主論文

# Non-IgG1 Nature of Cutaneous Basophil Hypersensitivity Factor in Contact Sensitivity

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**Abstract.** Cutaneous basophil hypersensitivity (CBH)-inducing factor was demonstrated in immune sera obtained from dinitrofluorobenzene (DNFB)-sensitized animals 2 weeks after sensitization (DNP-GPS-2W). It showed hapten specificity and worked dose dependently. It was fractionated into an non-γ-globulin fraction by Sephadex G-150 gel filtration following ammonium sulfate desalting and CM-cellulose chromatography. The factor was eluted into a fraction of a little smaller molecular weight than bovine serum albumin on Sephadex G-150 gel filtration. It passed through an antiguinea pig IgG1 column and was absorbed to a DNP-BGG column. On SDS-PAGE it failed to show any staining band because of low protein concentration. From these results CBH factor appearing in circulation in contact-sensitized animals was thought to be a somewhat different molecule from that of Askenase's factor, i.e. IgG1 antibody.

## Introduction

Cutaneous basophil hypersensitivity (CBH) is one of the delayed onset skin reactions in which a prominent infiltration of basophil leukocytes is observed [1, 2]. The mechanism triggering CBH and its immunological roles have been analyzed for the last 10 years. CBH reaction can readily be induced in guinea pigs as well as in human by sensitizing them with soluble protein antigens with or without Freund's incomplete adjuvant [3]. Prominent infiltration of basophils has also been demonstrated in inflammatory sites of contact sensitivity [4–6].

Several authors proposed that CBH is a reaction mediated by T lymphocytes [7–9] and that basophils which appeared in reaction sites will play a role to neutralize the inflammatory reaction mediated by classical delayed hypersensitivity [10]. CBH has been transferred with lymphoid cells of sensitized animals [11]. On the contrary, *Askenase* and co-workers [5, 12, 13] demonstrated that CBH was transferred to normal recipients with immune sera, especially with IgG1 antibody.

We have been analyzing the effects of humoral factors on contact sensitivity. IgG1 antihapten antibody generated in sera of contact-sensitized guinea pigs suppressed elicitation of contact sensitivity [14, 15]. This antibody failed to induce CBH reaction when transferred into normal recipients, although immune serum of contact sensitivity could induce CBH reaction [14]. Therefore, experiments were designed to separate CBH-inducing factor from the immune serum in this study.

## Materials and Methods

*Animals.* Hartley female guinea pigs weighing 300–500 g were used throughout the experiments. They were fed with pelleted food and water ad libitum.

*Chemicals.* The following chemicals were from commercial sources: 1-chloro-2,4-dinitrobenzene (DNCB), 1-fluoro-2,4-dinitrobenzene (DNFB) and dinitrobenzene sulfonate (DNBS) from Nakarai Chem. Co. Ltd., Kyoto; 1-chloro-2,4,6-trinitrobenzene (TNCB) from Tokyo Chem. Co. Ltd., Tokyo, and oxazolone (Ox), bovine γ-globulin (BGG) and bovine serum albumin (BSA) from Sigma, St. Louis, Mo. Dinitrophenylated BGG (DNP-BGG) was prepared according to the method described by *Little and Eisen* [16].

*Sensitization.* Guinea pigs were sensitized with a topical application of 0.025 ml of 10% DNFB in acetone, 3% TNCB in acetone or 3% Ox in ethanol. They were skin-tested 1–3 weeks after sensitization with 0.025 ml of 0.1% DNCB, 0.1% TNCB or 0.2% Ox, respectively, and bled on the next day. Sera obtained were donated as immune sera for passive transfer.

*Passive Transfer.* Normal guinea pigs were injected intravenously with 3–5 ml of the immune sera. They were skin-tested on their right pinna 30 min after the injection. Ear thickness was assessed before and 24 h after the skin test by using an engineer's micrometer (Peacock G1, Ozaki Co. Ltd., Tokyo) [5]. The tested sites were then biopsied for histological examination. Skin pieces were

fixed in Helly's fixative and stained with Giemsa solution according to the method described by *Askenase* et al. [12]. Cell counts were made in 5 × 1,000 fields near the dermoepidermal junction. Several specimens were fixed with osmic acid and processed for electron microscopic examination. A Hitachi type HU-2 electron microscope was used for observation.

*CM-Cellulose Chromatography.* Pooled immune sera (30 ml) were treated in 50% saturation of ammonium sulfate. The subsequent supernatant was dialyzed against 0.1 *M* acetate buffer, pH 5.0, and applied onto a CM-cellulose column (Whatman CM 32, 3 × 20 cm) equilibrated with 0.1 *M* acetate buffer, pH 5.0. The column was eluted with a linear gradient of 0.1–0.25 *M* acetate buffer, pH 5.0. Collected fractions were dialyzed against 0.01 *M* phosphate-buffered saline (PBS), pH 7.4, and concentrated to the original volume under negative pressure.

*Sephadex G-150 Gel Filtration.* After CM-cellulose chromatography the fraction containing CBH factor was further concentrated and applied onto a Sephadex G-150 column (1.5 × 100 cm) equilibrated with PBS. Elution was performed with PBS.

*Preparation of Antiguinea Pig IgG1 Antibody [14].* Pooled normal guinea pig sera were treated with 33% saturation of ammonium sulfate. The subsequent precipitate, after being dialyzed against 0.005 *M* phosphate buffer (PB), pH 8.0, was applied onto a DEAE-cellulose column (Whatman DE 32) equilibrated with the same buffer. Elution was performed stepwise with 0.005 *M* PB, pH 8.0, 0.04 *M* PB, pH 6.0 and 0.1 *M* PB, pH 6.0. The fraction eluted with the final buffer was rechromatographed with the same DEAE-cellulose column. The fraction thus obtained was injected with Freund's complete adjuvant into footpads of rabbits (2 mg protein/animal). 4 weeks later the rabbits were given an injection of the same dose of the fraction. They were exsanguinated 10 days after the last injection. Sera obtained showed a single precipitin line against IgG1 fraction on immunoelectrophoresis.

*Affinity Chromatography.* Affinity columns for DNP-BGG and antiguinea pig IgG1 were prepared by the method described by *Omen* et al. [17] and Katayama and Nighioka [14]. Elution from the affinity columns was performed with 1 *M* NaCl.

*SDS Disc Gel Electrophoresis.* SDS gel electrophoresis was performed according to the method of *Weber and Osborn* [18] using BSA (67,000), ovalbumin (45,000), myoglobulin (19,800) and cytochrome c (12,400) (Mann Chem. Inc.) as moolecular markers.

*Protein Estimation.* The amount of protein was estimated by the Folin-Ciocalteu reaction using BSA as the standard [18].

*Statistical Analysis.* Results were expressed as mean ± SE. Statistical analyses were performed by the Student's t test.

## Results

### *CBH Factors in the Immune Sera*

Immune sera of 2 weeks sensitization (DNP-GPS-2W), when injected into nonsensitized recipients, induced ear swelling by DNCB skintesting. The tested sites showed only swelling without erythematous reaction. Neither immune sera of 1 week sensitization (DNP-GPS-1W) nor those of 3 weeks sensitization (DNP-GPS-3W) induced any ear swelling (table I). Swelling induced by an injection of DNP-GPS-2W

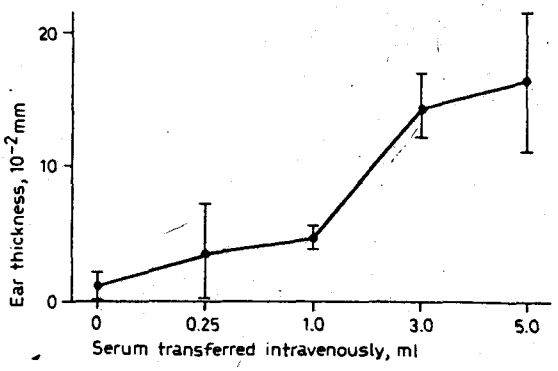


**Fig. 1.** Dose-response curve of CBH factor in immune sera.

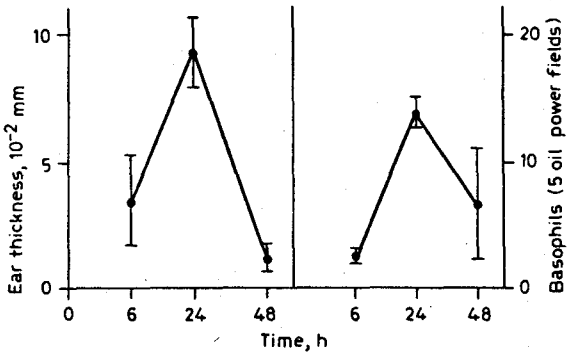


**Fig. 2.** Time course of CBH reaction. 3 ml of DNP-GPS-2W were injected into a recipient. Increment or ear thickness (left) and basophil infiltration (right) reached a peak at 24 h after skin testing.

was a dose-dependent reaction. It appeared with an injection of 1.0 ml DNP-GPS-2W and reached at plateau with more than 3 ml of the sera (fig. 1).

Histologically the swollen sites contained a significant number of basophils which were confirmed under electron microscopy by their granules of characteristic crystalline lattice material [2]. Strong basophil infiltration (14.0 ± 1.2) was noted in the swollen site induced by DNP-GPS-2W. Much less infiltration of basophils was observed in the recipients receiving DNP-GPS-1W or DNP-GPS-3W. Eosinophil infiltration was stronger in the sites induced by either DNP-GPS-1W or DNP-GPS-3W than in those induced by DNP-GPS-2W. There was no significant difference of mononuclear cell infiltration among those recipient groups (table I).

