Acta Dermatovener (Stockholm) 57: 23–28, 1977

STUDIES ON CARRIER PROTEIN IN CONTACT DERMATITIS: MACROPHAGE MIGRATION INHIBITION BY SOLUBLE EPIDERMAL SUBSTANCES AS CARRIER PROTEINS

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Abstract. The migration of peritoneal exudate cells from picrylchloride-sensitive guinea pigs can be inhibited by the water-soluble proteins present in the picrylchloride-treated guinea pig epidermis. Further examination of these proteins revealed that antigens were present in fractions with the molecular weights ranging between 10 000 and 200 000.

Key words: Contact dermatitis; Carrier protein; Macrophage migration inhibition; Soluble epidermal proteins

Present knowledge of allergic contact dermatitis explains the mechanism of sensitization as follows. After first contact with the skin, simple chemicals (haptens) bind with substances in the epidermis and/or dermis as they penetrate the skin. The bound complexes are conveyed by lymphatic vessels to lymph nodes where they stimulate immunoblasts.

The search for the hapten–protein complexes responsible for antigenicity has proceeded mostly by utilizing in vivo sensitization methods, but recently some workers have introduced an in vitro system into this field, viz. lymphocyte stimulation or macrophage migration inhibition tests.

Despite these efforts, the carrier substances in allergic contact dermatitis still remain ill defined. Nevertheless, many reports suggest that active immunogenic substances originate from the epidermis.

In our previous investigations, one of us attemped to isolate and identify carrier substances of picrylchloride contact allergy in guinea pigs by utilizing the in vitro macrophage migration inhibition technique as an indicator of a specific immunological reaction, and obtained the following results (6).

1. Hapten-conjugated homologous or heterologous serum did not stimulate immune lymphocytes to produce migration inhibitory factor (MIF).

2. Picrylchloride-painted guinea pig epidermis and its insoluble subcellular fractions (so-called nuclear, mitochondrial, and microsomal fractions) all produced MIF.

3. By repeated freezing and thawing of the picrylchloride-painted epidermis, active hapten–protein conjugates were released into the soluble fraction.

The experiment reported in this paper is an attempt to extend the analysis of allergenic hapten–protein conjugates solubilized from the picrylchloride-painted epidermis by the standardized macrophage migration inhibition test.

MATERIALS AND METHODS

1. *Animals*

Hartley female guinea pigs weighing about 400 g were used throughout the experiments.

2. *Immunization of animals*

Guinea pigs were immunized with 40 μg of sodium picrylsulfonate (TNBS) in Freund's complete adjuvant. According to preliminary experiments, this achieved sensitization of almost all animals. Animals were judged as sensitized when erythema was induced 48 hours after epicutaneous application of 0.1% picrylchloride in acetone solution.

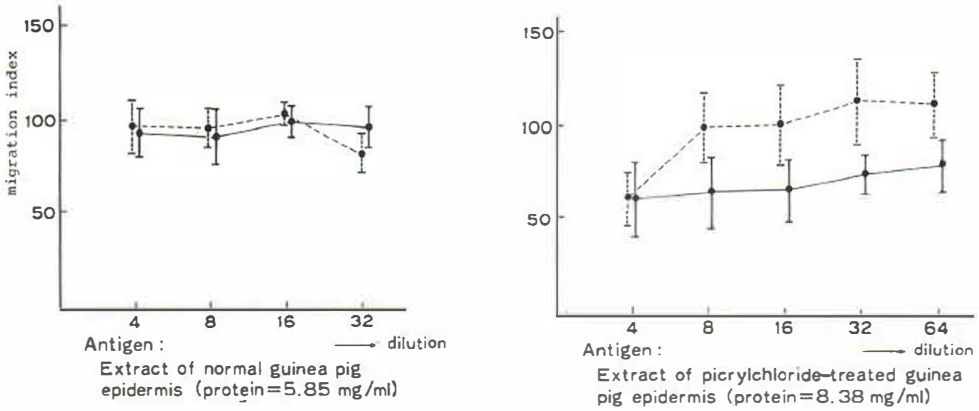

Fig. 1. Macrophage migration indices (means±S.D.) of PEC from normal (- - -) and from picrylchloride-sensitive (—) guinea pigs.

