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To cite this version:

D. Soury, G. Barratt, S. Ah-Leung, P. Legrand, H. Chacun, et al.. Skin localization of cow’s milk proteins delivered by a new ready-to-use atopy patch test.. Pharmaceutical Research / Pharmaceutical Research (Dordrecht), 2005, 22 (9), pp.1530-6. 10.1007/s11095-005-5881-4 . hal-00385449

HAL Id: hal-00385449
https://hal.archives-ouvertes.fr/hal-00385449
Submitted on 25 May 2009

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Research Paper
Skin Localization of Cow’s Milk Proteins Delivered by a New Ready-to-Use Atopy Patch Test

D. Soury,1,3 G. Barratt,1 S. Ah-Leung,1 P. Legrand,1 H. Chacun1 and G. Ponchel1

Purpose. Atopy patch tests (APT) allow the detection of delayed allergies at the skin level. The localization of b-lactoglobulin delivered into the skin by an innovative ready-to-use APT (E-patch) was investigated and the efficacy and safety of this device were assessed.

Methods. The E-patch containing b-lactoglobulin was placed for 24 h in contact with hairless rat skin mounted in a Franz diffusion cell. Transdermal passage was monitored by measurement of b-lactoglobulin concentration in the external receiver compartment by radioactivity assays. An iterative skin stripping allowed measurement of the b-lactoglobulin penetrating the first external skin layer.

Results. After 24 h, 92% of b-lactoglobulin remained on the skin. The iterative skin stripplings showed a 135-fold higher concentration of b-lactoglobulin in the stratum corneum than that found in the epidermis/dermis. Analysis of the solution in the receiver compartment by radioactivity assays or immunossays indicates that intact protein did not cross the skin.

Conclusions. The E-patch system allows native b-lactoglobulin to concentrate in the stratum corneum, in the vicinity of immunological cells, but does not lead to its systemic delivery. Therefore, it is suggested that this delivery system creates ideal conditions for promoting a positive topical response with reduced risk of systemic anaphylactic reactions caused by the native form of the b-lactoglobulin APT.

KEY WORDS: atopy patch test; b-lactoglobulin; delayed food allergy; milk; skin

INTRODUCTION

Food allergy is a common problem in infants and young children. In particular, hypersensitivity to cow’s milk occurs in 2Y3% of infants (1,2). The recommended test, or Bgold standard, in the diagnosis of food allergy is the double-blind, placebo-controlled food challenge (3). New tools, such as the patch test [atopy patch test (APT)], which provides a prolonged contact of 48 h of allergen with the skin, have been introduced for the screening and diagnosis of delayed forms of milk allergy (4Y7).

In every allergy, there always is a first contact between the organism and the allergen: the sensitization step, followed by manifestations of allergy occurring after a second contact. These manifestations are either immediate after the first contact with the allergen (less than 2 h) or delayed (more than 2 h) and the site of these manifestations can be different from the site of sensitization. Indeed, for example, in food allergy, provoked via the gastrointestinal tract, the manifestations could be gastrointestinal reactions (nausea and vomiting, diarrhea), or anaphylactic reactions, or respiratory reactions (asthma, rhinitis), or cutaneous reactions (urticaria, atopic dermatitis) (8).

Delayed allergy (type IV hypersensitivity) is a cellular immune response to an allergen. The first contact of the allergen with the organism, the sensitization, is necessary. The allergenic substance is captured by immune cells such as macrophages or Langerhans cells (in the skin). Immune cells migrate via the afferent lymphatics to the draining lymph nodes where they present allergen to T lymphocytes. An allergic response is thus induced with production of memory T lymphocytes. After this sensitization, when a second contact with the allergen occurs on the skin, allergen is again captured by local Langerhans cells that recruit circulating memory T lymphocytes. These lymphocytes release mediators that provoke the allergic reaction. Because this reaction occurs between 48 and 72 h after the second contact with the allergen, it is known as a delayed allergic reaction (9Y11).

Atopy patch tests have been shown to be a valuable complementary tool in the diagnosis of food allergy in children with late-phase clinical reactions (12,13). Moreover, this test has the advantages of being noninvasive and used on the skin. Indeed, the skin is at one and the same time a barrier of the organism with a local immune system and a possible route of administration via transdermal passage of drugs (14).

So allergens put on the skin can come into contact with immune cells however at the same time, their passage...
through the skin can be limited by the very low permeability of the skin. In general, APTs are prepared with a concentrate

one page of a document
b-lactoglobulin A, and glycine and alanine in b-lactoglobulin B (21). Because this protein is well characterized and available in both native and radiolabeled forms, it has been chosen as a marker both to study skin localization and to detect any potential transdermal passage.

