

Epicutaneous Application of Protein Antigens Incorporated into Cosmetic Cream Induces Antigen-Nonspecific Unresponsiveness in Mice and Affects the Cell-Mediated Immune Response

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Key Words

Epicutaneous immunization · Nonspecific unresponsiveness · $T\alpha\beta$ regulatory cells · TGF- β

Abstract

Background: Protein antigens applied epicutaneously by the patch method induce allergic dermatitis mediated by IgE antibodies in mice and simultaneously significant suppression of Th1-mediated delayed-type hypersensitivity (DTH) reactions. **Methods:** We developed a method in which protein antigens (calf collagen, elastin, keratin, TNP-substituted mouse Ig) were homogenized with neutral cream. Animals treated epicutaneously with such preparations were tested for contact sensitivity to TNP hapten or DTH hypersensitivity to hemocyanin. Antigen specificity of induced unresponsiveness was tested in vivo in 'transfer-in' and 'transfer-out' experiments. The influence of skin-induced regulatory cells on in vitro [^3H]thymidine uptake by immune cells as well as the possible mode of their action using anticytokine antibodies were tested. **Results:** Our procedure in which different protein antigens are applied on the skin in the form of cream induces a state of antigen-nonspecific unresponsiveness affecting cell-mediated immune responses in mice. Cream alone has no such effect. Suppression is transferable in vivo by TCR- $\alpha\beta$ lymphocytes, while $T\gamma\delta$ cells show no activity. In vitro, lymphoid cells of skin-tolerized mice suppress [^3H]thymidine incorporation by immune cells and this effect can be abolished by adding anti-TGF- β but neither anti-IL-4 nor anti-IL-10 antibodies.

Conclusions: Lack of antigen-specificity of unresponsiveness induced by epicutaneous deposition of cream containing protein antigens resembles 'determinant spreading' in oral tolerance induced by antigen feeding. This may suggest that similar immunoregulatory mechanisms operate on these two bodily surfaces.

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Introduction

Both skin and mucosa constantly exposed to many antigens play a crucial role in body protection from different pathogens present in the external world. While development of the immune response to pathogens is of vital importance to the macroorganism, response to innocuous antigens is not required and even more, sometimes it can

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1018–2438/02/1281–0008\$18.50/0

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be harmful and lead to allergy. It is well known that immunization with an antigen via the digestive tract or nasal mucosa leads to a state of profound immunosuppression [1–3] which seems to play an important role in avoiding the development of the immune response to nonpathogenic antigens. One can speculate that similarly to mucosal tolerance, skin exposition to irrelevant protein antigens will lead to development of immunological tolerance.

Indeed it was shown that although protein antigens or haptens applied epicutaneously by the patch method induce allergic dermatitis [4, 5] and production of specific Th2-cell-mediated IgE antibodies in mice [6], they simultaneously significantly suppress Th1-mediated delayed hypersensitivity type (DTH) reactions to the same antigen. In our preliminary experiments using a similar procedure we tried to establish the antigenic specificity of suppression induced by epicutaneous deposition of antigen and unexpectedly we have found that such manipulation leads to systemic antigen-nonspecific unresponsiveness. To avoid the cumbersome and inconvenient patch method, we developed a technique based on daily application of neutral cosmetic Nivea cream containing different proteins as additives on the shaved skin. We also used commercial skin care cream containing protein additives produced by a well-known manufacturer. Our experiments show that this procedure leads to significant antigen-nonspecific suppression of cell-mediated immunity and is mediated by T α β cells producing TGF- β .

Materials and Methods

Animals

Six- to seven-week-old female CBA/J inbred mice from the breeding unit of the Department of Immunology, College of Medicine, Jagellonian University, Cracow were used. Mice were fed with autoclaved food and water. The experiments were carried out according to the guidelines of the Animal Care and Use Committee of the Jagellonian University, College of Medicine. Each treatment group consisted of 5 mice and each experiment was performed at least twice.