Ear swelling was observed at 6 after skintesting, reached a peak at 24 h and almost disappeared at 48 h. Basophil infiltration showed a similar time course although a moderate infiltration of basophils remained in the sites at 48 h after skintesting (fig. 2). Ear swelling with basophil infiltration was not observed when

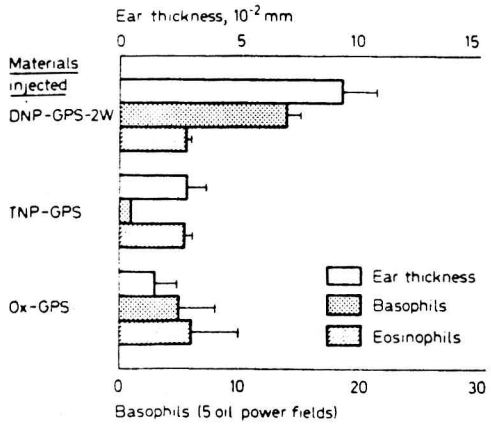


**Fig. 3.** Hapten specificity of CBH reaction. DNP-GPS, TNP-GPS and Ox-GPS were obtained from guinea pigs 2 weeks after sensitization. 3 ml of each sera were transferred into a recipient.

recipients receiving DNP-GPS-2W were skin-tested with TNCB or Ox. Immune sera obtained 2 weeks after sensitization with TNCB or Ox (TNCB-GPS or Ox-GPS) could induce ear swelling with basophil infiltration by skintesting with either TNCB or Ox; however, ear swelling with basophil infiltration was not observed by DNCB skintesting (fig. 3).

*Purification of CBH Factor*

Factor-inducing ear swelling with basophil infiltration was fractionated into a supernatant fraction by treatment with 50% saturation of ammonium sulfate (table I). The supernatant-containing CBH factor was first fractionated on CM-cellulose chromatography. The elution profile and activity-inducing ear swelling with basophil infiltration is shown in figure 4. The activity was found in Fr 2. 3 ml of Fr 2.

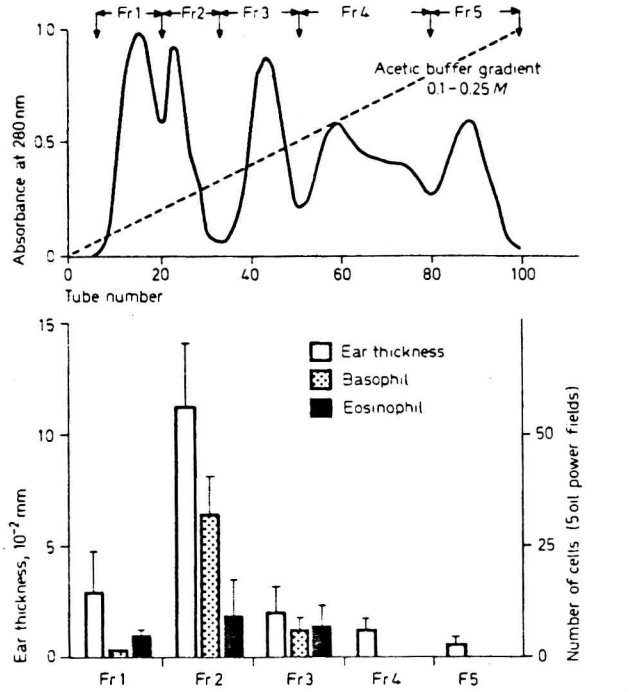


**Fig 4.** CM-cellulose chromatography of CBH factor. Supernatant obtained from 30 ml of DNP-GPS-2W by ammonium sulfate precipitation was applied onto a CM-cellulose column (3×20 cm) equilibrated with 0.1 *M* acetate buffer, pH 5.0. Elution was performed with a linear gradient of 0.1–0.25 *M* acetate buffer, ph 5.0. The eluate was concentrated to original volume of the sera and its 3 ml (7 mg/ml for Fr 2) were injected into a recipient.

(7 mg/ml) induced ear swelling with marked basophil infiltration. Fr 2. was then applied onto a Sephadex G-150 column. The CBH factor was eluted into the second peak which corresponded to a fraction of a little smaller molecular weight than BSA (fig. 5). 15 mg of the fraction induced ear swelling.

**Table I.** CBH reaction induced by immune sera

| Sera injected | 24-hour reaction in the ear of recipients | | | |
|---|---|---|---|---|
| | ear swelling $\times 10^{-2}$ mm ± SE | basophils[1] | eosinophils[1] | mononuclears[1] |
| DNP-GPS-1W | 1.2 ± 1.3 | 6.0 ± 0.7 | 7.0 ± 0.5 | 64.0 ± 10.5 |
| DNP-GPS-2W | 9.4 ± 1.2* | 14.0 ± 1.2* | 3.5 ± 0.3 | 62.5 ± 9.6 |
| DNP-GPS-3W | 2.4 ± 1.7 | 3.5 ± 0.3 | 6.0 ± 0.7 | 57.1 ± 7.1 |
| DNP-GPS-2W | | | | |
| 50% $AmSO_4$ Ppt | 0.9 ± 0.5 | 3.0 ± 0 | 0 ± 0 | 122.0 ± 0 |
| 50% $AmSO_4$ Sup | 14.7 ± 2.5* | 33.3 ± 7.6* | 4.3 ± 1.3 | 124.7 ± 4.8 |
| PBS | 1.3 ± 0.7 | 0 ± 0 | 1.5 ± 1.1 | 44.5 ± 7.4 |

* Statistically significant at level of $p < 0.001$.

[1] Cells/5 oil power fields ± SE.

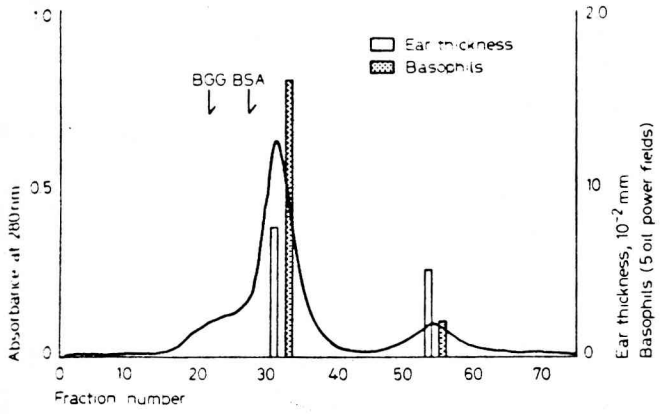


**Fig. 5.** Sephadex G-150 gel filtration of CBH factor. Fr 2 from the CM-cellulose column was applied onto a Sephadex G-150 column (3×100 cm). PBS was used for elution. The fractions were concentrated to the original serum volume and 3 ml of each (5 mg/ml for the second peak) were injected into a recipient.

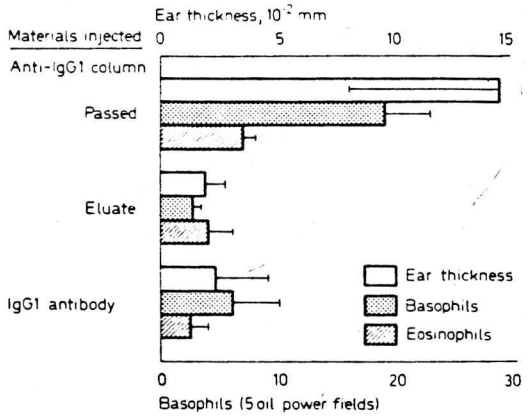


**Fig. 6.** Affinity chromatography of CBH factor. CBH factor from the Sephadex column was passed through an antiguinea pig IgG1-coated Sepharose column. IgG1 antihapten antibody having suppressive activity on contact sensitivity [14] was also transferred into a recipient. Fractions from a 10-ml equivalent of the original sera were concentrated to 3 ml and injected into a recipient.

**Table II.** DNP-BGG affinity chromatography of CBH factor

| Materials injected | 24-hour reaction in the ear of recipients | | | |
|---|---|---|---|---|
| | ear swelling $\times 10^{-2}$ mm ± SE | baso-phils[1] | eosino-phils[1] | mono-nuclears[1] |
| DNP-BGG column | | | | |
| Passed | 2.0 ± 1.9 | 1 | 5 | 83 |
| Eluate | 11.5 ± 0.5* | 28 | 10 | 124 |
| DNP-GPS-2W | | | | |
| 50% $AmSO_4$ ppt | 14.6 ± 2.5* | 33 | 4 | 124 |
| PBS Sup | 1.3 ± 0.7 | 0 | 2 | 47 |

* Statistically significant at level of $p < 0.001$.
[1] Cells/5 oil power fields (mean of 4 experiments).

The second peak from Sephadex gel filtration was absorbed to a DNP-BGG bound Sepharose columns. The factor was retained in the column and then eluted with 1 *M* NaCl (table II). Finally, the second peak was applied onto an antiguinea pig IgG1 antibody-bound Sepharose column. The factor passed through the column. The eluate from the column showed no activity (fig. 6). IgG1 separated from DNP-GPS-2W which showed suppressive activity to contact sensitivity [14], when injected intravenously into nonsentitized animals, showed no activity to induce CBH reaction.

The eluate from the DNP-BGG column, despite an extremely low protein concentration, was concentrated and applied to SDS disc gel electrophoresis. No staining band was observed in the disc gels. The second peak from Sephadex gel filtration before and after treatment of DNP-BGG columns was also electrophoresed on SDS disc gels. At least 7 staining bands were noted in both gel columns, however, there was no elimination of staining bands between gel columns electrophoresed with both preparations.

