the chambers. The concentrations used for induction and challenge are shown in Table I.

Buehler test

The original procedure was followed, comprising topical induction for 6 h on days 0, 7 and 21 and subsequent challenge and rechallenge at weeks 5 and 7 (2, 3, 11, 15, 16). A multiple-dose design was used with 5 concentrations for induction and 1 or 2 for challenge. The 30 animals were divided into 1 control group of 5 animals and 5 test groups of 5 animals.

Dose finding. The concentration ranges for induction and challenge were determined from a pilot study utilizing 4 naive guinea pigs tested with a range of test concentrations of each chemical. Readings were taken 3 and 24 h after removal of the patches. The highest concentrations tolerated systemically and not producing skin necrosis were selected for induction. The highest non-irritating concentration and 1 or 2 lower concentrations were used for challenge.

Induction and challenge. Test solution (450 µl) or petrolatum preparation was applied to 25 mm Hill Top® Chambers (Hill Top Biolabs., Cincinnati, OH, USA). The animals were placed in restrainers (Hill Top Biolabs.) for 6 h. Chambers were occluded

Table I. *Concentration ranges and vehicles used in the GPMT and Buehler test for induction and challenge*

		Concentration (%) used for			
		GPMT		Buehler test	
Test chemical	Vehicle	Induction	Challenge (top.)	Induction (top.)	Challenge (top.)
Chloraniline	id. PG	0.01–10	1 and 3	1–50	10 and 30
	top. Pet.	0.1–0			
Chlorhexidine	id. saline	0.001–1	0.1 and 0.3	0.3–20	1 and 3
	top. saline	0.01–10			
Eugenol	id. PG	0.01–10	1 and 3	0.3–20	3 and 10
	top. Pet.	0.1–20			
Formaldehyde	id. saline	0.003–3	0.3 and 1	0.3–30	0.3 and 1
	top. saline	0.03–3			
Mercaptobenzothiazole	id. PG	0.003–3	1 and 10	1–50	10 and 30
	top. Pet	0.03–30			
Neomycin sulphate	id. saline	0.01–10	1, 3 and 10	1–50	10 and 30
	top. saline	0.1–50			

id. = intradermal; top. = topical; PG = propylene glycol; Pet. = petrolatum.

Table II. *Results of dose–response GPMT and Buehler test with formaldehyde*[a]

Test protocol				Group 1 Induction concentration (%)	2	3	4	5	6
	Challenge concentration (%)	Day	Intradermal	Control	0.003	0.03	0.1	0.3	3
			Topical	—	0.03	0.3	1	3	3
GPMT	1	2		0/5	0/5	4/5	3/5	4/5	3/5
		3		0/5	0/5	3/5	3/5	4/5	3/5
			Topical	Control	0.3	1	3	10 (3)[b]	30 (3)[b]
Buehler test	0.3	2		1/5	1/5	3/5	2/5	2/5	1/5
		3		0/5	1/5	3/5	2/5	3/5	1/5

[a]GPMT: vehicle: saline. Buehler test: vehicle: saline.
[b]Induction dose reduced because of skin irritation.

using a rubber dental dam pulled tight and fastened to the bottom of the restrainer with metal clips.

Patch test reading (both methods). The challenge and rechallenge reactions were read blindly after 2 and 3 days using the grading scale (1). Reactions graded 2+and 3+were regarded as positive (14, 18).

Statistics

Logistic regression analysis was used for analysis of the dose–response data (14, 18). The general strategy for the dose–response analysis is as follows. If there are no positive responses in the control group the standard logistic dose–response model, $\log\{p(x)/[1-p(x)]\} = a + b\ \log(x)$, is used, giving the relationship between the probability $p(x)$ and the dose x, where a is the intercept and b the slope of the linear logistic relationship. The fit of the model is tested by means of a χ^2 test with n−p degrees of freedom, where n is the number of dosed groups and p the number of parameters in the model. The significance of the dose–response relationship is tested by means of a χ^2 test with p−1 degrees of freedom. The dose sensitising 50% of the animals (ED_{50}) is calculated as $ED_{50} = \exp(-a/b)$. The estimated maximal sensitization rate (EMS) is defined from the dose–response curve as the highest estimated response rate within the applied dose range. It is possible to extend the model either to estimate and test a non-monotonous dose–response model with respect to 1 induction mode or to include positive responses in the control group (14). A PC program designed for analysis of multiple dose–response data was used (18).

