Induction of Type IV Hypersensitivity to Contact Allergens in Guinea Pigs by *In vitro* Haptenized Allogenic Peritoneal Exudate Cells

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The induction of type IV hypersensitivity to contact allergens in guinea pigs has been studied by using allogenic peritoneal exudate cells (>90 % macrophages), which had been incubated primarily in vitro with dinitrochlorobenzene, formaldehyde, potassium dichromate, nickel II sulphate or *para*-aminobenzoic acid. In these guinea pig sensitization experiments Freund's complete adjuvant was used. In all haptens investigated the sensitization rates of the presented method were parallel to the known contact allergenicity in humans and, apart from the potassium dichromate results, comparable with those of the guinea pig maximization test. Because of its alternative immunization procedure, in which only few or no allergen molecules escape the effective presentation pathway, the authors conclude that this method could be developed into a predictive test assay for the evaluation of the contact allergenicity of water-soluble substances. *Key words: Animal assay; Contact allergy; Contact dermatitis; Macrophage; Predictive test.*

A lot of substances which come into contact with human skin must be regarded as possible sensitizers. To minimize or avoid the resulting hazard of contact allergy it is advisable to use predictive test methods evaluating the allergenicity of substances or compounds before their application. The tests most widely used for this purpose are guinea pig sensitization assays of a similar kind: an induction phase, followed by a resting period of about 2 weeks and subsequently a challenge test to prove whether sensitization has occurred or not. For induction, the hitherto most propagated test methods use "direct" application of the substance in question by intradermal injection, epicutaneous application or both (1, 2). However, in these application routes non-definable amounts of allergen may bypass the Langerhans' cells (LC). If the immune system is faced by allergens without involvement of the LC, specific unresponsiveness is often induced (3–5). Thus, it seems to be important to have induction procedures in which all allergen molecules are presented by sufficient accessory immune cells. Because of the technically complicated preparation of LC, we decided to choose the functionally and ontogenetically similar macrophages (paraffin oil-induced peritoneal macrophages). This way of inducing contact allergy has already been studied in some allergens in inbred guinea pigs by von Blomberg et al. (6). In the present study, we investigated the question whether the use of outbred guinea pigs and the application of allogenic haptenized peritoneal exudate cells (PEC), respectively, also cause a type IV hypersensitivity or not.

The following aspects have been studied: 1) the agreement of the sensitization results of our experiments with the known allergenicity of the tested substances in humans and with "reference assays" in animals; 2) the influence of some methodological factors on the sensitization rates (duration of the resting period between induction and challenge, different numbers of haptenized PEC); and 3) the reproducibility of the experiments.

After promising preliminary experiments with potassium dichromate (7) we used five different haptens in our study: one weak sensitizer – *para*-aminobenzoic acid (PABA) (Ferak Berlin, Germany) – three substances of medium sensitizing potential nickel II sulphate (Laborchemie Apolda, Germany), potassium dichromate (Laborchemie Apolda, Germany), formaldehyde (Laborchemie Apolda, Germany) – and one very strong sensitizer – dinitrochlorobenzene (DNCB) (Berlin-Chemie Berlin, Germany).

MATERIAL AND METHODS

Animals

Outbred guinea pigs (Dunkin-Hartley, Marx, Falkenberg) of either sex, weighing 300–400 g, single-housed in plastic cages, were used.

Peritoneal exudate cell production

Under aseptic conditions 10 ml of subliquid paraffin oil were injected into the peritoneal cavity of every animal in PEC breeding (32 animals in total). After 7–10 days PEC were harvested in cooled (2–4°C) Eagle medium (Institut für Immunpräparate und Nährmedien Berlin, Germany) and washed twice by centrifugation (380 g, 10 min). Then the cells were counted and probes for cell differentiation were taken. Two staining techniques were applied to identify the PEC: the Pappenheim method and a method for staining of the non-specific alphanaphthyl acetate esterase (8). Only samples with at least 90% macrophages were used.

Haptenization procedure

PEC (in a concentration of 10^7 cells/ml) were incubated at 37°C for 30 min in Eagle medium in the presence of a maximum non-toxic concentration of allergen (unless otherwise stated – see Table I). The maximum non-toxic concentrations were determined in preliminary experiments. In order to dissolve DNCB we added 0.1 % acetone (Laborchemie Apolda, Germany). During haptenization procedure the cells were kept in suspension by careful motion. The cells were then washed twice again. Subsequently we estimated the cell vitality by the trypan blue method (9). Only cell suspensions with at least 90 % vital cells were applied.