## Discussion

CBH-inducing factor has been analyzed by *Askenase* et al. [13]. They demonstrated that IgG1 antibody raised in hyperimmunized guinea pigs was responsible for CBH reaction. The reaction induced by DNP-GPS-2W in this study was equivalent to the reaction observed by them. It was expressed as swelling without erythema accompanying a prominent infiltration of basophils in addition to eosinophils and mononuclear cells [11]. Both swelling and basophil infiltration reached a peak at 24 h after skin test although their time courses were not completely identical. The factor appeared in circultation at 2 weeks after sensitization and disappeared at 3 weeks when animals were sensitized with one dose of topical DNFB. It showed hapten specificity and dose dependency. Unlike the finding of *Askenase* et al. [13], our factor was fractionated into non-$\gamma$-globulin fraction through ammonium sulfate desalting, CM-cellulose chromatography and Sephadex G-150 gel filtration. It did not precipitate by

treatment of 50% saturation of ammonium sulfate and was eluted from a Sephadex G-150 column into a fraction of a little smaller molecular weight than BSA. Especially it did not bind to antiguinea pig IgG1 antibody. IgG1 antihapten antibody separated from DNP-GPS-2W failed to induce a CBH reaction although it could induce suppression of the contact sensitivity reaction [14, 15]. It is possible that our CBH inducing factor is a different molecule from that found by *Askenase* et al. [13].

*Dvorak* et al. [7] and others [6, 9] reported that CBH was a T cell-mediated reaction and that the responsible factor was T lymphocytes and/or their products. Basophil chemotactic factors have been recognized in the culture supernatants of antigen-stimulated lymphoid cells [8, 20]. One had a molecular weight of 12,500 dalton and the other sedimented coincidently with BSA on sucrose density gradient centrifugation. In man many basophil chemotactic factors have been demonstrated by means of in vitro techniques: these include complement components such as C5a or C567, diffusates from passively sensitized lungs, kallikrein and supernatants from sensitized lymphocytes stimulated with specific antigens [21]. Our CBH-inducing factor may be one of those basophil-influencing factors. It was eluted into a fraction near BSA marker and had binding site to DNP residues in its molecule. With these results it may be one of the lymphokines, especially T cell products, although it is difficult to exclude a possibility that it is a split product of immunoglobulin.

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主論文

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# Suppression of Contact Sensitivity by IgG1 Antihapten Antibody in Contact-Sensitized Guinea Pigs

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**The guinea pig sera obtained 2 weeks after 1-fluoro-2,4-dinitrobenzene sensitization had ability to suppress elicitation of contact sensitivity when transferred intravenously into contact sensitized guinea pigs. The activity was found only in the sera obtained 2 weeks after the sensitization, whereas the sera obtained 1 and 3 weeks after the sensitization had no effect on contact sensitivity. Pretreatment of cyclophosphamide abolished the generation of the suppressive factor. The suppressive factor was hapten-specific. It was eluted from a Sephadex G-150 column into 7S γ-globulin fraction and from DEAE ion exchange cellulose column into IgG1 fraction. The suppressive activity was absorbed on a dinitrophenylated bovine gamma globulin Sepharose 4B column and was eluted by either acidic buffered solution or dinitrophenol from the affinity column. It was absorbed by antiguinea pig IgG1-Sepharose and not by antiguinea pig IgG2-Sepharose. Therefore, the suppressive activity found in the sera of 1-fluoro-2,4-dinitrobenzene sensitized guinea pigs was attributed to IgG1 antihapten antibody.**

Contact sensitivity is known to be an expression of T lymphocyte activities. The topical application of contact sensitizing agents, such as dinitrofluorobenzene (DNFB) or picrylchloride (TNCB), induces effector T cell, suppressor T and B cells as well as helper T cell for hapten specific antibody response [1,2]. It appears that the hapten-induced inflammatory reaction results from combinations of different activities of these lymphocytes.

The suppressor T cells are known to act both on the efferent and afferent arc of contact sensitivity [3-11], and they generate soluble factors which act on the efferent arc [3,4,6]. The suppressor B cells act only on the efferent arc [12-16]. However, the soluble factors of the suppressor B cells have not yet been demonstrated. It is likely that the soluble factors including antihapten antibodies derived from those activated lymphocytes circulate in the sensitized animals so that they modify the hapten-induced inflammatory reaction.

Animals, when sensitized with contact sensitizing agents, show the maximal skin reactivity on 5th to 7th days after the sensitization and declining reactivity thereafter. The reduction of skin reactivity may be caused by the activity of both suppressor T and B lymphocytes. In this report, therefore, experiments were designed to analyze any humoral factors which affect the skin reactivity of the sensitized animals. IgG1 antihapten antibody which appeared in the sera of 2 weeks sensitization had ability to depress contact sensitivity. The antibody was detected only after the peak of the immune response and seemed to be responsible for the depression of contact sensitivity seen 2 weeks after sensitization.

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Abbreviations:
CY: Cyclophosphamide
DNCB: 1-chloro-2,4-dinitrobenzene
DNFB: 1-fluoro-2,4-dinitrobenzene
DNP-BSA: Dinitrophenylated bovine serum albumin
DNP-BGG: Dinitrophenylated bovine gamma globulin
Ox: 4-Ethoxymethylene-2-phenyloxazolone
PB: Phosphate buffer
7.4-PBS: Phosphate buffered saline pH 7.4
PCA: Passive cutaneous anaphylaxis
PHA: Passive hemagglutination
TNCB: 1-chloro-2,4,6-trinitrobenzene

## MATERIALS AND METHODS

### *Animals*

Outbred Hartley female guinea pigs weighing 300-400 gm were used throughout the experiment. They were fed with pelleted food and water ad libitum.

### *Reagents*

1-Chloro-2,4-dinitrobenzene (DNCB), 1-fluoro-2,4-dinitrobenzene (DNFB) and dinitrobenzene sulfonate were purchased from Nakarai Chemicals Co., Kyoto. 1-Chloro-2,4,6-trinitrobenzene (TNCB) was obtained from Tokyo Chem. Co., Ltd., Tokyo. 4-Ethoxymethylene-2-phenyloxazolone (oxazolone, Ox) was obtained from Sigma Chem. Co. Dinitrophenylated bovine γ-globulin (DNP-BGG) was prepared by the method described by Little and Eisen [17]. Cyclophosphamide (CY) was a gift of Shionogi Pharmaceutical Co., Osaka. It was dissolved in distilled water at the concentration of 100 mg/ml. 300 mg/kg of CY was injected into an animal intraperitoneally 3 days before sensitization.

### *Sensitization of Guinea Pigs*

Guinea pigs were sensitized with a topical application of 0.025 ml of 10% DNFB, 3% TNCB in acetone or 0.025 ml of 3% Ox in ethanol. A week later they were tested with 0.025 ml of 0.1% DNCB, 0.1% TNCB acetone solution or 0.2% Ox in ethanol, respectively. The animals which showed positive skin test of 2.0 reaction (see serum transfer) were used as recipients of the serum transfer. At the same time, the animals with positive skin tests were bled 1 day after the test and the sera obtained were donated as the immune sera of one week sensitization. They were skin-tested again at either 2 or 3 weeks after the sensitization. The animals which showed reduced skin reaction as compared with that of the 1st skin test were bled and the sera obtained were used as the immune sera of 2 weeks or 3 weeks sensitization, respectively.

### *Serum Transfer*

The contact sensitized guinea pigs received 3 ml of the immune sera intravenously 1 day after the first skin test. They were skin tested again by dropping 0.025 ml of 0.1% DNCB solution on their shaved flank skin one hour after the serum injection. The tests were read 24 hr later and evaluated as follows [18]: no reaction, 0; some red spots in the tested area, 0.5; slight reddening, 1.0; marked reddening and slight swelling, 2.0; marked reddening and marked swelling, 3.0. The chromatographed fractions equivalent to 3 ml of original sera were also injected intravenously into the contact sensitized animals.

### *Gel Filtration of the Immune Sera*

Ten ml of the immune sera were treated with 34% saturated ammonium sulfate at 4°C. The resultant precipitate was dissolved in 0.01 M phosphate, 0.15 M NaCl, pH 7.4 (7.4-PBS) and applied to a Sephadex G-150 column (3 × 76 cm) equilibrated with 7.4-PBS. The effluent was collected and pooled. The pooled fractions were concentrated to the original volume of the applied sera and 3 ml of the fractions were injected into the recipients.

TABLE I. *Suppression of contact sensitivity by immune sera*

| Sera injected (weeks) | Anti-DNP titer PCA | Anti-DNP titer PHA | Recipients | Positive skin test | (Mean score) |
|---|---|---|---|---|---|
| DNFB immune (1 w.) | 0 | ×32 | DNFB sensitized | 5/5[a] | (2.0)[b] |
| (2 w.) | ×8 | ×512 | DNFB sensitized | 0/14 | (0.18) |
| | | | TNCB sensitized | 5/5 | (2.0) |
| | | | Ox sensitized | 5/5 | (2.0) |
| (3 w.) | 0 | ×128 | DNFB sensitized | 5/5 | (2.0) |
| TNCB immune (2 w.) | ND | ND | TNCB sensitized | 0/5 | (0.2) |
| | | | DNFB sensitized | 5/5 | (2.0) |
| Ox immune (2 w.) | ND | ND | Ox sensitized | 1/7 | (0.35) |
| | | | DNFB sensitized | 6/6 | (1.75) |
| Nonimmune | 0 | 0 | DNFB sensitized | 20/20 | (2.0) |

Guinea pigs were sensitized with DNFB, TNCB, or Ox. They were bled 1–3 weeks after sensitization. Three ml of the sera were injected intravenously into a recipient animal (skin reaction score, 2.0). The recipient was skin tested one hour after the injection. The reaction was read 24 hr later.

[a] Number of animals with positive skin test stronger than score 1.0/Number of recipients.

[b] Sum of skin reactivity score after serum transfer was divided by number of recipients.

