

## Alternate vehicles for diagnostic patch testing: an update

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**Aim.** The aim of the present study was to review the literature subsequent to 2001 for recent information on alternate vehicles for diagnostic patch testing. Aim. Patch testing is a standard tool in dermatology used in particular in the diagnostic process of allergic contact dermatitis. While petrolatum is employed in most cases, the way vehicles can influence results may not be neglected. Alternate vehicles may clarify hitherto negative or doubtful results.

**Methods.** The authors searched the most important medical databases using as search terms "contact dermatitis", "patch test" and "vehicle".

**Results.** Data obtained by local lymph node assay and *in vitro* percutaneous absorption experiments suggest methods to improve penetration and immunologic response by either adding substances to petrolatum or replacing it altogether. Still, an adequate hypoirritant substitute for petrolatum remains to be discovered. In addition, one study reveals the lack of a general recommendation as to which quantity of petrolatum, and therefore dose, to apply. In the meantime, a negative or unclear patch test in a patient with allergen exposition and maybe even a history of contact dermatitis might be repeated using the scratch method, a higher allergen concentration or sodium lauryl sulphate either in the vehicle or as a control. The authors review the literature subsequent to 2001 for recent evolution of knowledge on vehicles.

**Conclusion.** Little conclusive research has been done on alternate vehicles in patch testing. However, the authors recognize some interesting tendencies as to either improve the character-

istics of petrolatum as a vehicle by adding substances that may heighten the immunologic response or replace it.

**KEYWORDS:** Skin - Vehicles - Petrolatum - Patch tests - Dermatitis, contact.

Patch testing, a standard tool in dermatology, is typically performed using petrolatum as a vehicle; alternate vehicles may confer advantages for certain substances. Generally, enhanced thermodynamic activity increases percutaneous absorption,<sup>1</sup> hence improving the solubility of a substance in its vehicle may render results in hitherto negative or unclear patch tests. In addition, alternate vehicles may be more accurate than petrolatum to measure into the patch.

Tanglertsampan *et al.*<sup>2</sup> and Lee *et al.*<sup>3</sup> summarized publications on alternate vehicles in patch testing until 1992 and 2002, respectively. The authors review the literature subsequent to 2001 for recent information.

### Materials and methods

The authors searched the medical databases Pubmed, Embase and the Science Citation Index using as search

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TABLE I.— *Recent publications on vehicles in patch testing.*

| Source                                   | Methods                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | Results                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              | Notes                 |
|------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| D'Arpino<br>2003 <sup>4</sup>            | Piroxicam was dissolved in five vehicles (propylene glycol, Transcutol P®, petrolatum and petrolatum + Transcutol P® 10%) to the point of obtaining a saturated solution. The investigation was performed in vitro using diffusion cells.                                                                                                                                                                                                                                                      | Piroxicam penetration through human skin was clearly enhanced by propylene glycol and petrolatum + Transcutol P 10%, whereas petrolatum alone inhibited the diffusion of piroxicam.                                                                                                                                                                                                                                                                                                                                  | Human <i>in vitro</i> |
| Chaudhari<br>2007 <sup>5</sup>           | Review of contact dermatitis due to ophthalmic allergens                                                                                                                                                                                                                                                                                                                                                                                                                                       | 15 new allergens have been reported and patch tested since their previous review. However, the manufacturers often fail to provide the necessary drug samples. Standardized ophthalmic trays for patch testing are required. Negative and doubtful patch tests might be repeated with the scratch method, an increased drug concentration or addition of sodium lauryl sulphate.                                                                                                                                     | Review                |
| Betts<br>2007 <sup>6</sup>               | Local lymph node assays (LLNA)                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | A 1:3 mixture of ethanol (EtOH) and diethyl phthalate (DEP) is frequently used in the assessment of the sensitization potential of fragrances. EtOH: DEP is a suitable vehicle in local lymph node assay compared to the usual vehicle, a mixture of 4:1 acetone and olive oil. EtOH: DEP did not elicit unwanted increases in background proliferative responses.                                                                                                                                                   | Mouse                 |
| Imai<br>2006 <sup>7</sup>                | Mice were sensitized to fluorescein isothiocyanate (FITC) dissolved in acetone, acetone/di-methyl phthalate, acetone/di-ethyl phthalate, acetone/di-n-propyl phthalate, acetone/di-butyl phthalate, acetone/di-(2-ethylhexyl) phthalate and acetone/di-isononyl phthalate 1:1. 24 hours later lymph nodes were pooled and examined for FITC-presenting cells via flowcytometry. 14 days later mice were challenged with a solution of FITC in acetone/di-butyl phthalate on the right auricle. | During the process of sensitization to FITC, di-n-propyl phthalate as well as di-butyl-phthalate exert strong adjuvant effects associated with enhancement of trafficking of antigen-presenting dendritic cells from the skin to draining lymph nodes. Extent of the effect was dependent on the length of the alkyl chain, and DPP was the strongest among those tested, the strongest therefore being carbon number 3 followed by carbon number 4. The shorter alkyl chains had some effect, the longer ones less. | Mouse                 |
| Bruze<br>2007 <sup>8</sup>               | Three samples each of patch tests prepared by three technicians were collected and weighed.                                                                                                                                                                                                                                                                                                                                                                                                    | The individual technician could keep the variation within a limited range, while the inter-individual variation was significant. This may be due to the lack of a general recommendation as to which quantity of petrolatum, and therefore dose, to apply.                                                                                                                                                                                                                                                           |                       |
| Schnuch<br>2006 <sup>9</sup>             | Retrospective analysis of the patch test data of the Information Network of Departments of Dermatology between 1992 and 2004.                                                                                                                                                                                                                                                                                                                                                                  | True allergic patch test reactions to white petrolatum are extremely rare and probably due to an individually increased susceptibility to allergens and/or irritants. This is in agreement with considering petrolatum as an almost non-sensitizer.                                                                                                                                                                                                                                                                  | Human <i>in vivo</i>  |
| Geier<br>2003 <sup>10</sup>              | Patch tests were performed using standard Finn Chambers on Scanpor® on 1600 patients. Sodium lauryl sulphate was employed at a concentration of 0.5% in aqueous solution.                                                                                                                                                                                                                                                                                                                      | Patch testing with the known irritant sodium lauryl sulphate facilitates the interpretation of patch test readings. In the presence of a positive reaction to SLS, macular erythematous test reactions to problematic allergens can be interpreted more confidently as irritant, especially if there is no history and no known exposure to the allergen.                                                                                                                                                            | Human <i>in vivo</i>  |
| Virgili et Corraza<br>2005 <sup>11</sup> | In patients with negative patch test reactions, pre-treatment of the skin with SLS 0.5% for 24 h was performed in the sites of patch tests with patients' own ophthalmic products in 15 selected patients.                                                                                                                                                                                                                                                                                     | In patients previously negative to their own products tested with conventional patch tests, SLS pre-treatment showed 6 new relevant positive reactions and induced a stronger positive reaction in 1 patient. SLS pre-treatment could be proposed as an alternative promising method, which may increase sensitivity of patch tests with patients' own products.                                                                                                                                                     | Human <i>in vivo</i>  |

terms “contact dermatitis”, “patch test\*” and “vehicle” for the period June 2001-July 2007.

## Results

Table I summarizes the results.

## Discussion

Since Lee *et al.*<sup>2</sup> published their review on vehicles in patch testing in 2002 few related studies have been published. Although the shortcomings of petrolatum, such as false negative results and inconsistent dosing due to inexact measurement, are widely recognized no substitute has been developed.

The *in vitro* experiments performed by d'Arpino *et al.*<sup>4</sup> and the research by Betts *et al.*<sup>6</sup> and Imai *et al.*<sup>7</sup> on enhancing results in the local lymph node assay suggest ways of possibly improving the outcome of patch testing and avoiding false negative results. This may be achieved by supplying petrolatum with additives such as Transcutol P (diethylene glycol monoethyl ether), sodium lauryl sulphate or phthalates.

The ultimate goal, however, should be to replace petrolatum with a substance that would offer better characteristics in terms of solubility and thermodynamic qualities and ease of measurement while remaining hypopirritant. As mentioned by Chaudhari,<sup>5</sup> ophthalmics especially may require a higher flux and penetration in order to render positive patch test results even with a positive history of allergic contact dermatitis.

Hansen's solubility parameters, “Aa user's handbook”,<sup>11</sup> provides further useful information on how ideal solubility in terms of thermodynamics can be achieved. Smith<sup>12</sup> provides still more suggestions on enhancing penetration.

In order to address the problem of dosing this substance ought to be a semi-solid which could be measured using micropipettes while not being as runny as an aqueous solution.

## Conclusions

Although little conclusive research has been done on alternate vehicles in patch testing, the authors recognize some interesting tendencies as to either improve

the characteristics of petrolatum as a vehicle by adding substances that may heighten the immunologic response or replace it.

In the meantime, petrolatum is an adequate standard although technicians still lack an official recommendation as to which quantity of a given allergen suspension to apply, an issue that ought to be resolved.<sup>7, 13</sup>

In any case a negative or unclear patch test in a patient with allergen exposition and maybe even a history of contact dermatitis might be repeated using the scratch method, a higher allergen concentration or sodium laureth sulfate either in the vehicle or as a control.

## Riassunto

*Veicoli alternativi per il patch test diagnostico: un aggiornamento*

**Obiettivo.** L'obiettivo di questo studio è stato quello di rivedere la letteratura scientifica successiva al 2001 per reperire informazioni recenti sui veicoli alternativi per il patch test diagnostico. Il patch test è uno strumento standard utilizzato in dermatologia, in particolare durante il processo diagnostico delle dermatiti allergiche da contatto. Nella maggior parte dei casi viene impiegato come veicolo il petrolatum, ed il modo in cui i veicoli possono influenzare i risultati non deve essere trascurato. I veicoli alternativi possono chiarire risultati sinora negativi o dubbi.

**Metodi.** Gli autori hanno condotto la ricerca consultando i principali database medici, utilizzando come parole chiave i termini “dermatite da contatto”, “patch test” e “veicolo”.

**Risultati.** I dati ottenuti con la valutazione linfonodale locale e con gli esperimenti di assorbimento percutaneo *in vitro* suggeriscono metodi per migliorare la penetrazione e la risposta immunologica, aggiungendo delle sostanze al petrolatum o sostituendolo del tutto. Al momento, tuttavia, non è ancora stato scoperto un adeguato sostituto ipopirritante del petrolatum e, di conseguenza, la dose da applicare. Nel frattempo un patch test negativo o dubbio in un paziente con esposizione ad allergeni e probabilmente con un'anamnesi positiva per dermatite da contatto potrebbe essere ripetuto utilizzando il metodo della scarificazione, con una concentrazione maggiore di allergene o di sodio lauril solfato o nel veicolo o come controllo. Gli autori rivedono la letteratura scientifica successiva al 2001 alla ricerca di recenti evoluzioni sui veicoli alternativi.

**Conclusione.** Sono state concluse poche ricerche sui veicoli alternativi per il patch test. Tuttavia, gli autori individuano alcune tendenze interessanti per migliorare le caratteristiche del petrolatum come veicolo attraverso l'aggiunta di sostanze che possono incrementare la risposta immunologica o sostituirla.

PAROLE CHIAVE: Cute - Patch tests - Dermatiti da contatto.

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### Vaccination strategies based on the mimotope concept

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Specific immunotherapies are in broad use for many diseases like allergies, cancer, autoimmune diseases or parasitic infections. Although clinical trials show successful application of these therapies, several disadvantages hinder the complete success. High production costs and repeated administrations represent the practical problems, while the possibly occurring side effects are the therapeutic troubles. To avoid these problems, the target specificity should be considered more intensely. Epitopes, the particular parts of antigens/allergens where they bind specific antibodies, are useful targets. To generate an epitope-specific vaccination, mimotopes can be identified via the biopanning technology. Mimotopes are small peptides mimicking the epitopes in the structural as well as in the immunological point of view. They are able to induce antigen-specific antibodies in active immunization form. These antibodies are directed against the natural antigen/allergen, and therefore they are able to block the outbreak of the diseases. Current research focuses on the development of mimotopes to achieve an epitope-specific induction of blocking antibodies, e.g. for allergy treatment. In cancer therapy, studies with mimotopes show successful interference with tumor cell growth in immunizations of mice. Also in the case of autoimmune diseases and parasitic infections this method was applied, targeting different molecules, which are key mediators in the disease mechanisms. Through the mimotope treatment via the specific antibody production, the disease symptoms could be hampered. This review gives an overview of the use of the mimotope concept and also of related therapeutic trials for the treatment of allergy, cancer, autoimmune and infectious diseases.

**KEY WORDS:** Vaccination - Allergy and immunology - Immunotherapy - Epitopes.

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#### Current immunotherapies

##### *Current treatment of allergy*

To date, allergen-specific immunotherapy (SIT) is the only specific and curative approach for IgE-mediated hypersensitivity. The most commonly used immunotherapy is the hyposensitization, already successfully applied against mite, birch and grass pollen,<sup>1</sup> and *Hymenoptera* venom<sup>2</sup> allergies. During this treatment, allergen extracts are applied in increasing doses. This means that specific immunotherapy is the confrontation of a hypersensitive organism with the disease eliciting allergen, and therefore bears the risk of side effects, such as large local symptoms, anaphylactic reactions or even death.<sup>3-6</sup> In addition, using the whole allergen extract, there is the possibility of new IgE induction against irrelevant components in the extract. This phenomenon was observed previously, when *de novo* IgE responses were observed not only against irrelevant allergens, but also against irrelevant epitopes in grass pollen immunotherapy.<sup>7, 8</sup> These data were confirmed by Moverare *et al.*, who reported the development of new IgE reactivities using birch pollen extract in specific immunotherapy.<sup>9</sup> For all these rea-

TABLE I.—*Examples of monoclonal antibodies, which are clinically applied for the treatment of different cancer types or autoimmune diseases.*

| Name        | Disease                                         | Targets                            |
|-------------|-------------------------------------------------|------------------------------------|
| Rituximab   | Non-Hodgkin lymphoma                            | CD20 on B-lymphocytes              |
| Trastuzumab | Breast cancer                                   | HER2 receptor                      |
| Alemtuzumab | Chronic lymphocytic leukaemia (CLL)             | CD52 on T- and B-lymphocytes       |
| Cetuximab   | Colorectal cancer<br>Head and neck cancers      | Epidermal growth factor-receptor   |
| Bevacizumab | Colorectal cancer<br>Non-small cell lung cancer | Vascular endothelial growth factor |
| Panitumumab | Colorectal cancer                               | Epidermal growth factor-receptor   |
| Infliximab  | Rheumatoid arthritis<br>Crohn's disease         | TNF- $\alpha$                      |
| Etanercept  | Rheumatoid arthritis                            | TNF- $\alpha$ and TNF- $\beta$     |

sons, trials are going on to improve safety and to avoid potential hazardous side effects during these specific immunotherapies.

Several laboratories started to use DNA technology to create recombinant allergens <sup>10</sup> or hypoallergenic allergen derivatives, where point mutations or site-directed mutagenesis <sup>11</sup> have been applied. A different approach of immunotherapy is based on the application of peptides lacking B-cell epitopes with simultaneous preservation of T-cell epitopes of allergens. Clinical studies were already performed using this alternative for Fel d 1 from cat <sup>12</sup>, and Api m 1, the been venom allergen.<sup>13</sup> These peptides had been screened for reduced IgE-crosslinking activity in basophil histamine release assays, but could still induce numerous late phase adverse reactions occurring minutes to hours after the application of the peptide due to the booster of allergen-specific T-cells.<sup>14</sup>

As recombinant and hypoallergenic allergens still possess the whole protein structure, similar to the natural one, they also present other epitopes maintaining the possibility for novel IgE antibody production. Therefore, the most promising solution for this problem would be an epitope-specific treatment.

*Current treatment of cancer and autoimmune diseases*

Monoclonal antibodies (mAb) are essential and successfully applied in treatment of cancer and autoimmune diseases.

After the development of the hybridoma technology by Köhler and Milstein in 1975,<sup>15</sup> a number of tumor-specific antibodies have been approved and

eight anti-cancer mAbs have been ratified by the Food and Drug Administration (FDA).

Autoimmune diseases are the results of the breakdown of the mechanism normally responsible for maintaining self-tolerance in B-cells, T-cells or both, involving pathological changes through humoral as well as cellular-mediated mechanisms. Novel therapies focus on the interaction of the co-stimulatory signals, where monoclonal antibodies block the receptor or the ligands, or delete autoreactive lymphocytes and block the progression of autoimmune diseases.

Table I provides a list of the most important monoclonal antibodies clinically applied against cancer and autoimmune diseases.

Although wide research is going on to develop better and more specific monoclonal antibodies with optimized therapeutic strategies, there are still some limitations in the daily practice. One is the high required amount of the applied antibodies, which accounts <sup>5</sup> for the high productions costs. A related problem is the repeated administration of the therapy, which is an economic problem as well as a physiological stress for the patients. To overcome these problems, a more effective strategy needs to be generated and will most probably be found in an active (instead of a passive) immunotherapeutic treatment.

**Future prospect:  
epitope-specific immunotherapy with mimotopes**

A very promising, alternative strategy for all the above described therapies may be the epitope-specific immunotherapy. This strategy aims to direct the immune

response solely towards structures relevant for antibody recognition, *i.e.* the epitope of an antigen/allergen. Based on the exclusive epitope-specificity, this therapy reduces the unwanted side-effects. Also, the production costs can be lower, compared with the available possibilities for passive immunotherapies.

Theoretically as well as technically a very direct approach to identify epitopes is the generation of mimotopes,<sup>16</sup> which are either small peptides or Fab molecules mimicking structurally and also biologically the epitopes of the antigens/allergens.

### Phage display – peptide mimotopes

Phage display libraries consist of filamentous bacteriophages, where the minor coat protein pIII or the major coat protein pVIII of the phage particle are used for inserting foreign or random peptide sequences. With the random peptide sequence inserted in the gene 3 (g3) section of the phage genome, 3-5 copies of the peptide will be presented on the phage surface (Figure 1A), located in the pIII minor protein at one end of the filamentous phage particle.<sup>17</sup> Inserting the random peptide sequence in gene 8 (g8), multiple presentation can be achieved (up to 2 700 copies). Different libraries may carry diverse lengths of the represented peptides starting from 6 to 25 amino acids.<sup>18-21</sup> The structural presentation of peptides can be either linear or circular. For circular peptides, cysteine residues are inserted at both ends of the foreign sequence, causing a disulfid bond between the two cysteine residues, thereby forming a cyclic peptide. Peptide libraries can carry inserts in high diversity with up to 10<sup>9</sup> different peptides,<sup>22</sup> where each phage particle presents a certain peptide on the surface.

### Phage display - Fab-mimotopes

An alternative approach for the epitope-specific immunotherapy is based on the network hypothesis from Niels Kaj Jerne. He proposed the network theory in 1974: the immune system responds to a given antigen besides the production of antibodies (Ab1) with the appearance of anti-idiotypic antibodies (Ab2).<sup>23</sup> He suggested a fraction of these anti-idiotypic antibodies (*i.e.* anti-Ids) to be anti-paratopic and to mimic the antigen, presenting “internal images” of the antigen's epitope (*i.e.* Ab2 $\beta$ ). Moreover, upon immunizations with anti-Ids, anti-anti-Ids may be generated (Ab3). They are again directed towards the initial anti-

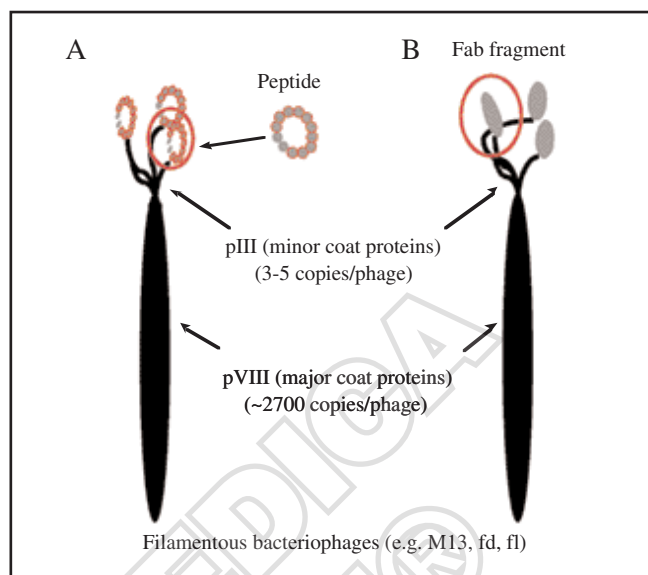


Figure 1.—Schematic presentation of the two different phage display libraries. Panel A shows the random peptide library, where each phage clone represents a different peptide (small rings) on the pIII minor coat protein. In panel B, the grey ovals symbolize the inserted heavy and light chain PCR products of the Fab parts, expressed also on pIII.

gen, thereby possibly potentiating a protective immune response.<sup>24, 25</sup> In Fab-libraries, the heavy and light chain PCR products, derived from RNA of the B-cell repertoire of allergic individuals, are inserted into the phagemid vector (Figure 1B), containing also the pIII minor coat protein of the filamentous bacteriophage.<sup>26</sup>

### Specific antibodies

For the mimotope selection with the help of biopanning, the required partner is the specific antibody for the desired or targeted antigen/allergen.

To select mimotopes for cancer or autoimmune diseases, specific antibodies already used in the daily therapy can be perfect tools. In the case of allergic diseases, there is a need of screening, high purification and concentration of allergen-specific IgE antibodies from human allergic patients' sera. This can be done by using affinity chromatography columns, coupled with the respective allergen. These allergen-specific antibodies, purified from allergic patients' sera, or monoclonal antibodies towards tumor antigen, virus antigen or autoimmune disease antigens, recognize the structural epitopes of the natural antigens and can be used as selection tools.

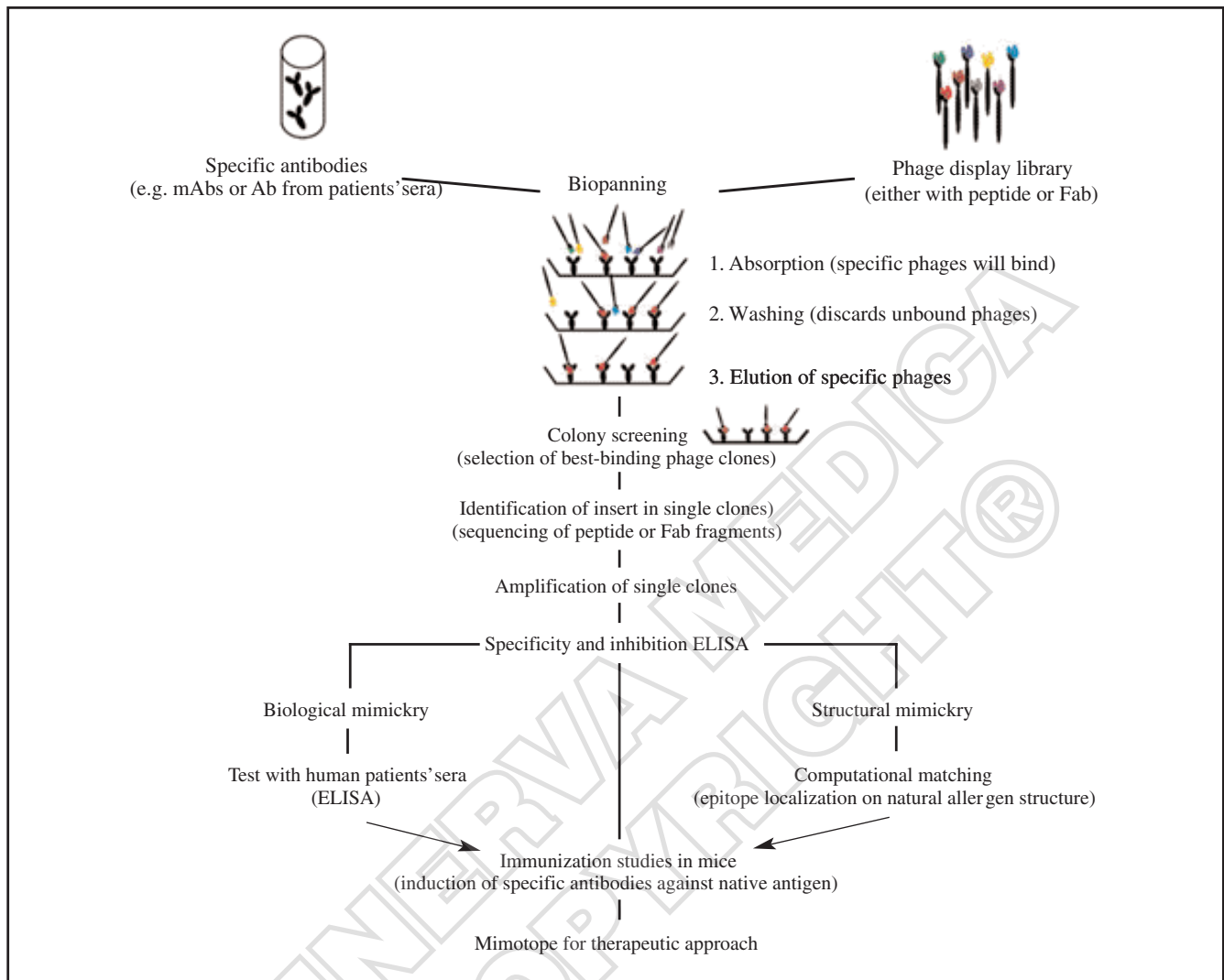


Figure 2.—The most important evaluation steps of the mimotope approach. After the proof of specificity, antibodies are applied with phage libraries in biopanning for the generation of mimotopes. Followed by colony screening, specificity and inhibition ELISAs are required together with human patients' sera test for the extensive mimotope selection. To examine the structural mimicry of the mimotopes computational matching can be used, which is also a further step affirming the selection of the best mimotope. Immunization studies in mice provide the final proof of the mimicking potential. The induced blocking antibodies are directed against the natural antigen with appropriateness for therapeutic trials of allergy, cancer, autoimmune or infectious diseases.

### Biopanning

As already mentioned, phage display libraries present billions of different candidates for the specific binding. To select from this huge repertoire the one which mimics best the epitope of the antigen/allergen, the biopanning procedure is applied. In this method, antigen-specific antibodies are used to screen the phage library. Allergen- or antigen-specific anti-

bodies are coated on microtiter plates, and are incubated with the original library, containing the high diversity of the phages presenting the different peptides or Fabs. During the procedure (Figure 2), phages carrying the specific peptides toward the antibody will bind, while unspecific phages will be washed away. Bound phages can be eluted either by acidic pH or by competitive elution with the native antigen. There-

after, they will be amplified and used for the next biopanning round.

To get the most specific mimotopes with high and specific binding ability to the antibodies, biopanning rounds with antigen-specific antibodies are repeated several times to amplify the best binding partners.

### *Selection of clones*

To verify the success of the biopanning, each round is tested by specificity ELISA, where the quantity of bound specific phages is monitored within the rounds using unspecific isotyp as controls. An increase in the number of specific phage particles from round to round gives a hint for the successful selection and amplification of specific phages. Single phage clones can therefore be screened from the amplification of the last biopanning round with the panning antibody. To prove the biological relevance of the finally selected (*i.e.* highest binding of a single clone) mimotopes, inhibition studies and tests with human allergic patients' sera are required.

Keeping the background information of the network hypothesis in mind, the above described biopanning procedure can also be performed with combinatorial Fab-antibody libraries. Upon selection with an allergen specific antibody (Ab1), anti-idiotypic Fab-fragments (Ab2) can be identified. If Ab1 is allergen-specific IgE, the selected Ab2 should be a mimic of the IgE epitope.

### *Epitope-localization with mimotopes*

To point out the structural mimicry of the mimotopes, and also to improve the selection criteria, computational matching can give an accurate answer about the structural characteristics of the epitope on the allergen. In a software program, biochemical and structural features of the antigens/allergens are compared with the amino acids of the mimotopes. The best fitting mimotopes with the highest superimpositions are given as the best-matching mimotope areas on the antigens/allergens. Extrapolating the 3D-matched amino acids on the primary sequence of the allergens, the discontinuity of B-cell epitopes can be confirmed, since amino acids separated on the primary sequence come together on the surface and form the epitope of the allergen.