3. *Preparation of epidermal extract*

Epidermis from normal guinea pigs was obtained as follows. After exsanguinating the animals, hair was plucked and whole skin was removed. The epidermis was mechanically separated from the skin by using a scalpel. To obtain picrylchloride-treated epidermis, a 3% solution of picrylchloride in acetone mixed with olive oil (1 : 1) was painted onto the skin (about 0.1 ml/cm²) of depilated animals. Animals were killed 18 hours later and the epidermis was obtained as described. The scraped epidermis was treated immediately in order to obtain the soluble fraction as described by Parker et al. (9) with slight modifications. Briefly, the epidermis was washed with PBS (phosphate-buffered saline, 0.02 M, pH 7.0) until the supernatant became almost completely clear. The epidermis was then homogenized by pestle and mortar in 8 volumes of PBS. The homogenate was frozen and thawed several times and then filtered through double-layered gauze. The extract was centrifugated at 700 *g* for 20 minutes at 4°C and the supernatant centrifugated again at 105 000 *g* for 60 minutes at 4°C in an ultracentrifuge (Hitachi Model 65P). The final supernatant was used as soluble fraction of epidermis for further experimentation.

4. *Gel filtration of soluble epidermal proteins*

Sephadex G-200 (Pharmacia) was swollen in an excess of PBS and packed into a column (2.6×90 cm). The column was calibrated for the determination of molecular weights (MW) as described by Andrews (1) using blue dextran, bovine gamma globulin (MW=160 000), bovine serum albumin (MW=67 000) and egg white lysozyme (MW=14 300) purchased from Sigma Chemical Co.

The optical density of the eluate was read at 280 nm (protein) and at 348 nm (ε-*N*-trinitrophenyl-lysine) using Hitachi model 101 spectrophotometer.

Table I. *Mean macrophage migration indices of peritoneal exudate cells from normal and from picrylchloride sensitive guinea pigs*

Results are the mean migration indices and standard deviation of 40 observations on 10 animals in each group with treatment effects. The degree of significance was determined by Student *t*-test

Source of peritoneal exudate cells	Antigens added to peritoneal exudate cells				
	×4[a]	×8	×16	×32	×64
Extract of normal guinea pig epidermis (protein=5.85 mg/ml)					
Normal g.p.	97.6±14.2[b]	96.2±11.3	104.0±7.0	85.3± 9.4	
Picrylchloride sensitive g.p.	95.2±12.3	93.4±13.3	100.3±8.0	97.9±10.5	
Degree of significance	$P>0.05$	$P>0.05$	$P>0.05$		
Extract of picrylchloride treated guinea pig epidermis (protein=8.38 mg/ml)					
Normal g.p.	61.0±14.0	98.4±19.3	100.1±21.2	112.7±23.6	111.5±16.5
Picrylchloride sensitive g.p.	60.7±19.9	64.1±19.7	65.8±16.4	73.9±11.0	77.3±14.7
Degree of significance	$P>0.05$	$0.001<P<0.01$	$0.001<P<0.01$	$P<0.001$	$P<0.001$

[a] Dilution with medium.
[b] Migration index.

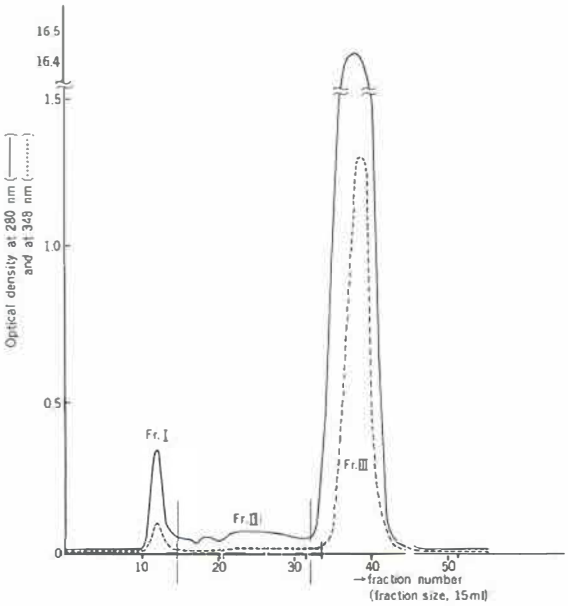

Fig. 2. Typical elution profile of the soluble protein fraction of picrylchloride-treated guinea pig epidermis on Sephadex G-200. V_0 (void volume)=135 ml, and V_t (total volume)=660 ml.