Reagents

The following reagents were used. Picryl chloride (2,4,6-trinitrophenyl chloride; PCL; Chemica Alta, Edmonton, Alberta, Canada); trinitrobenzene sulfonic acid (Eastman Kodak, Rochester, N.Y., USA); keyhole limpet hemocyanin (KLH; Calbiochem, San Diego, Calif., USA); carboxymethylcellulose sodium salt, mitomycin C (Sigma, St. Louis, Mo., USA); RPMI-1640, Hepes buffer (1 M), sodium pyruvate, fetal calf serum (FCS; Life Technologies, Grand Island, N.Y., USA); [^{3} H]-thymidine (Lacomed, Rez, Czech Republic). Low-tox rabbit complement (RC; Pel-Freez Biologicals, Brown Deer, Wisc., USA); purified anti-mouse mAbs-anti-IL-4 (clone 11B11),

anti-IL-10 (clone SXC1) and anti-TGF- β (clone HB 9849) were received by the courtesy of Dr. Charles A. Janeway, Jr., Yale University, (New Haven, Conn., USA); anti-TCR- δ mAb producing clone (UC7–13D5, hamster Ig) was received from Dr. J. Bluestone, University of Chicago (Chicago, Ill., USA) and anti-TCR- β -producing clone (H57–597, hamster Ig) from Dr. Ralph Kubo, Cytel Inc. (La Jolla, Calif., USA). Cells were cultured under standard conditions and supernatants were used. Calf collagen, elastin, and keratin hydrolysate were obtained from Proteina, Lodz, Poland; Nivea cream was obtained from Beiersdorf, Hamburg, Germany (cream 1), cosmetic cream 2 containing collagen and elastin was obtained from a well-known manufacturer; mouse immunoglobulins (mlg) were prepared from CBA/J mouse sera by the method of McKinney and Parkinson [7] and were substituted with TNP [8]. A preparation with the level of substitution TNP₂₀-mlg was used throughout.

Epicutaneous Exposure to Antigen and Induction of Cell-Mediated Immunity

Antigens dissolved in saline (containing 3 mg CM-cellulose in 1 ml to facilitate homogenization) were homogenized with Nivea cream (cream 1) at a ratio of 1:10 by repeated passage between two linked syringes. The final 1 ml of mixture contained 5 mg of antigen and was stable. Control cream 1 samples were prepared similarly, except that protein additives were omitted.

CBA/J mice were treated on the shaved dorsal skin (area 3 $\times$ 1.5 cm) for 7 days with 100 μ l of cream 1 containing 500 μ g of collagen, elastin, keratin, KLH or TNP-Ig or with cream 1 alone or were treated similarly with commercial protein-enriched cream 2. The cream was smeared on the skin with a cotton swab. On day 8, the mice were sensitized by topical application of 0.15 ml of 5% PCL in an acetone-ethanol mixture (1:3) to the shaved abdomen and chest. As a positive control, we used mice that were only actively immunized with PCL. Four days after skin painting with hapten, the mice were challenged with 10 μ l of 0.4% PCL in olive oil-acetone (1:1) onto each side of both ears. Subsequent contact sensitivity (CS) reaction estimated as increase in ear thickness was measured 24 h after the challenge, using an engineer's micrometer (Mitutoyo Kanagawa, Japan) and was expressed in units (U) of 10^{-3} cm $\pm$ SD [9]. In all experiments, a group of nonimmune control animals was also challenged on the ears with hapten and the resulting background increase in ear thickness ($\sim$ 2 U at 24 h) was subtracted from responses of experimental groups to yield net ear-swelling responses (tables 1, 2).

Alternatively, CBA/J mice treated epicutaneously with different creams for 7 days were injected into four divided sites of alcohol-cleansed unshaved abdominal skin with 100 μ g KLH dissolved in 200 μ l freshly opened sterile pyrogen-free 0.9% saline. Additionally, a group of naive animals or mice treated with cream 1 alone were immunized with KLH (positive control). Four days later, the mice were injected in both pinnae under ether anesthesia with 10 μ l sterile pyrogen-free saline containing 5 μ g KLH. Controls sham-immunized with saline alone were similarly ear challenged with KLH to obtain background responses. The size of the reaction was measured 24 h later as described above.

Adoptive Cell Transfer of CS Responses and Cell-Mixing Assay (Transfer Out)

Donors of CS-immune effector cells underwent contact sensitization with 5% PCL. Immune axillar and inguinal lymph nodes (LN) were harvested on day 4 and 7×10^7 cells were incubated for 30 min at 37°C in RPMI-1640 medium alone, washed and then injected

intravenously into normal syngeneic recipients (positive transfer). For the cell-mixing assay, 7×10^7 CS-effector immune cells from PCL CS donors were incubated for 30 min at 37°C with 5×10^7 LNC cells (LNC) from mice treated epicutaneously for 7 days with TNP-Ig or collagen in cream 1. After incubation, cells from each group were transferred intravenously into naive recipients. Mice were challenged with antigen within 30 min after transfer as described earlier and the CS reaction was measured 24 h later.