MATERIALS AND METHODS

Materials

b-lactoglobulin A-[methyl-14C], specific activity 29 mCi/mg was obtained from ARC (St. Louis, MO, USA) via Isobio (Hauts, Belgium). Commercial skim cow’s milk powder was distributed by Re’gilait (France). The phosphate buffer solution (PBS, pH 7.4) was supplied by Sigma (St. Louis, MO, USA).

Analysis of the Purity of the Radiolabeled Solution

The radiolabeled solution of b-lactoglobulin A-[methyl-14C], the solution of b-lactoglobulin A-[methyl-14C] mixed with Re’gilait, and the solution in the receiver compartment of the Franz diffusion cell at 24 h were analyzed by gel electrophoresis in 20% polyacrylamide in the presence of SDS and b-mercaptoethanol. The radioactivity on the gel was counted with a Berthold linear counter (Berthold France) and an autoradiograph of the gel was made by contact with a Kodak BioMax MR film (France).

Animals

Female hairless rats (OFA-hr/hr) used in the ex vivo permeation study were 8Y9 weeks old (weighing on average 220 g) and were purchased from Charles River Laboratories (Calco, Italy). Animals were housed in standard cages at room temperature on a 12-h light/dark cycle 1 week before experimentation, and standard laboratory food and water were given ad libitum.

Formulations

Although the E-patch formulation normally contains dry powder, for these experiments the patch tests contained a protein solution. This was chosen to minimize the effects of variability between the skins of rats and to compensate for a possible lack of TEWL in the ex vivo model, which in humans would have normally resulted in rapid dissolution of the milk components (18,19).

Patches were composed of a disk of occlusive polymer with a surface area of 78.5 mm2 surrounded by a foam border 1 mm thick, thus forming a chamber. An adhesive layer below the foam allowed the patches to stick to the skin.

For radioactivity studies, 25 ml of a radioactive solution of b-lactoglobulin A-[methyl-14C] mixed with a solution of Re’gilait milk powder in PBS was added to the patch to obtain 20 mg of cold b-lactoglobulin and 1 mCi of b-lactoglobulin A-[methyl-14C] (34.5 mg) per patch. The final concentration of total proteins in the patch was of 8 mg/ml.

For experiments in which b-lactoglobulin was measured by immunocassay, 25 ml of a solution of Re’gilait in PBS (8 mg/ml of proteins) was added to the patch so as to obtain 20 mg b-lactoglobulin per patch.

Diffusion Experiments

Dorsal skin was excised from hairless rats after euthanasia by CO2 inhalation. Any subcutaneous fat was carefully removed with a scalpel and several pieces were cut from each specimen. Each piece of skin was mounted on a Franz diffusion cell with a surface area for passage of 64 mm2. The epidermal side of the skin was exposed to ambient conditions whereas the dermal side was bathed with 0.01 M phosphate buffer solution (pH 7.4) containing 0.1% sodium azide. The receiver solution was stirred continuously with a small magnetic stirring bar. Care was taken to remove any air bubbles between the underside of the skin and the solution in the receiver compartment. The temperature of the receiver
compartment was maintained at 34°C. After overnight equilibration, 25 ml of radiolabeled solution or of unlabeled milk solution was added to a patch that was put on the epidermal surface of the skin on the cell. A minimum of six cells were used and experiments were carried out with sections of skin from different animals. All experiments were carried out with occluded donor compartments. Samples of the receiver compartment were withdrawn at intervals for radioactivity counting and replaced by fresh buffer. After 24 h, the experiments were stopped and the diffusion setup was dismantled.

Assay of Radiolabeled Markers
Before dismantling was complete, the patch and the skin were each rinsed carefully twice with 0.2 ml of PBS solution. All washings were collected and assayed for radioactivity, together with the patch. Following the rinsing procedure, the skin was put on a filter paper on a glass plate and a piece of adhesive tape, 1.9 cm wide and about 5 cm long, was pressed firmly to the skin surface with a mass of 150 g for 15 s. The tape was of sufficient size to cover the area of skin that was in contact with the formulation. Twelve such stripings (corresponding to the thickness of the stratum corneum) were carried out and each strip was analyzed separately for radiolabel. The remaining skin (that is, the rest of the epidermis and the dermis after striping of the stratum corneum) and the receiver compartment solution were also assayed for radiolabel.