5. *Preparation of antisera*

Rabbit antisera against the soluble proteins of guinea pig epidermis and against guinea pig whole serum were used in these experiments. They were prepared as follows; rabbits were injected in four foot pads with 4 mg of antigen emulsified in Freund's complete adjuvant, twice with a 5 week interval and, between 10 and 14 days after the last injection, the antisera were obtained.

The antisera against the soluble proteins of guinea pig epidermis were absorbed with insolubilized normal guinea pig serum before use. Insolubilized serum was prepared as described by Avrames et al. (2).

6. *Immunodiffusion and immunoelectrophoresis*

Agar diffusion experiments were carried out on glass plates covered with 1.0% agarose (Difco) in PBS. The wells were 2 mm in diameter and the distance between the wells was 3 mm.

The plates were left overnight in a moist room at 37°C. The gel then was washed, dried and stained in a conventional way.

Immunoelectrophoresis was as described in the previous paper (10). Electrophoresis was run for 60 min at 6 volt/cm.

7. *Estimation of protein content*

The protein content of the solutions was determined by biuret analysis.

8. *Estimation of hapten content*

To determine the number of trinitrophenyl groups per g protein, the molecular extinction coefficient of ε-*N*-trinitrophenyllysine (15 400 at 348 nm) was used (5).

9. *Macrophage migration inhibition assay*

The method used was essentially the same as that reported by David et al. (3). Thirty ml of sterilized liquid paraffin was injected intraperitoneally 2 weeks after immunization. After blood was taken by cardiac puncture, peritoneal exudate cells (PEC) were collected in Hanks' balanced salt solution.

The washed cell suspension was finally suspended in medium 199 containing 20% foetal calf serum and kanamycin (25 μg/ml). The PEC were put into a glass capillary tube (1 mm in diameter and 70 mm long).

The cell count per capillary tube was approximately 1.5×10^6. Two capillaries were set up per Petri dish and various kinds of antigen were added to this in 1 ml of medium 199 containing 20% foetal calf serum and kanamycin (25 μg/ml). The antigens were used after filtration through Millipore filters (0.45 μm). Petri dishes were incubated at 37°C in a CO_2-chamber for 18 hours. The migration area was enlarged 10 fold by a Nikon shadow scope and thin tracing paper was cut to the size of the migration area. Each cut paper was weighed in an analytical balance. Four capillaries were prepared for each antigen concentration. The migration index (%)

$$\frac{\text{mean migration area in presence of antigen}}{\text{mean migration area in absence of antigen}}\times100$$

was used to express the effect of antigen on macrophage migration.

Table II. *Molecular weight, ε-N-TNP lysine (mol)/protein (g) and results of immunodiffusions of picrylchloride-treated epidermis after separation on Sephadex column*

Fraction	Molecular weight	TNP-lysine (mol)/ protein (g)	Serum proteins[a]	Epidermal[a] proteins
I	>200 000	0.28×10^{-4}	Macroglobulins	–
II	200 000~ 10 000	0.20×10^{-4}	Albumin, Globulin	Six proteins
III	<10 000	0.70×10^{-4}	–	–

[a] Proteins were detected by immunodiffusion or immunoelectrophoresis.