Phenotype of Regulatory Cells

To phenotype regulatory cells, LNC isolated from mice tolerized with TNP-Ig in cream 1 were incubated with anti-TCR β mAb (H57-597), anti-TCR δ mAb (UC7-135D5) (1 ml of culture supernatant/ 10^7 cells) or medium alone for 45 min on ice. Then the cells were washed and incubated with rabbit complement (RC) for 60 min at 37°C and then washed and resuspended in PBS. After that, 7×10^7 PCL immune cells were incubated with medium alone, with 5×10^7 tolerized cells treated with RC alone or with 5×10^7 cells treated with anti-TCR β or anti-TCR δ mAb plus complement for 30 min at 37°C. The resultant cells were transferred into naive recipients that were challenged with PCL and tested for CS as described above.

Transfer into Tolerized Mice to Confirm Active Tolerance

To confirm the data from the cell-mixing assay ('transfer out'), 7×10^7 of 4-day PCL immune cells were transferred intravenously into recipients that were treated with cream 1 alone (positive transfer) or into mice that were tolerized via exposure to TNP-Ig or collagen in cream 1 as described above. Thereafter, mice from all groups were challenged with PCL and tested for CS.

Influence of Epicutaneous Tolerization on the *in vitro* Proliferation of PCL-Immune LNC

CBA/J mice were treated with cream 1 containing different proteins for 7 days. On the day after the last exposure, the mice were actively immunized by topical application of 5% PCL. As positive controls we used mice that were or were not treated with cream 1 alone. Four days after PCL immunization, a single cell suspension of axillar and inguinal LNC was prepared under aseptic conditions and 3×10^5 LNC were incubated in U-bottom 96-well microplates (Falcon, Oxnard, Calif., USA) in triplicates with threefold dilutions of TNP-Ig in 200 μ l of RPMI-1640 containing 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, 25 mM Hepes, 5×10^{-5} M 2-mercaptoethanol and 10% fetal calf serum for 48 h. Then 0.5 μ Ci/well of [^{3} H]-thymidine was added and the cells were incubated for an additional 18 h. [^{3} H]-thymidine incorporation was determined by β liquid scintillation counting. Results are presented as mean $\pm$ SE.

Proliferation Assay in the Presence of Regulatory Cells and Neutralizing Anti-Cytokine mAbs

To find the mechanism of suppression mediated by regulatory cells induced by epicutaneous immunization, 3×10^5 PCL immune LNC were cultured alone or were cocultured with 6×10^4 mitomycin-C-treated LNC from mice tolerized epicutaneously with TNP-Ig in cream 1. Conditions of culture were as described above. In some groups, immune and regulatory cells were cultured in the presence of 10 μ g/ml of neutralizing anti-IL-4, anti-IL-10 or anti-TGF- β mAb. [^{3} H]-thymidine incorporation was determined as previously by β liquid scintillation counting.

Table 1. Contact sensitivity and DTH reactions in mice treated epicutaneously with Nivea cream containing different proteins or with commercial cosmetic X cream

Epicutaneous treatment with vehicle (= Nivea cream, NC)	Contact sensitivity reaction to TNP ear swelling response units $\times 10^{-3}$ cm $\pm$ SD	DTH reaction to KLH units $\times 10^{-3}$ cm $\pm$ SD
No treatment	7.3 $\pm$ 1.68	12.6 $\pm$ 2.41
NC alone	7.4 $\pm$ 0.41 (100)	10.9 $\pm$ 2.40 (100)
NC + collagen	2.1 $\pm$ 1.26 (29)	2.1 $\pm$ 1.73 (19)
NC + elastin	2.8 $\pm$ 0.91 (38)	5.4 $\pm$ 1.70 (49)
NC + collagen + elastin	3.4 $\pm$ 1.08 (46)	ND
NC + keratin	3.4 $\pm$ 1.46 (46)	6.5 $\pm$ 2.07 (60)
NC + TNP-Ig	3.1 $\pm$ 0.66 (42)	3.4 $\pm$ 1.31 (31)
NC + KLH	3.1 $\pm$ 1.31 (42)	6.5 $\pm$ 1.20 (60)
X cream	2.2 $\pm$ 1.06 (30)	6.3 $\pm$ 1.18 (58)