Radioactivity was counted in a Beckman LS 6000 TA (France). Skin and strip samples were weighed and dissolved with Soluene (Perkin-Elmer, Coignieres, France) (1 ml), after which scintillation medium (Hionic Fluor, Perkin-Elmer) was added before counting. Liquid samples were counted in Ultima Gold scintillation medium (Perkin-Elmer). For analysis of the receiver compartment solution, an aliquot was assayed for total radiolabel and another aliquot was mixed with BSA (final concentration of BSA was 864 mg/ml in 810 ml), 90 ml of Tris 1 M SDS 1%, and 180 ml of trichloroacetic acid (TCA) 60%. This mixture was shaken and, after overnight equilibration, the mixture was centrifuged (Jouan MR 22i, France) at 734 g for 5 min and the supernatant was recovered for counting. Results are expressed as percentage of total radioactivity counted at the end of the experiment.

Assay by Two-Site Enzyme Immunoassay
This assay was used to determine the native and denatured b-lactoglobulin in the receiver compartment (23). One aliquot was assayed for native b-lactoglobulin and another for denatured b-lactoglobulin. The assays were performed in 96-well microtitre plates and were based on the use of pairs of monoclonal antibodies specific for either the native form or the reduced and carbamymethylated form of b-lactoglobulin. Detection limits for the native and the reduced b-lactoglobulin in the Re`giliait solution were both 0.32 ng/ml.

RESULTS
Purity of the Radiolabeled b-lactoglobulin A
The purity of the radiolabeled b-lactoglobulin A was assessed by SDS-polyacrylamide gel electrophoresis of the solution of b-lactoglobulin A-[methyl-14C] in buffer and mixed with milk.

Figure 1 shows the autoradiography of radiolabeled proteins present in the solution of b-lactoglobulin A-[methyl-14C] (A) or present in the mixture of b-lactoglobulin A-[methyl-14C] and unlabeled milk solution (B). Identical bands were found in the two solutions. Therefore, taking into account the reducing conditions of the electrophoresis other milk components did not seem to denature or associate with
the β-lactoglobulin A (other than by disulfide bonds). Two major bands were present: one band at 18 kDa, which corresponds to β-lactoglobulin A, and one band at 39 kDa, which seems to be the dimer of the β-lactoglobulin A of 36 kDa. Two other minor bands were observed around 13 and 11 kDa. The origin of these minor bands is unknown. β-lactoglobulin A was methylated using \( ^{14} \text{C}-\text{formaldehyde and sodium cyanoborohydride} \) according to the procedure of Dottavio-Martin and Ravel (24) and the resulting β-lactoglobulin A-[methyl-\( ^{14} \text{C} \)] was purified by extensive dialysis. There are two possible explanations for the presence of the minor bands: (a) the β-lactoglobulin A used for the synthesis might not have been pure but contaminated by other proteins of the lactoserum such as α-lactalbumin (14 kDa), or (b) the β-lactoglobulin A itself could have been denatured during or after radiolabeling.

To quantitate the results of autoradiography, radioactivity on the polyacrylamide gel was counted in a Berthold linear meter. The results are shown in Table I. For both solutions, the band corresponding to β-lactoglobulin represented about 78% of the total radioactivity, with the dimer about 18%, and the low-molecular-weight traces less than 4%. Hence, even if contaminants or impurities are present in the radioactive solution, they only represent a low percentage of the components of the solution.

Localization and Concentration of β-lactoglobulin A-[methyl-\( ^{14} \text{C} \)] in Hairless Rat Skin

Figure 2 shows the distribution of the β-lactoglobulin A-[methyl-\( ^{14} \text{C} \)] between the different compartments of the model after 24 h. The amount of radioactivity within the stratum corneum was determined by analyzing the 12 first tape stripplings of this surface, whereas the amount of radioactivity penetrating the deeper skin strata was determined by analyzing the remainder of the full thickness skin, i.e., the rest of the epidermis and the dermis. About 92% of the radioactivity remained on the skin under the patch. The rest of the radioactivity was approximately equally distributed between the two skin layers and the receiver compartment. The thickness of the two skin layers (18.4 ± 0.5 mm for the stratum corneum and 2.07 ± 0.07 mm for the rest of the skin) (25), the volume under the patch (78.5 mm³), and the volume of the receiver compartment (11 ml) were used to calculate the concentration of β-lactoglobulin A in each location. β-lactoglobulin A was essentially sequestered in the stratum corneum: it was 1.6 times more concentrated than under the patch on the skin and 135 times more than in the rest of the skin (epidermis/dermis) (Fig. 3).

