Topical immunization: Mechanistic insight and novel delivery systems

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Topical application of antigen and adjuvant directly on intact skin, termed as Topical Immunization (TI) or Transcutaneous Immunization (TCI), is a novel and emerging method of vaccine delivery because it is safe and convenient. Moreover, skin is potentially rich site for immunization. Immune response elicited by TI depends upon structure and composition of the skin of target species. TI induces potent, functional immune responses vis-à-vis offers significant practical advantages for vaccine delivery. Various routes of carrier entry into the skin include the intercellular pathway, the transcorneocyte pathway and the trans-appandageal pathway. Among various approaches for topical immunization, namely physical, chemical and vesicular, latter is gaining wide attention. Vesicular carriers, i.e. liposome, niosome, transfersomes and virossome, elicit immune response by different mechanisms. Some lipids directly lower the skin permeability barrier, which resides primarily in the stratum corneum. Hence, specially designed lipid vesicles, used as topical delivery system, are attracting increasing attention and can be used for TI. The review covers brief immunology of skin and an insight into delivery concepts of topical immunization with emphasis on vesicular systems.

Keywords: topical immunization, transcutaneous immunization, novel delivery systems, liposomes, niosomes, transfersomes, ethosomes

Introduction

The skin is an immunologically rich site and thus offers an attractive vaccination route. Many pathogenic bacterial and viral pathogens gain entry into the body via skin or mucosal surface. Vaccination seems to be a viable and cost effective means for the prevention of such diseases and it has always been a principal aim with vaccinologist, to be able to promote, simultaneously, protective immune response both systemically and at mucosal surface. Dermal and transdermal delivery of protein bioactives face enormous challenges and at the same time has very significant potential for the non-invasive treatment of both localized and systemic diseases.

Several efforts have been initiated to develop potential technique to deliver peptides and proteins by routes other than parenteral and oral. Among these is the transdermal route, which has been shown to provide possibility of bypassing gastrointestinal degradation and hepatic first pass elimination as well as achieving better patient compliance. Moreover, the skin also lacks proteolytic enzymes. All these factors render the skin an appealing site for the administration of therapeutically important peptides and proteins.

Immunization was successfully done over 200 years ago by Jenner and since then vaccines have been one of the potential tools in the prevention of diseases. The development of safer and more effective means for delivery of vaccines is high priority for both human and animal health. A new route of antigen delivery is its application directly upon intact skin. This novel pain free route of immunization is termed as Topical Immunization (TI) or Transcutaneous immunization (TCI), which has the potential to reduce the complication of skin penetration by injection and at the same time this is novel and more effective means for vaccines delivery.

Langerhans cells and other Antigen Presenting Cells (APCs) are known to be immunologically dominant in the dermis and these are found in abundance along the transdermal penetration pathway. These immunologically active cells in the skin are thought to be aligned specifically along the minute pores in the dermis through which the extracorporal pathogens are likely to invade the body. These characteristics favour the immunocompetence potential of the skin. Topical immunization will be more efficient than currently used parenteral routes provided that antigen can be transported efficiently into and through the viable skin. Furthermore, this route will diminish the

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variability of the subcutaneous and intramuscular immunization, which depends on injection site, injection depth, the local blood supply and body movement. Cholera toxin (CT), a potent adjuvant for oral and nasal immunization can induce both serum and mucosal immunoglobulin G (IgG) and IgA and can protect against toxin mediated mucosal diseases when administered by transcutaneous pathway. CT is a promising transcutaneous adjuvant7. TI induces secondary antibody responses to co-administered antigen as well as to CT. TI appears to induce potent, protective immune responses to both systemic and mucosal challenge and offers significant potential practical advantages for vaccine delivery8.

Topical Immunization
One of the major impediments in ensuring vaccine efficacy and compliance is its safe and effective delivery. At present, most of the vaccines are given by intramuscular injection9. However, they suffer from certain drawbacks, like requirement of periodic boosters, need of qualified personnel, patient inconvenience and possibility of transmission among patients of certain diseases, like AIDS, due to needle pricking. On the other extreme, topical immunization offers some potential advantages, i.e. it is a needle free method and ensues increased patient compliance and obviates complications related to physical skin penetration. TI provides access to skin immune system, which is dominated by potent Antigen Presenting Cells (APCs), Langerhans cells that can be manipulated by adjuvants to orchestrate specific, robust immune responses resulting in high IgA/IgG ratio in serum10-12. TI has potential for human vaccination and no serious vaccine related adverse reaction (systemically or locally) has been reported13. Furthermore, simple method of administration in TI excludes the requirement of any trained personnel for vaccination.