CBA/J mice (5 per group) were treated on the shaved dorsal skin (area 3×1.5 cm) for 7 days with 100 μ l of cream 1 (Nivea cream) containing 500 μ g of collagen, elastin, keratin, KLH or TNP-Ig or with cream 1 alone. When the mixture of collagen and elastin was used, concentration of proteins was 250 μ g each. Some mice were treated similarly with cosmetic cream 2 which contained collagen and elastin. On day 8, groups of mice were sensitized by topical application of PCL or immunized subcutaneously with KLH. Four days later, the animals were tested for cell-mediated immunity to corresponding antigens (PCL or KLH) by the ear-swelling test. Results are expressed as ear-swelling response in units $\times 10^{-3}$ cm $\pm$ SD. Percent responses are given in brackets (animals treated with cream 1 alone = 100% response). Nontreated controls were also included. ND = not done. Statistical differences between groups treated with cream 1 alone and groups treated before immunization with cream 1 containing proteins vary between $p < 0.005$ and $p < 0.05$.

Statistics

A double-tailed Student's *t* test was used to assess the significance of differences between groups, with $p < 0.05$ taken as a minimum level of significance.

Results

Epicutaneous immunization with protein antigens induces antigen-nonspecific suppression of DTH.

CBA/J mice were treated daily for 7 days by epicutaneous application of Nivea cream alone (cream 1) or containing as additives: calf collagen, elastin or their mixture, keratin, mouse Ig, TNP-Ig or KLH (500 μ g/0.1 ml) or were treated with cream 2 and then actively immunized with PCL or KLH. Table 1 shows that exposure to both homologous (TNP-Ig) or heterologous antigen (collagen,

Table 2. Epicutaneously induced suppressor cells inhibit the passive transfer of contact sensitivity by immune cells (transfer in and transfer out)

Group	Tr cells added to PCL immune cells induced by	Treatment of Tr cells	Adoptive CS reaction in recipients		
			normal	pretreated with TNP-Ig in Nivea cream	pretreated with collagen in Nivea cream
A	no Tr cells added	no treatment	6.0 ± 1.18 (100)	2.5 ± 0.84 (42)	1.5 ± 1.39 (25)
B	collagen induced	no treatment	0.6 ± 0.83 (10)	–	–
C	TNP-Ig induced	no treatment	2.1 ± 0.42 (35)	–	–
D	TNP-Ig induced	αTCRβ mAb + RC	6.1 ± 0.65 (100)	–	–
E	TNP-Ig induced	αTCRδ mAb + RC	2.5 ± 0.50 (42)	–	–

7×10^7 PCL-induced immune cells were transferred to normal recipients or to recipients treated epicutaneously for 7 days with TNP-Ig or collagen in cream 1 (Nivea cream; group A, transfer in). Alternatively, 7×10^7 PCL immune cells were incubated with 5×10^7 LNC from mice tolerized epicutaneously with TNP-Ig or collagen in cream 1 before transfer into normal recipients (transfer out, groups A, B, C). In groups D and E, Tr cells were treated with anti-TCRβ or anti-TCRδ mAb and complement before incubation with immune cells. All differences between group A and other groups are statistically significant, $p < 0.005$, except group D.

elastin, keratin or KLH) results in a decrease in CS to TNP of 54–71% compared with vehicle treatment (cream 1) alone or cream 1 containing mouse Ig. Also cream 2 containing collagen + elastin induced a deep suppression of CS of 70% in mice. Similar results were found in mice immunized with the high-molecular-weight antigen KLH. Again, mice tolerized with homologous (KLH) or heterologous (TNP-Ig, collagen, elastin or keratin) showed a significant decrease in DTH of 40–81% and cream 2 caused a reduction in DTH to KLH of 42%. These results are statistically significant. Significant suppression was observed even if the amount of protein was reduced (100 µg/0.1 ml of cream, instead of 500 µg) (results not shown).

Epicutaneously Induced Suppression of CS Can Be Transferred by T Cells

To find whether suppression of DTH in mice after epicutaneous exposition to antigen is transferable, we used two sets of experiments: transfer in and transfer out.