### *Molecular mimicry*

After the extensive selection of mimotope clones, immunization studies are carried out with peptide

mimotopes using carrier molecules, which make the small peptides more immunogenic. The most often used immunogenic carriers are keyhole limpet hemocyanin (KLH), tetanus toxoid (TT) and albumin binding protein (ABP). The immunological relevance can be confirmed, immunizing BALB/c mice with mimotope-carrier complexes. Specific mimotopes induce antibodies, which recognize the natural antigens/allergens. This can be examined by ELISA or in immunohistochemistry. The functional mimicry can be proven by inhibition studies or by antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) assays.

## **Mimotope-based vaccinations**

### *Advantages of mimotopes*

There are several advantages of synthetic mimotopes which make them attractive tools for vaccination strategies:

- since mimotopes are epitope-specific, the application of them avoids the induction of antibodies against undesired epitopes of antigens/allergens;
- they can be synthesized easily, therefore the productions costs are much lower then for the production of monoclonal antibodies;
- the synthesized peptides/Fabs are free from any infectious materials or toxins, therefore the usage of them is safe;
- to improve their immunogenicity they can be coupled to immunogenic carries.

### *Mimotopes in allergology*

In our research group at the Department of Pathophysiology of the Medical University of Vienna, one of the major research topics is the characterization of B-cell epitopes by generating peptide- and Fab-mimotopes of clinically relevant allergens. After successful proof of structural as well as biological relevance of them, they are applied in mouse models of allergy, to verify the therapeutic effect on allergic symptoms.

Peptide mimotopes were already generated by us for respiratory allergens such as birch pollen allergen Bet v 1<sup>27, 28</sup> and grass pollen allergen Phl p 5,<sup>29</sup> Der p 1 and Der p 2, the two major allergens from house dust mite,<sup>30</sup> for pan-allergen profilin<sup>31</sup> and for food allergens such as parvalbumin.<sup>32</sup>

After critical selection of mimotopes, using the above mentioned computational matching, the authors could demonstrate that B-cell epitopes are conformational and discontinuous in structure.<sup>30</sup> Mimotopes were 3D-matched on the structure of the natural allergen, where these amino acids identified separate parts in the primary sequence of the allergen, forming together a predominant discontinuous epitope patch. This finding has an important impact on cross-reactive allergens. Allergens belonging to one allergen group but derived from different species, like group 1 and 2 allergens in house dust mites, share high amino acid sequence and also structural homology. Our hypothesis based on this fact is that also B-cell epitopes may share high similarity within the group. This was confirmed by us, continuing the idea with house dust mite allergens. The amino acids matched with mimotopes are in the conserved regions of the allergens. Localizing the identified 3D-matched amino acids on the surface of other allergens, structurally highly similar group-specific epitopes could be detected.<sup>30</sup> Based on the epitope homology or cross-reactivity, the generated mimotopes could be used for epitope-specific immunotherapy for a whole allergen group.

For the proof of biological relevance, immunizations of BALB/c mice were performed and indeed could induce allergen-specific antibodies.<sup>27, 29</sup> The produced antibodies were further identified as blocking antibodies.<sup>33</sup>

Since the above mentioned selected mimotopes are short peptides with 9 amino acids, there is a need for a carrier molecule, for example keyhole limpet hemocyanin (KLH) to form complete antigens for immunization. Our hypothesis was that possibly by the selection of large mimicking structures the necessity of a carrier could be avoided, rendering even more specific and stronger immune responses. Therefore, we produced Fab-mimotopes of Phl p 5,<sup>35</sup> simultaneously being anti-idiotypes to allergen-specific IgE. The phage library used had been constructed from the IgG heavy and light chain PCR derived from mRNA of peripheral blood mononuclear cells (PBMCs) of patients allergic to grass pollen, and was used here for biopanning using Phl p 5-specific antibodies. The hypervariable regions of selected Fab-clones revealed partial sequence homology with solvent accessible antigenic sites of Phl p 5. Phagemid DNA derived from the phage clones was used to produce two different soluble recombinant anti-idiotypic Fab clones in *E. coli*. As a proof

of molecular mimicry, both Fabs induced anti-Phl p 5a-specific antibodies in immunization studies of BALB/c mice.<sup>34</sup>

Applying Phl p 5-mimotopes in a murine model of allergic asthma, the reduction of Th2 cytokines (IL-4, IL-5), the prevention of eosinophil infiltration and the drop of mucus production could be observed (Wallmann *et al.*, manuscript submitted).

Summarizing our results from mimotopes and continuing the examination of the mimicry and safety of our mimotopes, we strongly suggest their application in synthetic form also in human allergic patients.

### *Mimotopes in cancer therapy*

Since biopanning is a very powerful and easy handling technique, also research groups focusing on cancer immunotherapy successfully turned to this method.

Tumor-associated carbohydrate antigens are considered important targets in efforts to develop cancer vaccines, also for generating mimotopes. In the past decade, peptide mimotopes have been generated for several important sugar moieties, which are tumor antigens, such as Lewis Y,<sup>35</sup> sialyl-Lewis X,<sup>36</sup> the ganglioside antigens GD2<sup>35, 37</sup> and GD3.<sup>38</sup> Selected mimotopes have successfully been used in immunizations in peptide as well as in DNA form.<sup>35, 39-42</sup> Applying them in prophylactic or therapeutic studies, the induced immune response inhibited effectively the in vitro and in vivo growth of tumor cells expressing the corresponding target antigens, like GD2 and GD3.<sup>43</sup>

Another attractive target antigen in mimotope cancer immunotherapy is the high-molecular weight melanoma-associated antigen (HMW-MAA), with high frequency of expression in patients with melanoma. Panning experiments have been done with the anti-HMW-MAA monoclonal antibody (mAb) 225.28S also in the authors' research group.<sup>44</sup> This antibody mediates ADCC and has already been used for anti-idiotypic therapy trials in humans. Selected nonamer peptide mimotopes against HMW-MAA fused to albumin binding protein (ABP) as carrier showed immunogenic properties in immunization studies of BALB/c mice. These anti-mimotope antibodies recognized HMW-MAA of human melanoma cells, while sera of mice immunized with the carrier alone did not show any reaction.<sup>44</sup> In parallel, Wagner *et al.* performed a biopanning with the same antibody and library. The selected peptide was coupled to tetanus toxoid as a carrier. This group could also show the induc-

tion of HMW-MAA-specific antibodies, which inhibited the *in vitro* growth of a melanoma cell line up to 62%.<sup>45</sup> These highly concurrent studies show independently the relevance of this antigen in melanoma cancer and the efficacy of the mimotope therapy.

Since the daily medication practice uses monoclonal antibodies against growth factor receptors to hinder the signaling pathway, growth factor receptors have come also in the focus of mimotope approach as possible target molecules. The authors' group has focused on important growth factor receptors, using the clinically applied monoclonal antibodies cetuximab (recognizing epidermal growth factor receptor, EGFR) and trastuzumab (targeting human epidermal growth factor receptor 2, HER2).

Performing the biopanning with cetuximab, two mimotopes could be identified. One of them had higher binding capacity to the specific antibody and also induced antibodies more effectively, inhibiting better the cell growth of EGFR-overexpressing cells. However, testing both mimotopes for the biological activity in ADCC and CDC assays, the mimotope-induced antibodies showed equal potential.<sup>46</sup>

Focusing the mimotope approach onto HER2, biopanning experiments with trastuzumab resulted in 5 highly reactive clones, presenting cyclic decamer peptides.<sup>47</sup> Testing the immunogenicity of them by immunization of BALB/c mice coupled to tetanus toxoid, the induced antibodies recognized HER2 on SK-BR3 cell surface, which overexpress the antigen. Moreover, internalization studies confirmed the antitumor activity of the induced antibodies. Similarly as by trastuzumab, the receptor-antibody complex was internalized and moved into endocytic vesicles and accelerated degradation could be observed. To define the molecular binding sites, a computational algorithm was used, where mimotopes were matched on the surface of the antigen molecule HER2, and resulted in the localization of the known trastuzumab epitope.<sup>48</sup>

#### *Mimotopes in autoimmune diseases*

The biopanning technology has also been employed to study human autoimmune diseases, including rheumatoid arthritis,<sup>49</sup> multiple sclerosis,<sup>50</sup> autoimmune thrombocytopenia purpura<sup>51</sup> and autoimmune inner ear disorders, such as Cogan's syndrome.<sup>52</sup>

The major target molecules here are autoantigens. In the work of Gevorkian *et al.*, GPIIb/IIIa, the human platelet glycoprotein complex, was used as experi-

mental object for autoimmune thrombocytopenia purpura.<sup>51</sup> The glutamic acid decarboxylase (GAD65), major autoantigen of diabetes<sup>53</sup> and the islet tyrosine phosphatase-like protein IA-2/ICA512bdc, major autoantigen in type 1 diabetes<sup>54</sup> have also come into the interest of mimotope generation. With selected mimotopes the characterization of structural epitopes could be reached, and further, these mimotopes are possibilities for therapy application via induction of blocking antibodies.

An alternative option to treat autoimmune diseases is the intervention into several steps of the mechanism. One of the most important molecules is TNF- $\alpha$ , which plays a central role in infection, inflammation and autoimmune disease. Its functions are mediated by binding to the high affinity cell surface receptors. A potential approach to modulate excessive levels of serum TNF- $\alpha$  is the usage of soluble receptors as blocking molecules. To overcome the already mentioned problems of monoclonal antibody production, mimotopes were generated mimicking the binding sites of TNF- $\alpha$  receptor. The identified mimotope was able to inhibit TNF- $\alpha$ -mediated cytotoxicity in a mouse model and in a human cell line in a dose-dependent fashion. Furthermore, antibodies induced by mimotope immunization recognized the recombinant human TNF- $\alpha$  receptor, therefore hindering the interaction between TNF- $\alpha$  and its receptor.

Since the majority of human B-cell lineage malignancies express CD20,<sup>55</sup> a nonglycosylated phosphoprotein, this protein can be a target for immunotherapy of B-cell lymphomas. Rituximab (Rituxan® IDEC-C2B8) is already used as monoclonal antibody in the treatment of non-Hodgkin's lymphoma.<sup>56, 57</sup> Based on the high clinical importance of this antibody, but unfortunately with possible side-effects, several groups have focused on the generation of mimotopes with this antibody.<sup>58, 59</sup> Selected mimotopes induced high titers of specific antibodies, recognizing the native CD20. Therefore, these mimotopes have the potential to induce antibodies with the same specificity as rituximab in an active immunization.

#### *Mimotopes in infectious diseases*

Viruses, bacteria, fungal and parasitic infections affect a high percentage of the population around the world. As an alternative therapeutic approach, mimotopes were generated to define epitopes also for parasitic infectious diseases. Intensive investigations show

TABLE II.—List of generated mimotopes, targeting pathogenic antigens.

| Species name                    | Selecting antibody/<br>target molecule | References |
|---------------------------------|----------------------------------------|------------|
| <b>Bacteria</b>                 |                                        |            |
| <i>Streptococcus pneumoniae</i> | mAb T15                                | 61         |
| <i>Clamydophila pneumoniae</i>  | Patients' sera                         | 62         |
| <i>Salmonella typhimurium</i>   | Lipopolysaccharide                     | 63         |
| <i>Mycobacterium leprae</i>     | Phenolic glycolipid-I/III603.8         | 64         |
| <b>Viruses</b>                  |                                        |            |
| Hepatitis-A virus               | Patients' sera                         | 65         |
| Hepatitis-B virus               | Patients' sera                         | 66         |
|                                 | mAb 18/7                               | 67         |
| Hepatitis-C virus               | Patients' sera                         | 68, 69     |
| Respiratory syncytial virus     | mAb Mab-19                             | 70         |
| HIV                             | Patients' sera                         | 71, 72     |
| Japanese encephalitis virus     | mAb 2H2                                | 73         |
| <b>Parasites</b>                |                                        |            |
| <i>Plasmodium vivax</i>         | mAb D14-3                              | 74, 75     |
| <i>Plasmodium falciparum</i>    | mAb 18/2                               | 76         |
| <b>Fungi</b>                    |                                        |            |
| <i>Cryptococcus neoformans</i>  | Mouse monoclonal antibody              | 77, 78     |

the importance of this technical approach, since in the case of some common infectious agents (HIV, *Hepatitis-B*, *-C*), parallel research is going on. Table II gives an overview of identified mimotopes with the respective selecting antibodies and references.

## Conclusions

As the above presented literature summarizes, serious investigations focus on the development and generation of mimotopes in many therapeutic fields of medicine.

These "small" peptides have "big" advantages compared to the daily therapeutic strategies with monoclonal antibodies or with whole allergen extracts.<sup>60</sup> The most important positive aspects of them are the lower costs and the avoidance of unwanted side-effects. These are critical points, since they are most relevant for patients, who are suffering from serious diseases. The development of a potential vaccine necessitates the extensive selection of the specific mimotope (without cross-reactivity) as well as the selection of the type of the vaccine and the vaccination route.

The successful results from the different application fields strongly suggest that mimotopes are promising candidates for specific therapy of allergy, cancer, autoimmune as well as for infectious diseases.

## Riassunto

### Strategie di vaccinazione basate sul concetto di mimotopo

Specifiche immunoterapie sono utilizzate ampiamente per molte patologie quali allergie, cancro, patologie autoimmuni o infezioni parassitarie. Sebbene trials clinici dimostrino buoni esiti di tali terapie, diversi svantaggi ne impediscono un risultato completo. Alti costi di produzione e somministrazioni ripetute rappresentano problemi pratici, mentre i possibili effetti collaterali rappresentano i problemi terapeutici. Per evitare ciò, occorrerebbe pensare di più alla specificità del bersaglio. Epitopi, le parti specifiche di antigeni/allergeni dove essi si legano a specifici anticorpi, sono target utili. Per generare una vaccinazione epitopo-specifica, mimotopi possono essere identificati tramite tecnologia di "biopanning". I mimotopi sono piccoli peptidi che mimano gli epitopi nella struttura come pure dal punto di vista immunologico. Essi sono in grado di indurre anticorpi antigene-specifici in forma di immunizzazione attiva. Questi anticorpi sono diretti contro i naturali antigeni/allergeni e pertanto sono in grado di bloccare l'esplosione delle malattie. Attuali ricerche si concentrano sullo sviluppo di mimotopi per determinare un'induzione epitopo-specifica di anticorpi, per esempio nel trattamento delle allergie. Nella terapia del cancro, studi con mimotopi hanno mostrato un buon successo nell'interferenza con la crescita delle cellule neoplastiche nell'immunizzazione di topi. Anche nel caso di patologie autoimmuni e infezioni parassitarie è stato applicato questo metodo, avendo come target differenti molecole, mediatori chiave nei meccanismi patogenetici. Tramite il trattamento con mimotopi attraverso la produzione di specifici anticorpi, si potrebbe impedire l'insorgenza dei sintomi. Questa review fornisce una visione generale sull'impiego del concetto di mimotopo e su relativi trial di trattamento per quanto riguarda allergie, cancro, patologie autoimmuni e infettive.

PAROLE CHIAVE: Vaccini - Allergie - Immunologia - Immunoterapia - Epitopi.

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## A possible role for vaccination in the treatment of psoriasis?

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Psoriasis is a multifactorial immune skin disease whose etiology involves a strong genetic component, involving several genes encoding proteins involved in epidermal differentiation and immune, inflammatory and pathogen responses, in combination with microbial environmental factors. Although various microorganisms appear to provoke or aggravate the disease, including *Staphylococcus aureus*, *Malassezia* and *Candida albicans*, the association between *S. pyogenes* throat infections and guttate psoriasis is supported by the strongest clinical evidence. Furthermore, the identification of peptidoglycan-specific T cells in psoriatic skin lesions has led to the proposal that cell wall peptidoglycan may mediate the link between streptococcal infection in the tonsils and the subsequent induction of skin lesions. These findings suggest that psoriasis may be a possible candidate for therapeutic streptococcal vaccination. Current treatments for psoriasis have several limitations including toxicity and an increased risk of infection and malignancy. In contrast, vaccination could potentially induce long-term tolerance without the side effects caused by global immunosuppression. Future research will need to address the identity of the triggering microbial antigen(s); such knowledge could open the way for vaccination as a therapeutic tool for psoriasis.

**KEY WORDS:** Psoriasis - Vaccination - Streptococcal vaccines.

Psoriasis is a common skin disease that occurs worldwide but the highest incidence, 2-3%, is found in the Northern European countries. In the majority of individuals the disease presents as a few red scaly patches, but in a small number the skin involvement is extensive. Why some patients have extensive

and others minor involvement is not yet known. Psoriasis tends to be a persistent disease and its severity varies among patients. In addition, in approximately 10% of patients psoriasis is associated with an arthropathy. Although psoriasis itself has an extremely low mortality, its appearance causes psychological and social problems and several studies have shown that it affects the quality of life.

Histologically, the epidermis of a psoriasis lesion is characterised by hyperplasia of the keratinocytes, a poorly formed or absent granular layer and parakeratosis of the stratum corneum. These features are a result of increased mitosis of the epidermal basal cells and a failure of keratinocyte maturation, and are associated with increased numbers of activated epidermal CD4<sup>+</sup> T lymphocytes and Langerhans cells. In the dermis, a marked infiltrate consisting of predominately CD4<sup>+</sup> T cells, dendritic cells and macrophages is present, surrounding capillaries that are characteristically increased in size and tortuosity. These observations, together with various pieces of further evidence including the effectiveness of immunosuppressive treatments in psoriasis, the conversion of non-involved to lesional psoriatic skin in a mouse xenograft model after

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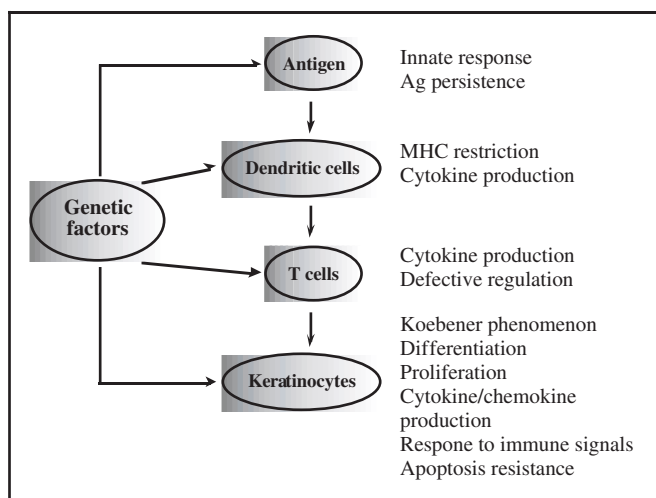


Figure 1.—Proposed sites affected by genetic factors in the psoriasis pathogenic pathway.

injection of CD4<sup>+</sup> T cells, and the effects of supernatants from skin T cell clones on keratinocyte proliferation, support a pivotal role for T cells in psoriasis pathogenesis.<sup>1</sup> Cytokines produced by activated T cells are likely to be responsible for the hyperproliferation of the epidermal layer. However, the nature of the antigen that activates T cells in psoriatic skin has yet to be determined.

Current immunosuppressive treatments, which include topical, systemic and ultraviolet light regimens, vary in their effectiveness and side effects. Furthermore, generally they do not alter the course or severity of the disease. Topical preparations are often ineffective in clearing the disease and exhibit tachyphylaxis, whilst the mostly effective ultraviolet light regimens are time-consuming for the patient who may have to travel long distances to obtain treatment. Systemic immunosuppressive drugs, such as cyclosporin and methotrexate, are also effective but have long term side effects, *e.g.* hepatotoxicity (methotrexate) and nephrotoxicity (cyclosporin). The latest treatments, termed “biological agents”, work by blocking specific immune pathways and cytokines implicated in psoriasis pathogenesis. Immunosuppression, particularly long term, induced by these various treatments is associated with an increased incidence of malignancy, as observed in immunosuppressed organ transplant patients. Thus there is a need for effective, long-lasting and safer treatments than are currently available. Vaccination is a possible therapeutic approach that

could fill these criteria. The evidence supporting the possible use of vaccination as a novel treatment for psoriasis is presented in this review.

## Etiology

The etiology of psoriasis involves both a strong genetic component, encompassing several genes encoding proteins involved in epidermal differentiation and immune, inflammatory and pathogen responses (Figure 1), and environmental factors, the most convincing evidence being for haemolytic streptococci.

In any discussion on the etiology of psoriasis it should be stressed that there are two distinct patterns of the disease, guttate (GP) and chronic plaque (CPP) psoriasis. GP is characterised by a sudden eruption of small red scaly papules on the trunk (and limbs), which, in two-thirds of patients, is associated with a preceding  $\beta$  hemolytic throat infection. New lesions may continue to develop for one month, remain for a second month and then gradually fade during the third month. In contrast, CPP occurs most commonly on the extensor elbows, knees and scalp and may either resolve spontaneously, remain the same for years, or gradually increase in size with the appearance of new lesions, sometimes coalescing to involve all the skin. Acute GP flares may occur in patients with stable CPP, often resolving after 3 months whilst the plaques remain stable. Although the two patterns of psoriasis are undoubtedly related, their different clinical features suggest that the etio-pathogenetic factors may be different.

## Psoriasis is an polygenic disease

It has been known for many years that genetic factors are important in psoriasis, following family and twin studies. Some studies have suggested that psoriasis is inherited as a Mendelian dominant with incomplete penetrance of the gene(s), whilst others have interpreted the family studies over generations to imply a recessive mode of inheritance with 90% inheritance. The findings of more recent studies have shown that psoriasis is a polygenic disorder. Alterations in genes may not only influence keratinocyte proliferation and maturation, but also responses to microorganisms via both the innate and adaptive immune systems, resulting in a failure to eradicate and the persistence of microorganisms in tonsils and/or skin.

### HLA-Cw6

Early genetic studies focused on the association of HLA alleles with psoriasis. The HLA genes, which are located within the major histocompatibility region (MHC) region on chromosome 6p21, express a high degree of polymorphism and association of several Class I and II antigens with psoriasis have been reported. The strongest and most consistent association of a HLA antigen in psoriasis is that of the Class I antigen, Cw6, the highest incidence being in Caucasians (36-84%, vs 10-15% in a control population).<sup>2</sup> Associations with various B antigens (B13, 17, 37 and 57) have also been reported, but are considered to be due to their linkage disequilibrium with Cw6. The presence of Cw6 correlates with early onset, guttate eruptions, a positive family history and more severe disease.<sup>3, 4</sup> However the association with GP is not absolute and this pattern of psoriasis may occur in patients who are Cw6 negative.<sup>5</sup>

The Class II antigens DR7 (60% in Caucasians vs 10% in a control population) and, to a lesser extent, DR4, have also shown an association with psoriasis. An association with DR15 has also been reported in which patients expressing this antigen had mild disease, late onset and no GP eruption.<sup>6</sup>

### Susceptibility loci

Since the mid 1990s several genome-wide linkage scans of families with psoriasis have been performed, resulting in at least 19 different putative loci for genetic susceptibility to the disease being reported on 15 different chromosomes.<sup>7</sup> Nine of the candidate loci with evidence of linkage have been designated as PSORS1-9 (Psoriasis susceptibility 1-9): PSORS1 on chromosome 6p21.3, PSORS2 – 17q25, PSORS3 – 4q34, PSORS4 – 1q21, PSORS5 – 3q21, PSORS6 – 19p13, PSORS7 – 1p35-p34, PSORS8 – 16q12-13 and PSORS9 – 4q31. These loci have been found in different ethnic groups, but are not all present in the various groups studied. Thus different combinations of genes may combine to produce psoriasis in different ethnic groups. The most consistently identified susceptibility locus in several independent samples is PSORS1, which is situated within the Class I MHC region of chromosome 6p21.3 containing Cw6. The PSORS1 locus confers significant risk for the disease and is estimated to account for between 35-50% of cases of early-onset psoriasis in the Caucasian popu-

lation. It is a major genetic risk factor for GP, but not for late-onset psoriasis, and is found in less than 20% of patients with psoriatic arthritis.<sup>8-10</sup> The PSORS1 region contains several genes, 3 of which have been extensively investigated and shown an association with psoriasis: HLA-C, coiled-coil  $\alpha$ -helical rod protein 1 (CCHCR1) and corneodesmosin (CDSN). Nair *et al.*<sup>11</sup> have recently concluded that Cw6 gene is the psoriasis susceptibility allele in this region, but this finding remains to be confirmed as other evidence supports the linkage disequilibrium of Cw6 with a nearby susceptibility variant.

Gene variations associated with psoriasis have been described in regions on PSORS2 and PSORS5 containing the ion transport molecules SLC9AR1 (solute carrier family 9, isoform A3, regulatory factor 1) and SLC12A8 (solute carrier family 12, isoform A8), respectively.<sup>12, 13</sup> SLC9AR1 is implicated in epithelial membrane function and immune synapse formation in T cells, and is adjacent to a putative binding site for the RUNX family of transcription factors. RUNX1 is essential for haematopoietic development, but is also present in polarised epithelial cells including keratinocytes. Interestingly, loss of a RUNX binding site has also been reported in systemic lupus erythematosus and rheumatoid arthritis in relation to different genes.

Recently JunB, a component of the AP-1 transcription factor that regulates cell proliferation, differentiation and cytokine expression, was proposed as a candidate gene for PSORS6. JunB expression is down-regulated in the epidermis of psoriatic skin lesions. Furthermore, inducible epidermal deletion of JunB and its proposed antagonist, c-Jun in mice led to the development of psoriasis-like skin disease and arthritis implicating the factor in psoriasis regulation.<sup>14</sup>

Various possible candidate genes are present in the PSORS4 region on 1q21, which is located within the epidermal differentiation cluster (EDC) region. These include genes coding for small proline-rich proteins and late envelope proteins (which code for precursor proteins of the cornified cell envelope), members of the S100A calcium-binding protein family who have a wide range of immunological functions, and the peptidoglycan receptor proteins (PGRP)-3 and -4.<sup>15</sup> A significant association between polymorphisms in or near the PGRP-3 and PGRP-4 genes with psoriasis was found using family-based analysis, but was not confirmed in an independent case-control sample.<sup>16</sup> Further evidence to support these genes as candidate pso-

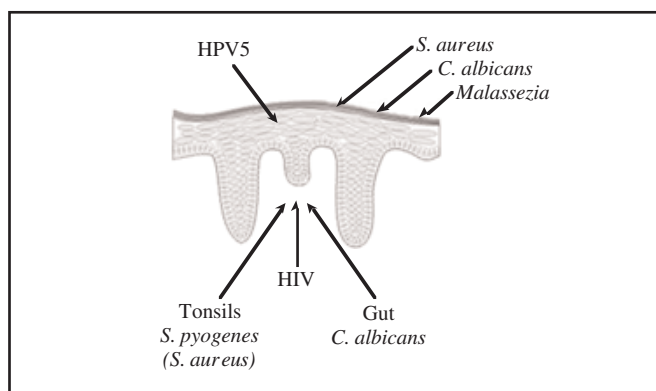


Figure 2.—Routes of entry of microorganisms that provoke or exacerbate psoriasis skin lesions. Reprinted from Clin. Dermatol., 25(6), Fry L, Baker BS, Triggering psoriasis: the role of infections and medications, p. 606-15, © 2007, with permission from Elsevier.<sup>17</sup>

riasis susceptibility genes on 1q21 has recently been reported (Kainu K *et al.*, personal communication).

### Micro-organisms associated with psoriasis

Various microorganisms are associated with the provocation of and/or exacerbation of psoriasis (Figure 2).<sup>17</sup> These include bacteria (*Streptococcus pyogenes*, *Staphylococcus aureus*), fungi (*Malassezia*, *Candida albicans*) and viruses (papillomaviruses, retroviruses). However the strongest evidence for the association of a microorganism with psoriasis is that of *S. pyogenes*, which has been implicated in both acute and chronic forms of the disease.