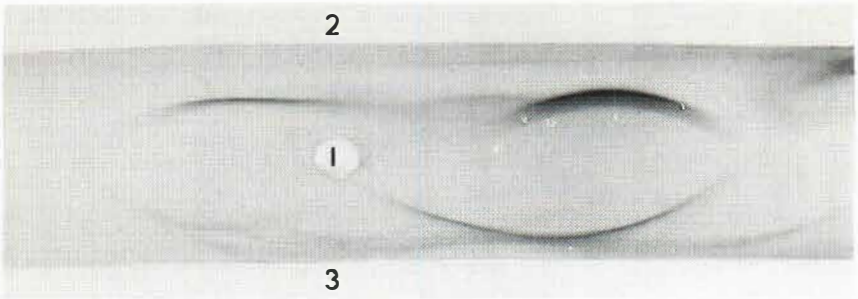

Fig. 3. Immunoelectrophoretic patterns of Fr. 11 developed against anti-guinea pig serum rabbit serum and against anti-guinea pig epidermis rabbit serum absorbed with guinea pig whole serum. *1:* Fr. 11 of Sephadex fractions. *2:* anti-guinea pig serum rabbit serum. *3:* anti-guinea pig epidermis rabbit serum.

RESULTS

1. *Migration inhibition of PEC from hypersensitive guinea pigs by normal and picrylchloride-treated epidermal extract*

In Table I and Fig. 1, it is shown how the soluble extract of picrylchloride-treated epidermis was able to inhibit the migration of PEC obtained from sensitized animals, whereas it had no effect on cells from normal controls. The difference was statistically highly significant ($P<0.001$) at dilutions of ×32 and ×64, and significant ($0.001<P<0.01$) at ×8 and ×16. At the dilution of ×4, migration of normal PEC was also inhibited, though this seemed due to cytotoxicity of the extract at this concentration.

2. *Gel filtration of picrylchloride-treated epidermal proteins*

The soluble protein fractions from picrylchloride-treated guinea pig epidermis were applied on the calibrated Sephadex column. The elution curve obtained is shown in Fig. 2. The eluate was divided into three parts. Fraction I (MW>200 000) and fraction II (MW 200 000–10 000) were concentrated by ultrafiltration using seamless cellulose tubes and were used for characterization and as antigens

Table III. *Mean macrophage migration indices of peritoneal exudate cells from normal and from picrylchloride-sensitive guinea pigs*

Results are the mean migration indices and standard deviation of 32 observations on 8 animals in each group

Source of peritoneal exudate cells	Antigens added to peritoneal exudate cells; Sephadex G-200 fractions of picrylchloride-treated guinea pig epidermis				
	×8[a]	×16	×32	×64	×128
Fr. I (protein=1.78 mg/ml)					
Normal g.p.	72.3± 6.0[b]	73.1± 5.7	83.1±10.9	86.9± 7.3	87.3±12.1
Picrylchloride-sensitive g.p.	68.8±19.2	75.7±15.8	75.5±14.9	81.9±11.7	83.9±12.8
Degree of significance (P)	$P>0.05$	$P>0.05$	$P>0.05$	$P>0.05$	$P>0.05$
Fr. II (protein=2.11 mg/ml)					
Normal g.p.	78.8± 8.5	79.8±1.4	91.4± 1.7	78.5± 4.5	79.6±2.6
Picrylchloride-sensitive g.p.	52.5±12.8	66.0±8.6	76.5±14.4	77.1±13.2	89.4±8.0
Degree of significance (P)	$P<0.001$	$P<0.001$	$0.01<P<0.02$	$P>0.05$	
Fr. III (protein=1.05 mg/ml)					
Normal g.p.	52.1± 4.1	88.1±17.6	117.0± 5.7	96.0±12.0	
Picrylchloride-sensitive g.p.	66.6±12.8	111.8±26.7	122.8±13.4	110.8± 7.6	

[a] Dilution with medium.
[b] Migration index.