The topical and oral routes are now considered as preferred routes for immunogen delivery. The topical route is having an edge over other routes because:

1. It prevents unnecessary invasion to body.
2. It prevents the problems related to degradation of peptidal vaccines as in the case of oral route.
3. It drains the antigens or carrier associated antigens to the lymphatic system and hence to lymph nodes.
4. It prevents unnecessary toxicity encountered in case of immunization by other routes.

Skin is the target site for topical immunization. TI enables efficient delivery of vaccine antigen to epidermis harbouring immunocompetent Langerhans cells14. Skin can normally allow the molecules not greater than 200-350 Da to penetrate when applied epicutaneously15. Therefore, for large molecule, some specialized carrier systems are needed to transport them across the skin in immunologically active form.

Immunology of Skin
The lymphoid cells, which constitute the immune system, are generally divided into two broad categories: B cells, which can differentiate into plasma cells that produce antigen specific antibodies, and T cells, which are further subdivided into a number of subsets based upon their function. These include: (1) cytotoxic T-Lymphocytes (CTLs), (2) delayed type hyper sensitivity cell (T_{DTH}), (3) T-helper cell (T_H), which help in the generation of either cell mediated immune responses or antibody mediated immune responses, and (4) Suppressor T (Ts) cells, which act as regulatory cells for both cell mediated and antibody mediated immune responses. Functionally associated with the lymphocytes are APCs, such as macrophages and dendritic cells, which act to "process" and "present" antigen to the various subpopulations of B and T cells16. The dendritic cells are found in nonlymphoid organs and tissues, in lymphoid organs and in the blood and lymph17.

The APCs are required for the induction, generation and regulation of immune responses. Antigens are presented to antigen specific T lymphocytes in conjunction with major histocompatibility complex expressed by APCs. The induction and elicitation phases of immune response that are initiated by the APCs cause both the activation and the clonal expansion of the antigen specific T cells.

Mature T cells are continually trafficking between the circulation and various different secondary lymphoid organs, such as the spleen, peripheral lymph nodes, mesenteric lymph nodes and Peyer's patches. The continual surveillance of different organ systems in the body by these recirculating lymphocytes thereby provides an enhanced protection against not only pathogens and toxins but also against neoplastically transformed somatic cells. This continual immuno-surveillance is of particular importance for those organ systems, such as skin and the gastrointestinal tract, which are in constant exposure with the numerous harmful agents in the immediate external environment.
The immunosurveillance capabilities of the immune system are further substantiated by the compartmentalization of subset of T lymphocytes into defined circulatory circuit. For example, an immunological circuit restricted to the skin is termed as skin associated lymphoid tissue (SALT). Compartmentalized immunological circuit thereby increases the probability of an interaction between antigen specific T cells and antigen bearing APCs by directing effector cells to the anatomical sites of antigen deposition.

The immunological effects of antigenic exposure are manifold with the simultaneous induction of both positive effector responses (which may be cellular or antibody mediated) and negative regulatory responses. In addition to the necessary cellular interactions a variety of lymphokines are required to elicit an immune response. Thus, the ultimate response detected is the result of a complex series of interactions between these two arms of immune response. Furthermore, the amount and route of antigen exposure also plays a major role in determining which of the two arms of the immune response is preferentially induced.

Skin is also an active participant in the host defence as it supports, or even creates, local immune or inflammatory reactions. Keratinocytes augment the local inflammation and produce cytokines but it is not yet clear whether such cells participate directly in the antigen presentation. Langerhans cells play the pivotal role in the immune response to antigenic protein in the skin. These APCs are rich in class II Major Histocompatibility Complex (MHC) molecules that are important for presenting antigen to T_{H} cells. These cells first bind the cutaneously encountered antigen and then process it. Langerhans cells carrying processed antigen migrate from the epidermis into lymphatic vessels and finally into regional lymph nodes. During this process, the Langerhans cells differentiate into dendritic cells, which offer the antigen to naive CD4^{+} T cells that have entered the lymph nodes through the high endothelial venules. In contrast, the other two types of APCs in the skin (macrophages and B lymphocytes) probably first require activations in order to present antigen and stimulate T cells. Antibodies may also be presented to T cells by the venular endothelial cells.