#### Bacteria: *S. pyogenes* throat infections

The first significant report of an association between psoriasis and streptococcal infections was made more than 50 years ago when a raised anti-streptolysin-0 (ASO) titre, together with a history of an acute sore throat 1 to 2 weeks prior to the eruption, was observed in two thirds of patients with GP.<sup>18</sup> The isolation of streptococcal organisms from the tonsils of psoriasis patients in several subsequent studies has further substantiated these observations.<sup>19</sup> Originally it was thought that only Lancefield Group A streptococci (*S. pyogenes*) was associated with GP, but streptococci of groups C and G have also been isolated from the tonsils of GP patients.<sup>20</sup> Group A (C and G) streptococci express one of several antigenically distinct M pro-

teins on their surface, but no association between particular M serotypes and the triggering of GP by *S. pyogenes* has yet been found.<sup>20, 21</sup> Similarly the evidence suggests that guttate flares in patients with established CPP are triggered by a streptococcal throat infection.<sup>22</sup> Furthermore, higher incidences of recurrent sore throats<sup>23, 24</sup> and of positive throat swabs<sup>2</sup> in CPP compared with controls suggest a further role for streptococcal organisms in the chronic form of the disease.

It has now been shown that, in streptococcal tonsillitis, the organisms can gain entry into the epithelial cells of the tonsils and persist at this site indefinitely, forming a reservoir of streptococcal antigens that can potentially maintain disease.<sup>25, 26</sup> This persistence is partly due to the fact that the antibiotics usually prescribed for streptococcal throat infection, such as penicillin, cannot penetrate the cell wall of the tonsillar epithelial cell to kill the organisms. Support for the possibility that psoriasis is maintained by a reservoir of streptococci in the tonsils is given by the observation that some patients have a significant improvement in their disease after tonsillectomy.<sup>27, 28</sup> However the psoriasis tends to recur in the majority of these patients, probably due to recolonization of the other lymphoid tissue in the oropharynx by streptococcal organisms. Furthermore it has been shown in patients with psoriasis and psoriatic arthritis that there are elevated levels of IgA antibodies directed against streptococcal peptidoglycan-polysaccharide cell wall complexes suggesting persistence of the antigen.<sup>29</sup> In contrast, evidence of the presence of streptococci in the skin of patients with psoriasis is lacking. A recent study using the polymerase chain reaction (PCR) technique to detect DNA specific for the mitogenic factor and/or streptolysin expressed by *S. pyogenes* found only 2 of 19 patients with CPP gave a positive result, despite the fact that almost half of them had raised anti-streptococcal antibodies or a positive throat swab indicating exposure to the organism.<sup>30</sup> However, a small number of case reports have described the eruption of GP lesions in perianal skin as a result of *S. pyogenes* infection, suggesting that the organism may be more likely to be detected in the skin in the acute rather than chronic form of the disease.<sup>31</sup>

#### Triggering of psoriasis by *S. pyogenes*

The appearance, distribution and natural history of GP and CPP are different; so it is likely the mechanisms by which streptococcal organisms induce or exacerbate these eruptions also vary.

In GP it has been suggested that the skin eruption is induced by streptococcal superantigens, T cell-activating toxins produced by *S. pyogenes* (as well as by *S. aureus* and other microorganisms) that bind to the variable region of the  $\beta$  chain ( $V\beta$ ) of the T cell receptor. Two characteristics of superantigens may contribute to the triggering of psoriasis. Firstly, superantigens can induce the expression of a skin homing receptor, cutaneous lymphocyte antigen (CLA) on T cells.<sup>32</sup> Thus superantigens released by streptococci in the tonsils may induce skin-seeking T cells in lymph nodes draining the pharynx, which then home to the skin where they are further activated. Secondly, superantigens stimulate T cells polyclonally on the basis of the  $V\beta$  family they express rather than according to their antigen specificity. Polyclonality of T cells expressing the T cell receptor  $V\beta 2$  has been reported in the very early skin lesions of patients with GP infected by streptococci producing streptococcal pyrogenic exotoxin-C, a stimulator of  $V\beta 2^+$  T cells, suggesting that the disease process was triggered by superantigen-induced T cell activation.<sup>33</sup> The initiation of GP may, therefore, be similar to that of scarlet fever. However, unlike the rash in scarlet fever that fades within 2 weeks, the GP skin lesions are maintained over several weeks before they begin to resolve. Furthermore, in CPP, the psoriatic plaques can persist over many years. This suggests that an antigen-specific T cell response may be involved in maintenance of the psoriatic process. This is supported by the presence of oligoclonal expansions of particular T cell receptor  $V\beta$  families in lesional skin of CPP patients.<sup>34</sup>

Antigen priming of T cells may occur in the skin-draining lymph nodes and/or in the tonsils, as suggested by the presence of the same clonal T cell receptor rearrangements in both lesional skin and tonsillar T cells expressing CLA in patients with recurrent psoriatic skin flares induced by streptococcal tonsillitis.<sup>35</sup> The nature of the antigen responsible for activation of these potentially pathogenic T cells in the skin is unknown, but has been proposed to be either a streptococcal antigen, or a skin-specific antigen with or without homology to a streptococcal antigen.

#### *T cell activating antigen: Streptococcal peptidoglycan*

Research has focused on the identity of the streptococcal antigen(s) responsible for T cell activation in psoriasis.<sup>36</sup> Increased numbers of interferon- $\gamma$ -pro-

ducing Th1 cells with specificity for group A streptococcal antigens have been isolated from the skin lesions of both GP and CPP patients, a subset of which recognizes streptococcal cell wall antigens.<sup>37-39</sup> Cell wall fractions containing proteins of 20-50 kDa were shown to be responsible for the skin T cell response.<sup>39</sup> These findings, and those of a deletion mutation study, excluded the larger molecular weight M protein, a major antigenic determinant in the streptococcal cell wall, as a candidate antigen.<sup>38, 39</sup> Valdimarsson *et al.* had previously postulated that streptococcal M protein triggered T cell activation in psoriasis, and that the disease process was subsequently maintained by a skin autoantigen, keratin 17, via molecular mimicry.<sup>40</sup> Although an increased frequency of interferon- $\gamma$ -producing CLA<sup>+</sup> CD8<sup>+</sup> T cells specific for keratin-derived peptides that shares sequences with streptococcal M proteins have been reported in the peripheral blood of HLA-Cw6<sup>+</sup> psoriatic patients compared with HLA-Cw6<sup>+</sup> healthy controls,<sup>41</sup> the presence of T cells specific for streptococcal M protein and/or keratin in psoriatic skin lesions has yet to be demonstrated.

Recently it was shown that at least half of the streptococcal cell wall-specific Th1 cells in psoriasis skin lesions were specific for streptococcal peptidoglycan (PG).<sup>42</sup> PG is a major constituent of the bacterial cell wall of Gram-positive bacteria such as *S. pyogenes* and *S. aureus*, and composed of repeating disaccharides of N-acetylglucosamine and N-acetylmuramic acid cross-linked by peptides. The response to streptococcal PG was self HLA-DR allele-restricted, demonstrating that PG could act as a classical antigen, in addition to its more commonly observed proinflammatory effects.<sup>43</sup> Additional support for the ability of PG to induce an antigen-specific T cell response was shown by the lack of cross-reactivity between these streptococcal PG-specific T cells and a further subset of T cells specific for staphylococcal PG present in the same psoriatic skin lesions.<sup>42</sup>

Furthermore, in close proximity to the PG-specific T cells in the dermis were increased numbers of macrophages containing PG (compared to non-lesional psoriatic or normal skin) in both GP and CPP lesions.<sup>42</sup> It was shown that, in most cases, the PG was not staphylococcal in origin, but it was not possible to confirm that the cell wall PG was streptococcal due to the lack of availability of a specific antibody. In support of the possibility that the PG was derived from streptococci, macrophages containing *S. pyogenes* or

PG have been observed in the crypts of tonsils after removal from patients with recurrent streptococcal tonsillitis.<sup>44</sup> Thus it is likely that at least some of the PG-containing macrophages observed in psoriatic skin cells have migrated to the skin from the tonsils, although the gut may be an additional source of the bacterial antigen.<sup>45</sup>

PG-containing macrophages are not specific to psoriasis or to inflammatory skin diseases, and have been detected in increased numbers in various chronic inflammatory diseases of other organs, including Crohn's disease,<sup>46</sup> rheumatoid arthritis<sup>47</sup> and multiple sclerosis.<sup>48</sup> However, the possible presence of PG-specific T cells in these conditions has not yet been investigated. Although streptococcal cell wall-reactive T cells have been isolated from non-psoriatic skin diseases such as eczema, lichen planus and pityriasis rosea, they differ from those of psoriatic skin lesions in that they proliferate, but do not produce IFN- $\gamma$  in response to streptococcal antigens.<sup>49, 37</sup> A lack of correlation between proliferative and IFN- $\gamma$  responses to streptococcal PG by psoriatic T cells implies different T cell epitopes might be involved in each case. These findings suggest that a Th1 cell clone(s) that recognises a streptococcal PG epitope(s) in the context of psoriasis-associated HLA alleles may be specific for psoriasis and contribute to disease immunopathogenesis.

#### *Bacteria: Staphylococcus aureus*

There is increased colonisation of psoriatic skin by *S. aureus* as compared to normal, healthy skin, and in at least half of the cases, the isolates secreted one or more staphylococcal enterotoxins.<sup>50, 51</sup> Patients with toxin-positive *S. aureus* colonisation had a significantly higher Psoriasis Area and Severity Index than those with toxin-negative *S. aureus* or who lacked bacterial colonisation, consistent with the proposal that exacerbation of psoriatic lesions by the organism is most likely mediated via superantigen(toxin)-induced T cell activation.<sup>51</sup>

As mentioned above, T cells specific for PG from *S. aureus* have been cultured from psoriatic skin lesions; however, macrophages carrying *S. aureus*-specific PG constituted only a very small proportion of the total number of PG-containing macrophages detected.<sup>42</sup> These findings suggest that activation of *S. aureus* PG-specific T cells may not be important in disease pathogenesis, and that the main role of *S. aureus* in

psoriasis may be that of an exacerbator of existing disease, via the production of superantigenic toxins.

#### *Fungi: Malassezia "Pityro sporum"*

*Malassezia* (the yeast phase of which was formerly known as *Pityrosporum*) are thick-walled yeast fungi that form part of the normal human skin flora and are found predominantly on the scalp, face and upper trunk, areas rich in sebaceous glands. This distribution is probably explained by the organism's requirement for exogenous fatty acids that results from a defect in fatty acid synthesis. The scalp is a common site for psoriasis, and several reports have suggested an association between *Malassezia* and the development of skin lesions. Treatment of patients with psoriasis with the oral antifungal drug, ketoconazole, produced marked improvement of their scalp lesions after a decrease in yeast cell numbers.<sup>52, 53</sup> Conversely, patch testing with sonicates of heat-killed *Malassezia* on the intact non-lesional skin of 10 patients with inactive psoriasis induced the formation of skin lesions clinically and histologically resembling psoriasis.<sup>54</sup>

In addition, *Malassezia* are implicated in so-called seborrhoeic eczema, but there is a school of thought developing who propose that this disorder should be referred to as seborrhoeic psoriasis. In support of this suggestion is the observation that this type of eruption on the face and scalp is often found in association with plaque psoriasis and has a higher incidence in family members of patients with psoriasis.

Various immune cell types have been shown to respond to *Malassezia* and its components in psoriasis. Antibodies that recognize N-acetyl glucosamine terminals of glycoproteins expressed by the organism, and T cells with differential specificity to the round and oval forms of *Malassezia*, isolated from scalp (and nonscalp) skin lesions, have been reported in psoriatic patients.<sup>55, 56</sup> In addition, soluble components produced by the fungi were demonstrated to act as chemoattractants for psoriatic polymorphonuclear leukocytes.<sup>57</sup> *Malassezia* can also upregulate keratinocyte expression of various molecules associated with hyperproliferation and cell migration of the epidermis, which are more highly expressed in psoriatic skin colonised with the organism than in non-colonised psoriatic skin.<sup>58</sup> More recently it has been shown that toll-like receptor (TLR)-2 mediates intracellular signalling in human keratinocytes in response to *Malassezia furfur*.<sup>59</sup> These findings suggest that these organisms may play a role

in the pathogenesis of psoriasis, via both the innate and adaptive immune systems.

#### *Fungi: Ringworm fungi*

Psoriasis of the nails, particularly toenails, can be difficult to distinguish from a fungal infection particularly when the main feature is subungual hyperkeratosis. However, an increased incidence of onychomycosis has been found in patients with psoriasis, and in family members of those with the disease. It has been proposed that fungal infection may induce the proliferative response of the nail bed in psoriasis, although it may be difficult to demonstrate the presence of the organism.

#### *Fungi: Candida*

The pathogenic yeast *Candida* is frequently found in intertrigenous psoriasis, but it is not known whether this is a secondary invader or whether the organism plays a role in either initiating or maintaining the lesions. Increased incidence of *C. albicans* in the faeces of psoriasis patients compared to controls has also been reported.<sup>60-62</sup> No correlation between the quantity of *Candida* in faeces and the PASI score was found, although there was a significant association between early age of onset of psoriasis and increased levels of the yeast.<sup>62</sup> Furthermore, more than half of a group of 50 psoriasis patients treated with the oral anti-fungal treatment, nystatin showed significant skin improvement, supporting a role for *Candida* in the exacerbation of psoriasis.<sup>63</sup> The mechanisms used by *Candida* to aggravate psoriasis are presently unknown, but may involve the production of superantigens that stimulate non-specific activation of T cells.<sup>64</sup>

#### *Viruses: Human papilloma virus (HPV)*

Human papillomaviruses (HPVs) have been associated with both benign and malignant skin tumours. In addition, it is now accepted that HPV is the aetiological agent for the mucosal tumour, carcinoma of the cervix.

One of the HPVs, HPV-5, has been found in approximately 90% of a large series of patients with psoriasis, but not in patients with atopic eczema, although HPV36 was detected in the latter.<sup>64</sup> Furthermore, HPV-5-specific antibodies reactive with the L1 capsid protein have been reported to be increased in psoriasis

patients.<sup>65</sup> HPV-5 is also associated with epidermodysplasia verruciformis, a susceptibility locus for which maps to chromosome 17qter in a region containing a susceptibility locus for psoriasis, PSORS2.<sup>66</sup> This may imply a susceptibility to HPV-5 if individuals have a mutation of a gene in the 17q region. It has also been proposed that HPV5 may be the putative antigen recognized by oligoclonal epidermal CD8<sup>+</sup> T cells in psoriatic skin lesions.<sup>67</sup>

#### *Viruses: Retroviruses (HIV)*

A common feature of HIV infected patients is that psoriasis may appear for the first time, or pre-existing disease is made worse and becomes difficult to treat. The virus appears to be an aetiological factor in these patients as treatment with antiretroviral drugs can improve the psoriasis.<sup>68</sup> Whether the virus itself, or one or more of the opportunistic infections (*e.g.* staphylococci in the skin or streptococci in the respiratory tract) associated with AIDS aggravates or precipitates the disease remains to be determined.

### **Innate immunity and psoriasis**

Recent evidence suggests that interaction between innate and adaptive immune responses are involved in the pathogenesis of psoriasis.<sup>69</sup> Furthermore, the innate immune system in psoriasis appears to be dysregulated, with upregulation and activation of various cellular and humoral components of the innate immune response.<sup>69, 70</sup>

The innate immune system is designed to provide a rapid response, without antigen specificity or long-lasting memory, to protect the body against microorganisms. This is achieved by recognition of conserved molecular patterns expressed by the organisms, by non-clonal receptors termed pattern-recognition receptors (PRRs). One such conserved component of Gram-positive bacteria is PG, whose inflammatory biological effects result from its recognition by two families of proteins, the NOD (nucleotide-binding oligomerization domain) proteins and peptidoglycan recognition proteins (PGRPs).

NOD1 and NOD2 are intracellular PG recognition molecules that recognise PG peptides containing meso-diaminopimelic acid (present in mainly Gram-negative bacteria), or muramyl dipeptide (in both Gram-positive and Gram-negative bacteria), respectively.<sup>71, 72</sup>

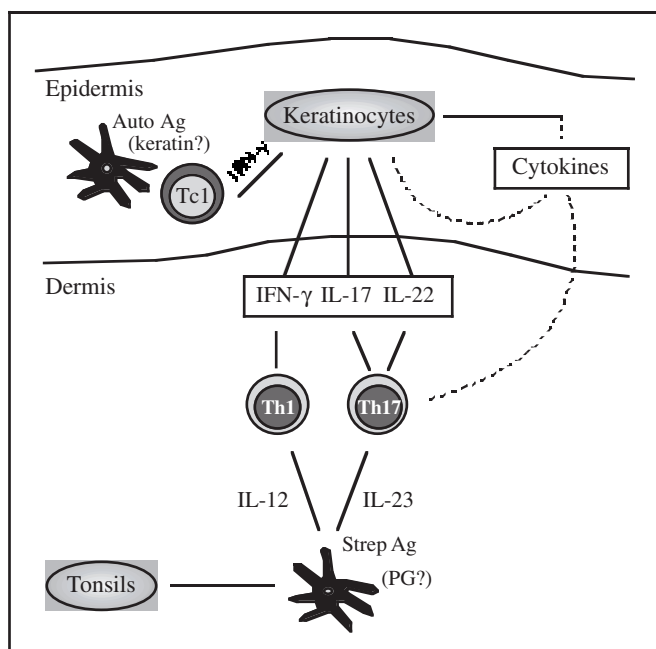


Figure 3.—Model for the pathogenesis of psoriasis.

Mutations in NOD2 contribute to disease susceptibility in Crohn's disease,<sup>73</sup> an inflammatory bowel disease in which the prevalence of psoriasis is significantly increased.<sup>74</sup> A susceptibility locus for psoriasis that overlaps with a susceptibility locus for Crohn's disease has been identified on chromosome 16q12,<sup>75</sup> but various studies that have investigated the Crohn's-associated NOD2 mutations in psoriasis patients have failed to find any association with the disease, with the exception of one study of psoriasis arthritis patients in Newfoundland.<sup>76-79</sup>

PG stimulation of NOD2 (or Toll-like receptor (TLR)-2) in keratinocytes induces the production of antimicrobial peptides that target Gram-positive and Gram-negative bacteria, and fungi.<sup>80</sup> In psoriasis, levels of human  $\beta$ -defensin (HBD) peptides, HBD-2 and HBD-3, and cathelicidin LL-37 are increased in the epidermis,<sup>81,82</sup> probably resulting from stimulation of the PRRs by bacteria colonising the surface of the skin, and/or by cytokines present in the epidermis. It has been shown that the abundance of HBD-2 in keratinocytes after induction by cytokines is correlated with its basal expression.<sup>83</sup> It is interesting in this respect that an association between risk of psoriasis and a higher genomic copy number for  $\beta$ -defensin genes has been recently reported.<sup>84</sup>

A family of four PGRPs (PGRP-1, 2, 3, 4) have been recently found in humans, the genes for three of which are located at chromosomal loci containing susceptibility genes for psoriasis. The genes for PGRP-3 and PGRP-4 are found in the region containing the PSORS4 locus on chromosome 1q21,<sup>85</sup> whilst the gene for PGRP-2 is located on chromosome 19p in a region containing the PSORS6 locus.<sup>86</sup> A recent study found an association between polymorphisms in or near the PGRP-3 and PGRP-4 genes and psoriasis using family based analysis, but not in an independent case control sample.<sup>87</sup> Another study just completed has also reported the association of polymorphisms of these genes in Irish and Finnish, but not in Swedish, families with psoriasis (Kainu K *et al.*, personal communication).

PGRP-3 and PGRP-4 are secreted by epithelial cells of the skin, gut and tonsils and, through interaction with cell wall PG, are directly bactericidal against pathogenic and non-pathogenic Gram-positive bacteria, but not normal bacterial flora.<sup>88</sup> PGRP-2 is an enzyme (N-acetylmuramoyl-L-alanine amidase), produced by the liver and secreted into the blood stream, which cleaves the stem peptide from the glycan chain of PG.<sup>89,90</sup> Mutations in the genes coding for these PGRPs could result in persistence of bacteria and/or an altered inflammatory response to PG. The recent demonstration of PG-specific T cells in close proximity to PG-carrying macrophages in the dermis of lesional skin suggests that PG could be an example of a bacterial component able to activate both innate and adaptive immune responses in patients with psoriasis.

### Model of immunopathogenesis of psoriasis

The immunopathogenic pathway leading to the development of psoriasis remains to be fully elucidated. However, the evidence suggests that interaction between CD4<sup>+</sup> and CD8<sup>+</sup> T cells, dendritic cells and keratinocytes leading to the production of a defined pattern of cytokines forms the basis of the disease process (Figure 3).

Activated CD4<sup>+</sup> and activated CD8<sup>+</sup> T cells coexist in equal numbers in the epidermis of CPP skin lesions,<sup>91</sup> and both subsets appear to play vital roles. It is proposed that initiation and maintenance of the psoriatic process requires antigen presentation to a pathogenic CD4<sup>+</sup> T cell subset by MHC Class II-pos-

itive dendritic cells in the epidermis and/or dermis, resulting in activation and cytokine production. The nature of the antigen is unknown, but the strong clinical association of psoriasis with streptococcal infections suggest that it is likely to be of streptococcal origin. The isolation of PG-specific T cells and PG-carrying macrophages in psoriatic skin lesions raises the possibility that PG may be a candidate for this role.

Once initiated, dominant epidermal CD8<sup>+</sup> T cell clones may contribute to the persistence of the disease process.<sup>92, 93</sup> It is unlikely that PG is the activating antigen for these IFN- $\gamma$ -producing cells as macrophages carrying PG are predominately observed within CD4<sup>+</sup> T cell dominated clusters in the dermis.<sup>42</sup> Circulating CD8<sup>+</sup> T cells that recognize peptides from keratin 17 and streptococcal M protein with sequence homology, and which express homing receptors for the skin, have been reported in psoriatic individuals.<sup>41</sup> Thus it is conceivable that a skin autoantigen(s), with or without cross-reactivity to a streptococcal antigen, drives epidermal CD8<sup>+</sup> T cell activation and maintains the chronicity of CPP skin lesions.

Production of IL-12 and IL-23 by dendritic cells in response to microbial stimulation induces two types of CD4<sup>+</sup> T cells in psoriasis; Th1 cells producing IFN- $\gamma$ , and the more recently identified Th17 cells which secrete IL-17 and IL-22.<sup>94</sup> These T cell-derived cytokines play a key role in the disease process through their effects on keratinocytes, including upregulation of adhesion and accessory molecule expression, enhanced production of Th1 cell-attracting chemokines and of various cytokines, regulation of proliferation and differentiation, and upregulation of S100 proteins and  $\beta$ -defensins.<sup>95-97</sup> In turn, cytokines produced by keratinocytes such as Interleukin-8 and transforming growth factor- $\alpha$  have autocrine effects on keratinocyte proliferation, whilst other keratinocyte-derived cytokines stimulate T cell growth and IFN- $\gamma$  production. This sets up a self-perpetuating cycle of events in which dendritic cells, activated T cells and keratinocytes, interact via the production of a defined mixture of cytokines and chemokines, thus maintaining the pathology of the psoriatic skin lesion. Furthermore, a lack of regulatory T cell function leading to unrestrained effector T cell proliferation, is a further contributory factor to the stability of skin lesions in CPP.<sup>98</sup> Recent evidence suggests that this may be due to inherent genetic programming passed down from bone-marrow-derived haematopoietic cells.<sup>99</sup>

### Treatment of psoriasis: a role for vaccination?

Until the mid-1980s, the use of both topical and systemic treatments for psoriasis were largely empirical. This changed with the discovery that T cells played an essential role in the disease process, leading to the development of evidence-based approaches to therapy. Immunosuppressive treatments such as corticosteroids and cyclosporin have been used with favourable results in the management of psoriasis, but have several limitations including toxicity and an increased risk of infection and malignancy. More recently, a new class of immuno-modulating biological agents (monoclonal antibodies and fusion proteins) that target specific steps in the disease pathway have been developed as a result of progress in the elucidation of the immunopathogenesis of psoriasis.<sup>100</sup> These agents are proving effective in various inflammatory diseases as they target common molecules with non-antigen specific functions. Although biological agents are more selective than immunosuppressive drugs, they are not without side effects and the possible consequences of long-term immunosuppression with these agents are unknown.

A therapeutic approach, such as vaccination, targeting the initiating antigen or pathogenic T cells would have the advantages of minimising side effects caused by global immunosuppression, and inducing long-term tolerance in psoriasis.

The use of vaccination as a therapeutic tool in psoriasis has been investigated in only a small number of studies. Vaccination of psoriasis patients with *M. vaccae* met with little success, but the basis for using such an approach was empirical, not evidence-based.<sup>101</sup> However components of *S. pyogenes*, especially PG, are strongly implicated in psoriasis, raising the possibility of vaccination against the organism as a therapeutic option. The aim of such an approach would be to stimulate the immune response to streptococci in psoriatic individuals so as to improve bacterial clearance and prevent persistence of streptococcal antigens that maintain the disease via T cell activation. However, there is also the possibility that vaccination against *S. pyogenes* could also stimulate expansion of the pathogenic T cell subset, resulting in disease exacerbation.

An alternative strategy that has been used in psoriasis (multiple sclerosis and rheumatoid arthritis) is T cell receptor peptide vaccination, which involves the

induction of a regulatory Th2 subset specific for portions of the T cell receptor of clonally expanded pathogenic Th1/Tc1 cells.<sup>102</sup> T cell receptor peptides corresponding to the mid region of the BV3 and BV13S1 genes, which are overexpressed in epidermal CD8<sup>+</sup> T cells in CPP skin lesions, were injected in low doses into a total of 177 psoriasis patients in two studies. Unsurprisingly, as CD8<sup>+</sup> T cells are likely to contribute to maintenance rather than initiation of the psoriatic process, only a modest clinical effect related to significant immunological responses to the peptides were reported.<sup>102</sup> Targeting of oligoclonal CD4<sup>+</sup> T cells, which are essential for initiation of the disease, would be predicted to produce a better clinical outcome.

The possible use of these vaccination approaches in psoriasis will be dependent upon identification of the initiating antigen(s), and characterisation of the pathogenic antigen-specific T cells, both of which are achievable in the near future.

It is salutary to reflect that, in 1896 in his Textbook of Dermatology, Radcliffe-Crocker stated that psoriasis was due to microorganisms in the skin. It has taken over a 100 years for evidence to support that prediction to accumulate. Future research will need to address the identity of the triggering microbial antigen(s); such knowledge could open the way for vaccination as a therapeutic tool for psoriasis.

### Riassunto

#### *Quale ruolo della vaccinazione nel trattamento della psoriasi?*

La psoriasi è una patologia cutanea immunitaria multifattoriale, la cui etiologia ha una forte componente genetica, con numerosi geni che codificano proteine coinvolte nella differenziazione dell'epidermide e le risposte immunitarie, infiammatorie e patogene, in combinazione con fattori ambientali microbici. Sebbene diversi microrganismi, tra cui lo *Staphylococcus aureus*, la *Malassezia* e la *Candida albicans*, sembrano provocare o aggravare la patologia, l'associazione tra infezioni delle vie respiratorie alte da *S. pyogenes* e la psoriasi guttata è supportata da evidenze cliniche molto forti. Inoltre, l'identificazione di cellule T peptidoglicano-specifiche nelle lesioni cutanee psoriasiche ha portato a proporre che il peptidoglicano della parete cellulare possa costituire il collegamento tra l'infezione streptococcica a livello tonsillare e la successiva induzione delle lesioni cutanee. Questi dati suggeriscono come la psoriasi possa essere una possibile candidata alla vaccinazione terapeutica streptococcica. Gli attuali trattamenti della psoria-

si hanno diversi limiti, tra cui la tossicità e un aumentato rischio di infezioni e neoplasie maligne. Al contrario, la vaccinazione potrebbe indurre una tolleranza a lungo termine senza gli effetti collaterali causati dall'immunosoppressione globale. Sono necessari ulteriori studi per valutare l'identità degli antigeni microbici trigger; tali conoscenze potranno aprire la via alla vaccinazione come arma terapeutica della psoriasi.