A generalization of Fisher's exact test of the same response rate in all groups was carried out by means of the program StatExact® (Cytel Software Corporation, Cambridge, MA, USA). Kruskal–Wallis 1-way analysis of variance was used to compare animal weight gain between the 2 test procedures.

RESULTS

The results for each chemical are reported separately and are also summarized in Table IV. There was no statistically significant difference between animal weight gain during the test period in the GPMT (16%) and Buehler test (18%).

Chloraniline

GPMT. Chloraniline sensitized significantly after challenge and rechallenge with 3% and 1% concentrations. After challenge with 3% chloraniline 1 control animal responded with a 2+reaction. The monotonous dose–response model with a background response rate gave an acceptable fit (χ^2 (3) = 0.4, $p > 0.5$) and the dose–response relationship was highly significant (χ^2 (2) = 17.3, $p < 0.001$). The challenge results were reproduced after rechallenge. The EMS was 0.9, intradermal ED_{50} was 0.1% and the threshold concentration for sensitization was < 0.01% intradermally.

Table III. *Results of dose–response GPMT and Buehler test with neomycin sulphate*[a]

Test protocol				Group 1 Induction concentration (%)	2	3	4	5	6
	Challenge concentration (%)	Day	Intradermal	Control	0.01	0.1	0.3	1	10
			Topical	—	0.1	1	3	10	50
GPMT	10	2		0/5	0/5	5/5	5/5	2/5	3/5
		3		0/5	0/5	5/5	4/5	3/5	4/5
			Topical	Control	1	3	10	30	50
Buehler test	10	2		0/5	0/5	0/5	0/5	0/5	0/5
		3		0/5	0/5	0/5	0/5	0/5	0/5
	30	2		0/5	0/5	0/5	1/5	0/5	0/5
		3		0/5	0/5	0/5	1/5	2/5	2/5

[a]GPMT: vehicle: saline. Buehler test: vehicle: saline.

Table IV. *GPMT and Buehler test data*

Chemical	GPMT				Buehler test		
	EMS[a]	ED_{50}[b] (%)	ED_{50}[c] (%)	Threshold id. (%)	EMS[a]	ED_{50}[c] (%)	Threshold top. (%)
Chloraniline	0.9	0.1	1	<0.01	0.9	0.09	—
Chlorhexidine	Not estimated				Equivocal		
Eugenol	1	0.26	2.6	0.01–0.1	Not estimated		
Formaldehyde	0.8	0.04	0.4	0.003–0.03	0.6	1	<0.3
Mercaptobenzothiazole	0.8	0.3	3	0.003–0.03	Not estimated		
Neomycin sulphate	0.9	0.03	0.3	0.01–0.1	0.5	—	3–10

[a]Estimated maximal sensitization rate.
[b]Intradermal concentration sensitizing 50% of the animals.
[c]Topical concentration sensitizing 50% of the animals.
id. = intradermal; top. = topical.

Buehler test. Challenge and rechallenge with 30% and 10% chloraniline gave similar results, with significant sensitization at day 2 but with only a few animals showing reaction at day 3. The EMS was 0.9 at day 2 and 0.3 at day 3.

Chlorhexidine

GPMT. No response was obtained in either test or control groups with chlorhexidine.

Buehler test. Positive reactions were seen after challenge and rechallenge with chlorhexidine but 1–2 of the 5 control animals showed confluent erythema (2+) regarded as an irritant reaction, even with the lowest challenge concentration (1%).

Eugenol

GPMT. Significant sensitization was obtained after challenge and rechallenge with 3% and 1% eugenol and no control animals were positive. After challenge with 3% eugenol the readings at days 2 and 3 followed a steep dose–response curve and the monotonous logistic model was acceptable (χ^2 (3) = 3.9, $p > 0.1$). The dose–response relationship was highly significant (χ^2 (1) = 10.68, $p < 0.01$). The EMS was 1 and the intradermal ED_{50} was 0.3%. The intradermal threshold concentration was between 0.01% and 0.1%.

Buehler test. No test or control animals were positive after challenge or rechallenge with eugenol.