**Skin Associated Lymphoid Tissue (SALT)**

The skin is an active and unique immunological microenvironment between the body and the environment, quite different from the other primary interfaces. The skin immune system (SIS) has been defined as the cutaneous complexity of interacting immune cells. Some of the most important constituents of SIS now recognized are summarized in Table 1.

### Immuno-competent Cells within the Skin

Skin cell populations that play a role in skin associated immune responses are Langerhans cells, dermal mast cells, epidermotropic blood-born cells, epidermal T cells and keratinocytes.

#### Langerhans Cells: Unique Dendritic Cells

Throughout the viable epidermis, immune competent dendritic cells called LCs cells are found. These LCs, despite only composing 1% of the cell population, cover nearly 20% of the surface area through their horizontal orientation and long protrusion, which form a meshwork that allows them to uptake antigen that they encounter. LCs express receptor for Fc portion (constant region) of immunoglobulin, the complement protein C3b and major histocompatibility gene complex encoded class II molecule. All these contribute to immunologic competence of LCs. Human LCs also express the CD4 and CD1 T-cell differentiation antigen. Birbeck granules, which are detected by transmission electron microscopy, serve as the definitive marker of LCs.

Functionally, LCs are very effective APCs. Studies conducted in vitro and in vivo have shown that these are capable of acting as APC for the induction and elicitation of humoral and cell mediated immune responses. LCs trap antigen in the epidermis and carry the antigen to the draining lymph nodes, where they present the peptide fragments as lymphoid dendritic cells, and then to T cells. Their location in the suprabasal cell layer of the epidermis indicates that LCs are primary sentinels for the processing and

<table>
<thead>
<tr>
<th>No.</th>
<th>Cellular</th>
<th>Humoral</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mainly related to innate immunity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Keratinocytes</td>
<td>Fibrinolysins</td>
</tr>
<tr>
<td></td>
<td>Tissue Macrophages</td>
<td>Antimicrobial peptides</td>
</tr>
<tr>
<td></td>
<td>Monocytes</td>
<td>Complement peptides</td>
</tr>
<tr>
<td></td>
<td>Granulocytes</td>
<td>Eicosanoids</td>
</tr>
<tr>
<td></td>
<td>Mast cells</td>
<td>Neuropeptides</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cytokines</td>
</tr>
<tr>
<td>2</td>
<td>Mainly related to acquired immunity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Langerhans cells</td>
<td>Secretary Immunoglobulins</td>
</tr>
<tr>
<td></td>
<td>Tissue dendritic cells</td>
<td>Interleukins</td>
</tr>
<tr>
<td></td>
<td>T cells</td>
<td>Interferons</td>
</tr>
<tr>
<td></td>
<td>Endothelial cells</td>
<td>Colony stimulating factors</td>
</tr>
</tbody>
</table>
presentation of antigen, which are introduced through the skin to the immune system of the host.

**Dermal Mast Cells**

The dermal mast cell population plays a central role in the different phases of contact hypersensitivity, inflammatory reaction to skin irritants and immediate type hypersensitivity response. Mast cells reside in close vicinity to the dermal capillaries. These cells synthesize and store in cytoplasmic granule a number of vasoactive compounds (e.g. serotonin, histamine, etc)\(^{16}\). When activated, the mast cells release the content of these storage granules into the interstitium, where the soluble mediators are able to activate endothelial cells to cause increased vascular permeability. Mast cells also secrete a variety of cytokines, including Interleukins (IL-1, IL-2, IL-3, IL-5, IL-6) and Tissue Necrosis Factor (TNF-\(\alpha\) and \(\beta\)). Thus, mast cells contribute to broad spectrum of immunologic and pathologic processes\(^{17}\).