Parole chiave: Psoriasi - Vaccini - Vaccino streptococcico.

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## A novel vaccine (Zostavax) to prevent herpes zoster

K. HOLCOMB, J. M. WEINBERG

*Varicella-zoster virus* is the causal agent of varicella and *herpes zoster* (HZ) in humans. HZ results from reactivation of latent *varicella-zoster virus* (VZV) within the sensory ganglia. The incidence and severity of HZ increase with advancing age; more than half of all persons in whom HZ develops are older than 60 years. The most frequent debilitating complication is postherpetic neuralgia, a neuropathic pain syndrome that persists or develops after the dermatomal rash has healed, and can be prolonged and disabling. There are many limitations of the current therapies for HZ and postherpetic neuralgia. A live attenuated VZV vaccine has been developed and recently approved by the United States Food and Drug Administration (FDA) and the European Union for the prevention of HZ in individuals 60 years of age and older. In a randomized, double-blind, placebo-controlled trial 38 546 adults of 60 years of age or older, the use of the HZ vaccine reduced the burden of illness due to HZ by 61.1% ( $P<0.001$ ), reduced the incidence of postherpetic neuralgia by 66.5% ( $P<0.001$ ), and reduced the incidence of HZ by 51.3% ( $P<0.001$ ). In this review, the authors will discuss the history of the use of the varicella vaccine in children, and the subsequent development of the new HZ vaccine.

**KEY WORDS:** Herpes zoster - Neuralgia, postherpetic - Vaccines.

*Herpes zoster* (HZ) is a reactivation of latent *varicella zoster virus* (VZV) from the dorsal root ganglion. It produces a characteristic rash accompanied by sensory changes which classically occur in a dermatomal pattern. HZ occurs in older adults and

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immunocompromised individuals due to VZV-specific decline in cell mediated immunity. The principal risk factor for HZ is prior history of VZV exposure (Table I). The risk for developing HZ increases with increasing age, with approximately 1 million cases of HZ occurring in the United States per year. Complications of HZ infection (Table II) include severe pain during the infection as well as postherpetic neuralgia, *herpes ophthalmicus* (which can lead to blindness), pneumonia, myelopathy, paresis, vasculopathy, and myocarditis. These complications have impact on health care costs and productivity and quality of life for those afflicted.

*Varicella virus* is a ubiquitous *herpes virus* that causes chickenpox in childhood. On resolution of the primary varicella infection, residual provirus segments travel from sensory nerve endings up sensory fibers, eventually lodging in the cranial or dorsal root ganglia. These viral fragments settle in neuronal or satellite cell nuclei, where they are protected from the high levels of antibody that persist in the circulation in response to the primary infection. This migration and colonization of virus along the neural route may in part explain why HZ primarily affects the sensory ganglia and its rash is distributed locally along a sensory nerve dermatome.

Conflict of interest. Dr. Weinberg is on the speakers' bureau for Merck..

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TABLE I.—*Risk factors and potential risk factors for VZV reactivation.*

- 
- Prior VZV exposure (chickenpox, vaccine)
  - Age >50 years
  - Immunocompromised state
  - Immunosuppressive drugs
  - HIV/AIDS
  - Bone marrow or organ transplant
  - Cancer
  - Chronic steroid therapy
  - Psychological stress
  - Trauma
- 

Modified from Arvin. A. Clin Microbiol Rev 1996;9:361-81.<sup>1</sup>

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Once inside the neuronal nucleus, the virus remains latent and does not multiply, although it retains the ability to revert to an infectious state at any time. It is unclear what induces reactivation of VZV, but it certainly depends on waning cell-mediated immunity to achieve populations sufficient to incite HZ disease. Furthermore, although memory CD4 and CD8 T cells are highly detectable in the young, who are largely resistant to HZ, they are substantially diminished among the elderly and in immunocompromised individuals.

A new vaccine (Zostavax) intended for the prevention of HZ was approved in the United States and European Union in 2006.<sup>3</sup> This vaccine is a more potent version of the varicella vaccine (Varivax), which is indicated for use in children.

### Use and impact of the varicella vaccine

The varicella vaccine (Varivax) was developed in Japan, and data was first reported in 1974 by Takahashi *et al.*<sup>4</sup> The live attenuated virus, known the Oka virus, was derived from a child with varicella virus infection. The Oka vaccine virus can be differentiated from wild-type virus found in the United States by restriction enzyme analysis and PCR.<sup>5</sup> It was approved for use in the United States in 1995 for use in healthy individuals.<sup>6</sup> In 1999, the Advisory Committee on Immunization Practices stated the varicella vaccine may be used in patients with humoral immunity deficiency, and that it may be considered in children with HIV, with no to mild HIV symptomology and a CD4% greater than or equal to 25%. It is also available free through a research protocol for children with acute lymphoblastic leukemia.<sup>7</sup> The vaccine has demonstrated a good record of safety. No serious adverse events have been noted since its release,<sup>8</sup> and only 3

TABLE II.—*Potential complications of VZV can contribute to chronic pain and impairment.*

- 
- Neurologic*
    - Postherpetic neuralgia
    - Motor paralysis
    - Meningoencephalitis
    - Transverse myelitis
    - Cerebral vasculitis
    - Cranial palsy
  - Ocular*
    - Lid ulceration
    - Conjunctivitis, keratitis, uveitis
    - Optic neuritis
    - Retinal necrosis
    - Secondary glaucoma
  - Visceral*
    - Pneumonitis
    - Myocarditis
    - Hepatitis
    - Esophagitis
- 

Adapted from Wood and Easterbrook. Washington, D.C.: IOS Press; 1995. pp. 193-209.<sup>2</sup>

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cases of transmission of the Oka virus have been reported out of over 15 million doses of vaccine.<sup>5</sup>

Postexposure studies of the varicella vaccine with widespread use in the United States have demonstrated approximately 84% efficacy.<sup>9</sup> Health care expenditure due to varicella infection decreased by \$ 100 million per year from 1993 to 2001, and total annual savings including non-medical expenditure (loss of time from school/work) is \$ 384 million.<sup>6, 10, 11</sup> From the prevaccination period to 2002, hospitalizations due to varicella declined by 88% (from 2.3 to 0.3 per 100 000 population) and ambulatory visits declined by 59% (from 215 to 89 per 100 000 population). Hospitalizations and ambulatory visits declined in all age groups, with the greatest declines among infants younger than 1 year.<sup>11</sup> In vaccinated patients who do develop varicella (breakthrough infection), the presentation is usually mild.<sup>12</sup>

While varicella infection has decreased, incidence of HZ has remained the same. There is concern that with less children developing wild-type varicella infection, there will be an increase in HZ incidence in adults. This is explained by the theory that exposure to infected individuals allows for cell-mediated immune boosting of adults and protection from HZ activation.<sup>13</sup> However, in studies in children with leukemia, and in adults who received 2 doses of the vaccine 3 months apart, decreased rates of HZ were observed.<sup>14-16</sup>

Clinical development of the HZ vaccine

Oxman *et al.*<sup>3</sup> enrolled 38 546 adults aged 60 years and above, in a randomized, double-blind, placebo-controlled study of an investigational live attenuated Oka/Merck VZV vaccine (“zoster vaccine”). The minimum potency of the HZ vaccine administered to subjects in the study was at least 14 times greater than the minimum potency currently licensed varicella vaccine. A preliminary study demonstrated that increased potencies were necessary in older adults in order to elicit a significant increase in the cell-mediated immunity to VZV.<sup>3</sup> Therefore, a high-potency vaccine was formulated for this study.

HZ was diagnosed according to standard clinical and laboratory criteria. In patients who developed HZ, the associated pain and discomfort were measured repeatedly for six months. The primary end point of the study was the burden of illness due to HZ, a measure affected by the incidence, severity, and duration of the associated pain and discomfort. The secondary end point was the incidence of postherpetic neuralgia.<sup>3</sup> The incidence of HZ was also evaluated.

There was no significant difference in demographics or general health status between the vaccine and placebo groups. Surveillance times ranged from 1 day to 4.9 years with the mean being 3.13 years. The vast majority of subjects (95.3%) completed the trial, with only 0.6% withdrawing and 4.1% dying during the study. These percentages were similar in both the vaccine and placebo groups.<sup>3</sup>

Subjects in the vaccine group received 0.5 mL subcutaneously of the live attenuated Oka/Merck VZV vaccine. The median potency of this dose was 24,600 plaque-forming units per dose. The average potency of the varicella vaccine is 1350 plaque-forming units.<sup>17</sup>

Burden of illness

The burden of illness (BOI) score, the primary end-point of this study, incorporates the incidence, severity, and duration of the pain and discomfort associated with HZ.<sup>18</sup> Each subject who had an episode of zoster rated their worst pain on a pain inventory. The rating was used to calculate a severity-of-illness score (ranging from 1 to 1 813 in this study); this score was defined as the area under the curve of *zoster* pain plotted against time during the 182-day observation period after rash onset. The BOI Score was the mean severity-of-illness score for each treatment group. In total,

TABLE III.—Effect of HZ vaccine on burden of illness in HZ.

|              | BOI Score*       |                  | Vaccine efficacy <sub>BOI</sub> (95% CI) (%) |
|--------------|------------------|------------------|----------------------------------------------|
|              | Vaccine N=19 254 | Placebo N=19 247 |                                              |
| All subjects | 2.21             | 5.68             | 61.1 (51.1-69.1)**                           |
| 60-69 yrs    | 1.5              | 4.33             | 65.5 (51.5-75.5)                             |
| ≥70 yrs      | 3.47             | 7.78             | 55.4 (39.9-66.9)                             |

\*Burden of Illness Score; \*\*P<0.001; Modified from Table II in Oxman MN *et al.* N Engl J Med 2005;352:2271-84.<sup>3</sup>

TABLE IV.—Effect of HZ vaccine on PHN.

|              | Vaccine N=19 254 |                    | Placebo N=19 247 |                    | Vaccine efficacy <sub>PHN</sub> (95% CI) |
|--------------|------------------|--------------------|------------------|--------------------|------------------------------------------|
|              | No. of confirmed | Incidence per 1000 | No. of confirmed | Incidence per 1000 |                                          |
| All subjects | 27/19 254        | 0.46               | 80/19 247        | 1.38               | 66.5* (47.5-79.2)                        |
| 60-69 yrs    | 8/10 370         | 0.26               | 23/10 356        | 0.74               | 65.7 (20.4-86.7)                         |
| ≥70 yrs      | 19/8 884         | 0.71               | 57/8 891         | 2.13               | 66.8 (43.3-81.3)                         |

\*P<0.001; Modified from Table III in Oxman MN *et al.* N Engl J Med 2005;352:2271-84.<sup>3</sup>

957 cases of confirmed zoster and 107 cases of postherpetic neuralgia were included in the burden of illness analysis.<sup>3</sup>

Vaccine efficacy with respect to the burden of illness due to HZ (VE<sub>BOI</sub>) was defined as the relative reduction in the burden-of-illness score in the vaccine group as compared with that in the placebo group. In the study by Oxman *et al.*<sup>3</sup>, the HZ BOI score was significantly reduced in the vaccine group as compared with the placebo group (P<0.001) (Table III). Overall, VE<sub>BOI</sub> was 61.1% (95% confidence interval [CI], 51.1 to 69.1) for the total study population, a result that met the prespecified criteria for success.

Incidence of postherpetic neuralgia

Vaccine efficacy with respect to the incidence of postherpetic neuralgia (VE<sub>PHN</sub>) was defined as the relative reduction in the incidence of postherpetic neuralgia in the vaccine group as compared with that in the placebo group.<sup>19</sup> There were 107 cases of postherpetic neuralgia, 27 in the vaccine group and 80 in the

TABLE V.—Effect of HZ vaccine on incidence of HZ.

|              | Vaccine<br>N=19 254          |                                     | Placebo<br>N=19 247          |                                     | Vaccine<br>efficacy <sub>HZ</sub><br>(%)<br>(95% CI) |
|--------------|------------------------------|-------------------------------------|------------------------------|-------------------------------------|------------------------------------------------------|
|              | No. of<br>confirmed<br>cases | Incidence<br>per 1000<br>person-yrs | No. of<br>confirmed<br>cases | Incidence<br>per 1000<br>person-yrs |                                                      |
| All subjects | 315/19 254                   | 5.42                                | 642/19 247                   | 11.12                               | 51.3<br>(44.2–67.6)                                  |
| 60–69 yrs    | 122/10 370                   | 3.90                                | 334/10 356                   | 10.79                               | 63.9                                                 |
| ≥70 yrs      | 193/8 884                    | 7.18                                | 308/8 891                    | 11.50                               | 37.6                                                 |

Adapted from Table II in Oxman MN *et al.* N Engl J Med. 2005;352:2271–2284

placebo group (0.46 case vs 1.38 cases per 1 000 person-years, respectively;  $P<0.001$ ) (Table IV). Overall, the  $VE_{PHN}$  was 66.5 percent (95% CI, 47.5 to 79.2). In a time-to-event analysis, the cumulative incidence of postherpetic neuralgia was significantly lower in the vaccine group than in the placebo group ( $P<0.001$ ).<sup>3</sup>

### Incidence of HZ

The overall incidence of HZ per 1 000 person-years was significantly reduced by the zoster vaccine, from 11.12 per 1 000 person-years in the placebo group to 5.42 per 1 000 person-years in the vaccine group ( $P<0.001$ ) (Table V). The  $VE_{HZ}$  was 51.3% (95% CI, 44.2 to 57.6). In a time-to-event analysis, the cumulative incidence of herpes zoster was significantly lower in the vaccine group than in the placebo group ( $P<0.001$ ). The  $VE_{HZ}$  was 37.6% among subjects 70 years of age or older and 63.9% among younger subjects ( $P<0.001$ ). There was no difference in  $VE_{HZ}$  according to sex.<sup>3</sup>

### Adverse events

Over the entire study period, the numbers and percentages of deaths were similar in both the vaccine and placebo groups. During the first 42 days after vaccination, the number and types of serious adverse events were similar in the two groups, as was the distribution of serious adverse events according to body system. During this period, varicella-like rashes at the injection site occurred more frequently among those in the vaccine group than among those in the placebo group, but varicella-like rashes at other sites occurred at similar rates in the two groups. There were 7 confirmed cases of herpes zoster in the vaccine group and

24 in the placebo group during the first 42 days after vaccination.<sup>3</sup>

In an adverse-events substudy performed, a significantly greater number of subjects in the vaccine group had one or more adverse events than in the placebo group, reflecting a greater frequency of adverse events at the injection site among subjects in the vaccine group. In the vaccine group, the most frequent adverse events at the injection site were erythema (in 35.8% of the vaccine group), pain or tenderness (in 34.5%), swelling (in 26.2%), and pruritus (in 7.1%).<sup>3</sup>

There were 5 serious adverse events during the study believed to be vaccine related. After unblinding, 2 of the events were in the vaccine group and 3 in the placebo group (no significant difference). The 2 reactions in the vaccine group were an asthma exacerbation and a new diagnosis of polymyalgia rheumatica. There was a diagnosis of polymyalgia rheumatica in the placebo group as well. Throughout the entire duration of the clinical trials, there was a significant difference in total serious adverse events between the two groups (1.9% in the vaccine group and 1.3 percent in the placebo group.) These events were each reviewed *post hoc*, and no clinically meaningful difference between the vaccine and placebo groups was identified by the writing committee.<sup>3</sup>

### Discussion

This novel HZ vaccine is an important new prevention strategy in the treatment of HZ. Current strategies for treating HZ demonstrate variable efficacy and do not prevent its appearance. The newly approved vaccine is a more potent form of the VZV vaccine currently approved for use in the prevention of varicella in children. The HZ vaccine decreases the incidence of HZ and burden of illness in adults aged 60 years and older, and appears more efficacious in patients aged 60–69 than in those over 70 years. Importantly, the incidence of postherpetic neuralgia is significantly reduced in patients who receive HZ vaccine, irrespective of age or sex. The duration of postherpetic neuralgia is also significantly reduced. The HZ vaccine has demonstrated a favorable safety profile. Most treatment-related adverse events are related to the site of injection and are generally mild. The HZ offers a significant step forward in the treatment of HZ and its sequelae.

Based on the results of the Shingles Prevention Study, the United States Food and Drug Administration (FDA) and European Union approved the HZ vac-

cine for the prevention of herpes zoster in adults age 60 years and older. The US Advisory Committee on Immunization Practices of the Centers for Disease Control and Prevention now recommends standard delivery of the HZ vaccine to immunocompetent adults age 60 years and older with a history of varicella.

### Conclusions

Given that as many as half of the 1 million cases of herpes zoster occur in the age group for which the vaccine is indicated, and that the vaccine reduces the incidence of herpes zoster by slightly over 50%, wide application of the HZ vaccine in the indicated population could prevent as many as 250 000 cases of HZ every year.<sup>3</sup>

### Riassunto

*Un nuovo vaccino (Zovastax) per prevenire l'herpes zoster*

Il virus varicella-Zoster è l'agente eziologico della varicella e dell'herpes zoster (HZ) nel genere umano. L'HZ è dovuto alla riattivazione del virus Varicella-Zoster (VZV) latente all'interno dei gangli sensitivi. L'incidenza e la gravità dell'HZ aumentano con l'avanzare dell'età; oltre la metà delle persone in cui compare HZ hanno un'età superiore a 60 anni. La complicità debilitante più frequente è rappresentata dalla nevralgia post-erpetica, una sindrome neuropatica dolorosa che persiste o si sviluppa dopo la guarigione dell'esantema dermatomero e che può essere di lunga durata e disabilitante. Vi sono molte limitazioni circa le terapie attuali per l'HZ e la nevralgia post-erpetica. È stato sviluppato un vaccino con VZV vivo attenuato che recentemente è stato approvato dalla Food and Drug Administration (FDA) Statunitense e dall'Unione Europea per la prevenzione dell'HZ in soggetti di 60 o più anni di età. In uno studio randomizzato, in doppio cieco versus placebo in 38 546 adulti di 60 o più anni l'utilizzo del vaccino ha ridotto il carico patologico dovuto all'HZ del 66,5% ( $P < 0,001$ ) e ha ridotto l'incidenza dell'HZ del 51,3% ( $P < 0,001$ ). In questa review gli autori discutono l'utilizzo storico del vaccino anti-varicella nei bambini e il successivo sviluppo del nuovo vaccino anti-zoster.

**PAROLE CHIAVE:** Herpes zoster – Nevralgia post-erpetica – Vaccini.

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# Vaccinations against cutaneous *Leishmania* infection

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Cutaneous leishmaniasis is an endemic disease with increasing incidence, even in Europe. Recently, it has attracted more attention due to reactivation in immunocompromised hosts, e.g. in the context of HIV. Therapeutic options range from topical treatment to systemic therapy for more complex cases. A vaccine does not exist at present. Despite of several attempts, vaccine generation has proven to be difficult even though protective immunity against this obligate intracellular protozoan parasite is dependent on the development of antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells capable of releasing IFN $\gamma$ . IFN $\gamma$ , in turn, activates phagocytic host cells to generate oxidative radicals and to eliminate the parasite. This review will describe the basic immunology leading to the development of protective immunity in infected individuals. In addition, the authors will focus on highlighting the different approaches utilized for vaccine development and describe what a efficient vaccine may consist of. Combined intensive research in the fields of basic parasitology and immunology may allow for the generation of an efficacious vaccine against this important human pathogen in the near future.

**KEY WORDS:** Vaccination - Leishmaniasis - Leishmaniasis, therapy - Immunity.

Infections with *Leishmania* parasites are worldwide increasing in number, central Europe is no exception. Infections often first become apparent after return from an endemic region. Depending on the *Leishmania* species and the host immune status, cutaneous, mucocutaneous or visceral leishmaniasis may develop (Table I).<sup>1, 2</sup>

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*Leishmania* infections are transmitted by the bite of a sand fly.<sup>2, 3</sup> The initial infection of tissue macrophages (M $\Phi$ ) most often occurs in the absence of inflammation, because the parasite invades phagocytic cells without notice of the immune system. Within M $\Phi$ , *Leishmania* parasites silently replicate and thus the infection is established. At this time, clinical symptoms are not apparent yet. In the further course of the following weeks post infection, inflammatory cells are recruited to the infected skin - neutrophils, more M $\Phi$ , dendritic cells (DC) - and become infected with *Leishmania*.<sup>3</sup> Infection of DC by the parasites leads to cell activation and DC migrate to the draining lymph nodes to present *Leishmania* antigen to naïve T and B cells.<sup>4, 5</sup> The development of antigen-specific lymphocytes is critical both for the formation of a tissue granuloma (responsible for a restriction of the infection to the site of inoculation) and ultimately for eliminating the parasite from the host organism.<sup>6</sup>

How does cellular immunity against *Leishmania* develop (Figure 1)? Healing and lesion resolution is primarily triggered by tissue infiltrating T cells capable of producing interferon (IFN)- $\gamma$ .<sup>6</sup> IFN $\gamma$  activates infect-

TABLE I.—Dependent of the subspecies of *Leishmania*, typical forms of disease associated with characteristic immune responses develop.

| Leishmania spp.              | Form of Leishmaniasis                 | Immune response |
|------------------------------|---------------------------------------|-----------------|
| <i>L. donovani</i>           | Visceral disease (Kala Azar)          | Th2-predominant |
| <i>L. bras. brasiliensis</i> | Mucocutaneous disease (Espundia)      | Mixed response  |
| <i>L. major</i>              | Dermatotropic disease (Oriental sore) | Th1-predominant |
| <i>L. tropica</i>            |                                       |                 |
| <i>L. mexicana</i>           |                                       |                 |
| <i>L. brasiliensis</i>       |                                       |                 |

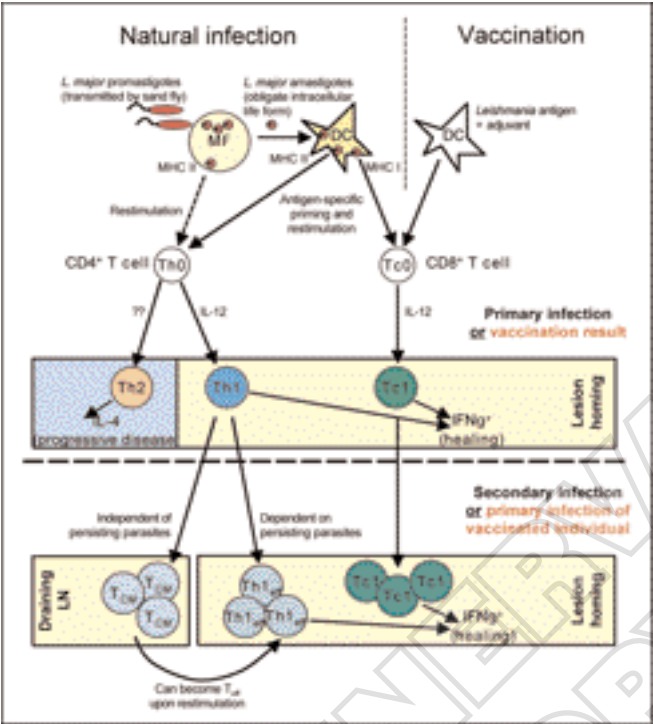


Figure 1.—Upon infection with *L. major* or, optimally, vaccination, protective Th1/Tc1-dependent immunity develops. *L. major* parasites are inoculated into a mammalian host by the bite of a sand fly. The promastigote life form is then internalized by macrophages (MΦ), in which they transform into obligate intracellular amastigotes. Amastigotes released into the tissue infect dendritic cells (DC) capable of priming *Leishmania*-specific CD4<sup>+</sup> and CD8<sup>+</sup> Th1 and Tc1 cells, respectively. Alternatively, targeting DC with antigen in the presence of adjuvant leads to induction of Th1/Tc1 immunity directed against *Leishmania* in a vaccination approach. Healing and lesion resolution in leishmaniasis is dependent on the release of IFNγ from *Leishmania*-specific T cells (Th1/Tc1) homing to the lesion. If in an immunocompromised host Th2 immunity develops, progressive disease associated with visceralization is observed. Upon restimulation of antigen-specific T cells in an immune host (due to a resolved primary infection or a successful vaccination), IFNγ is rapidly released from antigen-specific effector Th1 cells (Th1<sub>eff</sub>) and Tc1 cells. Thus, infection is prevented. The maintenance of Th1<sub>eff</sub> is dependent on a few life parasites persisting in the host. In addition, central memory cells (T<sub>CM</sub>) capable of becoming T<sub>eff</sub> after proliferation in the draining lymph node upon re-challenge can also maintain protective immunity. T<sub>CM</sub> development is independent of persisting parasites and can be induced by recombinant vaccines.

ed MΦ to produce oxidative radicals, especially nitric oxides (NO), which efficiently kill the parasite. Which T cell subtypes produce IFNγ is dependent on the priming environment. Infection of DC leads to the synthesis of IL-12, the most important cytokine responsible for induction of MHC class II-dependent CD4<sup>+</sup> T cells of a T helper type 1 (Th1) phenotype.<sup>6</sup> These cells primarily produce IL-2 and IFNγ upon antigen-specific restimulation. In addition, antigen presentation in the MHC class I pathway also induces priming of IFNγ-producing cytotoxic T cells (CD8<sup>+</sup>, Tc1 cells). Previously, it was demonstrated that both, IFNγ-secreting *Leishmania*-specific CD4 as well as CD8 cells contribute to protection against natural infection with this pathogen.<sup>7-11</sup> If, however, Th1/Tc1 development is hampered in an individual (e.g. due to low CD4 counts in HIV-infected humans or due to inadequate development of Th2 immune responses characterized by production of IL-4, IL-5 and IL-13 as observed in patients with allergic and some autoimmune diseases), progressive disease with a non-healing phenotype and even visceralization of the infection can be observed.<sup>1</sup> Interestingly, in the most recent years, it was also demonstrated that *Leishmania*-specific regulatory T cells suppress full elimination of the parasite from the host (thus maintaining protective memory responses via IL-10).<sup>12</sup>

In mice recovered from infection with *L. major*, the persistence of viable parasites in secondary lymphoid organs was important for the development of protective memory responses, because sterile cure in IL-10-deficient mice resulted in loss of long-term immunity against re-infection.<sup>13</sup> However, it has been shown recently that anti-*Leishmania* CD4<sup>+</sup> T cells include parasite-dependent T effector cells as well as parasite-independent central memory T cells.<sup>14</sup> Thus, the continued presence of live parasites may not absolutely be required for the maintenance of protective memory immune responses. It is of note to mention that

CD4<sup>+</sup> T cells that secrete IFN $\gamma$  have only limited capacity to develop into memory cells compared with IL-2- or IL-2- and IFN $\gamma$ -producing cells.<sup>15, 16</sup> A recent study showed that the degree of protection against *L. major* infection in mice can be predicted by the frequency of CD4<sup>+</sup> T cells simultaneously producing IFN $\gamma$ , IL-2 and TNF $\alpha$ .<sup>17</sup>

Most of the above mentioned knowledge has been investigated in detail in a mouse model that closely mimics natural *L. major* infection *in vivo* and the critical findings have already been verified in infected humans.<sup>6</sup> Due to the specific T cell response, immunocompetent humans heal their infection with *L. major* after several months and develop life-long protective immunity against the same subspecies of *Leishmania*. Specific antibodies initially contribute to DC infection thus promoting establishment of T cell-dependent protection.<sup>18</sup> Later on, they worsen disease outcome due to alternative M $\Phi$  activation via Fc receptors leading to pathological IL-10 release responsible for parasite persistence.<sup>19</sup>

Therapeutic options range from topical treatment of simple cutaneous leishmaniasis to systemic therapy, which is needed for more complex cases of cutaneous as well as mucocutaneous and visceral disease.<sup>20</sup> The efficacy of each therapy is dependent on its species- and stage-specific effect on *Leishmania* growth. In addition, most therapeutics have in part even severe side effects and are relative cost intensive making treatment in endemic regions problematic and difficult. The immunological facts described above indicate that due to the development of life-long immunity after resolution of infection, the development of a vaccine should be possible.<sup>21</sup> Despite of several attempts, this has proven to be difficult. This review will focus on describing the different approaches used so far and what a future efficient vaccine may consist of.