Immunization

Guinea pigs received totally 1.5×10^7 or 1.5×10^8 haptenized cells by six subcutaneous injections (4 into their extremities, 2 behind their ears). To increase the sensitivity of the method, in addition, all animals

Table I. *Sensitization rates (sensitivity/total number of animals) of experimental sensitization of guinea pigs by subcutaneous injection of allogenic peritoneal exudate cells (PEC) in vitro haptenated with p-aminobenzoic acid (PABA), nickel II sulphate, potassium dichromate, formaldehyde or dinitrochlorobenzene (DNCB)*

Substance	Number of animals	Number of injected PEC per animal	Hapten concentration in PEC incubation	Challenge test concentration	Sensitization rates (animals with positive test results/total number of animals tested) after			
					1 week	2 weeks	4 weeks	8 weeks
PABA	20	1.5×10^7	$1.4 \times 10^{-4}\%$ (10 µM)	2.0% aqu.[a]	–	0/20	0/20	1/20
Nickel II sulphate	20	1.5×10^7	0.3% (10 mM)[b]	0.5% aqu.[b]	2/20	5/20	9/20	14/20
	10	1.5×10^8	0.3% (10 mM)[b]	0.5% aqu.[b]	3/10	4/10	4/9[c]	5/9[c]
Potassium dichromate	20	1.5×10^7	0.1% (3.5 mM)	0.5% aqu.	3/20	9/20	–	–
	20	1.5×10^7	0.1% (3.5 mM)	0.5% aqu.	1/20	8/20	–	–
	18	1.5×10^7	0.1% (3.5 mM)	0.5% aqu.	5/18	9/18	–	–
	16	1.5×10^8	0.1% (3.5 mM)	0.5% aqu.	3/16	5/15[c]	–	–
Formaldehyde	10	1.5×10^7	$1.5 \times 10^{-3}\%$ (500 µM)	1.0% aqu.	0/10	0/10	–	0/10
	20	1.5×10^7	$1.5 \times 10^{-4}\%$ (50 µM)	1.0% aqu.	4/20	12/20	11/20	16/20
	12	1.5×10^8	$1.5 \times 10^{-4}\%$ (50 µM)	1.0% aqu.	2/12	5/12	6/12	7/12
DNCB	10	1.5×10^7	0.001% (50 µM)[d]	0.05% aqu.[e]	–	10/10	–	–

[a] plus 70% ethanol, [b] ($NiSO_4 \times 7\ H_2O$), [c] animal died during experiment, [d] plus 0.1% acetone, [e] plus 2% acetone.

were given a single subcutaneous injection of 0.1 ml undiluted Freund's complete adjuvant (Institut für Impfstoffe Dessau, Germany) into their neck.

Skin testing

After a resting period of 1–8 weeks, serial skin tests were performed. We used occlusive epicutaneous application of 100 µl of an aqueous solution (except of PABA: 70 % ethanol (Laborchemie Apolda, Germany)) of the allergen in the maximal non-irritative concentration (Table I). The test was performed on the shaved left or right flank. We used adhesive patch test tape (Leucotest R, Beiersdorf Hamburg, Germany) additionally fixed by a circular adhesive bandage. After 24 h the dressing was removed and the skin reaction was evaluated. A second evaluation was performed after 48 h. The reactions were read "blind" and evaluated in the following manner: 0 = no reaction, (+) = red or pink reaction without edema or papulae, + = erythema plus edema or erythema plus papulae, ++ = vesiculae or crustae plus erythema and edema, +++ necrosis of the epidermis. Only crescendo or lasting reactions of both readings were evaluated as positive test results, a reaction reaching merely the (+)-level was evaluated as negative in every case.

Control animals

A group of 10–20 animals for every experimental series were treated with unhaptenized PEC and FCA and tested in the same way as the "allergen animals". The control animals for the DNCB experiments were given cells incubated with 0.1% acetone.

RESULTS

Differentiation of PEC

The PEC samples used in sensitization experiments stained by the Pappenheim technique had the following composition:

macrophages: 91–98% (93.59±2.31 %)
neutrophilic granulocytes: 0–6% (2.59±1.85%)
eosinophilic granulocytes: 0–3% (0.88±0.87%)
lymphocytes: 0–4% (1.91±1.00%)
cells, not differentiable: 0–3% (1.03±1.12%)

By staining the non-specific alpha-naphthyl acetate esterase as a marker for dendritic cells, 94.37±2.42% of the total PEC were identified as macrophages.