**Epidermotropic Blood-born Cells**

Certain populations of T cells display epidermotropic properties. When activated, these cells tend to selectively recirculate to the skin sites. This in addition to possessing antigen specific activity through the expression of the T-cell antigen receptor. Epidermotropic T-cells also express receptors for skin endothelium-expressed cell adhesion molecule, which allows them to extravasate across the dermal vasculature\(^{16}\).

**Epidermal T-cells**

In an adult, the total number of T cells present in normal human skin is around 4 billion, over 90 % of which are localized in the dermal perivascular unit. Murine dendritic epidermal T cells possess natural killer cell-like activity. These are also responsible for the induction of T\(_c\)-cell (suppressor T-cell) responses to skin associated antigen\(^{16}\).

**Keratinocytes**

Keratinocytes augment local inflammation and produce cytokines but it is not yet clear whether such cells participate directly in the antigen presentation\(^{16}\). Keratinocytes are induced to express class-II antigens suggesting that these cells possess some immunological potential. These immunocompetent cells are targeted by means of topical delivery of immunogens in order to generate immunity. But since the skin, especially the stratum corneum acts as a barrier for transport of immunogen, a delivery system or any other physical method for transfer is necessary.

**Delivery Considerations**

In order for vaccine to be effective, it is important that it should be delivered to epidermis, where the APCs are localized. The stratum corneum is the main obstacle against effective transdermal delivery\(^9\). Each animal species possesses unique skin barriers that must be overcome to elicit a potent immune response by TI. In addition to antigen characteristics that may influence their delivery via the skin, several parameters important for optimizing TI for each species include differences in skin structure, the total surface area of the skin and the appropriate location for topical application\(^5\).

**Skin Composition**

For successful immunization by TI, it is important to understand the skin characteristics of each species of interest. Substantial variation occurs between species both in structural characterization and in lipid composition of skin. To facilitate the development of targeted and topically applied vaccine formulation, a thorough understanding of the composition of the stratum corneum is required. The stratum corneum has a complex structure composed of closely associated cellular and lipid components. In Table 2 the lipid composition of human stratum corneum is presented\(^2\). Each species has a unique composition of lipid in the stratum corneum, which needs to be taken into consideration when designing TI formulations. For TI to provide maximum efficacy, the vaccine component (adjuvant and a co-administered antigen) must penetrate the surface lipid, the sebum and the stratum corneum, containing both cells and lipids, for sufficient uptake and processing by immune cells, most likely LCs\(^5\).

<table>
<thead>
<tr>
<th>No.</th>
<th>Component</th>
<th>Wt %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Polar lipid</td>
<td>4.9</td>
</tr>
<tr>
<td>2</td>
<td>Cholesterol sulphate</td>
<td>1.5</td>
</tr>
<tr>
<td>3</td>
<td>Neutral Lipid</td>
<td>74.8</td>
</tr>
<tr>
<td></td>
<td>Free sterol</td>
<td>14.0</td>
</tr>
<tr>
<td></td>
<td>Free fatty acid</td>
<td>19.3</td>
</tr>
<tr>
<td></td>
<td>Triglycerides</td>
<td>25.3</td>
</tr>
<tr>
<td></td>
<td>Sterol or wax esters</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>Squalene</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>n-alkane</td>
<td>6.1</td>
</tr>
<tr>
<td>4</td>
<td>Sphingolipid</td>
<td>18.8</td>
</tr>
</tbody>
</table>
Transportation Route through Skin

Normal skin is impermeable to most substances. Stratum corneum contributes over 80% to this transport resistance. The stratum corneum (10-20 μm thick) consists of terminally differentiated keratinocytes called corneocytes. Corneocytes are 0.3 μm thick organized in parallel, partly overlapping multicellular stacks perpendicular to the skin surface. Group of 3 to 10 corneocytes stacks forms a cluster that is separated from the other cluster by clefts or gorges. Each gorge has a uniform width (4-6 μm) and depth (3-5 μm). Lipid material between the corneocytes is not only ample but also highly organized and this acts as an extra intercellular "glue" sealing the spaces between the cells in the skin. The multicellular lipids in the horny layer mainly encompass the relatively non-polar substances, such as free fatty acid, cholesterol and cholesteryl esters, in addition to more than a dozen ceramides. Owing to the fairly long aliphatic chains of the latter and due to the low overall lipid polarity in the skin, the intercorneocyte lipids are tightly packed and at least locally appear as the lipid multilamellae. All these contribute to the tightness and impermeability of the intact skin; hence, it is very difficult to bring molecule with a molecular mass greater than 200-350 Da size efficiently across the intact skin. Achieving the same task for the molecule greater than 750 Da size is practically impossible, even when these molecules have an ideal solubility in the skin. Only after elimination of the stratum corneum from the skin, e.g. by tape stripping or after lipid extraction from the horny layer (e.g. with ethanol or acetone), it does allow material transport across the skin with a dramatic increase.