LNC cells of mice actively skin-sensitized with PCL, transferred a good passive CS reaction into normal recipients while transfer into recipients tolerized with TNP-Ig or collagen resulted in a decrease in CS of 58 and 75%, respectively (transfer in) (table 2, group A). Addition to PCL-immune LNC from mice epicutaneously tolerized with TNP-Ig or collagen caused suppression of passive transfer of CS of 65 and 90%, respectively (transfer out) (table 2, compare group A with groups B and C).

Regulatory cells induced by TNP-Ig depleted of TCR αβ+ cells with anti-TCR-β mAb + RC lost the ability to suppress effector cells of CS, while treatment with anti-TCRδ mAb + RC causing depletion of TCRγδ+ cells did not affect their ability to suppress adoptive transfer of CS response (table 2, compare group A with groups D and E).

Antigen-Nonspecific TCR αβ+ Suppressor Cells Induced via Epicutaneous Immunization Inhibit Proliferation of CS Effector Cells via TGF-β

Epicutaneous immunization with TNP-Ig, collagen, elastin or keratin suspended in cream 1 before contact sensitization with PCL significantly suppresses in vitro proliferation of LNC restimulated with TNP-Ig as measured by [³H]-thymidine incorporation when compared to LNC isolated from mice treated with vehicle alone (cream 1) before PCL immunization. Similar suppression of proliferation was found in mice exposed to cream 2 before sensitization with PCL (fig. 1). Figure 2 shows that regulatory cells isolated from mice tolerized epicutaneously with TNP-Ig and treated with mitomycin C suppress proliferation of cocultured CS effector cells immune to PCL in vitro and the observed suppression could be overcome by addition of neutralizing anti-TGF-β mAb to the culture. Anti-IL-4 or anti-IL-10 mAb had no such effect.

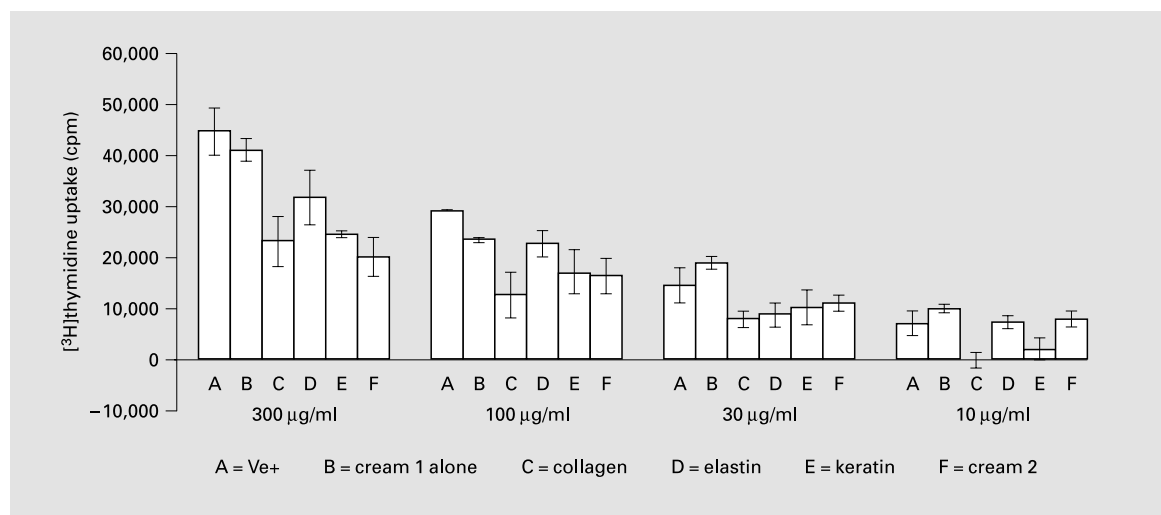
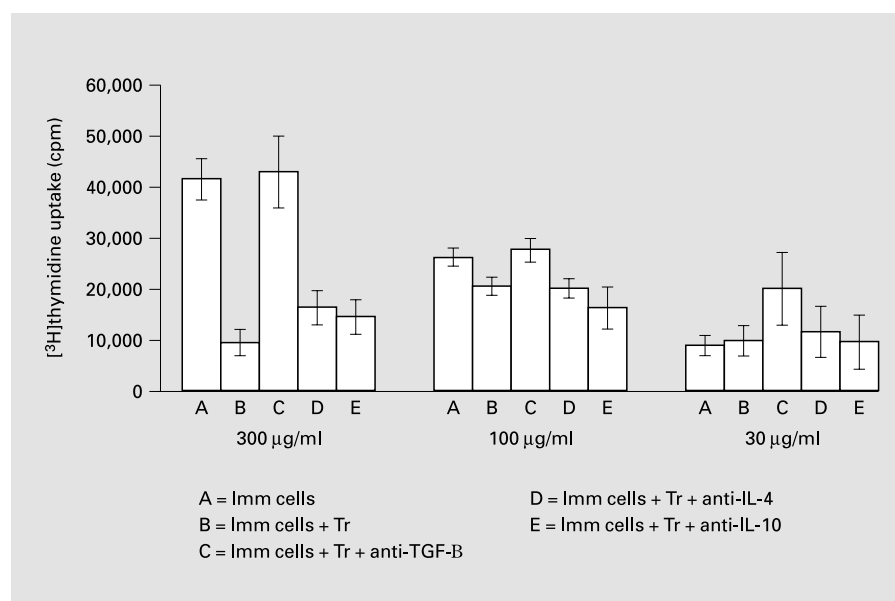