Formaldehyde

GPMT. Significant sensitization was seen at days 2 and 3. Challenge was performed with 0.3% and 1% formaldehyde. All controls were negative (Table II). The data obtained after challenge and rechallenge with 1% formaldehyde were used for statistical analysis. In this case the non-monotonous logistic model was chosen because it gave a significantly better fit compared with the monotonous model. The EMS was 0.8, the intradermal ED_{50} was 0.04% and the intradermal threshold concentration was 0.003–0.03% (Fig. 2).

Buehler test. The 2 highest concentrations of formaldehyde (10% and 30%) produced strong local irritation in the test animals and subsequent induction treatments were given with a lower concentration (3%).

Challenge was performed with 0.3% and 1% formaldehyde but 3 out of 5 control animals responded with a 1+ to 3+ reaction after challenge with 1% formaldehyde. In the initial dose-finding studies 1% formaldehyde was not an irritant. One control animal reacted to the 0.3% formaldehyde patch test at day 2 after challenge but the reaction had disappeared at day 3. The data obtained after challenge and rechallenge with 0.3% formaldehyde are used for statistical analysis. The fit of the non-monotonous logistic model was acceptable (χ^2 (2) = 1.2, $p > 0.5$). The EMS was 0.6 and the ED_{50} was 1%. The threshold concentration was <0.3% topically. Fig. 2 shows the fitted dose–response curves for the GPMT and Buehler data with formaldehyde.

Mercaptobenzothiazole

GPMT. Challenge and rechallenge with 1% and 10% mercaptobenzothiazole gave almost identical results. However, no intermediate responses (response rate 0–1) were obtained with the 10% challenge concentration and the logistic regression analysis could not be performed. For both challenge concentrations there was a significant difference between the control and test groups ($p < 0.05$, exact test). A monotonous dose–response curve could be estimated after challenge with 1% (χ^2 (3) = 5.9, $p > 0.05$) and as expected mercaptobenzothiazole was a sensitizer with an EMS of 0.8, an intradermal ED_{50} of 0.3% and an intradermal threshold concentration between 0.003% and 0.03%.

Buehler test. No sensitization was obtained after challenge with 30% mercaptobenzothiazole and 3 animals were positive after rechallenge with 50%.

Neomycin sulphate

GPMT. Neomycin sulphate was a significant sensitizer after challenge and rechallenge with 10% and 3% concentrations but only 3 animals reacted to 1% (Table III). Challenge results after day 3 gave an acceptable curve fit and the EMS was 0.9. The intradermal threshold concentration was

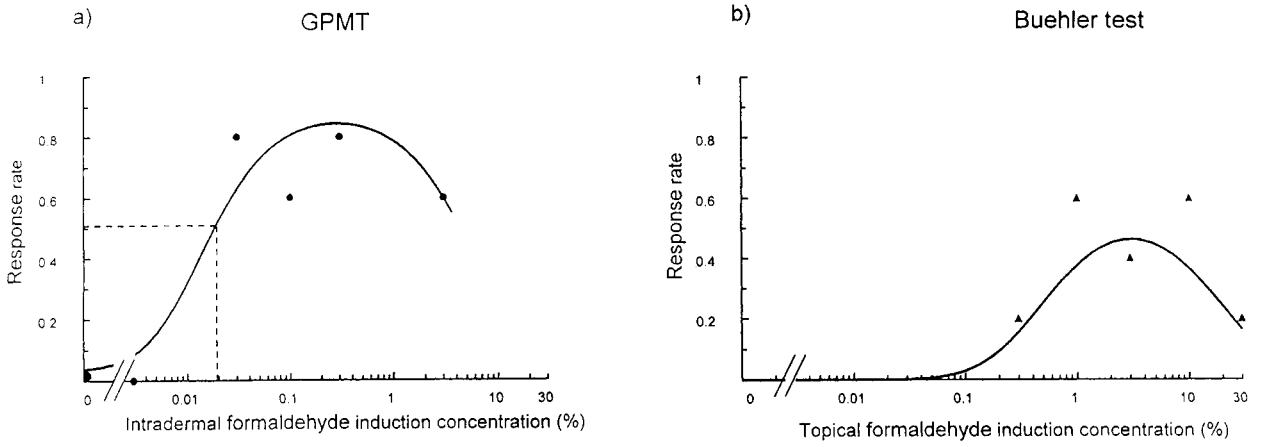

Fig. 2. Dose–response curves for formaldehyde. (a) GPMT readings at day 2 after challenge with 1% formaldehyde: (●), observed responses; (—), non-monotonous logistic curve fit. (b) Buehler test readings at day 3 after challenge with 0.3% formaldehyde: (▲), observed responses; (—), non-monotonous logistic curve fit.

between 0.01% and 0.1%, and the intradermal ED_{50} was 0.03%.