The possible macro routes comprise the trans-epidermal pathway (across the horny layer either intracellularly or intercellularly) or via hair follicles and sweat glands (the appendageal way) as shown in Fig.1.

The specific pathway that a vaccine antigen will follow depends on the physico-chemical characteristics of antigen as well as the method of delivery used. There are three possible pathways for penetration of compounds through the skin—the intercellular pathway, the transcorneocyte pathway and the transappendageal pathway.

**Intercellular Pathway**

In this pathway, the molecules travel through extracellular lipids, which form continuous pathways surrounding the corneocytes. Compounds delivered in liposomes generally use intercellular pathway for entry. Penetration of liposomes through extracellular lipids has been shown by liposome-skin interaction studies using a fluorescent lipid bilayer marker and confocal laser scanning microscopy.

**Transcorneocyte Pathway**

In this pathway, the delivery occurs through the corneocytes. Physical method of delivery such as gene gun and electroporation use the transcorneocyte pathway.

**Transappendageal Pathway**

In this route, the compound travels through hair follicles and sweat glands. This pathway allows the stratum corneum to be avoided by allowing the biomolecules to travel around it to the cells surrounding the hair follicles. Epithelial cells surrounding the hair follicles constitute a much less resistant barrier than the stratum corneum. Diameter
of hair follicles ranges from 50 to 100 μm and hair follicles range in density from 10% on areas such as scalp to 0.1% in area with low follicle density. The transappendageal route is thought to be the major route by which large molecules such as oligonucleotide or liposome-complexed DNA enter the skin\textsuperscript{11}.

**Transportation into and across the Skin**

Material administration on the skin creates a concentration gradient between the application site and the skin interior. This drives the applied molecule through the skin in proportion to the skin permeability and the involved skin area

\[
\text{Material flow} = \text{Skin permeability} \times \text{application area} \times \text{driving force}
\]

Any epicutaneously administered solute is thus pushed across the skin by its inward directed concentration gradient. Simultaneously, any such entity attracts water towards the skin surface while being itself attracted into the water rich (subcutaneous) compartment by intrinsic hydration pressure difference. In general, the efficacy of the permeant transport across the skin, hence, depends on the relative magnitude of the trans-barrier water and permeant flow (or permeability). The maximum useful permeant mass on the skin is limited by permeant solubility. This also restricts the maximum achievable material flow across the intact skin, when the permeant flux is driven merely by the permeant concentration gradient. This problem can be overcome mainly by three approaches:

1. By enlarging the application area.
2. By increasing the skin permeability using permeation enhancer.
3. By activating concentration-independent transport driving forces. This approach has been realized by applying an electric (in iontophoresis) or mechanical force on the skin (in various jet devices). Non-occlusively applied drug carrier on the skin is the most recent and an elegant variation of this principle\textsuperscript{15}.

For a lipid vesicle to be driven through an opening smaller than vesicle diameter, the activation energy rises with the cost of bilayer deformation. The latter is deduced from the standard model of membrane elasto-mechanics to increase with some power of the effective membrane elasticity. Lipid vesicle penetration through the skin is a function of membrane deformability and thus inversely proportional to the energy of elastic membrane deformation, which in turn is composition dependent.

\[
\text{Skin permeability} \propto \frac{\text{Pore density}}{\text{Membrane elastic energy}}
\]

Topical and transdermal delivery is a field of interest for delivery of bioactives and immunogens. Since stratum corneum is a barrier for transfer of drugs to the dermal and epidermal sites, number of approaches has been explored for efficient delivery of bioactives. These approaches include:

2. Chemical (Permeation enhancer)–Azones, Dimethyl formamide (DMF), Dimethyl sulphoxide (DMSO).
4. Topical Genetic Immunostimulation.