### Vaccination with whole parasites

In some endemic regions, artificially induced or provoked infections of children at body sites distant from face or extremities are used to avoid the development of disfiguring scars later in life. This approach is called "leishmanization".<sup>6</sup> Inoculation of live, wild-type *L. major* remains the only really successful vaccine in humans so far.<sup>22</sup> Interestingly, in this case, injection of *L. major* subcutaneously provided even better protection than intradermal vaccination against

reinfection<sup>22</sup> which was due to the rapid co-recruitment of IL-10-producing CD4<sup>+</sup> T cells to the rechallenge site together with effector Th1/Tc1 cells positive for IFN $\gamma$  after physiological intradermal antigen application. This fact may have to be considered when designing new vaccine approaches.

Because of the potential risk of parasite persistence and the fact that immunosuppression (e.g. due to HIV co-infection or diabetes) may occur in an individual later on, vaccination with wild type parasites is nowadays considered unethical. The ability to manipulate the *Leishmania* genome created new options to generate modified parasites with attenuated growth or infectability. For example, silent information regulatory 2 gene (SIR2)-deficient *L. infantum* were unable to replicate once transformed into the obligate intracellular life form.<sup>23</sup> Thus, after inoculation *in vivo*, these parasites persisted in the host for ~6 weeks, but did not establish an infection. In parallel, however, SIR2<sup>-/-</sup> *L. infantum* generated robust IFN $\gamma$  responses *in vivo* leading to protection against re-infection later on.<sup>23</sup> But again, if long-term memory responses can be maintained without persisting parasites as in this approach, is unclear. Other examples of genetically altered parasites were centrin<sup>-/-</sup> *L. donovani*<sup>24</sup> with reduced replication in both the promastigote and amastigote stage, glucose transporter gene family<sup>-/-</sup> *L. mexicana*,<sup>25</sup> leishmanolysin<sup>-/-</sup> *L. major*<sup>26</sup> and *Leishmania* phosphoglycan (LPG)1<sup>-/-</sup> or LPG2<sup>-/-</sup> *L. major*.<sup>27, 28</sup> Of concern was the fact that, after some time, the LPG2-deficient parasites partially reverted to virulence using a compensatory mutation.<sup>29</sup> More genetically modified parasites have been generated (compare e.g. Table II and Selvapandiyan *et al.*<sup>24</sup>). In addition, parasites non-pathogenic for humans such as *L. tarentolae* may be successful vaccine candidates eliciting cross-reactive immune responses (e.g. against *L. donovani*).<sup>30</sup>

In summary, using attenuated parasite strains two requirements would be met that are potentially important for vaccination: first, the parasite would persist after infection thus promoting long-term memory CD4 and CD8 responses, and – at the same time – not induce harm because of its persistence in the infected host.<sup>24</sup> However, several disadvantages of attenuated parasites are obvious: 1) similar to natural infections, protection against one subspecies of *Leishmania* using these strains does not (necessarily) induce cross-protection against other *Leishmania* strains; 2) induction of protection would require activation of intrinsic DC

TABLE II.—*Examples for life (mutant) parasites used for vaccination.*

| Organism used for vaccination                                            | Genetic or other alteration                                                                                              |
|--------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------|
| <i>Dermatotropic parasites</i>                                           |                                                                                                                          |
| — <i>L. major</i> [Tabbara 2005 22]                                      | Wild type parasites<br>±combination with CpG                                                                             |
| — <i>L. mexicana</i> [Burchmore 2003 25]                                 | Glucose transporter genes-/-<br>(reduced infection of MΦ <i>in vitro</i> )                                               |
| — <i>L. major</i> [Joshi 2002 26]                                        | Leishmanolysin-/-<br>(impaired lesion formation <i>in vivo</i> )                                                         |
| — <i>L. major</i> [Spath 2000 ???, Spath 2003,27 or 28??? Spath 2004 29] | LPG-/-<br>LPG1-/- no survival, LPG2-/- persisted <i>in vivo</i><br>and protected without disease, compensatory mutation) |
| <i>Viscerotropic parasites</i>                                           |                                                                                                                          |
| — <i>L. donovani</i> [Selvapandian 2006 24]                              | Centrin-/-<br>(reduced replication for limited time)                                                                     |
| — <i>L. tarentolae</i> [Breton 2005 30]                                  | Non-pathogenic for humans<br>Cross-reacts with <i>L. donovani</i>                                                        |
| — <i>L. infantum</i> [Silvestre 2007 23]                                 | SIR2-/-<br>(unable to replicate in host)                                                                                 |

which may be incapable of inducing protective immunity as observed in susceptible individuals such as BALB/c mice,<sup>1, 31</sup> and 3) the reproducibility and practicability of life vaccines is limited.

### Adjuvants in leishmaniasis

Adjuvants were originally used to enhance the immunogenicity of antigens and to increase resulting immune responses. Strong immunological memory to the immunizing antigen is the primary objective of a vaccine and successful adjuvants must therefore be capable of inducing long-term immunity. Moreover, adjuvants in *Leishmania* vaccines should modify the immune response towards protective Th1/Tc1 immunity. The most frequently used Th1-promoting adjuvants are IL-12 and CpG oligodeoxynucleotides (CpG ODN).

One drawback of cytokines as adjuvants is their relatively short half-life *in vivo* and even in leishmaniasis, sustained IL-12 production is necessary for long-term immunity.<sup>32, 33</sup> In mice vaccinated with killed promastigotes<sup>34, 35</sup> or recombinant LACK protein<sup>34</sup> plus IL-12 as adjuvant, only short-term protection against reinfection with *L. major* was observed. Long-lasting immunity was achieved when protein/rIL-12 were administered repeatedly or when antigen/adjuvant were delivered as plasmid DNA.<sup>34, 35</sup> In visceral leish-

maniasis, co-administration of IL-12 DNA and recombinant protein antigen also resulted in long-term immunity.<sup>36</sup>

Synthetic oligonucleotides with unmethylated CpG dinucleotide motifs are strong Th1-promoting adjuvants. CpG ODN have been shown to activate MΦ and DC via TLR9 to synthesize Th1-associated cytokines including IL-12, IL-18, TNF-α, IFN-α/β, and IFN-γ and to up-regulate costimulatory molecules such as CD40 and MHC class II.<sup>37-40</sup> In leishmaniasis, CpG efficiently induced IL-12-dependent protective immunity in otherwise susceptible BALB/c mice.<sup>41, 42</sup> Furthermore, coinjection of CpG ODN with live parasites resulted in significantly decreased course of diseases and maintained long-term, anti-*Leishmania* immunity.<sup>43</sup> This resulted in the induction of fewer regulatory T cells due to increased IL-6 production from local, infected DC resulting in even more promising vaccination efficacy.<sup>44</sup>

As described above, live vaccines are hardly applicable in humans and alternative vaccination strategies must be chosen. Mice vaccinated with killed promastigotes or recombinant leishmanial protein and CpG as adjuvant induced long-lasting, CD4<sup>+</sup> and CD8<sup>+</sup> T cell-dependent anti-*Leishmania* immune responses. Protection against infectious challenge was detected even six months after vaccination.<sup>45</sup> More recent approaches utilized CpG ODN coencapsulated in liposomes together with recombinant *Leishmania* anti-

TABLE III.—*List of antigens used for vaccines (for references compare text).*

| Protein used for vaccination                                             | Protein mixture of wild type parasites |
|--------------------------------------------------------------------------|----------------------------------------|
| Promastigote lysate                                                      | —                                      |
| Heat killed parasites                                                    | —                                      |
| Leishmania surface glycoprotein (gp) 63                                  | —                                      |
| Histone (H)1                                                             | —                                      |
| Promastigote surface antigen (PSA)-2                                     | —                                      |
| Cathepsin L-like cysteine proteinase (CP)                                | —                                      |
| Leishmania homologue of receptors for activated C kinase (p36/LACK)      | —                                      |
| L. major homologue of the eucaryotic thiol-specific antioxidant (TSA)    | Leish-111f (tri-fusion protein)        |
| L. major homologue of the eucaryotic stress-inducible protein-1 (LmSTI1) | —                                      |
| Leishmania elongation and initiation factor (LeIF)                       | —                                      |

gens<sup>46, 47</sup> and *in vivo* experiments in mice confirmed enhanced immunogenicity of the *Leishmania* antigen in these settings. In summary, Th1-promoting CpG is capable of inducing long-lasting, CD4<sup>+</sup> and CD8<sup>+</sup> T cell-dependent immune responses and is therefore the most promising adjuvant for vaccines against infection with *Leishmania spp.* so far.

Imiquimod, an immune response modifier which can be applied topically, was originally used for the treatment of genital warts caused by human papillomavirus infections.<sup>48</sup> Furthermore, it has been shown that imiquimod activates *Leishmania*-infected MΦ to release nitric oxide, thus killing intracellular parasites, and that it efficiently ameliorates the course of infection in murine leishmaniasis.<sup>49</sup> The exact mechanism by which imiquimod activates the immune response is unknown yet, but activation of immune cells by ligation of TLR7 seems to be pivotal resulting in the secretion of proinflammatory cytokines and the expression of costimulatory molecules.<sup>50</sup>

Thus, in the future, imiquimod, CpG motifs suitable for humans and/or other Toll-like receptor (TLR) agonists may serve as promising adjuvant candidates for the development of anti-*Leishmania* vaccination strategies.

Protein-based vaccines against leishmaniasis

As described above, a successful vaccine against leishmaniasis should induce *Leishmania*-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells,<sup>6</sup> sustained IL-12 production<sup>33</sup> as well as long-lasting memory immune responses. Non-live, protein-based vaccines which are capable of expanding memory T cells that can survive independently of life, persisting parasites and that are capable of producing both IL-2 and IFNγ might therefore also

be efficacious and become an alternative solution to live vaccines.

Parasite promastigote lysate in combination with BCG as adjuvant was one of the first safe, non-living vaccine tested in cutaneous leishmaniasis, but failed to confer substantial protection in humans.<sup>51, 52</sup> *In vivo* studies in experimental murine cutaneous leishmaniasis revealed that heat-killed parasites plus rIL-12 as adjuvant were able to mediate short-term protection, but completely failed to induce long-lasting immunity.<sup>35</sup> In addition to its missing potency and durability, vaccines based on promastigote lysate also possess low reproducibility, because of versatile variations among different parasites preparations. Therefore, well-defined, recombinant proteins may be more promising antigens for a successful vaccination.

Indeed, several *Leishmania*-derived proteins have already been identified as candidates for a second generation vaccine (Table III). These include the *Leishmania* surface glycoprotein 63 (gp63) and the histone H1, which have been tested in mice<sup>46, 53-55</sup> and monkey<sup>56, 57</sup> and which were able to improve the course of infection. Immunization with the promastigote surface antigen-2 (PSA-2) resulted in complete protection against infectious challenge with *L. major* in resistant mice, but mediated only partial protection in susceptible animals.<sup>58</sup> Cathepsin L-like cysteine proteinases (CP), which are mainly expressed and active in the amastigote life form, have received considerable attention.<sup>59</sup> Three classes of CP have been identified in *Leishmania*.<sup>60</sup> Vaccination with a recombinant hybrid protein of cysteine proteinase type I (CPB) and II (CPA) partially protected BALB/c mice against infection with *L. major*.<sup>61</sup> One of the most promising *Leishmania*-antigens appears to be the *Leishmania* homologue of receptors for activated C kinase (p36/LACK). It is a well-conserved 36 kDa protein, highly immuno-

genic,<sup>62</sup> expressed in both life forms of all *Leishmania* spp.<sup>63</sup> responsible for the rapid expansion of V $\beta$ 4-V $\alpha$ 8<sup>+</sup> CD4<sup>+</sup> T cells.<sup>64</sup> Furthermore, deletion experiments with *L. major* revealed that LACK is required for parasite viability as well as pathogenesis.<sup>65</sup> Consequently, LACK was utilized as target antigen in several vaccine strategies. Immunization of mice with rLACK protein in combination with rIL-12 resulted in partial protection of BALB/c mice against infectious challenge with *L. major*.<sup>33, 34, 63</sup>

It is now known that components of the sand fly saliva coinjected with parasite exacerbate the course of disease due, in part, to an upregulation of Th2 immune responses.<sup>66</sup> However, immunization with sand fly saliva prior to infection completely abrogated the saliva effect in BALB/c as well as C57BL/6 mice and moreover conferred protection against infection with *L. major* infections transmitted by sand fly bites.<sup>67</sup> In the meantime, two defined saliva proteins, SP15 and maxadilan, were identified and vaccinations of mice with these proteins protected animals against a challenge inoculation containing *L. major* and sand fly saliva.<sup>68, 69</sup>

All protein-based vaccines mentioned above were able to efficiently induce short-term immunity against *Leishmania* infection, but over all failed to elicit long-lasting memory immune responses as obtained by DNA-based vaccine strategies<sup>34, 70, 71</sup> due, in part, to insufficient induction of CD8<sup>+</sup> T cells<sup>35</sup> as well as poorly sustained presentation of antigen. Antigens capable of targeting DC *in vivo* would therefore be appropriate candidates for protein-based vaccines. Fusion proteins comprised of defined antigens and the HIV-1 TAT protein transduction domain are known to translocate directly into the cytosol of cells<sup>72-74</sup> and may thus be utilized to target skin DC *in vivo*. Moreover, utilizing TAT fusion proteins would additionally ensure antigen presentation via MHC class I as well as class II pathways<sup>75-77</sup> leading to induction of both CD4 and CD8 anti-*Leishmania* responses. Indeed, utilization of a fusion protein comprised of TAT and LACK as vaccine promoted significantly better protection against *L. major* as compared to LACK alone.<sup>78</sup>

To develop a recombinant vaccine that results in similar protection as obtained with live vaccines, more than just one antigen may have to be used. Such multi-antigen vaccines should contain highly immunogenic proteins present in both amastigote and promastigote life forms. For example, Leish-111f, a tri-

fusion protein consisting of TSA (*L. major* homologue of the eucaryotic thiol-specific antioxidant), LmSTI1 (*L. major* homologue of the eucaryotic stress-inducible protein-1) and LeIF (*Leishmania* elongation and initiation factor) is one example for a poly protein-based vaccine.<sup>79</sup> Mice immunized with Leish-111f together with MPL-SE (monophosphoryl lipid A plus squalene) as adjuvant were protected against challenge with *L. major* even three months after the last immunization.<sup>80, 81</sup> Currently, Leish-111f is tested in human trials and as a first step, a Phase I, double-blind, dose-escalation trial in normal volunteers has been performed in the USA.<sup>79</sup>

### Plasmid-based vaccines

DNA based vaccines are based on the direct injection of eucaryotic expression vectors, which encode the antigen of interest.<sup>82</sup> Regardless of the route of administration, the plasmid has to reach the nucleus of the transfected host cell in order to be transcribed to mRNA. The mRNA will then be translated inside the host cell and the resulting antigen is presented to the immune system in order to induce a full range of immune responses. It is important to note that the amount of antigen produced *in vivo* after DNA inoculation is usually in the pg to ng range. Given the relatively small amounts of protein synthesized by DNA vaccination, the efficient induction of immune responses must relate to the type of antigen presenting cell (APC) transfected and/or the immune-enhancing properties of the DNA itself. There are at least three different mechanisms by which DNA vaccines are processed and presented *in vivo* to elicit immune responses: 1) direct priming by somatic cells (*e.g.* myocytes or keratinocytes); 2) direct transfection of professional APC; and 3) "cross-priming", in which plasmid DNA transfects a somatic cell and/or professional APC and the secreted protein is processed by untransfected DC and presented to T cells.

It is well known that plasmid DNA derived from bacteria – in addition to carrying the antigen of choice – acts as non-specific adjuvant by stimulating a Th1 response.<sup>83</sup> However, several lines of evidence indicate that the protection induced by multicomponent DNA vaccines was not based on this non-specific immunostimulatory effect of the vector DNA alone, but rather on their antigen-specific sequences. In addition, DNA vaccinations are sometimes associated with low or

absent humoral responses. This is discussed controversially, but it seems to be a common feature of DNA vaccines that they favor cellular rather than humoral responses.<sup>70, 84-86</sup>

Even in the case of successful protective recombinant vaccines against cutaneous leishmaniasis, their plasmid DNA counterparts had the additional advantage of being more stable and easier to prepare.<sup>86</sup> As described above, recombinant protein vaccines in mice showed that protein antigens such as gp63,<sup>56</sup> PSA,<sup>87, 88</sup> or LACK<sup>89</sup> induced strong immune responses, but weak and short protection against *Leishmania* infection. In contrast, when these antigens were used as DNA vaccines they induced a stronger immune response and significantly better protection than the recombinant form. In BALB/c mice, a eukaryotic expression vector driving the gp63 gene under the control of CMV or RSV promoters was used.<sup>90, 91</sup> The NH36 DNA vaccine induced strong protection against visceral and cutaneous leishmaniasis, suggesting that this DNA vaccine represents a good candidate for use against several *Leishmania* species.<sup>86</sup> Administration of LACK-DNA was used in various DNA vaccination models leading to protective immunity of mice infected with *L. major*.<sup>84</sup> The vaccination effect was long-lasting, associated with antigen-specific IFN $\gamma$  production, and IL-12 dependent.<sup>33</sup> A recent study<sup>92</sup> compared the candidate DNA vaccines LACK, PSA2, Gp62, LeIF, two p20 and ribosomal like proteins, in addition to different truncated LACK variants. The most promising results were obtained with the LACK gene and it was even more protective when used as a p24 truncated form. Furthermore, the presence of a tandem repeat of immunostimulating sequences (ISS) in the plasmid backbone played an important adjuvant effect in the observed protective effect induced by the DNA vaccine encoding LACK p24.

Vaccination with plasmid DNA has been shown to induce protective immunity through both MHC class I- and class II-restricted T cell responses in a variety of experimental infection models.<sup>93</sup> Thus, in leishmaniasis, the ability of DNA vaccines to elicit MHC class I responses may be advantageous over conventional protein vaccinations in providing a more broad-based and potentially durable immune response. As such, long-lived LACK-responsive CD8<sup>+</sup> and CD4<sup>+</sup> T cells were induced by LACK DNA in BALB/c mice.<sup>34, 94</sup>

Several routes of administration for DNA based vaccinations were described so far. Intranasal vacci-

nation of LACK DNA was shown to be a feasible vaccination route and promoted rapid and durable protective immunity.<sup>95</sup> Mice injected intramuscularly developed significant resistance against challenge with *L. major* parasites associated with preferential Th1 response. Nonetheless, intradermal applications in the footpad or the ear were the major routes of administration of DNA vaccines against murine *L. major* infections.<sup>33, 35, 43, 95</sup>

A comparison of the degree of protection induced by three vaccination strategies (DNA/DNA, protein/protein and DNA/protein) against *L. major* infection using signal peptidase type I in BALB/c mice indicated that the DNA/DNA strategy led to more effective protection than the two other approaches and induced a 81% reduction in lesional parasite loads.<sup>96</sup> Among immunization protocols against other parasitic infections, e.g. malaria, vaccination strategies based on DNA priming followed by a boost with either protein or a viral vector encoding this antigen has proven successful. A recombinant vaccinia virus (rVV) expressing the same parasitic antigen as the initial DNA sequence has provided significant protection that correlated with activation of cellular immune response, especially CD8<sup>+</sup> T cells.<sup>97-99</sup> A recent study showed that vaccination of BALB/c mice by priming with LACK-DNA followed by a boost with modified vaccinia virus Ankara (MVA)-LACK triggered a Th1 immune response leading to high protection against *L. major*, which correlated with induction of high numbers of antigen specific CD8<sup>+</sup> T cells.<sup>100</sup> An additional plasmid DNA vaccination method is based on the usage of linear minimalistic, immunologically defined gene expression (MIDGE) vectors that contain only the minimal sequence required for gene expression, which was considered to be a good alternative to plasmid for immunization.<sup>101</sup>

Studies in different disease models showed that animals primed with a DNA vaccine and boosted with recombinant protein acquired a higher level of immune protection compared with DNA or recombinant protein regimens alone.<sup>102, 103</sup> Previous attempts to enhance immunity by use of a similar heterologous prime-boost vaccination strategy have also been successful against murine cutaneous leishmaniasis.<sup>104, 105</sup> In addition, a regimen of ORFF DNA priming followed by a booster dose of rORFF protein was effective in triggering protection against visceral leishmaniasis correlating with a skewing toward Th1 immunity.<sup>36</sup> The efficacy

may rely, in part, on the ability of DNA vaccines to generate high-affinity T cells, whose numbers are expanded after the following antigen boost. This mainly relies on their relatively low-level, but persistent, expression of immunogenic proteins *in vivo*. The resulting primed T cells may display higher average affinities for MHC peptide molecules after multiple exposures to antigen. Moreover, limiting the doses of antigen may select for T cells that have receptors of increased affinity.<sup>106, 107</sup>

Finally, the choice of antigens seems to be critical for the vaccination efficacy using plasmid DNA. Immunization with a large group of antigens encoded by a DNA library (1 000 to 3 000 clones) was effective in vaccinating mice against *Mycoplasma spp*<sup>108</sup> and *L. major*.<sup>109</sup> Another study demonstrated that immunization with sequential fractions of a cDNA library was a powerful strategy for identifying anti-infective vaccine candidates against visceral leishmaniasis.<sup>110</sup> Two different cocktail DNA vaccines composed on one hand of the *Leishmania* genes LACK, LmSTI1, and TSA, and, on the other hand, DNA encoding cysteine proteinases of *L. major* conferred protective, long-lasting immunity.<sup>35, 111</sup> In addition, immunization with different groups of cDNA encoding for histone proteins (subpool FL-O-B and FL-O-D) induced a strong Th1 response and protection against *L. donovani* challenge.<sup>110, 112</sup> It should be noted that immunization with full-length cDNA encodes potentially all possible T cell epitopes, which may be a reason for its efficacy.

Despite of such promising results, there are no DNA vaccines in active clinical and veterinary medical trials. Therefore, further investigations on different methods, such as DNA vaccines using a cocktail of antigens or prime/boost approaches have to be performed.

### DC-based vaccination strategies

Due to their ability to initiate and regulate adaptive immune responses, to induce both CD4<sup>+</sup> and CD8<sup>+</sup> T cells and to elicit long-lasting memory responses, DC are the most potent APC of the organism.<sup>113</sup> Since DC can be long-lived, sustained presentation of antigen and stimulation of T cell in the draining lymph nodes may be possible.<sup>114</sup> Thus, DC-based vaccines seem to represent a promising and successful strategy.

In the model of murine leishmaniasis, (epi-)dermal DC play a key role in initiating and maintaining pro-

TECTIVE anti-*Leishmania* immune responses. Upon Fc receptor-mediated phagocytosis of amastigotes,<sup>18</sup> DC become activated,<sup>5</sup> migrate to the draining lymph nodes<sup>115</sup> and initiate *Leishmania*-specific T cell priming. In murine cutaneous leishmaniasis, DC infected with viable parasites efficiently activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells and vaccinated against progressive infection in susceptible BALB/c mice, when injected intradermally.<sup>116</sup> Comparable results were obtained when mice were immunized intravenously with Langerhans cells (LC),<sup>117, 118</sup> bone marrow-derived DC<sup>119, 120</sup> or plasmacytoid DC,<sup>121</sup> which were pulsed with promastigote lysate.

Moreover, DC seem to be the source of secreted IL-12,<sup>5, 122</sup> which is required during T cell activation to enable the development of protective Th1 immunity. However, a recent study suggests an additional IL-12-independent, but CD70-dependent pathway of DC-mediated Th1 cell activation.<sup>123</sup> DC-derived IL-1 $\alpha$  additionally contributes to protective immunity in resistant C57BL/6 mice,<sup>31, 124</sup> whereas DC-derived IL-12p40 homodimer facilitates susceptibility in BALB/c mice.<sup>125</sup> In DC-based vaccination protocols, protection was dependent on DC-derived IL-12.<sup>117</sup> Protection mediated by lysate-pulsed DC could be enhanced, when DC were engineered by retroviral gene transfer techniques to secrete high levels of biologically active IL-12.<sup>126</sup> However, IL-12 expressed by the immunizing DC was not required, when DC were pre-stimulated *in vitro* by exposure to CpG ODN.<sup>120</sup>

The route of immunization is pivotal for development of protective immunity since homing of CD8<sup>+</sup> T cells into the skin was only detected when DC were administered i.d.<sup>127</sup> leading to the conclusion that a DC-based anti-*Leishmania* vaccine should preferentially be applied intradermally.

Since immunisation with DC infected with viable parasites or pulsed with promastigote lysate are not safe and therefore hardly applicable in humans out of ethical reasons, DC pulsed with defined, recombinant antigens would be more appropriate vaccine candidates, especially because of higher reproducibility. LC loaded with a mixture of the recombinant proteins LACK, gp63, PSA and LeIF or pulsed with the single antigens LACK or LeIF alone were able to mediate significant protection against infectious challenge with *L. major* in susceptible BALB/c mice.<sup>117</sup> The observed protection was comparable to the immunity obtained with promastigote lysate-pulsed LC. Thus, vaccines

based on single, defined proteins can be potentially as efficacious as immunisations with crude antigen mixtures as present in whole parasite preparations. Recently, a study suggested that pulsing DC with recombinant antigens in the presence of CpG ODN is beneficial for the development of protective immunity.<sup>128</sup> In addition, bone marrow-derived DC loaded with a mixture of histones (H2A, H2B, H3 and H4) of *L. infantum* and CpG ODN were capable of mediating protection against infectious challenge with *L. major*. Reduced lesion volumes were accompanied by significantly decreased lesional parasite burdens. However, DC pulsed in the absence of CpG failed to induce comparable anti-*Leishmania* immune responses.

Induction of CD8<sup>+</sup> T cells necessary for substantial protection against natural *L. major* infection<sup>7</sup> requires presentation of antigens via MHC class I molecules and, therefore, cytosolic distribution of proteins after antigen uptake by DC. DC pulsed with antigens ingest proteins predominantly by endocytosis, thus facilitating antigen presentation in the MHC class II pathway. Therefore, transduction of DC with TAT-fusion proteins or transfection of DC with RNA/DNA encoding for appropriate antigens as utilized routinely in tumor immunology<sup>75, 76, 129, 130</sup> would be promising strategies to enhance the induction of antigen-specific CD8<sup>+</sup> T cells.

Although DC-based immunisations have been shown to have a high potency and are very efficacious, they are hardly applicable in *Leishmania*-endemic countries. Isolating DC from humans or their generation from buffy coats are laborious and expensive procedures. Thus, DC-based vaccinations can only serve as proof-of-principle experiments for the development of protein-based vaccines. Ultimately, vaccines which are capable of targeting and activating DC *in vivo* would be highly desirable.

## Conclusions

In summary, none of the reported vaccination strategies have solved the problem of generating a sufficient vaccine against *Leishmania* infections so far. To tackle this question, a number of complex problems related to this parasite and the immunity directed against it have to be considered. The parasitic strain, the mouse model adopted, the parasite load, the route of administration, the candidate antigen and - in addition - the experimental procedure (e.g. high dose vs

low dose of parasite inoculation) may explain the different vaccination outcomes shown in the different reports.

Development of new vaccines that induce long-lasting Th1 immunity is based on a detailed understanding of the regulation of Th1/Tc1 effector and memory differentiation. First, the vaccine itself and/or an adjuvant must have the capacity to stimulate the appropriate cytokines. A vaccine that is "too" efficient and which generates mostly terminally differentiated IFN $\gamma$  single-positive CD4<sup>+</sup> T cells may mediate protection over a relatively short period of time, but would be limited in its capacity to mediate sustained protection.<sup>131</sup> A successful vaccine must thus induce an appropriate balance of Th1 lineage cells: first, cells that mediate protective functions and, second, those which can be maintained following subsequent antigen encounter (memory response). In addition, an efficacious vaccine must also induce *Leishmania*-specific CD8<sup>+</sup> T cells as well as CD4 cells.