TABLE II. *Effect of cyclophosphamide on generation of suppressive factor*

| Serum donor | Pretreatment of donor | Recipients | Positive skin test | (Mean score) |
|---|---|---|---|---|
| DNFB sensitized | | DNFB sensitized | 0/14 | (0.18) |
| DNFB sensitized | CY 300 mg/kg | DNFB sensitized | 5/5 | (2.0) |
| Nonsensitized | — | DNFB sensitized | 10/10 | (2.0) |
| DNFB sensitized | — | CY + DNFB sensitized[a] | 5/5 | (2.6) |

Guinea pigs were treated with intraperitoneal injection of CY (300 mg/kg) 3 days before sensitization. They were bled at 2 weeks after sensitization. Three ml of the sera were injected into a DNFB sensitized guinea pig. For skin test see footnote of Table I.

[a] The recipients were sensitized with DNFB after pretreatment with 300 mg/kg of CY. Their skin reactivity score were 3.0.

*DEAE Cellulose Chromatography of the Immune Sera*

The immune sera were chromatographed on DEAE cellulose according to the method described by Oliveira et al [19]. Briefly, the sera were precipitated by treatment with 34% saturated ammonium sulfate. After dialysis against 0.005 M phosphate buffer (PB), pH 8.0, the precipitate was applied to a DEAE cellulose column (2 × 10 cm, Whatman DE 32) equilibrated with the same buffer. The column was eluted with 0.005 M PB, pH 8.0, 0.04 M PB, pH 6.0 and then 0.1 M PB, pH 6.0. The eluate was collected and concentrated under negative pressure to the original volume of the applied sera. After dialysis against 7.4-PBS, 3 ml of the eluate was injected intravenously into a recipient.

*Antisera to Guinea Pig IgG1 and IgG2*

Pooled normal guinea pig sera were treated with 34% saturated ammonium sulfate and then chromatographed on DEAE cellulose (Whatman DE-32) according to the same method as described above [19]. In order to prepare the IgG1 fraction for immunization the fraction containing IgG1 from the DEAE cellulose column was rechromatographed and then passed through an anti IgG2-Sepharose column (see affinity chromatography). Rabbits were injected with either IgG1 or IgG2 in Freund's complete adjuvant (2 mg protein/animal) into 4 footpads. They were injected again with the same doses of the antigen 4 weeks later and exsanguinated at 10 days after the booster injection.

*Affinity Chromatography*

The affinity columns for DNP-BGG, antiguinea pig IgG1 and antiguinea pig IgG2 were prepared according to the method described by Omen, Ontjes, and Anfinsen [20]. Briefly, 15 ml of either 0.2% DNP-BGG, antiguinea pig IgG1 or antiguinea pig IgG2 were mixed with 15 ml of cyanogen bromide-activated Sepharose 4B. After stirring at 4°C overnight 0.1 M monoethanolamine was added to the mixture to cover unreacted sites of the Sepharose. The Sepharose was washed thoroughly with 7.4-PBS, 0.05 M glycine-HCl, pH 3.0 or 2 M NaCl, and then 7.4-PBS. The immune sera, after treatment of 34% saturated ammonium sulfate, were mixed with the Sepharose with constant stirring at 4°C overnight. The mixture was poured into a plastic column and the fraction passing through the column was collected. The column was washed thoroughly with 7.4-PBS and then eluted either with 0.05 M glycine-HCl, pH 3.0 or with 0.1 M dinitrophenol-0.1 M Tris solution for the DNP-BGG column and with 2 M NaCl for either antiguinea pig IgG1 or antiguinea pig IgG2 column. The eluate was dialyzed against 7.4-PBS, concentrated and then injected into the recipients.

*Titration of Antibody*

Passive cutaneous anaphylaxis (PCA) and passive hemagglutination (PHA) were performed to titrate antibody activity in the immune sera. The serially diluted sera or the concentrated eluate from the column were injected intradermally into nonsensitized guinea pigs. Four hours later 1.0 ml of 0.2% DNP-BGG in 1.0% Evans Blue (Merk Co., Ltd.) was injected intravenously. Blueing of the skin was assessed 30 min later [21]. DNP-BGG coated glutaraldehyde fixed sheep erythrocytes were mixed with the serially diluted sera or the eluate to titrate the hemagglutinating antibodies.

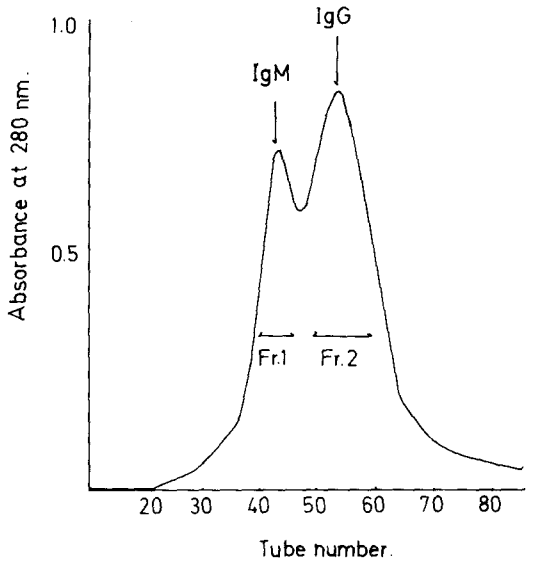
## RESULTS

### *1. Suppression of Contact Sensitivity by the Immune Sera*

The immune sera obtained 1 week after the sensitization showed no suppressive effect on contact sensitivity when passively transferred into the recipients whose skin reactivity score was 2.0. The sera of 2 weeks sensitization showed remarkable suppressive effect on DNFB contact sensitivity. All the DNFB sensitized guinea pigs which received the immune sera revealed reduced or negative skin reactivity to challenge dose of DNCB. Five out of 14 animals showed only some red spots in the tested area (score 0.5) and the others showed negative skin reaction (score 0). However, this suppressive effect was not detected in the immune sera of 3 weeks sensitization. The immune sera of 2 weeks sensitization had no suppressive effect on contact sensitivity of the TNCB or Ox sensitized guinea pigs. The immune sera obtained from guinea pigs sensitized with either TNCB or Ox 2 weeks previously had the same effect on contact sensitivity to the corresponding haptens; however, they had no effect on DNFB contact sensitivity (Table I). The antibody titer in the DNFB immune sera was highest in those from the animals of 2 weeks sensitization and the PCA anti DNP antibody was found only in those animals.

### *2. Effect of Cyclophosphamide on Generation of the Suppressive Factor*

In order to examine whether the generation of the factor was affected by pretreatment of cyclophosphamide (CY) which suppresses maturation of precursor of suppressor B cell [22] as well as suppressor T cell [23–25], the guinea pigs were injected 300 mg/kg of CY intraperitoneally. They were sensitized with topical DNFB 3 days later and then exsanguinated 2 weeks later. The sera obtained from CY pretreated animals, when transferred to DNFB sensitized guinea pigs, showed no effect on elicitation of contact sensitivity (Table II).




| Materials injected | Fr. 1 | Fr. 2 | Normal sera |
|---|---|---|---|
| Anti DNP titer (PHA) | x 256 | x 64 | 0 |
| Positive skin test | 4/4 | 0/4 | 20/20 |
| (Mean score) | (2.0) | (0.13) | (2.0) |

FIG 1. Sephadex G-150 gel filtration of the immune sera. The ammonium sulfate precipitate of 10 ml immune sera was applied onto a 3 × 76 cm Sephadex G-150 column. 7.4-PBS was used as the elution buffer. The eluate was pooled and concentrated to the original volume of the applied sera. Three ml of the fractions were injected to the recipient animals. For skin test see footnote of Table I.

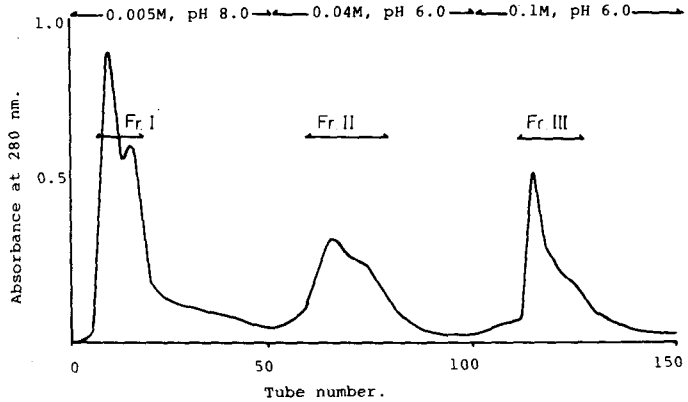



FIG 2. DEAE cellulose chromatography of the immune sera. The ammonium sulfate precipitate of 10 ml immune sera was dialyzed against 0.005 M PB, pH 8.0 and applied onto a 2 × 10 cm DE-32 column equilibrated with the same buffer. Elution was performed with 0.005 M PB, pH 8.0, 0.04 M PB, pH 6.0 and then 0.1 M PB, pH 6.0.

The immune sera, when transferred into CY pretreated guinea pigs whose skin reactivity score was 3.0, showed only slight suppressive effect on the inflammatory reaction (Table II).

### *3. Sephadex G-150 Gel Filtration of the Immune Sera*

The suppressive factor was precipitated in ammonium sulfate at 34% saturation. The precipitate was dissolved in 7.4-PBS and applied to a Sephadex G-150 column. The activity was detected only in the second fraction which corresponded to the same molecular size as IgG (Fig 1).

### *4. DEAE Cellulose Chromatography of the Immune Sera*

The elution profile of DEAE cellulose chromatography is shown in Fig 2. The greatest suppressive activity was eluted in fraction III. Although 8 out of 20 recipients showed positive skin reaction, average reaction score was reduced from 2.0 to 0.73. A little activity was found in fraction I. PHA titer and PCA titer of these fractions were shown in Table III. Fraction III contained both PCA and PHA antibodies while fraction I contained no PCA antibody but weak PHA antibody.