The physical approaches such as electroporation can be used for the transfer of bioactive molecules across the stratum corneum. Electroporation is used for the delivery of genes to the keratinocytes for immunization as well as for gene therapy. The majority of protocols to increase the permeability of the epidermis include utilization of chemicals, such as surfactants, alcohols and polyols. They increase the permeability of the stratum corneum by any of the following mechanisms or combination of them:

a) increasing the fluidity of skin lipids,
   b) hydrating the polar pathways,
   c) opening heterogenous multilaminate pathways, and
   d) keratolytic action.

These chemicals do not increase the permeability of the bioactives to the desired extent. Only up to five times, the permeability can be enhanced by this method. However, permeability is still less in case of high molecular weight molecules. The chronic use of these chemicals for permeability enhancement may have dangerous side effects. Selective methods for circumventing stratum corneum are given in Fig. 2.
Vesicular Delivery

Delivery devices, like jet injection, have already been shown to be effective but are not as practical in targeting vaccine antigen to the skin as simply applying or rubbing the antigen onto the skin. But lipid vesicle systems for topical delivery have attracted increasing attention in recent years. However, it is generally agreed that classical liposomes are of little or no value as carrier for transdermal antigen delivery because they do not deeply penetrate the skin. They rather remain confined to the upper layer of the stratum corneum. Only specially designed vesicles were shown to be capable of transdermal delivery.

Vesicles have been developed as possible carriers in transdermal targeting by facilitating the transdermal transport through specific interaction with skin. Skin-vesicle interaction is influenced by both, the chemical characteristics of vesicle, their composition and charge, and physical characteristics, such as physical state, lamellarity and size. In addition, interaction is also influenced by the mode and method of application such as occlusive and non-occlusive and more so the general skin conditions.

The vesicular approach is gaining wide acceptance now-a-days for TI. These vesicular carriers are targeted through different pathways in the skin, i.e. either through keratinocytes or through hair follicles. In one study, recombinant Pasteurella haemolytica leukotoxin (Lkt) and hen egg lysozyme (HEL), as model antigens, were used to investigate the ability of the topical administration of antigen to induce humoral and cellular responses in mice and to assess the immunomodulatory effects of IL-12 on antigen-specific immune response. Mice were immunized through transdermal route with Lkt or HEL, formulated in lipid based bi-phasic delivery systems (BPDs). Transdermal delivery of these antigens induced strong polarized Th2 responses characterized by enhancement of antigen-specific IgG1 antibody subclass and predominant induction of antigen specific IL-4 over IFN-γ in spleen and draining lymph node cells. The lipid vesicles are exploited for topical application since:

1. The lipidic components of vesicles having the lipophilic nature may serve as organic phase for poorly aqueous soluble substances.
2. Vesicles can be targeted intracellularly.
3. Vesicles can act as penetration enhancers because of lipophilic nature.

The lipid vesicles do not release the incorporated molecule instantaneously. They can act as local depot for delayed release of drug/immunogens. Vesicular carriers, utilized for topical administration of bioactives (drug/antigen), are liposomes, niosomes, transfersomes, ethosomes, reconstituted Sendai virus envelop (RSVE), adenovirus vector, herpes simplex virus (HSV) and ampiclon vector (Table 3).

Liposomal Delivery

Liposomes have great potential as drug delivery system, not only for intravenous delivery but also for the topical application of medicaments. The addition of phosphatidyl choline (PC) to dermal dosage forms enhances the percutaneous absorption. The amphipathic nature of liposome/lipid may allow them to be used as non-invasive delivery agent for vaccine/antigen. The composition of lipid and cholesterol determines the properties of liposomes. Thus for formulating vaccines with liposomes, the compatibility of antigen with the liposomes must be ascertained. Different antigens can have different chemical properties, which may influence the ultimate
formulation. The vaccine must be formulated specifically for an antigen exclusively because properties of proteins/antigens are greatly varied.