As discussed above, several vaccination approaches have been tested with limited success. From the information collected, one can conclude that two components appear to be critical for vaccine design against *Leishmania*: first, the choice of antigen and second, the route of administration combined with the adjuvant chosen. As antigens, whole (attenuated) parasites, parasite lysates, protein preparations, and plasmid DNA encoding for *Leishmania* proteins have been used. All of these have been combined to various degrees with DC or adjuvants targeting APC *in vivo* (such as skin-derived DC). Recently, new substances have been introduced (such as TLR-agonists) which may have superior adjuvant activity as compared to the ones previously tested. Interestingly, combinations of these strategies appeared to be most effective overall, so that a promising future vaccine against *Leishmania* infections may be comprised of several antigens in different application forms which require a differential prime-boost strategy to induce long-lasting protective immunity against this important human pathogen.

## Riassunto

*Vaccinazioni contro l'infezione cutanea da Leishmania*

La leishmaniosi cutanea è una patologia endemica con incidenza in crescita, anche in Europa. Recentemente, ha suscitato maggior attenzione a causa della riattivazione in ospiti immunocompromessi, per esempio nei soggetti HIV

positivi. Le opzioni terapeutiche variano dal trattamento topico alla terapia sistemica nei casi più complessi. Attualmente, non esiste un vaccino. Nonostante numerosi tentativi, la generazione di vaccini è risultata difficile, sebbene l'immunità che protegge contro questo parassita protozoiario intracellulare obbligato dipenda dallo sviluppo di cellule T CD4<sup>+</sup> e CD8<sup>+</sup> antigene-specifiche capaci di rilasciare INF $\gamma$ . L'INF $\gamma$ , a sua volta, attiva i fagociti dell'ospite a produrre radicali dell'ossigeno e ad eliminare il parassita. Questa review descrive le nozioni di immunologia di base per lo sviluppo dell'immunità protettiva in soggetti infetti. Inoltre, evidenzieremo i differenti approcci utilizzati per lo sviluppo del vaccino e descriveremo in cosa dovrebbe consistere un vaccino efficace. La ricerca intensiva combinata nel campo della parassitologia e dell'immunologia di base potrebbe permettere, nel prossimo futuro, la generazione di un vaccino efficace contro questo importante patogeno umano.

**PAROLE CHIAVE:** Vaccinazione - Leishmaniosi - Leishmaniosi, terapia - Immunologia.

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## Immunotherapeutic strategies for the treatment of malignant melanoma

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The incidence of cutaneous malignant melanoma is increasing at a faster rate than any other cancer worldwide. Despite new advances in surgical management of melanoma, this malignancy remains one of the most aggressive and intractable to treat among other solid tumors. Continuous search for better therapeutics led to the development of various immunological approaches applicable to the treatment of this melanocytic malignancy. Multiple peptide, dendritic cell, adjuvant, lymphocyte, and virus-based strategies were established and tested in preclinical and clinical studies with varying degrees of clinical success. However, the most recent investigations in melanoma immunotherapy have clearly demonstrated that complex vaccines and the combination of different approaches, such as the use of dendritic cell vaccines in conjunction with costimulatory molecules, are superior to conventional immunization protocols in induction of tumor-specific immune responses. These recent studies open new perspectives for the development of efficient melanoma immunotherapeutics suitable for the treatment of primary and metastatic disease.

**KEY WORDS:** Melanoma - T-lymphocytes, cytotoxic - Dendritic cells - Chemokines - Vaccines.

In the human body, pigment-producing cells (melanocytes) normally reside in the skin, eyes, ears, leptomeninges, oral and genital mucous membranes. Tumorigenic transformation of these cells leads to the development of a melanocytic malignancy also known as melanoma. Based on the site of occurrence,

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melanoma can be classified as skin, mucosal, and uveal. Out of these three, skin melanoma is the most frequent. Although it accounts for only 4% of all skin cancers, it causes the greatest number (77%) of skin cancer-related deaths worldwide.<sup>1</sup> Despite new advances in surgical management, which is curative in many cases of thin melanomas, this malignancy continues to be one of the most aggressive and intractable to treat among other solid tumors.

Better understanding of the human immune system over the last 50 years along with the discovery of tumor-associated antigens (TAAs) in the 1980s<sup>2</sup> led to the development of the two new closely related fields of modern biomedical science: tumor immunology and tumor immunotherapy. For malignancies like melanoma, which are often resistant to chemo and radiation therapy, especially on advance stages, immunotherapy is considered one of the most prominent treatments. To achieve effective immune-based melanoma rejection, several vaccine therapies utilizing peptide vaccination, tumor-associated antigen-pulsed dendritic cells (DCs), and tumor-infiltrating lymphocytes have been tested on pre-clinical animal

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models and in multiple clinical trials. However, the rapid progression of melanoma and its ability to escape treatment present serious challenges for the development of immunotherapeutics. This review will discuss some of these immunotherapeutic approaches developed during last decade and future directions of melanoma immunotherapy.

### Peptide vaccines

The discovery of tumor-associated antigens (TAAs) in the 1980s led to the rebirth of immunotherapeutic approaches for cancer treatment. In early 1990s, a number of genes encoding melanoma associated antigens and their peptide products that can be recognized by cytotoxic T lymphocytes (CTL) have been defined.<sup>3-8</sup> Most of them were identified as immunogenic epitopes from the variety of melanocyte/melanoma-specific proteins including Melan-A, tyrosinase, tyrosinase-related proteins, MART-1, and gp-100, which were found in association with major histocompatibility complex (MHC) class I molecules. Non-melanoma specific antigens, such as MAGE, NY-ESO-1 and E-cadherin and tumor-specific antigens derived from mutated genes, such as mutated p16, or sequences not transcribed under physiologic conditions, which are limited to tumor cells of individual patients were also found to be presented on MHC class I molecules.<sup>9</sup> Since these antigenic epitopes consisted of small peptide fragments, it was suggested that synthetic peptides could elicit a strong anti-tumor immune response when presented to the naïve T cell by antigen presenting cells (APCs). Despite the ability of the peptide vaccines to induce potent immune response against viral and bacterial infections, early studies on vaccination of tumor-bearing hosts with synthetic peptides deterred their use because of limited immunogenicity and their rapid, peptidases-mediated hydrolysis *in vivo*. To address this question, Blanchet *et al.* analyzed a series of 36 non-natural Melan-A/MART-1 peptide derivatives and identified 8 of them, which were protected against proteolysis and retained antigenic properties of the parental peptides. Three of the eight analogs were twice as potent as the parental peptide in stimulating *in vitro* Melan-A specific CTL responses.<sup>10</sup> Although being potent in induction of melanoma-specific cytotoxic responses *in vitro*, similar peptides showed limited success in clinical applications.<sup>9</sup>

To enhance the efficacy of peptide vaccines and the overall tumor-specific immune responses, several combinational strategies were proposed and tested. These strategies included the administration of peptide vaccines along with the co-stimulatory cytokines such as IL-2 and IL-12, with adjuvants, such as GM-CSF, Montanide ISA 51, or CpG-containing oligodeoxynucleotides. A concurrent use of peptides with cytokines and/or adjuvants enhanced the amplitude, duration, and polarity of peptide vaccinations and showed promise in animal model studies. However, in several clinical trials it was challenging to assess clinical impact of the peptide vaccination on metastatic melanoma because of a lack of effective "measurement" tools. For instance, when different cytokine adjuvants including IL-2 were used along with gp100-derived peptide, a lower level of immunity was detected, although clinical response was observed.<sup>11</sup> In other phase I and II clinical studies it was determined that despite initial immune response, antigen-positive melanoma cells continued to proliferate after a few months of treatment. Collectively, several clinical trials reported that in melanoma patients the responsiveness to the vaccination is time-limited and rarely sustained.

Another approach to enhance the immunogenicity of peptide vaccines was proposed and tested in early 1990s. As it was shown that expression of antigenic proteins is often downregulated in tumors after activation of immune response, it was suggested that introduction of more than one peptide into the vaccination should improve immune-mediated tumor targeting. Although, initial clinical studies did not show any significant benefits, very recently, Slingluff and co-workers re-visited and refined this approach and tested multipptide vaccines in phase II clinical trials.<sup>12</sup> In this study, 12 defined shared melanoma peptides from melanocytic differentiation proteins (tyrosinase and gp100) and cancer testis antigens (MAGE-A1, MAGE-A3, MAGE-A10, and NY-ESO-1), as well as five peptides that had not previously been evaluated for immunogenicity in humans were used. Although no definitive clinical assessment can be made after 2 years from the initiation of this trials, the up-to-date results appear promising. It is demonstrated that there is no increase in toxicity when using 12 peptides and no evidence of competition at MHC class I sites. Moreover, 10 out of 12 vaccines were immunogenic and induced potent T cell response in all treated patients.<sup>12</sup>

Although an ultimate combination of peptide vaccination therapy that results in cure and complete, long-lasting remission of the tumor has not yet been found, several recently developed approaches of combination peptide vaccines hold much promise<sup>12-14</sup> and support future investigations of complex vaccines for melanoma.

### Dendritic cell vaccines

Adaptive antigen specific immune responses solely depend on the activation of T- and B-lymphocytes, which is regulated by the APCs, including DCs. These cells have a unique ability to capture, process and present foreign antigens to the naive T-cells leading to their activation and clonal expansion.<sup>15, 16</sup> DCs are generated from bone-marrow progenitors, which give rise to immature DCs that circulate in the blood and lymphatic systems and reside in different tissues, where they are primarily involved in immune surveillance and protection from infection and maintenance of homeostasis.

Understanding of the immuno-regulatory functions of DCs and the molecular mechanisms involved in the capture, processing and presentation of antigens by DCs led to the hypothesis that DCs could be used for cancer immunotherapy. As many studies have shown that tumors express unique proteins, it was suggested that TAA-loaded DCs could be delivered to a tumor-bearing host to trigger a tumor-specific immune response.<sup>17, 18</sup> To explore this possibility, in the mid 1990's, several pre-clinical and clinical studies were conducted. Most of them demonstrated that autologous DCs pulsed with melanoma-associated antigens can trigger active melanoma specific immune responses against the tumor.<sup>19, 20</sup> Although consequent clinical investigations showed that the immune system of advanced (stage IV) melanoma patients allows only a transient, antigen-specific immune response,<sup>21</sup> the results of these pioneering investigations demonstrated the applicability of DCs as adjuvants for immunotherapy and opened new perspectives for the development of melanoma immunotherapeutics.

There are three major subsets of human DCs, which originate from CD34<sup>+</sup>, bone marrow-derived progenitors. Under physiological conditions, these progenitors differentiate into immature DCs that subsequently circulate via the blood to the peripheral tissues. Several phenotypically different DC populations reside

in the skin, the primary site of melanoma occurrence. CD14<sup>+</sup> precursor cells develop into CD14<sup>+</sup> CD1a<sup>+</sup> and langerin/CD207<sup>+</sup> Langerhans cells, which home to the epidermis.<sup>22, 23</sup> CD11c<sup>+</sup> and langerin/CD207<sup>-</sup> cells represent blood-derived myeloid DCs that indigenously reside in epidermis and primarily in the dermis of normal skin. PDCA-1<sup>+</sup> CD11c<sup>-</sup> or CD11c<sup>low</sup> CD123/BDCA-2<sup>+</sup> plasmacytoid DCs (pDCs) are home to the dermis, especially in pathologic conditions.<sup>24, 25</sup> Acquisition of antigens by these DCs induces a cascade of intracellular events that leads to the differentiation of DCs into terminally differentiated, mature APCs. Unlike immature DCs, which specialize in antigen capture and are characterized by weak T-cell priming, mature DCs can induce strong inflammatory T-cell responses.<sup>15, 26</sup> These observations were tested in several clinical studies, which confirmed that activation of potent, melanoma-specific T cell-mediated immune responses could only be induced by properly matured DCs.<sup>21, 27, 28</sup>

For most of these clinical studies, autologous DCs were obtained from blood-derived monocytes or CD34<sup>+</sup> stem cells by *ex vivo* differentiation in the presence of granulocyte monocyte-colony stimulating factor (GM-CSF) in combination with tumor necrosis factor alpha (TNF $\alpha$ ). Then, these cells were used for the *in vitro* priming with TAA, maturation, and transplantation into the tumor-bearing host. In studies using mature monocyte-derived DCs, vaccination of melanoma-bearing patients led to significant expansion of antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells and broad T-cell immunity.<sup>20, 29-31</sup> However, despite persistent T-cell activation with antigen-loaded mature DCs, in most cases of stage IV melanoma, tumor-specific immune responses were transient and sufficient only for targeting of individual tumors/metastases. Furthermore, it was observed that persistent and growing tumors develop escape mechanisms and establish a state of specific T-cell tolerance that in most cases cannot be overcome by conventional vaccination.<sup>32</sup> Nevertheless, clinical studies on stage II melanoma patients demonstrated that after surgical removal, vaccination with peptide-pulsed DCs resulted in strong and long-lasting MelanA/MART-1 specific T cell responses.<sup>32, 33</sup> Collectively, these studies demonstrated that DC-based vaccination of melanoma patients is a safe approach, which is suitable for the stimulation of an efficient anti-tumor response in patients at earlier stages of the disease. These studies also revealed the

necessity for a better understanding of the molecular mechanisms, which allow established tumors to escape immune-mediated rejection.

One of the major challenges in immunotherapy for malignant melanoma (as well as all other cancers) is that most TAA are self-antigens.<sup>34</sup> Generally, these antigens include melanocyte-specific proteins such as gp100, MART-1, tyrosinase and Melan-A, which are expressed in both normal and malignant melanocytes. Therefore, to launch an efficient immune-mediated targeting of melanoma, the immune system must overcome tolerance against self. Multiple studies have demonstrated that a population of CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells (Treg) is involved in suppression of active immune responses against self and prevention of autoimmunity.<sup>35</sup> Therefore, downregulation of anti-tumor responses and poor clinical outcome of DC-based vaccination in advanced-stage melanoma patients can be explained, in part, by activation of Treg cells, which naturally suppresses anti-tumor immune responses.<sup>36</sup> However, suppressive mechanisms that allow tumors to escape immune-mediated rejection are not limited to the Treg cells only.

A more detailed analysis of immuno-targeted tumors demonstrated that often tumors are characterized by the down-regulation of MHC molecules, tumor-associated antigens,<sup>37, 38</sup> costimulatory molecules and/or intracellular molecules involved in antigen presentation such as TAP1 and 2.<sup>39</sup> In addition, tumors are able to secrete immuno-suppressive cytokines such as IL-10 or TGFβ thus repressing an inflammatory response.<sup>40, 41</sup> For instance, IL-10 was originally described as a "cytokine synthesis inhibitory factor" that down-regulates cell-mediated immunity.<sup>42</sup> In physiologic conditions, it is produced by T cells, macrophages, B lymphocytes, DCs, and keratinocytes.<sup>43</sup> This cytokine inhibits T cell proliferation and suppresses the production of various cytokines. Also, IL-10 has inhibitory effects on IFNγ and TNFα expression by NK cells, IL-1, IL-6, IL-8, TNF, MMP-9, reactive nitrogen oxide production by macrophages, and on IL-12 secretion by DCs.<sup>44-47</sup> In addition, IL-10 is involved in induction of Treg in both human and mouse. CD4<sup>+</sup> T cells, repeatedly stimulated in the presence of IL-10, differentiate into a new subset of CD4<sup>+</sup> T cells, termed Tr1 (T regulatory 1) cells.<sup>48</sup> These T cells have a poor proliferative response, secrete high levels of IL-10, and down-regulate Th1 and Th2 responses *in vitro* and *in vivo*.<sup>49, 50</sup> In particular, the differentiation of Tr1 cells

is controlled by DCs, which produce IL-10 and express tolerogenic molecules such as B7-H1.<sup>51-53</sup> Several pre-clinical studies demonstrated that IL10 directly suppress immunity against allogeneic tumors [54]. In addition, pretreatment of Langerhans cells with IL-10 abrogated their ability to present tumor antigen.<sup>55</sup> Moreover, other studies showed that the functional domain of human IL-10 down-regulates expression of MHC class I and TAP 1/2 transporters in human melanoma cells.<sup>56</sup> Therefore, tumor cells with elevated expression of IL-10 have more chances to escape immune-mediated eradication. Although, the molecular mechanism that regulates IL-10 expression in tumors is not well-understood, there is considerable evidence that IL-10 is expressed by a variety of human cancer cells including melanoma.<sup>57-59</sup> Collectively, these findings demonstrate that immuno-modulatory cytokines such as IL-10 can reprogram immature DCs into tolerogenic DCs, which support the development of suppressive T cells rather than effector T cells and result in the overall inhibition of tumor-specific immune responses.<sup>60</sup>

To facilitate tumor immunotargeting, it is necessary to overcome tolerance against self, reduce the activity of immunomodulatory cytokines, and facilitate TAA presentation. To achieve these goals, several strategies can be employed. To eliminate CD4<sup>+</sup> CD25<sup>+</sup> Treg cells and break self-tolerance, it was suggested to utilize recombinant proteins, where cytotoxic molecules are fused to cell-type specific receptor ligand. This approach was recently tested in pilot clinical studies using IL-2-diphtheria toxin fusion protein (Ontak) for the targeting of Treg cells in melanoma patients.<sup>61</sup> Unfortunately, none of the patients enrolled in this study experienced an objective clinical response, suggesting that further optimization of fusion protein structure and localized delivery is required to make this strategy practical. Alternatively, CD4<sup>+</sup> CD25<sup>+</sup> Treg cells can be depleted using systemic administration of anti-CD25 antibodies. Although this approach is feasible, in clinical settings complete depletion of Treg raises a high risk of the development of autoimmune diseases. Moreover, because the CD25 may also be expressed on activated T cells, inhibition of these cells may block tumor-specific immune responses. Other strategies may employ upregulation of MHC molecule expression in MHC-negative melanomas using IFNγ,<sup>38, 62</sup> or *in vivo* gene transfer of MHC transactivators.<sup>63</sup> Alternatively, targeted inhibition of cytokines,

such as IL-10 using cytokine-specific antibodies or soluble receptors may lead to inhibition of immune response modulation. The enhancement of anti-tumor immunity could also be achieved by the optimization of tissue resident DC activation through Toll-like receptors ligands (TLRL) as adjuvants. Stimulation of DCs with TLRL may significantly enhance the proliferation of naïve and effector T cells and abrogate the negative influence of Treg cells.<sup>64, 65</sup> Another alternative strategy to enhance tumor-specific immune responses after vaccination with TAA-loaded DCs might be to enhance tumor infiltration with naïve CD4<sup>+</sup> and CD8<sup>+</sup> T-cells to allow more effective priming of these cells. Perhaps, a combination of these strategies applied concurrently with DCs-based vaccination is necessary to achieve clinically significant outcome of the treatment.

### Chemokines

Over the past few years, there has been growing interest in chemokines within the fields of tumor immunology and immunotherapy. Chemokines are small protein molecules that are involved in immune and inflammatory responses. One of them, secondary lymphoid chemokine, CCL21 (also known as SLC, C6kine), which is expressed in high endothelial venules and within T-cell zones of secondary lymphoid organs such as lymph nodes, strongly recruits naïve T-cells and maturing DCs to these organs via a mechanism known as chemoattraction.<sup>66</sup> This mechanism is based on the CCL21-mediated activation of G-protein coupled receptor, CCR7, which is expressed in mature DCs and T-cells. Interaction between the ligand (CCL21) and the receptor (CCR7) triggers a cascade of intracellular events that promotes cytoskeletal rearrangement, changes in the expression of several adhesion molecules, and results in directional migration of the CCR7-positive cells along the CCL21 gradient.

Based on the chemoattraction properties of CCL21, it has been suggested that this chemokine can recruit APCs and different subsets of T cells to solid tumors, facilitating antigen recognition, presentation, and activation of tumor-specific immune responses. Chemoattraction properties of CCL21 have been tested on several animal models. Recently it has been shown that the presence of the recombinant CCL21 administered via direct intratumoral injection induces strong cytotoxic,

CD8<sup>+</sup> lymphocyte-dependent antitumor responses that has led to temporal inhibition of melanoma and Lewis lung carcinoma growth.<sup>67, 68</sup> It has also been shown that constitutive expression of the chemokine within experimental colon carcinoma significantly reduced tumor growth *in vivo* due to the enhanced infiltration of DCs and CD8<sup>+</sup> T-cells in the tumor mass. Spleenocytes isolated from treated mice showed greatly enhanced cytotoxic T lymphocyte (CTL) activity against colon carcinoma.<sup>69</sup> In addition, Kirk and *et al.* exploited the chemoattraction properties of CCL21 in developing DC-based vaccines. By using adenoviral gene transfer, they expressed the chemokine in TAA-pulsed DCs and showed that immunization of the pre-existing mouse melanomas with these genetically altered DCs resulted in inhibition of tumor growth. Distal site immunization with CCL21-expressing DCs pulsed with melanoma lysates in tumor bearing mice elicited an anti-tumor response, whereas control DCs did not.<sup>67</sup> As most of these studies were conducted on small tumors and complete eradication of the tumors was reported only in several cases, utilization of the recombinant CCL21, or genetically-altered CCL21-expressing DCs may not be sufficient in the clinical setting.

However, recent studies from our group demonstrated that elevated level of tumor-derived CCL21 is required for the effective recruitment of CD11c<sup>+</sup> DCs, CD4<sup>+</sup> and CD8<sup>+</sup> naïve T cells to deeper recesses of the tumor mass and activation of systemic cytotoxic immune responses.<sup>70</sup> Using mouse B16 melanoma model it was shown that CCL21-stimulated melanoma-specific immune responses were sufficient for eradication of primary experimental melanomas and generation of functional CD4<sup>+</sup> and CD8<sup>+</sup> memory T-cells permitting continuous melanoma-specific protective immunity against secondary and even tertiary tumors.

Overall, these studies showed that chemokines can be effective in facilitating tumor-specific immune responses. It may be hypothesized that application of these natural adjuvants, in combination with DC-based, peptide, or DNA vaccines may lead to a highly favorable immunotherapeutic outcome. These findings also suggest that targeted, tumor-specific expression of chemokines (CCL21 in particular) could be used to increase infiltration of accessible tumors, such as cutaneous melanomas, with T cells, which further can be isolated from the excised tumors and used for T cell-mediated tumor immunotargeting.

### T cell-mediated tumor immunotargeting

A continuous search for better immuno-therapeutics has led to the development of the T cell-based cancer vaccines, which have been selected based on their ability to induce strong T cell expansion *in vivo*. Recently developed strategies utilizing the adoptive transfer of *ex vivo* expanded tumor-reactive/tumor infiltrating lymphocytes (TIL) to patients with cancers, particularly with melanoma, has offered a powerful proof that in some cases tumor-reactive CD8<sup>+</sup> T cells can mediate objective clinical responses.<sup>71-73</sup>

Primary cutaneous melanomas are developed from transformed melanocytes that normally reside in the basal layer of human epidermis. At first, melanoma undergoes radial expansion and then invades the dermis, forming the so-called vertical growth phase/metastatic tumor. At early stages of development, cutaneous melanomas are surrounded by stroma and overlying epidermis, which are naturally infiltrated with APC, lymphocytes and macrophages. However, as melanoma progresses slowly, it "acclimates" the immune system, renders immune cells anergic and escapes immune-mediated destruction. As a result, even with application of active vaccination strategies, only a small portion of T cells in tumor infiltrates remain tumor antigen reactive. Nevertheless, mere existence of antigen-reactive TILs suggested that these cells can be isolated from excised tumors, propagated *ex vivo*, and transferred back into a tumor-bearing host. To test this hypothesis, studies on different animal tumor models were conducted, and various cellular characteristics, required for therapeutic efficacy of the adoptive cell transfer were defined.<sup>74-77</sup> Subsequent studies with melanoma patients demonstrated that TILs generated from melanoma lesions contained both CD8<sup>+</sup> and CD4<sup>+</sup> T cells and were highly lytic against their autologous tumors.<sup>78-80</sup> In the clinical setting, about 34% of immuno-competent patients with melanoma who were treated with bulk TILs and high-dose IL-2 therapy achieved objective clinical responses.<sup>81</sup>

However, despite progress in the "formulation" of T cell-based vaccines, approximately 50% of cases of adoptive cell transfer did not demonstrate any objective response,<sup>36</sup> suggesting that the generation of a large population of tumor-reactive cytolytic CD8<sup>+</sup> T-cells alone is insufficient to mediate clinically significant tumor rejection. Therefore, further refinement

of T cell-based vaccines, such as the optimization of *in vitro* TILs cultures,<sup>82</sup> *in vitro* generation of tumor-reactive TILs with T cell receptors that recognize specific tumor-associated peptides bound on class I MHC molecules,<sup>83</sup> and engineering of tumor-targeting lymphocytes in patients without *ex vivo* cultures, are required to achieve effective tumor immunotherapy.

Another critical question related to TIL-mediated tumor immuno-targeting is the acquisition of tumor-specific immunologic memory. Several groups have recently demonstrated the critical importance of both CD4<sup>+</sup> and CD8<sup>+</sup> memory T-cells in the induction and maintenance of tumor-specific immunity. Memory T cells are direct descendants of naïve T-cells that encounter antigen in the appropriate context of co-stimulatory signals. With respect to the CD4<sup>+</sup> T cells, it has been demonstrated that CD4<sup>+</sup>CD25<sup>-</sup> T-helper (T<sub>h</sub>) cells facilitate CD8<sup>+</sup> T cell activation, survival and function.<sup>84</sup> In the absence of the CD4<sup>+</sup>CD25<sup>-</sup> T<sub>h</sub> cells, memory CD8<sup>+</sup> T cells exhibit impaired functionality and inability to control secondary tumor challenge.<sup>85</sup> In contrast, CD4<sup>+</sup> CD25<sup>+</sup> regulatory T-cells (T<sub>reg</sub>) have been shown to suppress T cells, negatively regulate CD8<sup>+</sup> memory T-cells, and control immunologic tolerance to self-antigens.<sup>84</sup>

It is known that CD8<sup>+</sup> memory T-cells are heterogeneous with respect to phenotypic markers, effector function, and tissue homing capabilities. CD8<sup>+</sup> memory T-cells have been divided into two broad categories: central memory T cells (T<sub>CM</sub>) and effector memory T-cells (T<sub>EM</sub>). T<sub>CM</sub> are antigen-experienced cells that constitutively express two surface molecules - CD26L and CCR7. In contrast, T<sub>EM</sub> are antigen experienced T-cells in which these markers are significantly down-regulated. These cells have an ability to populate peripheral tissues and inflammatory sites. It has been suggested that these two distinctive populations of memory T-cells have different functions: T<sub>EM</sub> cells function as sentinels for the immediate protection from a peripheral challenge, while T<sub>CM</sub> cells provide protection against a systemic challenge and can generate a second wave of effector cells. In addition to the ability of T<sub>CM</sub> to preferentially migrate to secondary lymphoid organs, due to the expression of the CCR7 (receptor for the secondary lymphoid chemokine, CCL21) and CD26L, these cells are also capable of secreting interleukine-2 (IL-2). In mice and non-human primates, CD8<sup>+</sup> T<sub>CM</sub> cells have been shown to be superior mediators of host protection against

viral and bacterial challenges as compared to T<sub>EM</sub> cells.<sup>86, 87</sup> It is also been shown that adoptively transferred tumor reactive CD8<sup>+</sup> T<sub>CM</sub> cells are superior mediators of therapeutic antitumor immunity to an established cancer compared to T<sub>EM</sub> cells, when given in combination with systemically administered tumor antigen vaccine.<sup>36</sup> T<sub>CM</sub> cells also have greater proliferative capacity upon antigen re-encounter compared with T<sub>EM</sub> cells. However, the superiority of the T<sub>CM</sub> cells has not been uniformly observed. The question of which of these two T cell memory populations should be targeted in future vaccine trials is a subject of considerable interest.