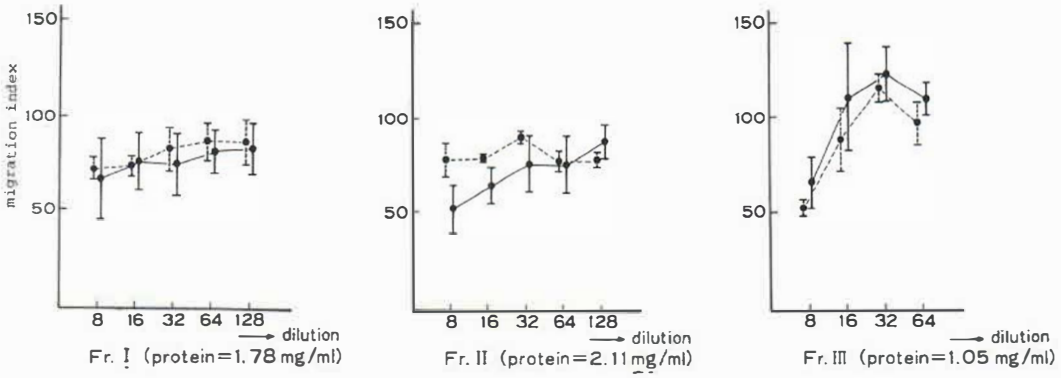

Fig. 4. Macrophage migration indices (means±S.D.) of PEC from normal (---) and from picrylchloride-sensitive (—) guinea pigs. Antigens are Sephadex G-200 fractions of picrylchloride-treated guinea pig epidermis.

of macrophage migration test. Fraction III (MW<10 000) was represented by the eluate at the peak point.

3. *Characterization of the Sephadex fractions of the picrylchloride-treated epidermal proteins* (Table II)

The presence of serum and epidermal proteins was determined by immunoelectrophoresis. Fractions I and II contained serum proteins. Fraction II contained at least six proteins of epidermal origin (Fig. 3). Fraction III was shown to contain no antigenic substances originating from serum or epidermis.

ε-*N*-trinitrophenyllysine(mol)/protein (g) (hapten/protein ratio) was as follows: Fr. I: 0.28×10^{-4}, Fr. II: 0.20×10^{-4}, Fr. III: 0.70×10^{-4}.

4. *Migration inhibition of PEC from hypersensitive guinea pigs by Sephadex fractions of picrylchloride-treated epidermal extract*

As shown in Table III and Fig. 4, significant inhibition of the migration of PEC from sensitized guinea pigs took place with Fr. II only. At ×8 and ×16 dilutions of this fraction, migration of sensitive cells was inhibited to a significantly ($P<0.001$) lower level than that of normal cells. With Frs. I and III, significant migration inhibition of sensitive cells did not take place at any dilution.

DISCUSSION

These experiments demonstrate that the water-soluble proteins of picrylchloride-treated guinea pig epidermis inhibit the migration of PEC from guinea pigs sensitized to picrylchloride. On further examination of these water-soluble conjugates by Sephadex column chromatography, only Fr. II (MW 200 000–10 000) is found to inhibit the migration of sensitized PEC. This Fr. II contained six antigenic proteins of epidermal origin; Frs. I and III contained no such antigenic substances. Therefore it is possible that one of the epidermal proteins behaved as the immunogenic carrier in these contact sensitivity experiments.

As serum proteins were detected in the epidermal extract by immunoelectrophoresis, it may also be possible that trinitrophenyl (TNP)-conjugates of serum proteins were immunogenic to sensitized PEC. However, in our previous experiments, the normal guinea pig serum conjugated with TNBS did not stimulate the regional lymph node cells of sensitized guinea pigs to produce migration inhibitory factor (MIF).

Free hapten or TNP-amino acids may be capable of stimulating immune lymphocytes to produce MIF. But, since Fr. III did not show any migration inhibition effect, this possibility can be discounted. This is compatible with our previous finding that PEC from picrylchloride-sensitive guinea pigs did not respond to free hapten (TNBS), TNP-lysine, or TNP-glycine added at various concentrations.

Inhibition of migration of normal macrophages by antigen–antibody complexes has been reported recently (4). The possibility that this type of migration inhibition took place in our experiments is discounted by our previous experimental results that TNP–BSA and TNP-guinea pig serum did not stimulate immune lymphocytes to produce MIF.

Furthermore, as reported previously (7), no hemagglutinating antibody could be detected in the culture medium under same conditions in order to test macrophage migration inhibition using the hen egg lysozyme system.

Nishioka et al. (8) showed that DNCB-microsomes could elicit the release of MIF from sensitized lymphocytes taken from a DNCB-sensitive guinea pig. Under our experimental conditions the epidermis was repeatedly frozen and thawed before fractionation. It is therefore possible that carrier proteins might be solubilized from cellular organelles under these conditions.

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Received December 29, 1975

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