Figure 4 illustrates the distribution of β-lactoglobulin A-[methyl-\( ^{14} \text{C} \)] in the 12 first layers of the epidermis, which comprise the stratum corneum. Most were found in the two first strips, 1 and 0.5%, respectively, representing about 58% of the total radioactivity counted in the stratum corneum. Starting from the third strip, the amount of radioactivity decreased with about 8% of the total radioactivity in the stratum corneum in each strip until the sixth strip. Thereafter, radioactivity was about 2% per strip of the total radioactivity in the stratum corneum.

Lack of Passage of Intact β-lactoglobulin Across Hairless Rat Skin

The percentage of total β-lactoglobulin A-[methyl-\( ^{14} \text{C} \)] detected in the receiver compartment solution was followed
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Results were obtained after separation of the proteins on a 20% polyacrylamide gel in the presence of SDS and *"*mercaptoethanol and integration of the measurement of radioactivity by a Berthold linear meter.

Table 1. Distribution by Percentage of Total Radioactivity in the Solution of *"*Lactoglobulin A-[methyl-14C] in Buffer (Lactoglobulin A) (A) or Mixed with Unlabelled Milk (Lactoglobulin A in Milk) (B)

<table>
<thead>
<tr>
<th>Component</th>
<th>A (%)</th>
<th>B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer (18 kDa)</td>
<td>78.7</td>
<td>77.5</td>
</tr>
<tr>
<td>Dimers (59 kDa)</td>
<td>17.7</td>
<td>18.8</td>
</tr>
<tr>
<td>High molecular weight (&gt;51 kDa)</td>
<td>1.2</td>
<td>2.1</td>
</tr>
<tr>
<td>Low molecular weight (&lt;13 kDa)</td>
<td>2.1</td>
<td>1.6</td>
</tr>
</tbody>
</table>

DISCUSSION
There is already considerable evidence in the literature that proteins and peptides applied to bare skin can provoke systemic immune responses, leading to the proliferation of specific T cells of both Th1 and Th2 phenotypes and the production of IgG and IgA antibodies (26,27). Proteins as large as tetanus toxoid (150 kDa) and bovine serum albumin (68 kDa) can be used as immunogens in this way, especially when cholera toxin, itself a protein of 68 kDa, is used as an adjuvant (28). Furthermore, a specific circulating IgE response to ovalbumin (45 kDa) has been obtained after its application onto non-sensitized animal skin (29). All this evidence indicates that sufficient numbers of protein molecules are able to reach the Langerhans cells present in the epidermis. Langerhans cells are crucial for the development of cutaneous immune responses to allergens (10). They play a role in the sensitization and manifestation of delayed allergy. Following topical exposure to sensitizing chemicals, Langerhans cells capture the allergen and are induced to migrate from the skin, via the afferent lymphatics, to the draining lymph nodes. They are therefore essential for the effective presentation of topical allergens to T lymphocytes and the induction of allergic responses. After a second contact, Langerhans cells capture allergen and recruit T lymphocytes to induce a local allergic reaction (10,11).

None of the studies of transdermally induced immune responses mentioned above have attempted to localize the antigens within the skin. The present study suggests that β-lactoglobulin A concentrated in the stratum corneum.

About 92% of the radioactivity remained on the surface of the skin after 24 h, but 3% was recovered in the 12 strips corresponding to the stratum corneum. This represents a significant concentration of radioactivity within this compartment. A similar accumulation of radioactivity in the stratum corneum was observed when radioactive IFN-α was applied to hairless guinea pig skin (30). In the present study, preliminary experiments have shown that the purity of the radiolabeled β-lactoglobulin A was high (>90%) but that minor impurities could have crossed the skin. Moreover, no metabolism of the β-lactoglobulin A occurring in the skin could be detected. Therefore it could be suggested that native β-lactoglobulin A accumulated in the vicinity of Langerhans cells and other immune cells, making it possible to induce an immune reaction. It is very likely that low or very low amounts of proteins are enough to induce an immune response. Nevertheless, during the penetration of the β-lactoglobulin A through the skin, allergenic residues of β-lactoglobulin A could have been created and could have also accumulated in the vicinity of immune cells, thus inducing an immune response. Whatever the mechanism, the clinical results show a clear immune response in the skin when allergen is applied by APT (1,5,6,13,15Y17,31,32).