Sensitization experiments

The results of the sensitization experiments are shown in Table I. As expected, the weak sensitizer PABA exerted the lowest sensitization rate (one animal out of 20 after 8 weeks). The ubiquitous hapten nickel 11 sulphate induced altogether 30% (9/30) and 66% (19/29) positive results after 2 and 8 weeks, respectively, while the percentage of sensitization was 42% (31/73) for potassium dichromate after 2 weeks. Four test series with 20, 20, 18 and 16 guinea pigs gave similar results, indicating a good reproducibility of the experiments. In the highest hapten concentration used, the common hapten formaldehyde induced no positive test result although this concentration seemed to be non-toxic. The other formaldehyde series performed with a ten times lower hapten concentration showed allergic eczematous test reactions in 53% (17/32) and 72% (23/32) of the animals after 2 and 8 weeks, respectively. DNCB acted as a positive control. All animals reacted positively in the patch test after 2 weeks.

In the control animals of all series we did not find any positive epicutaneous test results.

The time course of the test results

The sensitization rates increased significantly (chi-squared test, $p < 0.05$) until the second week in all series of the nickel II sulphate, potassium dichromate and formaldehyde experiments using 1.5×10^7 haptenized cells (except of the disregarded first series of the formaldehyde experiments). In nickel II sulphate but not in the other haptens we found an additional significant increase (chi-squared test, $p < 0.05$) until the 8th week (Table I).

The influence of the number of injected cells

In nickel II sulphate, potassium dichromate and formaldehyde all series using the higher number of cells for immunization (1.5×10^8 per animal) did not show significantly higher or lower sensitization rates than the corresponding series using 1.5×10^7 cells per animal. In contrast to the series carried out with 1.5 10^7 PEC per animal, in the series using the higher number of cells for immunization there was no influence of the duration of the resting period between immunization and challenge.

DISCUSSION

The most important antigen presenting cell (APC) in contact dermatitis is the LC (10, 11). LC can take up and process antigen, migrate most likely under the influence of TNF alpha to the draining lymph nodes, mature under stimulation by GM-CSF (12), are capable of expressing certain cytokines (13) and to present antigen sufficiently in complex with MHC molecules to initiate T-cell response. What we have done in our experiments is to replace the APC function of LC by macrophages in the primary phase of a type IV immune reaction. The other cells of the PEC used do not play an important role as APC. Some of them may have an APC function in special cases (14) but when few, as in our experiments, they are not expected to have a significant influence.

The principal usefulness of macrophages as APC also in type IV reactions to contact allergens is widely accepted (6, 7, 10, 11, 14, 15). Despite of some differences, macrophages and LC have a functional analogy in their ability of antigen handling, presentation and T-cell stimulation, and they most likely share the same bone marrow stem cell (16, 17). However, in this study allogenic macrophages were used. The question is whether MHC molecules must be identical between APC and T-lymphocytes as a prerequisite for a successful cooperation. The identity of the MHC molecules seems to be necessary only for the secondary immune reaction, but not for the primary one (14, 18–20). This study does not give a conclusive answer to this question. The results can be interpreted as a possibility to induce contact allergy with allogenic macrophages in outbred guinea pigs. On the other hand, it is possible that allogenic macrophages exert only a carrier function for haptens, i.e. after bringing the hapten into the organisms of the animals PEC are recognized as foreign cells and are killed consequently. The hapten may then be processed by accessory immune cells of the host. But the completely negative test result of the first formaldehyde series (PEC incubated in the higher formaldehyde concentration) speaks against this carrier hypothesis. A more likely explanation for these negative test results has been provided by investigations showing that a low concentration aldehyde treatment (0.0012 to 0.005%) inhibits the expression of HLA class II antigens in human monocytes (21).

Another fact speaking against the carrier hypothesis is that such low doses of allergen carried by the haptenized PEC were capable of inducing the sensitization rates achieved. Even the amount of allergen carried hypothetically by the PEC before cell washing (simply the volume of the injected cells multiplied by the hapten concentration during haptenization procedure) was about 10 (nickel II sulphate) to 1000 (formaldehyde) times lower than for instance the lowest published intradermal induction doses in the guinea pig maximization test (22–24). Because of this fact and the difficulty in exact quantification of the low amounts of allergen finally bound by the PEC (after cell washing twice), we have not included control experiments with groups of animals treated with comparable doses of a non-cell-bound hapten.