### *5. Affinity Chromatography of the Immune Sera*

As it has been shown from the chromatographic studies that the suppressive factor could be in the IgG1 γ-globulin fraction, we tried to demonstrate whether the active factor had binding sites for DNP residues. The immune sera were applied on a DNP-BGG-Sepharose column. The fraction passed the column showed no suppressive effect when transferred to the recipients. The suppressive effect was found in the eluate from the column (Table IV).

### *6. Absorption of Suppressive Activity by Anti IgG1-Sepharose*

The active immune sera were applied to either anti IgG1- or anti IgG2-Sepharose as described above. As shown in Table V, the suppressive activity disappeared in the fraction passed through the anti IgG1-Sepharose column which contained only PHA antihapten antibody. The activity was recovered in the eluate from the column. The fraction passed through the anti IgG2-Sepharose column retained the activity and the eluate from the column had no activity on the recipients. PHA antihapten antibody was removed after the anti IgG2 column and the fraction containing only PCA antibody was able to suppress contact sensitivity (Table V).

TABLE IV. *Absorption of suppressive activity by DNP-BGG Sepharose*

| Materials injected | Anti-DNP Titer PCA | Anti-DNP Titer PHA | Recipients | Positive skin test | (Mean score) |
|---|---|---|---|---|---|
| DNP-BGG column | | | | | |
| Passed | 0 | 0 | DNFB sensitized | 5/5 | (1.60) |
| Eluate | ×4 | ×32 | DNFB sensitized | 1/5 | (0.40) |
| Immune sera | ×8 | ×512 | DNFB sensitized | 0/14 | (0.18) |
| Nonimmune sera | 0 | 0 | DNFB sensitized | 20/20 | (2.0) |

TABLE III. *Suppression of contact sensitivity by fractions from DEAE cellulose chromatography*

| Fraction injected | Anti-DNP titer PCA | Anti-DNP titer PHA | Recipients | Positive skin test | (Mean score) |
|---|---|---|---|---|---|
| Immune sera Fr. I | 0 | ×16 | DNFB sensitized | 15/20 | (1.08) |
| Fr. II | 0 | 0 | DNFB sensitized | 16/16 | (1.69) |
| Fr. III | ×8 | ×64 | DNFB sensitized | 8/20 | (0.73) |
| Nonimmune sera | 0 | 0 | DNFB sensitized | 20/20 | (2.0) |

The immune sera obtained from DNFB sensitized guinea pigs 2 weeks after sensitization were chromatographed on DEAE cellulose ion exchange chromatography (Fig 2). The fractions obtained were concentrated to the original volume of the applied sera. Three ml of them were injected intravenously into a DNFB sensitized guinea pig. For skin test see footnote of Table I.

TABLE V. *Absorption of suppressive activity by anti guinea pig IgG1- and IgG2- Sepharose*

| Materials injected | Anti-DNP Titer | | Recipients | Positive skin test | (Mean score) |
|---|---|---|---|---|---|
| | PCA | PHA | | | |
| Anti-IgG1 column | | | | | |
| Passed | 0 | ×256 | DNFB sensitized | 5/5 | (2.0) |
| Eluate | ×32 | ×2 | DNFB sensitized | 1/5 | (0.7) |
| Anti-IgG2 | | | | | |
| Passed | ×32 | 0 | DNFB sensitized | 0/5 | (0) |
| Eluate | ×1 | ×128 | DNFB sensitized | 5/5 | (2.0) |
| Immune sera | ×8 | ×512 | DNFB sensitized | 0/14 | (0.18) |

## DISCUSSION

It was demonstrated in this study that antihapten IgG1 antibody suppress the hapten-induced inflammatory reaction. The antibody appeared in the circulation in the animals 2 weeks after the sensitization and disappeared 1 week later. It was not produced by CY pretreated animals. The suppressive activity was not expressed when it was transferred into CY pretreated animals. It is specific in activity in that it is able to distinguish DNP from TNP or Ox residues.

In contact sensitivity it is well known that antihapten antibody is detected in the sera of contact sensitized animals [1,2]. The role of antihapten antibodies has not been discussed until recently, although it was expected that the antibodies play some role in contact sensitivity. Asherson and Loewi [24] reported antibody requirement in passive transfer of contact sensitivity. They explained that the antibody was necessary to fix antigens at the reaction site long enough for the sensitized lymphoid cells to produce a detectable reaction. Haynes et al [25] showed that IgG1 antihapten antibody can transfer cutaneous basophil hypersensitivity in guinea pigs. These antibodies played a role in the positive expression of contact sensitivity whereas our IgG1 antibody worked on the negative expression of contact sensitivity.

B cell tolerance proposed by Turk et al [16,26] is one of the negative control mechanism of contact sensitivity, where B cell products, i.e., antibodies, appear to play a role. However, relationship between B cell tolerance and antihapten antibody remains unknown. Crowle and his colleagues [27,28] showed that the humoral antibodies have the ability to inhibit the induction phase of delayed hypersensitivity to nonreplicating antigens. The activity was found in IgG1 antibody of immunized guinea pigs [29]. Their findings seemed equivalent to ours in contact sensitivity; however, our IgG1 antibody worked on the efferent arc of contact sensitivity which is also the target of suppressor B cells. The production of the antihapten antibody was suppressed by CY pretreatment. CY is known to work on suppressor B cells as well as on suppressor T cells to enhance delayed hypersensitivity reaction and to suppress antibody production with the amount of CY used in this study [22-25]. It is not easy to explain the relationship between B cell tolerance and the antihapten antibody at present. Further studies are indicated to elucidate the relationship.

The IgG1 antihapten antibody was found in the sera of guinea pigs at 2 weeks after the sensitization. The animals showed reduced skin reactivity as compared with that in guinea pigs of 1 week sensitization. The reduction of skin reactivity could be due to the action of the antibody. However, the antibody activity was not detected in the animals of 3 weeks sensitization which showed reduced skin reactivity. The skin reactivity in the latter case is not easy to explain relative to the absence of IgG1 antihapten antibody; possibly the reaction is modified by other factors such as an antireceptor antibody in contact sensitized mice [33] or direct action of suppressor T and B cells. At present we believe that the IgG1 antihapten antibody is one of the responsible factors which depresses the skin reaction seen 2 weeks after sensitization.

The exact mechanism of action of our IgG1 antibody is not known. However, it may bind to the surface of macrophages or lymphocytes through its cytophilic nature and attenuate the antigen. The cells involved in the response may be sensitive to CY treatment as the antihapten antibody was ineffective in the CY pretreated animals. Work to elucidate the mechanism is under study in our laboratory.

Recently induction of helper T cells for hapten specific antibody response was demonstrated in a contact sensitivity [1,2]. IgG1 antihapten antibody is a prerequisite for passive transfer of cutaneous basophil hypersensitivity which is thought to be a modulated form of contact sensitivity [25]. Our IgG1 antihapten antibody appears to be in somewhat different subclass of IgG1 antibodies, as it is unable to induce cutaneous basophil hypersensitivity when injected into normal guinea pigs (unpublished data). And it may be one of the products of B lymphocytes stimulated by the helper T cells.

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# POSSIBLE BINDING OF EPIDERMOLYTIC TOXIN TO A SUBCELLULAR FRACTION OF THE EPIDERMIS

KIYOSHI NISHIOKA, ICHIRO KATAYAMA AND SHIGEHARU SANO

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ABSTRACT

An *in vitro* method for assessing the activity of epidermolytic toxin (ET) was developed in this study. It was almost equal in sensitivity to the *in vivo* assay system in which ET was injected subcutaneously into new-born mice. By using the *in vitro* method it was found that ET could induce epidermal splitting after incubation with the skin only for 5 minutes. No epidermolytic activity remained in the reaction mixture. The activity of ET was not abolished by proteinase inhibitors such as Trasylol, ε-aminocaproic acid or soy bean trypsin inhibitor. It was abolished by an extract from the epidermis of new-born mice. The inhibitory activity of the extract was preserved by heating at 56°C for 30 minutes. It was destroyed by heating at 80°C for 30 minutes. The activity was found in a subcellular fraction which should contain mitochondria and lysosomes from the epidermal cells. A reduction in the amount of ET was observed in acrylamide gel electrophoresis after incubation of ET with the subcellular fraction. The possible mode of action of ET was discussed.

Phage group II staphylococci produce an epidermolytic toxin (ET) having the ability to cause intraepidermal splitting just below the stratum granulosum. The chemical properties of ET have been analyzed in several laboratories (1-6). It is a protein with a molecular weight between 23,000 and 33,000 daltons, and has an isoelectric point at either pH 6.7-7.0 or 4.0-4.5. Kondo and his colleagues (4) showed that ET contained at least 4 different molecules of the same molecular weight with different electrophoretic mobilities. Two ETs separated in our laboratory showed the same antigenicity, but had different molecular weights and electrophoretic mobilities (6).

On the other hand, the mechanism of its biological action is still a matter of speculation. No proteolytic activity of ET has been demonstrated. Its activity is not inhibited by proteinase inhibitors but only by anti-ET serum (7-9). An organ culture system was employed to analyze the biological action of ET, but only indirect evidence was obtained by the system (10-12). Development of *in vitro* assay systems to assess the activity should be one of the important steps in analyzing the biological action of ET because it is difficult to exclude indirect factors in the *in vivo* assay system.

In this report we have developed an *in vitro* method for assessing the activity of ET. The activity of ET was inhibited by a subcellular fraction of the new-born mice epidermis.