### Micro-organisms and viruses

The history of cancer immunotherapy dates far back to the 1800s. "Coley's mixed toxins," an extract of *Streptococcus* and *Serratia* were employed to induce systemic anti-cancer responses for many years until the mid 1930s.<sup>88</sup> It was hypothesized that these toxins induce the immune system to fight cancer. The use of microorganisms to boost immune responses resurfaced in the 1960s, when it was shown that Bacilli Calmette-Guerin (BCG) and *Corynebacterium parvum* (*C. parvum*) could generate immune responses against tumors.<sup>89, 90</sup> Morton *et al.* demonstrated that postoperative systemic BCG adjuvant immunotherapy in patients with stage II melanoma resulted in a decreased size of primary lesions and regression of distant metastases, as well as increased survival in some patients with cutaneous melanoma.<sup>91, 92</sup> The use of certain viruses as immunologic modifiers in the treatment of specific cancers began to gain popularity in the 1970s. Influenza virus-modified tumor lysates were of particular interest. In 1977, Wallack *et al.* described the generation of an alternative system for tumor cell modification that employed vaccinia virus-lysed tumor cells.<sup>93</sup> It was suggested that vaccinia modifies membrane-associated tumor antigens and, therefore, enhances the expression of antigen-chaperoned heat shock proteins, induction of tumor-specific CTL and overall immunogenicity of cancers. Since then, vaccinia-based melanoma immuno-targeting was tested in multiple pre-clinical and clinical studies. Some of these studies explored the possibility of using vaccinia for the re-expression of B7.1 co-stimulatory molecules in the tumor microenvironment to overcome T cell tolerance. Others utilized a DC transduced with a modified

vaccinia virus encoding a human tyrosinase gene.<sup>94, 95</sup> Very recently, Adamina *et al.* proposed to conduct a phase I/II controlled clinical trial investigating the effectiveness of a novel vaccination protocols. In this study, it is planned to use five melanoma epitopes, two costimulatory molecules CD80 and CD86, and the CD40 ligand, encoded in a recombinant vaccinia virus, for the induction of tumor-specific immune responses.<sup>96</sup> If successful, these clinical studies will open new perspectives in the use of vaccinia-based complex vaccines for tumor immunotherapy.

### DNA vaccination

The original idea of DNA vaccination emanated from the observations that intramuscular injection of plasmid DNA encoding influenza A virus protein resulted in the induction of specific humoral and cellular responses that protect against viral challenge.<sup>97</sup> These findings have led to the development of simple and potentially powerful technology of DNA vaccination. Initial studies on DNA vaccination were carried out using an intramuscular route of vaccine administration. Later, DNA vaccination through skin was suggested to be superior over the intramuscular route. Skin has evolved as a barrier to prevent the entry of pathogens, with efficient immune surveillance complex including Langerhans cells, dendritic cells, lymphocytes, and cell types that actively participate in innate immunity. Skin also is rich in lymphatic vasculature to drain body fluids, and this network provides an efficient route for the trafficking of APC and T lymphocytes. Depending on the physical methods of into-skin DNA delivery, DNA-based vaccines can be targeted to specific locations in the skin,<sup>98</sup> and in conjunction with traditional or genetic adjuvants, they can elicit specific immune responses.<sup>99</sup> DNA vaccination approach has several advantages: 1) multiple expression vectors coding for different proteins (*e.g.* antigen and costimulatory molecules) can be concurrently delivered into skin; 2) the use of cell-type-specific promoters can provide specificity of protein expression; 3) protein expression from designed plasmids can be controlled by inducible promoters, the use of ubiquitous chromatin opening elements (UCOE), or chemically (*e.g.* sodium butyrate). These attractive characteristics of DNA vaccines have prompted extensive research in the field within last 5 years. Multiple studies on pre-clinical animal models of melanoma and other can-

cers have been conducted. Studies on canine model of aggressive and metastatic melanoma (stages II-IV) presented by Bergman and *et al.*, demonstrated that xenogeneic DNA vaccination of dogs with DNA coding for human tyrosinase led to the overall clinical response in most of vaccinated dogs.<sup>100</sup> A long-term survival of dogs with advanced stage IV disease with bulky lung metastases (on average 400 days) was observed. Vaccinated dogs with stage II/III disease also had long-term survivals (on average 500 days) with no evidence of melanoma on necropsy. Overall, median survival time for all treated dogs was 389 days. Other recent canine model study<sup>101</sup> showed that xenogeneic DNA vaccination induces melanoma-specific antibody response, which coincides with observed clinical responses. However, up to date only a few human clinical trials on DNA vaccination were conducted. One of such study, aimed at evaluation of immune response in patients with hormone-refractory prostate cancer showed that DNA vaccination with a prostate-specific antigen (PSA) encoding plasmid vector, given with GM-CSF and IL-2 is safe and in doses of up to 900 µg, and that vaccination can induce cellular and humoral immune responses against PSA protein.<sup>102</sup> Unfortunately, for this and other few human clinical trials, the follow-up reports on patient survival or characterization of immune responses are not available at present time. Nevertheless, an active research and optimization of the DNA vaccines continues. Several recent studies on tumor animal models demonstrated that DNA vaccination, especially when used synergistically with other treatments, can be successful in eliciting tumor-specific immune responses and protective immunity against the tumors.<sup>103-108</sup> However, the effectiveness of these DNA vaccines in human clinical trials remains to be seen.

### Conclusions

During last decade, various melanoma-specific immunotherapeutics have been developed and tested in pre-clinical and clinical studies with varying degrees of clinical success. Recent identification of multiple costimulatory and co-inhibitory molecules, chemokines as natural adjuvants, and better understanding of molecular mechanisms involved in the induction and maintenance or suppression of an anti-tumor immune response has allowed for the fine-tuning of immunotherapeutics and led to the development of

novel, combinational approaches for melanoma immunotargeting, many of which already demonstrated promising clinical results. These advances in vaccine formulation provide us with the hope that in the near future melanoma immunotherapy will become curable for all melanoma patients.

### Riassunto

#### *Strategie immunoterapeutiche per il trattamento del melanoma*

L'incidenza del melanoma cutaneo è in aumento con una crescita più rapida rispetto a qualsiasi altra neoplasia maligna. Nonostante le innovazioni nel trattamento chirurgico del melanoma, questa neoplasia rimane una delle più aggressive e difficili da trattare tra i tumori solidi. Continue ricerche per migliorare il suo trattamento hanno portato allo sviluppo di diversi approcci immunologici da applicare nel trattamento del melanoma. Multiple strategie basate su peptidi, cellule dendritiche, linfociti e virus sono state testate in studi preclinici e clinici con diversi gradi di successo clinico. Comunque, le più recenti ricerche nel campo dell'immunoterapia del melanoma hanno chiaramente dimostrato che vaccini complessi e la combinazione di diversi approcci, come l'utilizzo di vaccini di cellule dendritiche insieme a molecole di costimolazione, sono superiori ai protocolli di immunizzazione convenzionali nell'induzione delle risposte immunitarie tumore-specifiche. Questi recenti studi aprono nuove prospettive per lo sviluppo di efficaci immunoterapie per il trattamento della malattia primitiva e metastatica.

Parole chiave: Melanoma - Linfociti T citotossici - Cellule dendritiche - Chemochine - Vaccini.

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## Terbinafine-induced acute generalized exanthematous pustulosis

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Terbinafine is an allylamine antifungal agent, effective in the treatment of dermatomycoses. Many cutaneous adverse reactions have been reported (in about 3% of treated patients). Furthermore terbinafine has been associated with pustular eruptions, as well as the induction and exacerbation of pre-existing psoriasis and acute generalized exanthematous pustulosis (AGEP). AGEP is an uncommon aseptic pustular eruption, classified for many years as a pustular psoriasis, that usually follows recent administration of oral or parenteral drugs. The disease is most frequently triggered by antibiotics, most of all aminopenicillins and macrolides. Characteristic AGEP features include the sudden onset of fever above 38 °C with widespread erythematous eruption, rapidly progressing to a fine, non-follicular, micropustular rash. Leucocytosis is generally present, sometimes associated with eosinophilia. The illness usually resolves spontaneously with the fever and the pustulation clearing within 15 days, sometimes followed by desquamation. Histopathology shows non-follicular spongiotic pustules in the epidermis filled with neutrophils, a mixed perivascular infiltrate of neutrophils and occasional eosinophils with papillary dermal oedema. On this subject, Sideroff et al. recently elaborated a validation score based on morphology, histological criteria, and disease course. The pathogenetic mechanism which leads to the induction of AGEP by some medicines has still not been clarified, but T cells seem to play a crucial role. The authors report a case of a patient with terbinafine-induced AGEP and a review of the literature about this topic. The case illustrates once again the role of terbinafine in AGEP and reminds us that early diagnosis of AGEP is important to avoid

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unnecessary investigations and/or the administration of antibiotics.

**KEY WORDS:** Dermatomycoses - Terbinafine - Drug eruptions.

Acute generalized exanthematous pustulosis (AGEP) is an uncommon aseptic pustular eruption that usually follows recent administration of oral or parenteral drugs; it is therefore considered to be a clinical reaction pattern.<sup>1</sup> The disease is most frequently triggered by antibiotics, most of all aminopenicillins and macrolides.<sup>2</sup> Characteristic features include the sudden onset of fever above 38 °C with widespread erythematous, scarlatiniform eruption, rapidly progressing to a fine, non-follicular, micropustular rash.<sup>3</sup> Leucocytosis is generally present (blood neutrophils above  $7 \times 10^9 \text{ L}^{-1}$ ), sometimes associated with eosinophilia. The illness usually resolves spontaneously with the fever and the pustulation clearing within 15 days, sometimes followed by desquamation. As regards differential diagnosis, AGEP can be difficult to distinguish from other pustular dermatoses, in particular from pustular psoriasis. On this subject, Sideroff et al. recently elaborated a validation score based on morphology, histological criteria, and disease course.<sup>3</sup>

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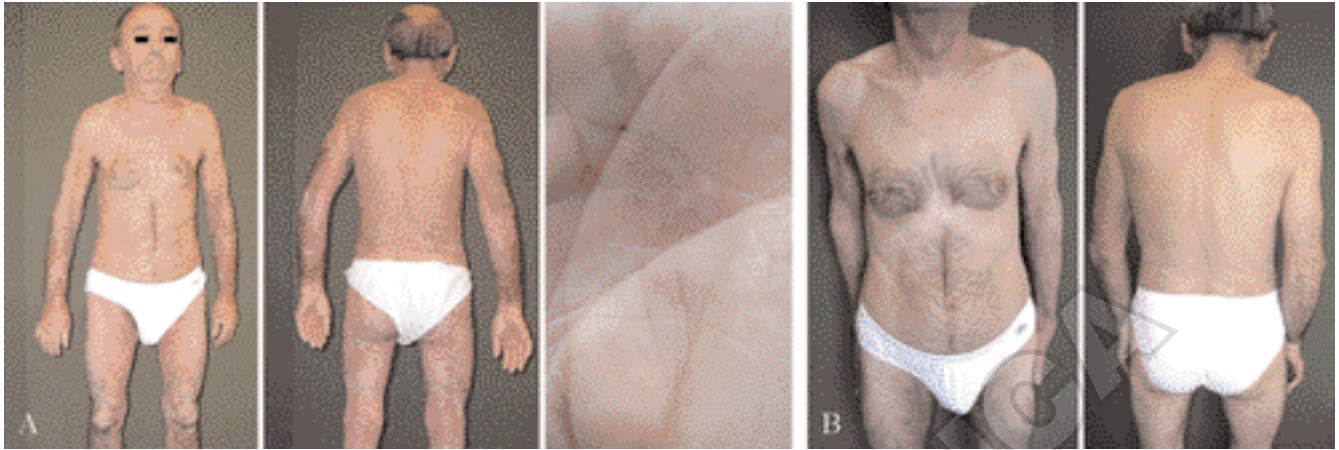


Figure 1.—(A) Terbinafine-induced acute generalized exanthematous pustulosis (AGEP) clinical appearance: erythematous background and desquamation with multiple pustules (hand detail); (B) patient appearance 15 days after terbinafine suspension and antipyretic treatment with paracetamol (100 mg day).

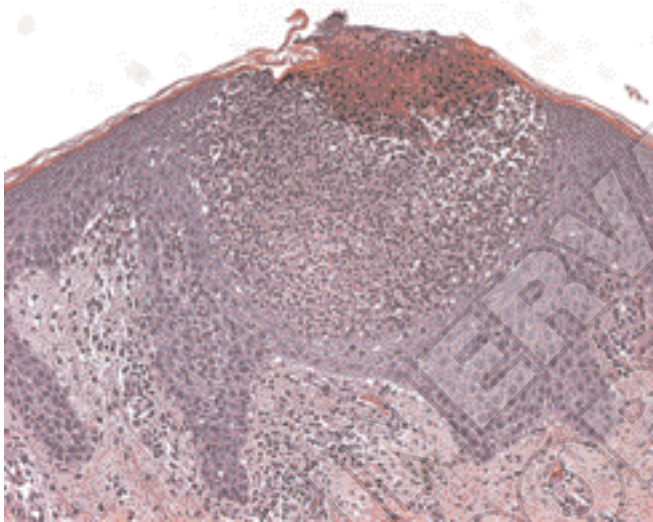


Figure 2.—[Haematoxylin-Eosin  $\times 100$ ] Subcorneal and intraepidermal pustule with peripheral edema, neutrophils and scattered eosinophils in the upper dermal infiltrate.

onychomycosis.<sup>4</sup> The most common reported side effects involve the gastrointestinal, hepatic, cutaneous and central nervous systems. Many cutaneous adverse reactions have been reported (in about 3% of treated patients) including erythema, pruritus, urticaria, fixed drug eruption and alopecia. Rarer but severe cutaneous reactions include Stevens-Johnson syndrome, toxic epidermal necrolysis, erythroderma with severe desquamation, erythema multiforme, severe urticarial erup-

tion, pityriasis rosea<sup>5-6</sup> and drug hypersensitivity syndrome.<sup>7</sup> Moreover, terbinafine has been associated with pustular drug eruptions, as well as the induction and exacerbation of pre-existing psoriasis and AGEP.<sup>5-10</sup> The authors report a case of a patient with terbinafine-induced AGEP.

### Case report

A 68-year-old male presented a 4 day history of a rapidly spreading, non scaling and non pruritic generalized erythematopustular eruption. The rash developed four days after starting oral terbinafine (250 mg/day) for treatment of onychomycosis due to the *Trichophyton rubrum* species (confirmed by positive culture). The patient had suffered from minimal plaque psoriasis for about 20 years and from chronic myeloid leukemia for 7 years. The patient had not had previous therapy with terbinafine and reported no adverse reaction to other drugs. Physical examination revealed large, non scaly, erythematous plaques, with multiple pustules spread over the entire body surface (Figure 1A). Particularly, papules and pustules were sparsely distributed over the trunk, buttocks and thighs. Palms and soles, face and mucous membranes were spared. Body temperature was 38.5 °C, white blood cells count was  $17.50 \times 10^9 \text{ L}^{-1}$ , with  $14.22 \times 10^9 \text{ L}^{-1}$  neutrophils and  $0.7 \times 10^9 \text{ L}^{-1}$  eosinophils. Histopathology of a punch biopsy taken from affected skin of the forearm showed non-follicular spongiotic pustules in the epidermis filled with neutrophils. Papillary dermal edema with a mixed perivascular infiltrate of neutrophils and occasional eosinophils were also evident (Figure 2). These findings were consistent with the clinical diagnosis of

AGEP. Based on the scoring system devised by Sideroff *et al.* for the diagnosis of AGEP, in which a score of more than 8 confirms the diagnosis, the patient of the case observed in this study scored 11 (Table I). Terbinafine therapy was interrupted and treatment with paracetamol (1 000 mg/day) was started. Skin eruption completely resolved within 15 days (Figure 1B). No relapse was observed at a 12 month follow-up.

TABLE I.—Scoring system for the diagnosis of AGEP in the patient observed.

| Characteristic of our patient                                                         | Score |
|---------------------------------------------------------------------------------------|-------|
| Development of small sterile pustules                                                 | 2     |
| Erythematous background                                                               | 2     |
| Distribution                                                                          | 2     |
| Postpustular desquamation                                                             | 0     |
| Absence of mucosal involvement                                                        | 0     |
| Acute onset (≤10 d)                                                                   | 0     |
| Resolution ≤15 d                                                                      | 0     |
| Fever ≥38 °C                                                                          | 1     |
| PMN ≥7 000/mm <sup>3</sup>                                                            | 1     |
| Spongiform subcorneal pustules with papillary Oedema on histopathological examination | 3     |

Modified from Sideroff A. *et al.*<sup>3</sup>

Discussion

AGEP is considered to be an uncommon pustular eruption most often triggered by systemic drugs.<sup>1</sup> It was classified for many years as a pustular psoriasis. In 1968 Baker and Ryan assumed that this disease represented a distinct entity.<sup>11</sup> In 1991, in a report of 63 cases, Roujeau *et al.* differentiated AGEP from acute generalized pustular psoriasis (AGPP) for the first time.<sup>2</sup> All the same, the differential diagnosis between these two pustular dermatoses is even now particularly strenuous because AGEP frequently occurs in psoriatic patients, as in the case described here, and it can subsequently simulate an exacerbation of psoriasis, as well as of psoriasis *de novo*.<sup>9</sup> Latency periods between administration of drug and onset of AGEP are typically short, most often starting within 1-2 days after drug intake. In contrast, the development of psoriasis *de novo* or its exacerbation often requires weeks.<sup>1</sup> Regarding the differential diagnosis between AGEP and AGPP, the distribution pattern in AGPP is more generalized than in AGEP, where there is predominance in the folds. The duration of both fever and pustules are

TABLE II.—Anti-infectives vs non-anti-infectives as causative drugs for AGEP.

| Anti-infectives as causative drugs for AGEP |                          | Non anti-infective drugs as causes of AGEP |
|---------------------------------------------|--------------------------|--------------------------------------------|
| <i>Antibiotics:</i>                         | Acetylsalicylic acid     | Lansoprazole                               |
| β-lactam antibiotics                        | Allopurinol              | Nadoxolol                                  |
| Macrolides                                  | Amoxapine                | Nifedipine                                 |
| Cephalosporins                              | Azathioprine             | Nimesulide                                 |
| Quinolones                                  | Buphenine                | Mercury                                    |
| Tetracyclins                                | Bufexamac                | Paracetamol                                |
| Chloramphenicol                             | Calcium-channel blockers | Prostaglandin                              |
| Gentamycin                                  | Carbamazepine            | Piperazine ethionamide                     |
| Imipenem                                    | Carbutamide              | Pneumococcal vaccine                       |
| Isionazid                                   | Celecoxib                | Pseudoephedrine                            |
| Metronidazol                                | Chemotherapy (high dose) | Quinidine                                  |
| Trimethoprim                                | Chromium picolinate      | Sulbutiamine                               |
| Sulfamethoxazole                            | Cimetidine               | Sulfasalazine                              |
| Vancomycin                                  | Clemastine               | Thalidomide                                |
| <i>Antimycotics:</i>                        | Clobazam                 | Thallium                                   |
| Griseofulvin                                | Clozapine                | Topical agents                             |
| Itraconazol                                 | Dexamethasone            | Valdecoxib                                 |
| Nystatin                                    | Disulfiram               | PUVA                                       |
| Terbinafine                                 | Enalapril                | Herbal remedy (Ginkgo Biloba)              |
| <i>Other anti-infectives:</i>               | Eprazinone               |                                            |
| Hydroxychloroquine                          | Fenoterol                |                                            |
| Diaphenylsulfone                            | Furosemide               |                                            |
| Nifuroxazide                                |                          |                                            |
| Pyrimethamine                               |                          |                                            |
| Protease inhibitors                         |                          |                                            |

Modified from Sideroff A. *et al.*<sup>3</sup>

shorter in AGEF (mean duration of 9.4 days) than in AGPP. Furthermore AGEF is very frequent subsequent to recent drug administration and usually there is a positive history of drug reactions. Some histopathological differences have also been reported. In particular in AGEF histopathology shows non-follicular spongiotic pustules in the epidermis filled with neutrophils, a mixed perivascular infiltrate of neutrophils and occasional eosinophils with papillary dermal edema. Instead in AGPP histological findings include non spongiotic subcorneal and/or intraepidermal pustules, papillomatosis and acanthosis. All the same, considering the multiple similarities between these two eruptions, a study group (EuroSCAR Study Group) has recently been formed, and has elaborated a diagnostic algorithm to use in those cases where the diagnosis is dubious.<sup>3</sup>

In front of cases like the one described here, however, other differential diagnoses need to be taken into consideration, in particular, the following: subcorneal pustular dermatosis (Sneddon-Wilkinson syndrome), pustular vasculitis, toxic epidermal necrolysis (TEN), pustular erythema multiforme, bullous impetigo, Staphylococcal scalded skin syndrome (SSSS), drug hypersensitivity syndrome (DRESS), atypical Sweet's syndrome (acute febrile neutrophilic dermatosis), foliaceous pemphigus, infantile chronic acropustulosis, pyoderma vegetans, chicken pox, dermatophyte infections, generalized contact dermatitis, acne vulgaris and bacterial folliculitis. In the present case all these diseases were fairly easily excluded based on clinical, histopathological and anamnestic findings.<sup>1,4</sup>

The optimum management of AGEF has not been agreed upon. In the past, many cases have been reported in which short term oral corticosteroids were used in those cases where AGEF lasted for longer than 15 days.<sup>12</sup> Most authors currently suggest using only symptomatic therapy. In the present case, in fact, the authors obtained complete resolution of the case with the use of an antipyretic alone.

The pathogenetic mechanism which leads to the induction of AGEF by some medicines has still not been clarified, but T cells seem to play a crucial role.<sup>1</sup> In fact previous studies in patients with AGEF have revealed a high rate of strongly positive patch tests to drugs compared with patients with other drug eruptions.<sup>13-16</sup> Britshcigi *et al.* confirmed that an involvement of T cells could be implied by positive skin patch tests and lymphocyte transformation test (LTTs).<sup>17</sup> Their

analysis of cytokine/chemokine profiles revealed that IL-8 is produced significantly more by drug-specific T cells from patients with AGEF compared with drug-specific T cells from patients that had non-AGEF exanthemas.<sup>17</sup>

The onset of AGEF as a consequence of treatment with terbinafine and other oral antifungal medicaments has been recently described.<sup>18-28</sup> Considering that antimycotics are widely prescribed for various fungal skin infections, AGEF seems to be a very rare adverse reaction. However, in a recent review from France, antimycotics accounted for about 10% of all AGEF cases, with terbinafine heading the list.<sup>29</sup> It is therefore likely that with the increasing use of oral terbinafine an increasing number of significant adverse cutaneous drug reactions to terbinafine (including cases of AGEF) can be expected.<sup>4</sup> Patients should be alerted to the risk of developing an adverse cutaneous drug reaction to terbinafine and advised to seek medical help promptly if they experience any adverse reaction. Recently, a case of AGEF with a relapse after reintroduction of terbinafine, eight years after the initial AGEF, has also been described,<sup>30</sup> so patients should also be alerted about the risk of developing a relapse after reintroduction of a drug that had previously caused AGEF.

## Conclusions

This case illustrates once again the role of terbinafine in AGEF and reminds that early diagnosis of AGEF is important to avoid unnecessary investigations and/or the administration of antibiotics.

## Riassunto

*La terbinafina e la pustolosi esantematica acuta generalizzata*

La terbinafina è un antimicotico derivato dell'allilamina efficace nel trattamento di alcune dermatomicosi. In circa il 3% dei pazienti trattati con tale farmaco sono state segnalate reazioni cutanee avverse. L'assunzione di terbinafina è stata inoltre associata alla esacerbazione di una preesistente psoriasi volgare ed, in casi molto rari, alla induzione di pustolosi esantematica acuta generalizzata (*acute generalized exanthematous pustulosis*, AGEF). L'AGEF è una dermatite pustolosa di non frequente osservazione, associata, nella gran parte dei casi descritti in letteratura, alla assunzione di farmaci per via sistemica. Fra questi ultimi, quelli più frequentemente associati alla insorgenza dell'AGEF sono gli antibiotici, soprattutto i  $\beta$ -lattamici ed i macrolidi. Sideroff

*et al.* hanno recentemente proposto una scala di punteggio che costituisce un valido ausilio diagnostico, basato su criteri morfologici, istologici e clinici. Gli elementi clinici peculiari consistono in un'eruzione eritematosa generalizzata accompagnata da febbre ( $>38^{\circ}\text{C}$ ), che rapidamente evolve in un rash papulo-pustoloso non-follicolare. Gli esami ematochimici di routine mettono in luce nella fase acuta della dermatosi, una leucocitosi neutrofila, talvolta associata ad ipereosinofilia. La malattia per definizione risolve spontaneamente entro 15 giorni dalla insorgenza dell'esantema. In alcuni casi la residua desquamazione può protrarsi per un periodo di tempo più lungo. L'esame istopatologico è caratterizzato dalla presenza di pustole intraepidermiche, non follicolari ed amicrobiche, contenenti numerosi neutrofili. Il quadro patologico può inoltre essere corredato da un infiltrato perivascolare neutrofilico, con occasionali eosinofili ed edema del derma papillare. Gli autori riportano il caso di un paziente affetto dal classico quadro clinico di AGEP indotta dall'assunzione di terbinafina.

**PAROLE CHIAVE:** Dermatomicosi - Terbinafina - Reazioni cutanee avverse ai farmaci.

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## Zuska's disease

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Cutaneous signs and symptoms in subareolar abscesses of the breast or lactiferous fistula (Zuska's disease, ZD) are common and frequent, but generally dermatologist ignore this clinical entity. An epithelial squamous metaplasia causes plugging and obstruction of the ducts is a pathogenetic event. Subsequent inflammatory reaction and infection produce local and general symptoms. Nipple retraction, recurrent episodes of erysipela and presence of painful nodules under the areola in a non-lactating woman are suspect. The presence of a milky draining sinus in the areola is characteristic. The diagnostic challenge is to differentiate these benign condition from a breast cancer. Treatment with antibiotics in the acute and chronic phase is mandatory, surgical removal of abscess and duct is sometimes resolutive. The authors describe a case of ZD in a pathologically obese woman treated with a long term penicillin schedule with no favorable effects.

**KEY WORDS:** Abscesses - Nipples - Erysipelas.

Subareolar abscesses of the breast or lactiferous fistula (Zuska's disease, ZD) is a rare recurring condition characterized by draining abscesses around the nipple of one or both breasts.<sup>1</sup> Since little is known

about the condition it is often misdiagnosed and inappropriately treated. Despite the frequency of cutaneous involvement in ZD, reports in dermatological literature are lacking. A bizarre long-lasting history of recurrent inflammatory painful subareolar nodular lesions, discharging fistulas of the nipple and an erysipela-like reaction is commonly referred. The presence of an obstructive mammary duct squamous metaplasia is probably causative and a subsequent infection confirms the disease.<sup>2-4</sup> Its managements—underestimated, not only by dermatologists—is difficult because of the high risks of therapy failure and relapse.

### Case report

RE, a 60-year old Italian woman, presented an erysipela-like manifestation on her left breast. The eruption had started one month before with malaise, low grade fever and a painful nodule under the areola.

The patient referred a two year troublesome history of similar episodes and a consistent number of consultations, examinations and tests without a clear-cut diagnosis. Of all the previous scheduled treatments only systemic antibiotics improved the condition.

She suffered from pathological obesity with an hypothy-

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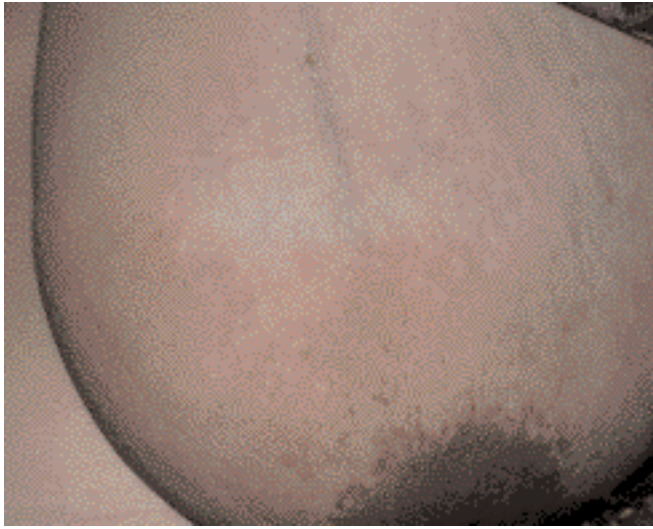


Figure 1.—Left breast erysipelas: a pale red erithematous-edematous, "peau d'orange"-like eruption.