Fig. 1. The influence of epicutaneous tolerization on the in vitro proliferation of PCL immune lymph node cells. CBA/J mice were treated epicutaneously with Nivea cream (cream 1) alone, cream 1 containing different proteins or cream 2 for 7 days and then skin-sensitized with PCL (see legend to table 1). Four days later, axillar and inguinal LNC (3×10^5 per well) were cultured in U-bottom 96-well microplates in triplicates in RPMI-1640 medium supplemented with FCS, *L*-glutamine, Hepes and 2-mercaptoethanol. Threefold dilutions of TNP-Ig were added to the wells (consecutively 300, 100,

30 and 10 µg/ml). After addition of 0.5 µCi/well of [^{3} H]-thymidine, the cells were incubated for 18 h and then [^{3} H]-thymidine incorporation was measured in a β scintillation counter. +Ve refers to mice which were only skin-sensitized with PCL. Subsequent bars represent groups of animals treated with cream 1 alone, or cream 1 supplemented with different proteins and then sensitized with PCL. Results are expressed as mean $\pm$ SE. Statistical significance: 300 µg/ml, A versus C, E and F, $p < 0.05$; 100 µg/ml, A versus C, E and F, $p < 0.05$. Differences in other groups are nonsignificant.

Fig. 2. Proliferation assay in the presence of regulatory cells and neutralizing anti-cytokine antibodies. 3×10^5 LNC from PCL-sensitized animals (Imm cells) were cultured in triplicates alone or with addition of 6×10^4 mitomycin-C-treated LNC from mice epicutaneously immunized with TNP-Ig in cream 1 for 7 days (Tr). Conditions of culture were as described in the legend to figure 1. In some groups, 10 µg/ml of neutralizing anti-IL-4, anti-IL-10 or anti-TGF- β mAb were added. [^{3} H]-thymidine incorporation was determined as described in the legend to figure 1. Imm cells = PCL immune LNC of mice treated with cream 1 alone. In all other groups, Tr cells of TNP-Ig tolerized animals were added. Statistical significance: 300 µg/ml A versus B, D and E, $p < 0.05$. Differences in other groups are nonsignificant.



Discussion

The immune system has at its command effective mechanisms that control the effector responses of lymphocytes in order to prevent both unwanted immune responses to nonpathogenic antigens and to silent inflammatory responses to allow their resolution. There is increasing evidence that regulatory T cells (Tr) are involved in maintenance of tolerance to foreign antigens entering the organism via the digestive tract [1–3]. Our experiments show that this is also true for antigens penetrating through nondamaged skin.