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## MATERIALS AND METHODS

*Preparation of ET:* ET was obtained from the culture supernatant of the phage group II *Staphylococcus aureus* isolated from the lesion of a patient with staphylococcal scalded skin syndrome (6). The culture supernatant was precipitated in 50% saturated ammonium sulfate. The resultant supernatant was again precipitated in 100% saturated ammonium sulfate. The final precipitate, after being dissolved in 0.05 M Tris-HCl, pH 7.4, was applied to a Sephadex G-75 column (5×100 cm). The third fraction from the Sephadex column was col-

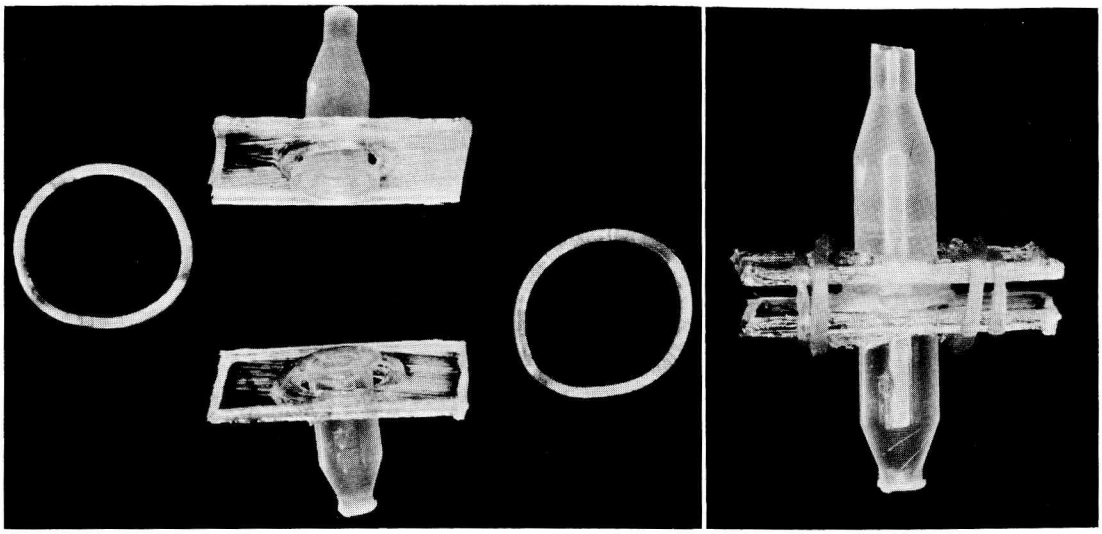

Fig. 1. Microchamber.
Upper and lower cups with plastic boards. They were fixed with rubber bands after insertion of a piece of skin between them (right).

lected and then passed through DEAE Sephadex A-25 (1.5×20 cm) equilibrated with 0.01 M Tris-HCl, pH 7.4. The fraction which passed through the column was collected and used as the ET preparation.

*In vivo assay of the epidermolytic activity:* Newborn mice 2–4 days old (ICR strain) were used for the assay (6). Subcutaneously, 0.05 ml of ET preparation were injected into the dorsal skin of a newborn mouse. The Nikolsky phenomenon was examined every hour after the injection by a gentle rubbing of the treated skin.

*In vitro assay of the epidermolytic activity:* The microchamber (Fig. 1) consisted of two plastic cups with an inside diameter of 4 mm and two pieces of plastic board (1×3 cm) with a 6 mm diameter hole at their centers. Small pieces of excised skin were placed just between the two cups. The cups were fixed by the plastic boards with rubber bands. ET preparation was injected into the upper cup which remained in contact with the dermal side of the skin. Incubation was performed at 37°C for 3 hours in a moist box keeping the chambers with the dermal side upright. Following incubation, the skin pieces were removed from the chambers and a gentle rubbing was applied to the epidermal side to examine the Nikolsky phenomenon.

*Inhibitors of proteolytic enzymes:* Trasylol (Bayer), soy-bean trypsin inhibitor (SBTI, Sigma Chem.) and ε-aminocaproic acid (EACA, Nakarai Chemicals, Kyoto) were used to inhibit the biological activity of ET. Anti-ET serum was prepared by immunizing rabbits with ET in Freund's complete adjuvant (6).

*Subcellular fractions of new-born mice epidermis:* The epidermis was separated from the excised skin of new-born mice by treating skin pieces in 1 M NaSCN at 0°C for 1 hour. The separated epidermis was washed with 0.01 M phosphate, 0.15 M NaCl, pH 7.4 (PBS) and then floated on 0.25 M sucrose, 0.05 M Tris-HCl, pH 7.4. The epidermis was homogenized by using a Polytron® homogenizer. The homogenate was fractionated by differential centrifugation. It was centrifuged at 1,000×G for 10 minutes. The resultant supernatant (Sup. I) was centrifuged at 10,000×G for 30 minutes to obtain the precipitate (Ppt. II). The supernatant was then centrifuged at 105,000×G for 60 minutes (Ppt. III and Sup. III).

*Acrylamide gel electrophoresis:* Acrylamide gel

disc electrophoresis of the ET preparation was performed according to the method described by Davis and Ornstein (13). ET was loaded on a 7.5% acrylamide gel column and was run at 3 mA/gel for 60-90 minutes with 0.005 M tris-glycine, pH 8.8, as the running solution. The gel was stained with 0.1% Coomassie brilliant blue. After staining, the gel column was analyzed semiquantitatively using a dual-wavelength TLC scanner (CS-910, Shimazu Co. Ltd., Tokyo).

## RESULTS

*In vitro detection of epidermolytic activity:*

After incubating the chambers at 37°C for 3 hours, 0.1 μg of ET was able to induce a positive Nikolsky sign on a piece of excised skin (Fig. 2). This amount of ET was also the minimum dose which induced a positive Nikolsky sign on a new-born mouse 3 hours after subcutaneous injection. When the skin pieces showing a positive Nikolsky sign were examined histologically, separation of the horny layer was noted just below the granular layer of the epidermis (Fig. 3). This finding was equivalent to that found in the skin of new-born mice treated with ET.

The chamber method was performed using frozen skin of new-born mice to determine whether the epidermolytic activity could be detected in dead skin. The skin of new-born mice was stored at −20°C for 7 days. The thawed skin was cut into pieces and used for the chamber method. These skin pieces showed a positive Nikolsky sign without ET after 3 hours incubation at 37°C. This separation was established histologically as being at the dermoepidermal junction. The positive Nikolsky sign provoked by ET was observed with higher amounts of ET (more than 0.5 μg ET) after incubation at 37°C for less than 2 hours.

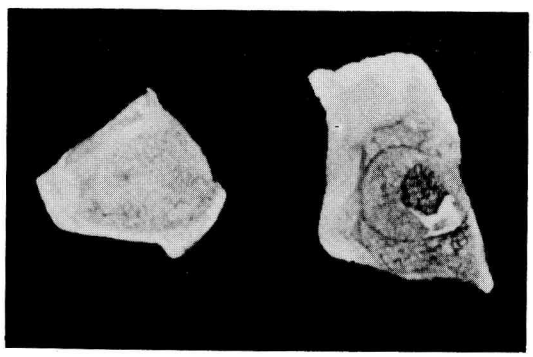

Fig. 2. Positive Nikolsky sign.
Negative (left) and positive (right) Nikolsky sign on pieces of the skin.

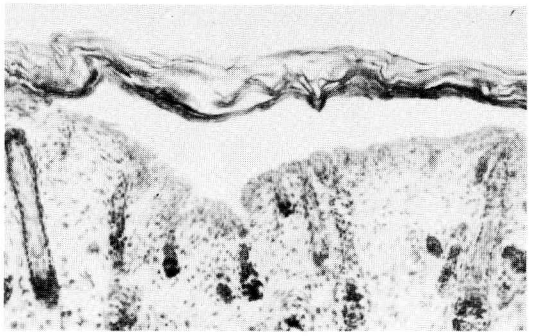

Fig. 3. Intraepidermal splitting.

*Minimum exposure time of ET:*

As it was posible to wash out ET from the skin in the chamber method, ET was removed from the upper chamber at various times after the initiation of incubation at 37°C. Positive Nikolsky signs were observed on skin with which 0.1 μg of ET had been incubated for longer than 5 minutes (Table 1).

*Inhibition of the epidermolytic activity by proteinase inhibitors:*

ET was introduced into the upper chamber with various proteinase inhibitors. Trasylol, SBTI and EACA had no effect on the epidermolytic activity, but anti-ET serum inhibited it (Table 2).

*Inhibition of epidermolytic activity by epidermal extract from new-born mice:*

The epidermolytic activity of ET was abolished when ET was preincubated with the epidermal extract of new-born mice. The inhibitory activity of the extract was preserved after heating at 56°C for 30 minutes but was destroyed by heating at 80°C for 30 minutes. This

Table 1. Minimum contact time of ET required to induce Nikolsky sign

| Incubation period | Nikolsky sign |
|---|---|
| 0 min. | neg. |
| 3 | neg. |
| 5 | pos. |
| 10 | pos. |
| 30 | pos. |

0.1 μg of ET was added to the upper chamber and incubated for various periods. Then ET in the upper chamber was washed out with PBS. Nikolsky sign was assessed after incubation for 3 hours at 37°C.

Table 2. Effect of proteinase inhibitors on ET

| ET added | Inhibitor added | Nikolsky sign |
|---|---|---|
| — | — | neg. |
| 0.1 μg | — | pos. |
| 0.1 | 250 u. Trasylol | pos. |
| 0.1 | 100 μg SBTI | pos. |
| 0.1 | 250 μg EACA | pos. |
| 0.1 | Anti-ET (×10 diluted) | neg. |

Quadruplicate chambers were prepared to assess the activity. ET and inhibitor were added to the upper chamber. Nikolsky sign was examined after 3 hours incubation.

inhibitory activity, when fractionated, was found in Ppt. II but not in Ppt. III or Sup. III (Table 3).

*Disc electrophoresis of the incubation mixture:*

ET was incubated with the subcellular fractions of the epidermis at 37°C for 30 minutes. The mixture was centrifuged at 3,000×G for 30 minutes at 4°C. The resultant supernatant was applied onto an acrylamide gel column. A single staining band of ET appeared in the gel column. However, a weaker staining band was noted in the gel to which the reaction mixture of ET and Ppt. II had been applied as compared with the staining band in the gel to which the reaction mixture of ET and PBS was applied. This apparent difference in the amount of ET recovered in the supernatant of the reaction mixture was demonstrated semiquantitatively by using a TLC scanner (Fig. 4).