An important question to discuss is whether it is possible to obtain reliable and realistic sensitization rates in bypassing the epidermis. Now it is known that especially keratinocytes beside their barrier function take an active part in immunological processes by expression of a variety of cytokines, adhesion molecules and MHC (25, 26). In fact, this disadvantage is connected with the advantage of the method presented, namely that only few or no allergen molecules bypass the APC. From our results we can conclude that this induction method leads to realistic sensitization rates, too. It could be shown that according to the known sensitizing potentials of the haptens in man the sensitizing rates found in this assay were in parallel. This means that DNCB as a compulsory sensitizer (100% after 2 weeks) was followed by formaldehyde, potassium dichromate and nickel II sulphate as medium sensitizers, while PABA as a weak sensitizer reacted only in one animal after 8 weeks. To evaluate the significance of the method presented we considered it necessary to compare it with other predictive guinea pig tests. Numerous tests with different procedures and varying sensitization results have been published. We prefer the comparison with the guinea pig maximization test (GPMT) introduced by Magnusson & Kligman in 1969 (27) and the Tierexperimenteller Nachweistest (TINA-test) developed in our department by Ziegler & Süss (28) because the first-mentioned method is internationally accepted, in the second method we are experienced and both methods use an occlusive patch testing as we did.

Comparison with other results

1. Para-aminobenzoic acid. Goodwin et al. (23) studied PABA with three different methods: the modified Draise test, the GPMT and the single injection adjuvant technique. The sensitization rates were 0% (0/10, 0/10, 0/20) and corresponded with those of our study. In the TINA-test 4 of 23 animals (18%) reacted positively to PABA. This result does not differ significantly from ours (chi-squared test $p > 5\%$).

2. Nickel II sulphate. Achieving sensitization rates of maximally 40%, Rohold et al. (22) have demonstrated that in nickel sulphate the results of the GPMT correlate especially with the intradermal induction concentration. This may be an explanation of the different results of different authors mentioned in this paper. Magnusson & Kligman reached 55% (11/20) (29); other authors had lower results: 35% (7/20) (30) or 23% (7/31) (31). Our results (30% and 66% after 2 and 8 weeks, respectively) are in agreement with the GPMT results already mentioned. On the other hand our results after 2 weeks but not after 8 weeks agreed with those of the TINA-test (8/33; 24%).

3. Potassium dichromate. Our results (31/73, 42 % at 2 weeks) do not differ significantly (chi-squared test, $p < 0.05$) from those of the TINA-test (28/48; 58%). However, in the GPMT the sensitization rates were significantly higher: 75% (18/24) (29) and 100% (10/10) (23) (chi-squared test, $p < 0.05$).

4. Formaldehyde. Andersen et al. (24) studied the dependency of the sensitization rates of formaldehyde on the induction and the eliciting concentration in the GPMT. They found 17 positive reactions in 19 animals (89%). In the same test concentration (1%), but 10 times higher induction concentrations (intradermal 1%, epicutaneous 5%) they observed 50 % positive reactions (10/20). Our results observed again with 1 % test concentration are between these results (17/32; 53% after 2 weeks and 23/32; 72% after 8 weeks). Magnusson & Kligman (29) reported on 16/20 positive animals (80%). 100% positive animals were found in the GPMT (10/10) by Goodwin et al. (23).

5. Dinitrochlorobenzene. DNCB proved to be a very strong allergen in guinea pig tests. In the GPMT the following results were reported: 15/20 (32), 10/10 (23) and again 10/10 (33). The 15/20 results may be explained by the relatively low challenge concentration (0.01%). We also found a sensitization rate of 100% (10/10).

The test results depend on various factors influencing the sensitization procedure and the challenge process. One of them is the resting period between immunization and challenge. The test series with nickel II sulphate, potassium dichromate and formaldehyde show that sensitization rates after one week are low and not representative of the expected allergenic potential of the tested substances. On the other hand it should be noted that the sensitization rates of nickel II sulphate increased until the 8th week. A possible explanation could be that the serial patch tests could act like booster applications. In order to standardize the method a resting period of 2 weeks seems to be recommendable.

Another factor expected to influence the sensitization results might be the number of injected PEC. The present results suggest that an increase in the number of PEC does not necessarily increase the sensitization rate, however. 1.5×10^7 seems to be a recommendable number of PEC for immunization.

The three test series with potassium dichromate with 18 to 20 animals, each, using 1.5×10^7 haptenized PEC showed a good agreement of the sensitization rates, which confirms the good reproducibility of our method. The differences of the results after one week underline the recommendation not to test earlier than 2 weeks after induction.

In conclusion the sensitization rates yielded with this method corresponded in all tested haptens with the known allergenic potential in humans as well as to the test results observed with the GPMT (except of potassium dichromate) and the available results of the TINA-test (PABA, nickel II sulphate, potassium dichromate). This in vitro/in vivo method is suited for the detection of the contact sensitizing potency of various water-soluble substances.

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