To apply an antigen epicutaneously we have used a method in which the antigen was homogenized with cream 1 (Nivea cream) which is completely devoid of any tolerogenic activity per se. This method is simpler and less stressful to mice than the patch method since it does not require partial immobilization of animals by dressing and simultaneously allows gradual and continuous release of antigen from cream deposition. Our data show that epicutaneous immunization with a series of different antigens results in nonspecific suppression of both CS to TNP hapten and DTH to protein antigen like KLH. Further experiments showed that epicutaneous exposure to an antigen can induce Tr cells and observed that suppression of cell-mediated immune response was transferable and was mediated by TCR $\alpha\beta$ ⁺ cells. In our experiments TCR $\gamma\delta$ lymphocytes did not participate in skin-induced suppression, although in mucosa-induced tolerance they were shown to play a dual role depending on experimental conditions as up- and/or downregulatory cells [10, 11]. This was also observed in other systems studied [12, 13]. Experiments with neutralizing antibodies demonstrated that Tr cells induced by skin immunization suppressed proliferation of T effector cells of DTH in vitro via released TGF- β , while a lack of effect of anti-IL-4 and anti-IL-10 neutralizing antibodies suggests that these two cytokines do not play a crucial role in the observed suppression at least at its effector stage. Many T $\alpha\beta$ cell subpopulations can secrete TGF- β [14] and our preliminary experiments suggest that both CD4⁺ and CD8⁺ cells were involved in generalized downregulation of cell-mediated immune responses. Similar findings were also reported in mucosa-induced tolerance [1].

In classical immunological suppression, both suppressor and effector cells are specific for the same antigen. The tolerance spreading observed in our experiments is possibly akin to or identical with the phenomenon known as bystander suppression (determinant spreading) [15, 16] in which suppressor cells elicited by a particular antigen

secrete antigen-nonspecific cytokines that downregulate immune responses of other cell populations to their respective antigens. TGF- β may have a particular role here since it is present in the plasma and also binds to matrix components in many organs forming an available reservoir of this suppressor cytokine [17, 18]. TGF- β exerts multiple inhibitory effects on B cells, CD4⁺ (Th1 and Th2) and CD8⁺ cells, NK cells and macrophages [19, 20] and suppresses the production of IFN- γ , TNF- α and IL-2 by Th1 lymphocytes [21, 22].

In the well-studied model of oral tolerance, orally administered antigens appear to interact with gut-associated lymphoid tissues (GALT) and generate Tr cells that produce TGF- β , IL-4 and IL-10 [23] and also mediate bystander suppression [16]. Our data thus suggest that epicutaneous exposure to an antigen similarly to mucosal immunization results in the induction of T downregulatory cells that have much the same mechanisms of action and therefore underline the similarity of immune mechanisms operating on these two bodily surfaces. Peripheral tolerance induced by orally or epicutaneously applied antigens may have important implications for designing therapeutic schemes aimed at modulating immune responses to self-antigens involved in autoimmune diseases.

In our experiments, cosmetic cream 2 containing collagen and elastin significantly inhibited DTH responses in mice while neutral cream 1 was devoid of any suppressive activity unless supplemented with proteins. This difference between the two creams is impressive as it cannot be taken at its face value as a definitive proof of immunomodulatory effects of protein additives in cream 2. Since both creams differed not only in protein content but also in content of other constituents, there remains a remote possibility that some other ingredients in cream 2 were responsible for or contributed to the observed effects. To draw a firmer conclusion, it would be necessary to test several protein-enriched cosmetic creams produced by different manufacturers for immunoregulatory activity. Assuming, however, as we do, that the suppressive activity of cream 2 was solely or for the most part due to protein additives, one is tempted to ask whether protein-enriched cosmetic creams could influence the immune status of their human users as they do experimentally in mice. We are not aware of any information on this subject. The amounts of antigens we have used in mice on daily basis were proportionally not much different from the amounts used by humans during normal skin-care procedures. Although creams are used by humans for much more longer periods of time (1 week vs. many months), the anatomo-

my and physiology of mouse skin differs from its human counterpart in several respects. These differences include skin thickness which in the mouse would favor easier penetration of antigen, although the thick and hydrophobic horny layer of human skin is now frequently removed by peeling, thus making the skin more permeable to subsequently applied cosmetics. Moreover, mouse and human skin also have a somewhat different cellular composition, e.g. the presence of $\gamma\delta$ cells in mouse skin and their absence in human skin [23]. Thus, in summary, the possi-

ble extrapolation of this part of our results to humans must await further studies.

Acknowledgments

This work was supported by a grant from the Committee for Scientific Research (KBN, Warsaw) No. 4P0 5B 04 519. We express our gratitude to the Foundation for Polish Science for the purchase of equipment for column chromatography.

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