Table 3. Inhibition of epidermolytic activity by epidermal extracts and subcellular fractions

| ET preincubated with | Nikolsky sign |
|---|---|
| — | pos. |
| Epidermal extract | neg. |
| treated 56°C, 30 min. | neg. |
| treated 80°C, 30 min. | pos. |
| Subcellular fraction | |
| Ppt II | neg. |
| Ppt III | pos. |
| Sup III | pos. |

An equal volume of 4.0 μg/ml of ET and 1.0 mg/ml of epidermal extract or subcellular fractions was preincubated at 37°C for 30 minutes. Then 0.05 ml of the mixture were injected into the upper chamber. Nikolsky sign was examined after incubation at 37°C for 3 hours. Quadruplicate chambers were prepared for each experiment. Protein concentration of subcellular fractions was determined by Folin-Ciocalteu reaction using bovine serum albumin as the standard.

## DISCUSSION

The organ culture system was first introduced by McCallum (12) to analyze the biological activity of ET *in vitro*. However, greater amounts of ET were required to demonstrate the epidermal splitting after short-term incubation in the culture system than in the *in vivo* assay for epidermolytic activity (11). The *in vitro* assay system developed in this study was found to be as sensitive as the *in vivo* assay system. The chamber method was useful for studies of biological action of ET, because it was able 1) to prepare more than 4 chambers from the dorsal skin of one new-born mouse, 2) to add various inhibitors of ET in the upper chambers, 3) to stop the reaction easily by washing the reaction mixture out of the upper chamber, and 4) to use the frozen-thawed skin as the target organ when given a higher

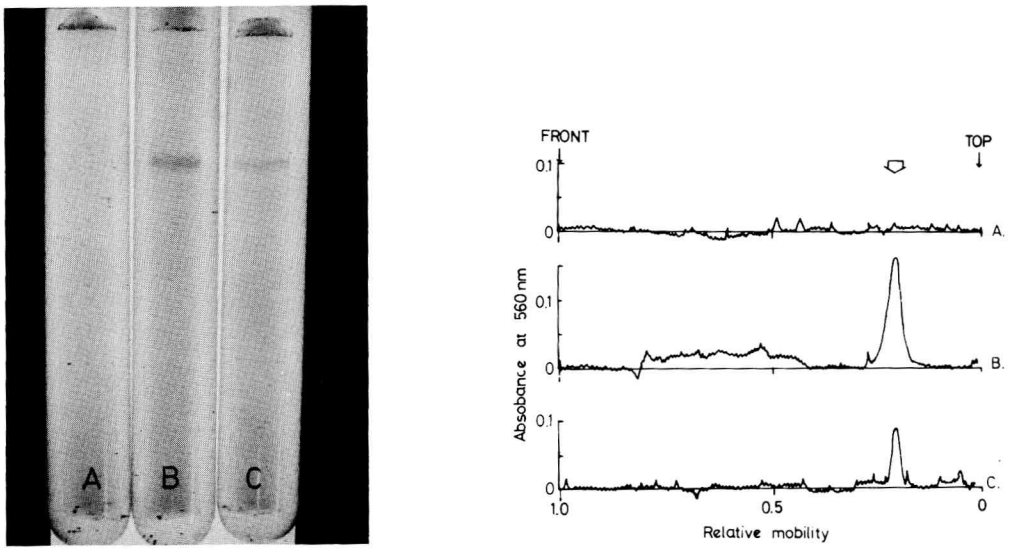


Fig. 4. Disc gel electrophoresis.
Subsellular fraction (Ppt II) alone (A), ET (B) and mixture of Ppt II and ET (C). They were preincubated at 37°C for 30 minutes and applyed to gel columns after centrifugation at 3,000×G for 30 minutes (left). The gel columns were analyzed densitometrically after staining with Coomassie brilliant blue. A lower peak of ET (⇨) was noted on C (right).

amount of ET.

Using the chamber method ET appeared to be easily permeable into the skin from the subcutaneous side. It took only 5 minutes to provoke a positive Nikolsky sign. The reaction mixture removed from the upper chamber had no ability to provoke a Nikolsky sign when transferred to the next chamber (data not shown). Therefore, ET should be absorbed into the skin within 5 minutes of incubation to induce intraepidermal splitting.

As has been already reported, the activity of ET was not inhibited by Trasylol or other proteinase inhibitors in the *in vivo* assay system (7-9). We have investigated the possibility of direct inhibition of the epidermolytic activity in the *in vitro* method. Neither Trasylol, soy-bean trypsin inhibitor nor EACA had any effect on the activity (Table 2), whereas anti-ET serum was able to inhibit the activity completely.

Although several possible modes of action of ET have already been postulated (14), no direct evidence to support those possibilities is available at present. Electron microscopic observations (7) have revealed that ET treated epidermal cells are not injured and stain normally with both ruthenium red and Concanavalin A. ET had no effect on either the binding site of pemphigus antibody or on HL-A antigens. The authors postulated that ET acts on extracellular sites on the epidermal cells. Wuepper et al. (14) reported the immunofluorescent binding of ET with epidermal cells. It is possible that ET binds to the surface of the epidermal cells or to the intracellular substance of the epidermis. The crude extract of the epidermis was able to inhibit the epidermolytic activity. The inhibitory activity of the extract was resistant to heat treatment at 56°C for 30 minutes, but was destroyed by heating to 80°C for 30 minutes. When the extract was fractionated by differential centrifugation, the inhibitory activity was found in the particulate fraction of the epidermis. Binding of ET to the par-

ticulate fraction was also apparent in the electrophoretic study (Fig. 4). Recently, the same laboratory which reported the positive binding of ET to epidermal cells has published the failure of binding of ET to cells such as erythrocytes, lymphocytes and epidermal cells and the detection of ET in blister fluid of patients with bullous impetigo (15). The authors suggested that binding of ET to cells is not a necessary step in provoking intraepidermal splitting. In our study, no inhibitory activity was detected in the first precipitate of the epidermal homogenate which contained the cell membrane, nuclear debris and non-homogenized cells. Although it is not easy to exclude the possibility of non-specific binding of ET to subcellular particles, it is possible that ET could be absorbed into the cells to bind with subcellular organelles to trigger the epidermal splitting because the particulate fraction contains various lysozomal enzymes. Further studies are indicated to elucidate a possible interaction between ET and lysozomal emzymes, although our ET-treated subcellular fraction failed to show any proteolytic activity (unpublished data).

## ACKNOWLEDGMENTS

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# Chronic pigmented purpura induced by chemical substances

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**Summary**

Two cases with chronic pigmented purpura are reported. In one case the skin condition was cleared by stopping the habitual intake of a thiamine preparation and it was provoked by taking the drug. Two out of three commercial thiamine preparations were able to induce skin lesions. In the other case skin lesions were cleared by masking the mouth and nose with sheets of gauze in the workplace, which was filled with dye-dust. As most cases with chronic pigmented purpura are treated non-specifically at present, we suggest that more effort should be focussed on detecting aetiological factors in patients' environments.

Chronic pigmented purpura includes four types of purpuric disease, namely, purpura annularis telangiectodes of Majocchi, progressive pigmentary dermatosis of Schamberg, pigmented purpuric lichenoid dermatitis of Gougerot and Blum and eczematid-like purpura of Doucas and Kapetanakis (Lever & Schamberg-Lever, 1975). They are closely related to one another so they cannot be differentiated on clinical or histological grounds. It is classified as chronic capillaritis of unknown cause in a British textbook (Champion, 1972). The treatment of chronic pigmented purpura is still non-specific and the disorder tends to run a chronic course. A drug, carbromal, has been observed as an aetiological factor in purpuric skin reactions of chronic pigmented purpura (Peterson & Manick, 1967; Rosenthal & Burnham, 1964). We have recent experience of two patients with chronic pigmented purpura whose skin condition was provoked by administering thiamine preparations in one case and was cleared by stopping inhalation of dye-dust in a factory in the other case.

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## Case reports

*Case* 1

H.M., a 45-year-old male office worker, had noticed a rash on both his lower legs since January 1974. The rash consisted of purpuric spots with slight itching. The rash spread gradually from both lower legs to the dorsa of both feet within 5 months. He had no prodromal signs before the onset of purpura. He was in the habit of taking a vitamin $B_1$ preparation whenever he felt tired. On his first examination, pin-head sized purpuric spots were scattered symmetrically over both his lower extremities. The spots tended to be grouped on his lower legs to form 2–3 cm diameter round purpuric patches with scaling and dark brownish pigmentation (Fig. 1). Laboratory values were as follows: RBC $486 \times 10^4$ /mm$^3$; haemoglobin 15·4 g/dl;

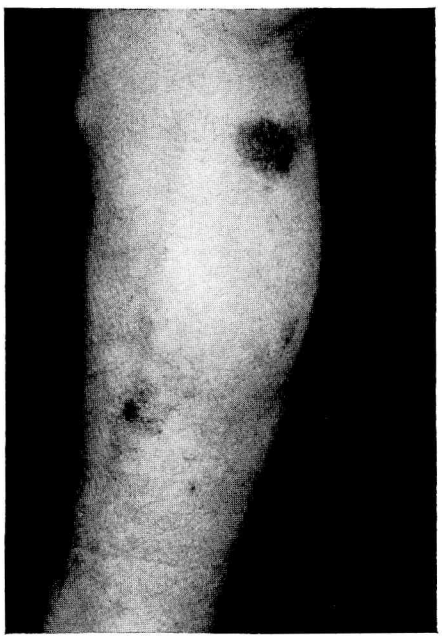


**Figure 1.** Confluent purpuric spots on the right lower leg of Case 1. Pin-head sized purpuric spots were scattered over the right lower leg and some of them were confluent to form scaly brownish pigmented purpuric patches.