Buehler test. Maximum 2 animals per test group were positive at day 3 after challenge with 30% neomycin sulphate. Rechallenge with 10% neomycin sulphate showed a steep monotonous dose–response curve. The EMS was 0.4 and the topical threshold concentration was between 3% and 10%. The sensitization rate was higher after rechallenge, with an EMS of 1. Fig. 3 shows the fitted dose–response curves for the GPMT and Buehler challenge data.

DISCUSSION

The experiments were performed simultaneously and test conditions—i.e. animal source, choice of vehicle and batch of test chemical—were kept as identical as possible. The sensitization assays were conducted with a multiple induction design as previously introduced (14). The multiple-dose design provides quantitative data wherein the EMS, ED_{50} and the concentration below which the allergen does not sensitize the animals (threshold concentration) can be estimated. These data provide better characterization of the allergenicity of the test substance compared with the hazard identification (low, moderate or strong sensitizing potential based on frequency of sensitization in the guinea pig test group) obtained with a routine GPMT using only 1 induction concentration.

The induction doses used in the GPMT covering a concentration range of a factor of 1000 were selected, with a factor of 3 between the middle doses and a factor of 10 between the highest and lowest doses. This design was chosen because previous dose–response studies have shown that a broad dose range covering several orders of concentration, from the very lowest to the highest tolerable concentration, may be the most appropriate for testing chemicals with unknown allergenic dose–response relationships (14). For the Buehler test a higher, more narrow induction dose range was used compared with the GPMT because only patch-test induction is used and the literature shows that higher induction concentrations are needed for sensitization with the Buehler test.

The Buehler test animals were restrained during treatment because it has been argued that wrapping alone is an inappropriate procedure for occlusion in the Buehler test (2, 19). Furthermore, patch testing in the Buehler test was conducted with 25 mm Hill Top chambers containing 450 µl of chemical, instead of 8 mm Finn chambers as used in the GPMT, because it has been shown that a larger patch-test chamber increased the sensitivity of the Buehler test (20). From diagnostic patch tests in eczema patients it is well known that larger amounts of the same concentration of test allergen give stronger reactions (21)

All of the test substances except chlorhexidine produced significant sensitization in the GPMT. Formaldehyde and neomycin sulphate were positive in the Buehler test but eugenol and mercaptobenzothiazole were negative and chloraniline gave positive reactions at day 2 only. It is questionable whether chloraniline is a sensitizer in the Buehler test because the reactions faded at day 3. The reactions at day 2 may have been due to irritation, although the control animals were negative.

Mercaptobenzothiazole is a moderate sensitizer in guinea pig assays and is therefore 1 of the 3 positive control substances recommended in OECD guideline #406. We could not sensitize with this compound in the Buehler test, but some laboratories have obtained positive Buehler tests (10).

Eugenol sensitized significantly in accordance with previous GPMT data (22), but was negative in the Buehler test. Basketter & Gerberick (23) also found negative or low responses (0% and 11% positive test animals) at 2 different laboratories even though they considered a grade "1" response as a positive challenge.

Chloraniline was a sensitizer in the GPMT, with an EMS of 0.9. Previously reported GPMTs with this chemical showed sensitization in 50% of the animals (24). In 2 dose–response studies with chloraniline with the Freunds Complete Adjuvant Test (FCAT) method EMS rates of 0.5 and 0.9 were found (Boman, personal communication, 1996). The loss of response in our Buehler test at day 3 was even more obvious after rechallenge, where the response rate in the test groups decreased from 4–5 out of 5 at day 2 to 0 out of 5 at day 3.