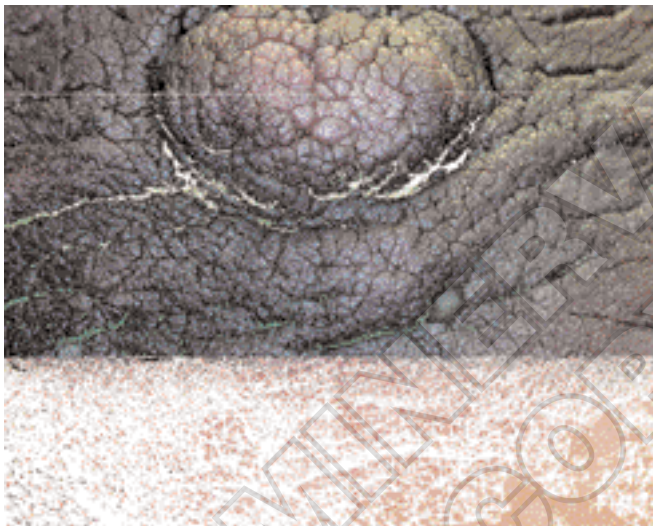


Figure 2.—Polilobulated tender painful node under the external-inferior face of the areola with a milky discharge which emerges on the skin.

roidism treated with substitutive opotherapy. A pale red erithematous-edematous, "peau d'orange"-like eruption affected her left breast (Figure 1); a 3 cm polilobulated tender painful node under the external-inferior face of the areola was present; on squeezing it, a milky discharge emerged on the skin (Figure 2). A sample taken for microbiological investigation demonstrated a *Staphylococcus Aureus* colonization. A 4 mm punch biopsy was obtained pointed to the lactiferous fistula.



Figure 3.—Iperplasia of the lining epithelium of the duct with hyperplasia and hypergranulosis EE 20x.

The histological findings revealed an iperplasia of the lining epithelium of the duct with hyperplasia and hypergranulosis (Figure 3). A simil-cystical enlargement was present on the glandular side of the specimen. Mammographic and ecographic findings showed an aspecific cellulitis-like aspect.

On the basis of the historical, histologic and clinical data, subareolar abscesses, draining fistula, nipple discharge, tenderness on palpation and mastalgia, the authors suggest a diagnosis of ZD. The authors treated the patient with an oral course of betalactamic antibiotics (Amoxicilline Velamox® tablets 1gr) 3 g a day for two weeks obtaining a rapid improvement of cutaneous and subareolar symptoms.

A two months scheduled,  $1.2 \times 10^6$ /weekly intramuscular injection of penicilline-based (Benzilpenicilline Diaminocilina® 1200000 U/L im fl) treatment has been attempted to reduce number and frequency of episodes, failed. Acute phase antibiotic essay guided therapy was planned in case of new episodes.

## Discussion

Chronic recurrent abscesses of the breast, lactiferous fistula (ZD) is a misdiagnosed disease of the breast. The incidence in the majority of reports is 1-2%<sup>5</sup> in women but in disease-oriented series it rises up to 10%.<sup>6</sup>

Non lactating and non pregnant women are frequently involved and two spikes of incidence in young and in postmenopausal woman are reported. Men are rarely affected at any age.

Epithelial squamous metaplasia with plugging of the duct is the first step of the syndrome. Duct engorgement and subsequent bacterial infection causes the majority of symptoms. No familiarity but some cigarette-smoking predisposition is referred.<sup>7</sup>

A typical clinical course of irregularly bilateral painful growing nodule recurrences under the areola of one or both sides is characteristic. Lymphnode swelling and general symptomatology with malaise and fever are common accompanying symptoms. A milky draining sinus in the areola is another typical sign and retraction or oozing eczematous reactions on the nipple are frequent. Erythematous eruption and erysipela-like reaction on the skin of the breast are often reported. Each episode lasts for weeks and a large number of episodes are referred before a correct diagnosis is established.

Clinical course is diagnostic; mammographic or ecographic investigations are unhelpful. Breast cancer is the most important differential diagnosis therefore a complete instrumental evaluation of the breast to exclude a neoplasm is necessary.

A fine needle aspiration of the mass can contribute to a correct diagnosis but its performance is difficult. The wide range of dysplastic and metaplastic squamous cells can create difficulties even to expert pathologists. In ZD, squamous cells are benign-looking and often mixed with anucleated squames. They are mitotically inactive and show a regular maturation pattern. The presence of abundant, foamy macrophages suggests a benign lesion.<sup>8,9</sup>

ZD treatment has been as yet undefined. Patients are frequently mastectomized due to cancer suspect. More conservative surgical options consist in excision of the abscessual mass together with the retroareolar ducts and fibroglandular tissue (Hadfield's procedure).<sup>1,2</sup> Restricted fistulectomy and duct excision or abscess drainage can resolve uncomplicated

episodes.<sup>10</sup> In case of repeated relapse more aggressive surgery is recommended. In recent years the opportunity of a surgical approach to ZD is discussed as a consequence of the increasing number of recurrences reported.<sup>11</sup> Antibiotic treatment in the acute phase is mandatory. Usefulness of a very conservative approach with a large-spectrum high dose antibiotic therapy combined or not with prolactin inhibitors is also reported.<sup>12</sup> The results show that an early conservative treatment is important to prevent abscesses formation.<sup>12</sup>

In the case observed a long term treatment with benzilpenicilline (1.2 × 10 UI/weekly im for two months) had no favorable effects.

## Conclusions

The evolution of benign conditions of the breast requires vigilance in relation to diagnosis, treatment, and have to be distinguished from breast cancer.

It is important to suspect the diagnosis of ZD in woman who present chronic recurrent swelling inflammatory breast masses and draining abscesses from the subareolar tissue

This case emphasizes the importance to make a correct diagnosis in the presence of this recurrent, troublesome clinical conditions especially to prevent unnecessary mutilating surgery with poor results and, importantly, exclusion of carcinoma.

## Riassunto

### *La malattia di Zuska*

L'ascesso sottoareolare della mammella o fistola galatofora (malattia di Zuska) è una rara condizione morbosa caratterizzata da ricorrenti ascessi e tragitti fistolosi attorno al capezzolo, mono o bilateralmente. Segni e sintomi cutanei sono di frequente riscontro, eppure, in genere, i dermatologi ignorano tale entità clinica. Una metaplasia squamosa dell'epitelio in grado di ostruire i dotti ghiandolari è alla base del processo patologico. I sintomi locali e sistemici sono dovuti al conseguente processo infiammatorio ed infettivo. La comparsa di noduli dolorosi al di sotto dell'areola, una retrazione del capezzolo, la presenza di una fistola da cui fuoriesce secreto lattiginoso, episodi ricorrenti di erisipela, devono indurre al sospetto diagnostico. La difficoltà diagnostica risiede nella distinzione di questa condizione benigna dal cancro della mammella. Il trattamento è spesso inefficace e raramente previene le ulteriori recidive. L'antibiotico terapia in fase acuta e cronica determina significativo se pur transitorio miglioramento. L'asportazione chirurgica degli

ascessi e delle fistole talora risulta la soluzione migliore. La gestione terapeutica nel suo complesso è quindi decisamente complessa perché gravata dalla estrema frequenza delle recidive e dalle mutilazioni per gli interventi chirurgici praticati. Gli Autori descrivono un caso di malattia di Zuska commentandone decorso clinico ed iniziative terapeutiche.

PAROLE CHIAVE: Ascessi - Capezzoli - Erisipela.

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# Randomized prospective study on the efficacy of a new revitalizing filler composed of hyaluronic acid (Wipeline®)

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L. A. DESSY, E. TRIGNANO, N. SCUDERI

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**Aim.** The aim of the study was to test the efficacy of a new revitalizing filler (Wipeline®) formed by a buffer physiological solution of hyaluronic acid (HA).

**Methods.** A prospective study was performed on 100 patients (aging between 40 and 70 years), with clear signs of premature facial aging. Patients were randomly assigned to two groups, one treated with a HA concentration of 1.6%, the other with a concentration of 2% in the tested product. The treatment protocol consisted of three sessions with a four weeks intervals between them. Visual Analogue Scale (VAS) and digital photos were used to evaluate results after 1, 3, 6 and 12 months from treatment end.

**Results.** An improvement of turgidity, elasticity and luminosity of the skin and a reduction of folds and wrinkles of the treated areas were observed in both groups. The higher concentrated solution of HA had a more prolonged effect and a greater filling effect. Products were well tolerated and no adverse reactions observed.

**Conclusion.** The efficacy of Wipeline® has been clinically supported. This revitalizing filler succeeded in increasing skin elasticity and tone by dermal hydration. The procedure is simple and little invasive. It represents a good treatment option to restore vitality and turgidity of skin presenting the signs of aging.

**KEY WORDS:** Hyaluronic acid - Skin aging - Skin care.

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Hyaluronic acid is the main component of glycosaminoglycans (GAG) that constitute the basic dermal matrix. It has a great ability to bind water molecules (until 500 times its weight) due to its high molecular weight; it also has an important anti-oxidant action. The sodium salt of hyaluronic acid is formed by repetitive units of the disaccharide formed by N-acetylglucosamine and D-glucuronic acid and is one of the chief component of extracellular matrix of most tissues, particularly of the skin.

The aging and photo-aging processes result in a reduction of hyaluronic acid (HA) production in the cutis. This causes a decrease of the cementing and moisturizing ability of the amorphous part of the dermis, resulting in skin aging, decreased deep hydration, and folds and wrinkles formation.

In order to correct aging effects, HA has been used for several years as absorbable filler and in different formulations to fill and wipe wrinkles and folds or to augment lips and zygomas.<sup>1-5</sup> Moreover, HA creates the physiological conditions in the extracellular matrix for proliferation, migrations and organization of dermal cells, protecting them from UV rays action, free radicals, xenobiotics and superficial cutaneous traumas.

The various HA based fillers can be of animal or non-animal origin, the so-called non-animal stabilized hyaluronic acid (NASHA).<sup>6,7</sup> Recently, a sub-category of HA based filler called revitalizing has been introduced (f.e. Ial System ACP, Restylane Vital).<sup>8</sup> As a consequence of an appropriate injection technique, this creates in the dermis a reserve of stabilized HA that is slowly and gradually released. Due to its high molecular weight, HA retains molecules of water, increasing the degree of cutaneous hydration, and consequently improving skin elasticity, tone and turgidity.

The authors present their experience in using a new revitalizing filler formed by a buffer physiological solution of HA called Wipeline® (Fasel S.r.l., Bologna, Italy) for facial rejuvenation.

### Materials and methods

From January to December 2006, at the Department of Cutaneous and Venereal Diseases and Plastic Surgery of the University "La Sapienza" of Rome, a prospective study was performed on 100 patients, ranging from 40 to 70 years of age, that were suitable for intradermal injection of a revitalizing filler in the face. Inclusion criteria were clear signs of premature aging of the lower face, as prominent nasolabial folds, relaxed soft tissues, and decreased skin hydration, turgidity and elasticity. Exclusion criteria from the study were the presence of connective tissue diseases, coagulation disorders, severe cardiopathy, phlebopathy, hypertension, neuropathy, allergy to filler components, viral, bacterial and traumatic dermatitis, and pregnancy. Further exclusion criteria were previous facelift or injection with permanent fillers, injection within six months of an absorbable fillers and the intention by the patient to undergo other cosmetic procedures in the following twelve months.

Patients were informed about indications, benefits and risks of treatment, and were proposed to be enrolled in the study protocol of injections with either one of the two available formulations of Wipeline® in a patient blinded design. Patients who accepted the study terms signed a proper consent form.

Patients were then randomly assigned to two groups of 50 patients each. One group was treated with Wipeline® 1.6%, the other with Wipeline® 2%. The treatment protocol consisted of three sessions with a four weeks interval between them.

The treated area was disinfected with an antiseptic solution and the filler was injected following the technique advised by the producer.

A self evaluation was requested to patients 1, 3, 6 and 12 months after the end of the treatment. A Visual Analogue Scale (VAS) was used to evaluate results. The patients gave a score between 1 and 10 (1 = absence of improvement; 10 = disappearance of the defect). Digital photograms of the treated area were taken before filler injection and during each follow-up visit.

A group of three doctors not involved in the study evaluated the patients before the treatment and at each follow-up by using previous photograms, without knowing which of the two formulations was used (evaluator-blinded design). A VAS scale was used to evaluate turgidity, elasticity and luminosity of the skin of treated areas giving a score between 1 (no improvement) and 10 (the best result obtainable). An overall evaluation was also expressed with the same scale.

The eventual onset of adverse reactions were also recorded from treatment beginning to follow up end.

### Wipeline® features

Wipeline® is a revitalizing filler formed by a buffer physiological solution of HA, obtained in a fermentative way without chemical modification. HA is in form of sodic salt, highly purified, characterized by weak bonds, and with a molecular weight of about 1 million Daltons. Its tridimensional molecular aspect allows it to have a long permanence time in the tissues, where it integrates without altering its biocompatibility. An other feature is the isovolemic degradation that allows to maintain the initial volume even during the phase of degradation. It means that as the hyaluronic acid degrades in smaller molecules, with a consequent decrease of its initial concentration, each molecule progressively binds more water, prolonging HA permanence time. Wipeline® is a viscous solution available in two formulations with a different concentration of hyaluronic acid: 1.6% (16 mg/mL) and 2% (20 mg/mL) in 1 mL syringes with a 27 gauge needle. The other components of Wipeline® are sodium chloride, sodium phosphate and water for injectable preparations. The technique of injection is a slow linear inoculation in the middle-deep dermis, as provided for by the intra-dermal injection technique named "hydro reserve". This procedure is neither a filling/corrective technique nor a skin bio-stimulation. The gel

TABLE I.—Self-assessment VAS of patients in group A.

| Months after treatment | 1  | 2  | 3  | 4  | 5  | 6  | 7 | 8 | 9 | 10 |
|------------------------|----|----|----|----|----|----|---|---|---|----|
| 1                      | —  | —  | —  | 6  | 14 | 20 | 6 | 1 | — | —  |
| 3                      | —  | 1  | 7  | 15 | 16 | 7  | 1 | — | — | —  |
| 6                      | 5  | 13 | 14 | 12 | 3  | —  | — | — | — | —  |
| 12                     | 44 | 3  | —  | —  | —  | —  | — | — | — | —  |

TABLE II.—Self-assessment VAS of patients in group B.

| Months after treatment | 1  | 2  | 3  | 4  | 5  | 6  | 7 | 8 | 9 | 10 |
|------------------------|----|----|----|----|----|----|---|---|---|----|
| 1                      | —  | —  | —  | 5  | 9  | 21 | 8 | 4 | — | 1  |
| 3                      | —  | —  | 3  | 12 | 17 | 11 | 5 | — | — | —  |
| 6                      | 2  | 10 | 13 | 15 | 5  | 3  | — | — | — | —  |
| 12                     | 44 | 4  | —  | —  | —  | —  | — | — | — | —  |

TABLE III.—External assessment by VAS of patients in group A.

| Months after treatment | Evaluation | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8 | 9 | 10 |
|------------------------|------------|----|----|----|----|----|----|----|---|---|----|
| 1                      | Turgidity  | —  | —  | —  | —  | 10 | 20 | 12 | 5 | — | —  |
|                        | Elasticity | —  | —  | —  | 12 | 16 | 16 | 3  | — | — | —  |
|                        | Luminosity | —  | —  | —  | —  | 5  | 21 | 14 | 7 | — | —  |
|                        | Overall    | —  | —  | —  | 6  | 17 | 23 | 4  | — | — | —  |
| 3                      | Turgidity  | —  | —  | —  | 11 | 18 | 11 | 5  | 2 | — | —  |
|                        | Elasticity | —  | 2  | 12 | 15 | 15 | 3  | —  | — | — | —  |
|                        | Luminosity | —  | —  | —  | 9  | 19 | 13 | 6  | — | — | —  |
|                        | Overall    | —  | 2  | 8  | 15 | 19 | 6  | —  | — | — | —  |
| 6                      | Turgidity  | 2  | 11 | 16 | 11 | 5  | 2  | —  | — | — | —  |
|                        | Elasticity | 12 | 16 | 16 | 3  | —  | —  | —  | — | — | —  |
|                        | Luminosity | 1  | 6  | 21 | 13 | 6  | —  | —  | — | — | —  |
|                        | Overall    | 3  | 12 | 15 | 11 | 4  | 2  | —  | — | — | —  |
| 12                     | Turgidity  | 47 | —  | —  | —  | —  | —  | —  | — | — | —  |
|                        | Elasticity | 46 | 1  | —  | —  | —  | —  | —  | — | — | —  |
|                        | Luminosity | 46 | 1  | —  | —  | —  | —  | —  | — | — | —  |
|                        | Overall    | 47 | —  | —  | —  | —  | —  | —  | — | — | —  |

TABLE IV.—External assessment by VAS of patients in group B.

| Months after treatment | Evaluation | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8 | 9 | 10 |
|------------------------|------------|----|----|----|----|----|----|----|---|---|----|
| 1                      | Turgidity  | —  | —  | —  | —  | 4  | 14 | 19 | 8 | 3 | —  |
|                        | Elasticity | —  | —  | —  | 10 | 15 | 19 | 4  | — | — | —  |
|                        | Luminosity | —  | —  | —  | —  | 4  | 21 | 15 | 6 | — | —  |
|                        | Overall    | —  | —  | —  | 5  | 10 | 24 | 8  | 3 | — | —  |
| 3                      | Turgidity  | —  | —  | —  | 6  | 13 | 17 | 5  | 7 | — | —  |
|                        | Elasticity | —  | —  | 3  | 15 | 16 | 12 | 2  | — | — | —  |
|                        | Luminosity | —  | —  | —  | 8  | 14 | 19 | 2  | — | — | —  |
|                        | Overall    | —  | —  | 3  | 12 | 20 | 11 | 2  | — | — | —  |
| 6                      | Turgidity  | —  | 5  | 15 | 15 | 11 | 4  | —  | — | — | —  |
|                        | Elasticity | 3  | 16 | 17 | 12 | —  | —  | —  | — | — | —  |
|                        | Luminosity | —  | 6  | 16 | 19 | 7  | —  | —  | — | — | —  |
|                        | Overall    | —  | 12 | 18 | 12 | 4  | 6  | 2  | — | — | —  |
| 12                     | Turgidity  | 46 | 2  | —  | —  | —  | —  | —  | — | — | —  |
|                        | Elasticity | 47 | 1  | —  | —  | —  | —  | —  | — | — | —  |
|                        | Luminosity | 46 | 2  | —  | —  | —  | —  | —  | — | — | —  |
|                        | Overall    | 46 | 2  | —  | —  | —  | —  | —  | — | — | —  |



Figure 1.—A) frontal view of a patient of group A before Wipeline® 1.6% infiltration in the cheeks, nasolabial and orbital folds; B) follow-up visit after one month; C) follow-up visit after six months.

must be injected tangentially to the skin surface in the deep dermis, realising micro-drops while extracting the needle.

## Results

In the study, 78 women and 22 men were recruited. The average patients' age was 54 years.

Tables I, II show patients' evaluations of groups A and B respectively during follow up visits. Table III and IV show external observers' evaluations of both groups.

In both groups, there was an improvement of turgidity, elasticity and luminosity of the skin and a reduction of folds and wrinkles in the treated areas (Table III-IV, Figures 1-3). In group B, the filler (Wipeline® 2%) had a slightly more prolonged effect, as underlined by the evaluations at the six months follow-up, (Tables

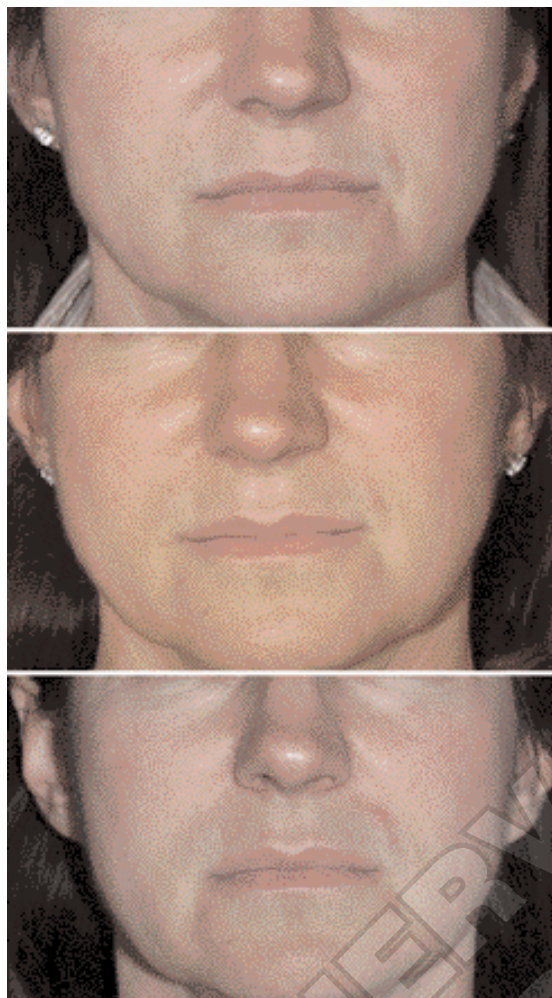


Figure 2.—A) frontal view of a patient of group B before Wipeline® 2% infiltration in the cheeks and nasolabial folds; B) follow-up visit after one month; C) follow-up visit after six months.

II) if compared to group A (Tables I, III). Wipeline® 2% had also a greater filling effect in group B, compared to Wipeline® 1.6% in group A (Table III, -IV Figure 3).

Fillers were well tolerated and no adverse reactions observed, apart from 4 patients (2 in each group), who suffered from a localized erythema healed in 24 hours; 3 cases of ecchymosis in group A, healed in 3-5 days; 1 case of hematoma of the left cheek in group B, probably caused by puncture of the facial artery, healed in 8 days.

Two patients (1 in each group) have not completed the treatment protocol for a subjective intolerance to

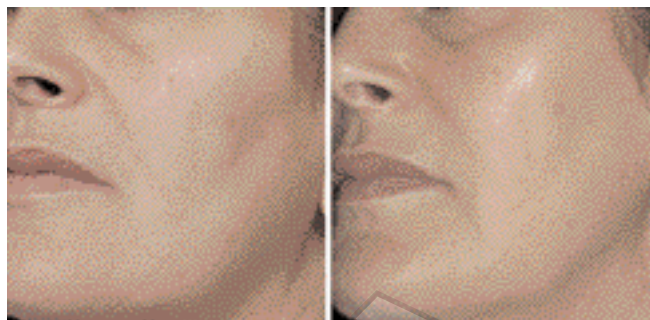


Figure 3.—A) 3/4 view of a patient of group B before Wipeline® 2% infiltration in the cheeks and nasolabial folds; B) follow-up visit after one month.

the pain caused by the injections during the first application. Three patients (2 of group A and 1 of group B) did not attend the follow-up visits.

### Discussion

HA is a polysaccharide normally present in the human body, whose main function is to maintain a proper tissue hydration due to its intrinsic ability to bind a large amount of water. Furthermore, inside the extracellular matrix, HA creates the physiological conditions for proliferation, migration and organization of dermal cells, protecting them from UV rays, free radicals, xenobiotics and superficial cutaneous traumas. The HA sodium salt is formed by the repetition of disaccharide units composed of N-acetylglucosamine and D-glucuronic acid and is a basic component of extracellular matrix of most tissues, particularly the skin.

HA based fillers have been used for several years to fill and correct wrinkles or to augment zygomas and lips.<sup>2, 3, 5, 9</sup> HA-based fillers can be of animal or non-animal origin, the so-called NASHA.<sup>6, 7</sup> Recently a subcategory of HA based fillers has been introduced, the so-called revitalizing fillers (f.e. Ial System ACP, Restylane Vital).<sup>8</sup> As a consequence of a proper injection technique, they create in the dermis a reserve of gradually released and stabilized HA of high molecular weight, which binds water molecules, increasing skin hydration with a consequent improvement in elasticity, tone and turgidity.

The results of the authors' clinical experience have been very satisfactory. Wipeline® improves skin deep hydration and reduces folds and wrinkles of the cheek and of the connection areas among cheeks, nose and lips.

Both groups were satisfied with results. The level of satisfaction was subjective and depended on the defect extent and skin type.

In particular, group A, treated with Wipeline® 1.6%, showed an improvement of turgidity and skin texture one month after treatment. This effect remained unchanged after three months, remarkably decreased after six months, and completely disappeared after one year. In group B, treated with Wipeline® 2%, a greater zygoma augmentation and nasolabial folds reduction was produced (Figure 3), in addition to the effects noted in group A. These effects are due to the main differences between Wipeline® 1.6% and 2% that are the more cohesiveness and less malleability of the second one. Moreover, Wipeline® 2% produced a more enduring effect due to its higher concentration of HA. However, also the effect of Wipeline® 2% completely disappeared after a year.

The shortness of treatment program promoted its conclusion. Effects become evident in 4/5 days (as reported by patients and confirmed by external observers).

Wipeline® proved to be effective in mitigating the signs of the aging process. The viscoelastic and hydrating properties of HA allow tissues rehydration and create the best conditions to prevent and oppose the effects of the aging process and to help tissue remodelling. Moreover, Wipeline® intradermal injection allows to bring directly in the skin the optimum quantity of HA necessary to oppose the cytotoxic action of free radicals on fibroblasts.

The authors' clinical experience on Wipeline® leads them to advice the use of the 1.6% formulation two times a year in the early phases of skin aging process with an hydration alteration and initial appearance of fine wrinkles. Instead, the authors advice the use of the 2% formulation every 6-8 months in advanced aged skin, with pronounced folds and wrinkles.

Finally, being Wipeline® a biocompatible product, it is possible to combine it with other ancillary procedures (such as radiofrequency, intense pulsed light and other riabsorbable fillers) or in preparation of invasive surgical procedures.

### Conclusions

The efficacy of Wipeline® has been clinically tested. It represents a low invasive solution to increase skin elasticity and tone and to hydrate dermis. It is advisable for every subject presenting signs of skin aging.

### Riassunto

*Studio prospettico randomizzato sull'efficacia di un nuovo filler rivitalizzante composto da acido ialuronico (Wipeline®)*

**Obiettivo.** Gli autori hanno testato l'efficacia di un nuovo filler rivitalizzante (Wipeline®) formato da una soluzione fisiologica tamponata di acido ialuronico (*hyaluronic acid*, HA).

**Metodi.** Su 100 pazienti di età compresa tra 40 e 70 anni, con chiari segni di invecchiamento precoce del viso, è stato eseguito uno studio prospettico. I pazienti sono stati assegnati in modo random a due gruppi, uno trattato con il prodotto testato con una concentrazione di HA pari all'1,6%, l'altro con il prodotto testato con una concentrazione del 2%. Il protocollo di trattamento prevedeva tre sessioni, ognuno ad intervalli di quattro settimane. Per valutare i risultati dopo 1, 3, 6 e 12 mesi dalla fine del trattamento sono state utilizzate la Scala Visiva Analogica (*Visual Analogue Scale*, VAS) e foto digitali.

**Risultati.** In entrambi i gruppi si è avuto un miglioramento del turgore, dell'elasticità e della luminosità della cute e una riduzione dei solchi e delle rughe delle aree trattate. La soluzione a maggiore concentrazione di HA ha evidenziato un effetto più prolungato e un maggior effetto-riempimento. I prodotti sono stati ben tollerati e non sono stati osservati effetti collaterali.

**Conclusioni.** L'efficacia di Wipeline® è stata confermata clinicamente. Questo filler rivitalizzante è stato in grado di aumentare l'elasticità e il tono della cute attraverso l'idratazione del derma. La procedura è semplice e minimamente invasiva. Essa rappresenta una buona opzione terapeutica per restituire vitalità e turgore alla cute che presenti segni di invecchiamento.

**Parole chiave:** Acido ialuronico - Invecchiamento della pelle - Cura della pelle.

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