WBC 9200/mm$^3$, with 81% neutrophils (25% band form and 56% segmented form), 15% lymphocytes, 3% monocytes and 1% eosinophils; platelets $27{\cdot}3 \times 10^4$/mm$^3$; normal bleeding and coagulation times; negative cuff test. His liver and renal function test showed values within the normal range. Serum protein electrophoresis showed a normal pattern. Antistreptolysin titre, C-reactive protein and rheumatoid arthritis tests showed negative findings. The biopsied specimen from the purpuric patch showed intercellular oedema with occasional spongiotic bulla formation in the epidermis. Remarkable lymphocytic infiltration was noted in

the papillary layer and the upper dermis. Destruction of papillary capillaries and extravasation of erythrocytes was observed. The extravasated erythrocytes were packed in the papillary layer (Fig. 2).

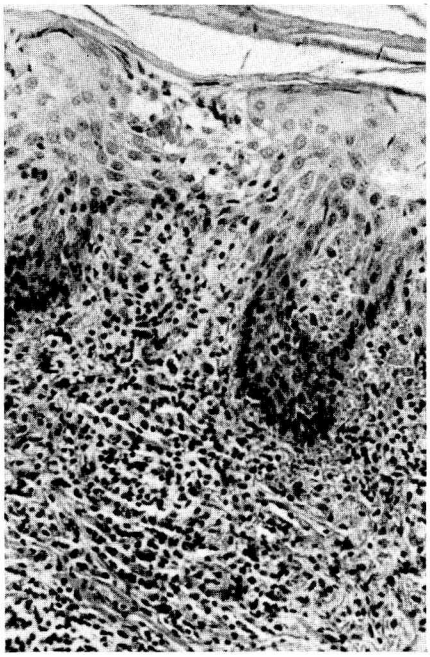


**Figure 2.** Histology of a purpuric patch.

He was treated with penicillin and tranexamic acid (Transamin). One week after the first examination the rash spread to his buttocks, and it then subsided gradually over the following 2 months. However, it recurred and persisted thereafter in spite of treatment. As we noted his habit of taking a vitamin $B_1$ preparation, we advised him to stop taking the drug. The activity of his skin lesions was reduced rapidly after stopping this habit. We attempted, therefore, to provoke his skin lesions by giving the drug. When he took 25 mg of thiamine propyldisulfide (TPD), his skin condition showed no special changes. He developed a purpuric reaction, however, after taking TPD for seven days (total dose was 175 mg TPD) (Fig. 3). He was also given other vitamin $B_1$ preparations. He took 25 mg of O, S-dicarbethoxythiamine hydrochloride and O-benzoyl thiamine disulfide for 7 days respectively. He responded to the former $B_1$ preparation by developing purpuric spots. However, he did not respond to the latter $B_1$ preparation (Fig. 4). Lymphocyte transformation tests against these vitamin $B_1$ preparations gave negative results.

*Case 2*

M.I., a 46-year-old male dye factory worker. He had developed skin lesions on his lower legs

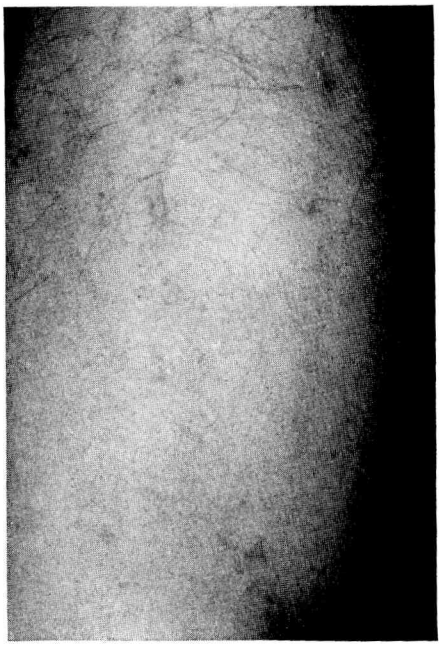
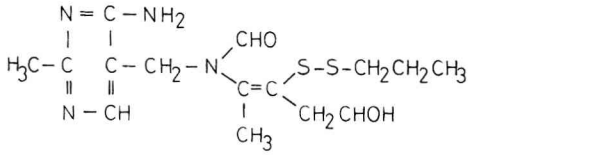
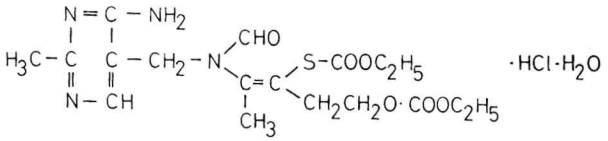


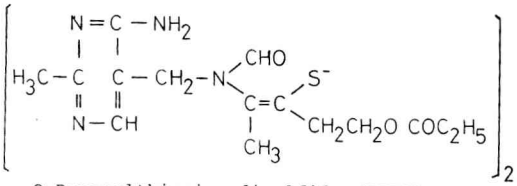
**Figure 3.** Purpuric spots induced by administration of TPD.



Thiamine propyldisulfide (TPD)



O, S-Dicarbethoxythiamine hydrochloride (DECT)



O-Benzoylthiamine disulfide (BTDS)

**Figure 4.** Chemical structures of thiamine preparations.

since 1974. Initially the lesions were limited in distribution to around his ankles. They spread over both lower extremities in February 1977. He visited a dermatologist and was given an oral corticosteroid. His skin lesions subsided within 2 weeks, but they reappeared when the amount of corticosteroid was reduced. There were pin-head sized purpuric spots over both his lower extremities. The spots were grouped to form thumb-nail sized scaly annular purpuric patches on the lower legs. He had no special past history or family history. His laboratory examination showed normal values for RBC, WBC and platelet counts. Both bleeding and coagulation times were normal. His liver and renal function test showed values within normal ranges. Anti-streptolysin titre, C-reactive protein and rheumatoid arthritis tests were all negative. The histopathology of his skin lesions showed exactly the same pattern as that in Case 1 except for spongiosis. The activity of the rash was reduced by giving a non-steroidal anti-inflammatory agent (sodium diclofenac) and it was completely suppressed within 1 month by this treatment combined with rest. When he went back to work, his skin lesions reappeared on the lower legs. They were resistant to non-steroidal anti-inflammatory agents. We advised him to avoid chemicals in the factory by masking his mouth and nose with sheets of gauze. The activity of his skin lesions was reduced rapidly. Unfortunately we could not get his collaboration for further studies on responsible factors.

## Discussion

Chronic capillaritis has been classified into two categories in a British textbook; one is capillaritis provoked by several aetiological factors such as drugs, clothing and so on, and the other is capillaritis of unknown cause (Champion, 1972). The diseases included in chronic pigmented purpura are members of the latter group. The two cases reported in this paper would be diagnosed as chronic capillaritis of unknown cause or chronic pigmented purpura from their clinical findings. In Case 1 the skin condition was diagnosed rather as eczematid-like purpura. It was provoked by taking a vitamin $B_1$ preparation and disappeared after withdrawal of the drug. Two out of three thiamine preparations were able to provoke purpuric spots on his lower legs. A single dose of the drug was not sufficient for provocation and it was necessary to give the drug for at least 7 days to provoke the lesions. As we were not able to analyse the components of the $B_1$ tablet, it is difficult to say that thiamine itself is responsible for the purpuric reaction. However, it is plausible that thiamine preparation is the responsible factor because a different coloured tablet of thiamine, O,S-dicarbethoxythiamine hydrochloride, was also able to provoke the skin lesions. It has been reported that a thiamine preparation was able to provoke contact dermatitis (Hjorth, 1958) and toxic erythema (Miura, 1958). However, vascular damage by thiamine has not been reported before.

In Case 2 the skin condition was diagnosed as annular telangiectatic purpura of Majocchi. We were not able to analyse the responsible chemicals. He was working in a dye factory. His workplace was filled with dust from sulphated and azo dyes. He complained that his mouth and nose were covered with dye-dust by working even half a day in the factory. As his skin lesions cleared when using a mask, it is possible that the dust penetrated from his mucous membrane to damage the capillary endothelium.

Few drugs have been listed as substances which cause damage to the capillary endothelium

(Champion, 1972). Carbromal was reported to induce a purpuric reaction similar to Schamberg's disease (Rosenthal & Burnham, 1964; Peterson & Manick, 1967). Meprobamate can cross-react with carbromal to provoke the purpuric reaction (Marcussen, 1958; Peterson & Manick, 1967). However, these drugs were listed as special cases of drug-induced capillary damage.

The mechanism underlying chronic pigmented purpura produced by bromcarbromide was speculated upon after an electron microscopic observation of the lesion (Berger & Hagedorn, 1973). The authors observed a perivascular monocytic infiltration similar to that in the tuberculin reaction and speculated that chronic pigmented purpura is an allergic reaction of delayed type. This idea is attractive as deposition of immunoglobulin and complement components has not been demonstrated in the affected blood vessels. However, it is difficult to attribute the purpuric reaction to an allergic reaction of delayed type from the present evidence accumulated in chronic pigmented purpura (Rosenthal & Burnham, 1964). Capillary damage can also be produced either by toxic substances or by an allergic reaction mediated by humoral antibodies. Although the mechanism involved in these two cases is not easy to explain at present, it is plausible that a toxic effect of vitamin $B_1$ preparation could be responsible for the production of vascular lesions in Case 1 because of both the necessity for amounts of $B_1$ preparations greater than one dose for provocation and negative lymphocyte transformation tests to them. Therefore, aetiological factors and their mode of action should be elucidated when similar cases with chronic pigmented purpura are accumulated in the future.

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