Induction with 30% and 10% formaldehyde in the Buehler test caused severe irritation and the induction doses were subsequently reduced. Formaldehyde sensitized significantly

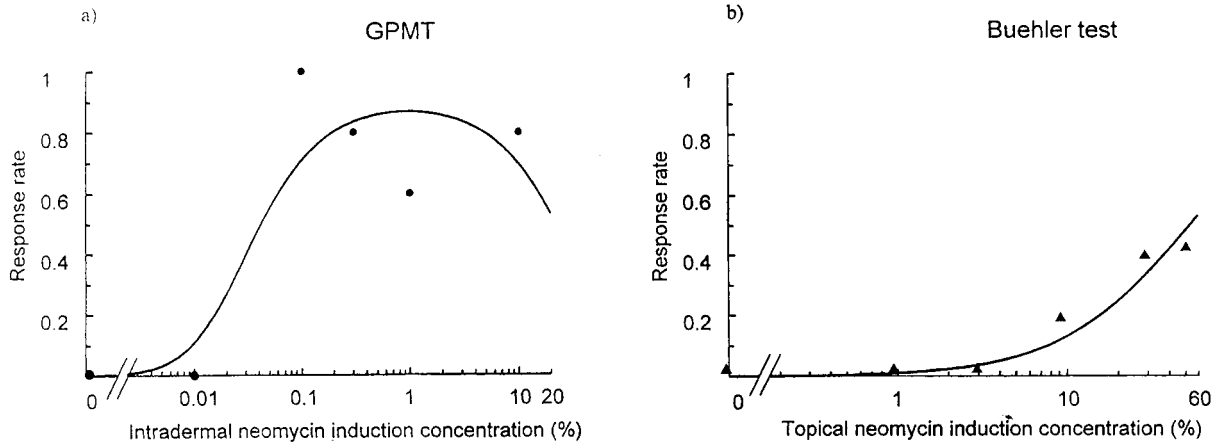

Fig. 3. Dose–response curves for neomycin sulphate. (a) GPMT readings at day 3 after challenge with 10% neomycin sulphate: (●), observed responses; (—), non-monotonous logistic curve fit. (b) Buehler test readings at day 3 after challenge with 30% neomycin sulphate: (▲), observed responses; (—), monotonous logistic curve fit.

in both tests but if the induction doses are compared as shown in Table II higher induction concentrations were needed to sensitize the animals in the Buehler test compared with the GPMT, in spite of the larger patch-test chambers used in the Buehler test. Previously reported Buehler tests with formaldehyde have given sensitization rates varying between 0% and 70% (7, 19, 25).

Neomycin sulphate sensitized significantly in both tests. Goodwin et al. (24) tested neomycin sulphate in a standard GPMT and found a sensitization rate of 0.3, while the EMS was 0.9 in the present GPMT study.

Chlorhexidine gave negative results in the dose–response GPMT and questionable positives in the Buehler test where positive control animals were observed. Goodwin et al. (24) observed sensitization to chlorhexidine in 2 out of 10 animals. However, a surprisingly high challenge dose of 12.5% was used (compared with 0.3–0.1% in the present study) and no information was given concerning responses in the control group. No Buehler test data have been published with this compound.

In general, a higher induction concentration was needed in the Buehler test to show allergenicity compared with the GPMT and for some allergens the Buehler test protocol was not sensitive enough to detect allergenicity. The difference in sensitivity may be related to several methodological differences in the test procedures, i.e. induction modes, topical treatment time and the use of FCA in the GPMT. Because the GPMT includes intradermal induction the limitation of skin penetration from topical administration is bypassed. Consequently, the GPMT may be less dependent on the choice of vehicle compared with the Buehler test. Magnusson & Kligman (1) demonstrated that the use of FCA was essential for the sensitivity of the GPMT and no alternative to FCA has been developed and validated to date (26). However, FCA may induce false-positive reactions due to hyperirritable skin, which may overestimate the allergenic potency of a compound (27, 28). In the present dose–response GPMT study chlorhexidine was negative and previous dose–response studies with lidocaine, propyl paraben and methyl acrylic acid were also negative (14, 29). The false positive results caused by hyperirritable skin can be counteracted by pilot studies in FCA-treated animals, rechallenge and the use of several challenge concentrations and these techniques facilitates the interpretation of GPMT results (17). The impact of FCA on the animals in the GPMT is of concern because severe inflammation may develop at the injection site. In this study the impact of the procedures on the animals' welfare was evaluated by comparing the average weight gain of the animals in the 2 test procedures but no significant difference was found.

In conclusion the Buehler test was less sensitive than the GPMT in a systematic comparison, where all details in each test were selected to give a maximal response. This finding could have implications for future guidelines for guinea pig tests of contact allergy.

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