When developing a diagnostic test for allergy, it is important to consider the possibility of systemic delivery of allergen proteins through the skin, which could obviously induce a systemic anaphylactic response. From this point of view, the present study suggested that the passage of β-lactoglobulin A in native form through the skin was almost non-existent. Indeed, no percutaneous absorption leading to the transfer of allergen across the skin from the external environment as far as to the blood has ever been proved (33). Radioactive material from the patch was shown to cross rat skin over a 24-h period. However, the results obtained after acid precipitation of the receiver phase and by immunossay and electrophoresis indicate that this radioactivity did not originate from intact protein. Electrophoresis of the radioactive
mother solution showed that radioactivity in the receiver compartment could be attributed to low-molecular-weight impurities. Analysis by electrophoresis indicated that the radiolabeled solution of b-lactoglobulin A-[methyl-14C] was essentially composed of radiolabeled b-lactoglobulin A monomers and dimers accounting for 96% of the total of radiolabeled material. However, 4% of radiolabeled impurities

Fig. 4. b-Lactoglobulin A-[methyl-14C] detected in the 12 first layers of the stratum corneum after iterative stripping. These 12 strips allowed "b-lactoglobulin A-[methyl-14C] to be detected in the stratum corneum, which is the first layer of the epidermis and which was in contact with the radiolabeled "b-lactoglobulin (n = 3). The total amount of "b-lactoglobulin A-[methyl-14C] reached 100% at the end of the experiment after 24 h.

The absence of b-lactoglobulin A in native form in the receiver compartment is not unexpected because proteins, as large hydrophilic molecules, penetrate the skin barrier poorly. The topical delivery of IFN-α, a soluble protein with a molecular mass of about 20 kDa, similar to that of lactoglobulin, in different formulations has been followed (30). After 24 h, no IFN-α could be detected in the receiver compartment, whatever the formulation.

Concerning these first results on b-lactoglobulin A, it is important to bear in mind that this study, which could not be performed on infant’s skin for obvious ethical reasons, necessitated the use of an in vitro experimental diffusion model. Permeability data about infant’s skin are quite poorly documented and no specific in vitro models have been established. On one hand, porcine ear skin has been shown to be a valid model for the human skin barrier by comparing the permeability of marker molecules (34).

Furthermore, porcine ear skin can be differentially tapestipped to mimic the barrier properties of skin in premature neonates (35), known to have higher skin permeability than infants. On the other hand, Fasano et al. (36) have shown that, under infinite dose and occlusive conditions, rat skin permeability was higher than that of human skin. Therefore, as infant’s skin permeability is higher than adults’ skin permeability but lower than that of premature neonates, rat skin was preferred to tape-stripped porcine ear skin because of its high permeability compared with human skin under experimental conditions close to those adopted in this study (infinite dose and occlusive conditions). Therefore, the results were thought to be relevant to the clinical situation in infants.

To summarize, with the E-patch system, the native form of the b-lactoglobulin A could not cross hairless rat skin but putative degradation products of this protein may do so, probably because of their low molecular mass. Therefore, this suggests that only negligible amounts of residues of lactoglobulin A could cross hairless rat skin during the 24 h after the patch is applied.

b-Lactoglobulin A is a good marker of cow’s milk allergy. The study of this model protein demonstrates that the native form of b-lactoglobulin A could not cross the skin but concentrates in the vicinity of immune cells. This result is important because it suggests that the E-patch would probably not promote systemic delivery of intact proteins, thereby reducing the possibility of systemic anaphylactic shocks. This is in agreement with the fact that no incident of
anaphylactic shock has been described during clinical trials of APT (1,5,15,31).

Therefore, it seems that ideal conditions for promoting a topical response for a positive test to milk proteins are created. This study also gives a first appreciation of the efficacy and the safety of the E-patch system. Nevertheless, cow’s milk also contains other major proteins having similar or larger molecular masses: between 20 and 24 kDa for caseins, 14 kDa for α-lactalbumin, and 60 kDa for albumin. Their localization and transdermal delivery through hairless rat skin should be studied to complete our observations.

CONCLUSIONS

The E-patch system allows b-lactoglobulin A to concentrate in the stratum corneum, in the vicinity of Langerhans cells and other immunological cells, but does not lead to systemic delivery of intact b-lactoglobulin A. Therefore, it seems to create ideal conditions for milk allergens to promote a topical response for a positive diagnostic test with reduced risk of systemic anaphylactic reactions caused by the native form of the b-lactoglobulin A.

ACKNOWLEDGMENTS

This work was supported by the CNRS, UPS, INRACEA, and by DBV Technologies (Boulogne Billancourt, France). The authors would like to thank Dr. Michel Renoir (UMR CNRS 8612) for help with electrophoretic analysis of b-lactoglobulin purity and Dr. Herve Bernard (INRA) for immunodosage. ANRT supported D. Sourry (CIFRE).

REFERENCES

15. O. M. Kokki, K. Turjanmaa, and E. Isolauri. Differences in Skin Localization of Cow’s Milk Proteins 1535 skin-prick and patch-test reactivity are related to the heterogeneity...
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