A major limitation with the use of liposomal delivery system for vaccine antigen is toxicity to cells of immune system. Cationic lipids are highly toxic to phagocytic cells like macrophages. This toxicity may be due to destabilization of lysosomal membrane by cationic lipids.

Initially, liposomes were developed as a model for biological membrane because they characteristically mimic the lipid composition and structure of the biomembrane(s). Liposomes containing stratum corneum lipids have been tested earlier for their better skin penetration properties. Recently, two distinctive mechanisms are being explored for enhanced topical localization and/or targeting of liposome encapsulated bioactives: the transdermal pathways and transappendegeal pathways.

For a liposomal formulation to be effective, especially for hydrophilic compounds, it is essential that the suspension undergoes significant dehydration. Lipid concentration scarcely exceeds 100 mg/ml, the bulk aqueous medium constitute roughly 90% of the formulation. Thus, without a high degree of dehydration, no advantages over a simple aqueous solution can be expected by employing liposomal system, especially if the drug action is desirable within a few hours after application. Two interdependent factors control the extent of dehydration of a liposomal suspension. The first is the phase transition temperature (Tm). The second, often one that affects Tm, is the presence of components that either affect bilayer packing (e.g. cholesterol) or those that are humectants/cryoprotectants. The combined effect of the two factors will determine how much water could be retained by liposomal bilayers following dehydration under non-occluded conditions. The extent and rate of dehydration of liposomal bilayers control the transfer of antigen irrespective of its hydrophilic or hydrophobic nature into the skin. The mechanism of action following dehydration is different for hydrophobic and hydrophilic antigen.

### Transfer of Hydrophobic Bioactives

Hydrophobic bioactives are encapsulated or intercalated within the lipid bilayer of the liposomes. The transfer of bioactive from the lipid bilayers into the skin can occur as long as the bilayers are in a liquid crystalline state. If the liquid crystalline phase is altered to gel state, transport of the bioactive will cease or be negligibly low. Dehydration of suspension

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**Table 3—Summary of applications of novel delivery systems in transdermal delivery of bioactives**

<table>
<thead>
<tr>
<th>No</th>
<th>Delivery system</th>
<th>Bioactive(s)</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Liposomes</td>
<td>Triamicinolone</td>
<td>3-5 times enhanced accumulation within epidermis and dermis</td>
<td>Mezei et al.</td>
</tr>
<tr>
<td>2</td>
<td>Liposomes</td>
<td>Progesterone</td>
<td>Unsaturated fatty acid contribute to a fluidization of lipid domain within stratum corneum, results in facilitated transdermal flux of progesterone</td>
<td>Knepp et al.</td>
</tr>
<tr>
<td>3</td>
<td>Liposomes</td>
<td>Betamethasone dipropionate</td>
<td>Improved anti-inflammatory action</td>
<td>Korting et al.</td>
</tr>
<tr>
<td>4</td>
<td>Liposomes</td>
<td>Interferon-α (INF-α)</td>
<td>Improved treatment of cutaneous virus infection</td>
<td>Lieb et al.</td>
</tr>
<tr>
<td>5</td>
<td>Liposomes</td>
<td>Recombinant glycoprotein D antigen of herpes simplex virus (HSV-1)</td>
<td>Successful treatment of genital infection in guinea pigs</td>
<td>Ho et al.</td>
</tr>
<tr>
<td>6</td>
<td>Liposomes</td>
<td>Betahistine</td>
<td>Higher plasma concentration of BH</td>
<td>Ogiso et al.</td>
</tr>
<tr>
<td>7</td>
<td>Transfersomes</td>
<td>Gap junction protein (GJP)</td>
<td>Higher specific antibody titre than those elicited by subcutaneous injection of GJP in transfersome</td>
<td>Paul et al.</td>
</tr>
<tr>
<td>8</td>
<td>Transfersomes</td>
<td>Bovine Serum Albumin (BSA)</td>
<td>Facilitated penetration of antigen</td>
<td>Chen et al.</td>
</tr>
<tr>
<td>9</td>
<td>Transfersomes</td>
<td>Lidocaine</td>
<td>Antipain effectiveness of dermally applied anesthetic transfersome was similar to that of corresponding subcutaneous injection</td>
<td>Planas et al.</td>
</tr>
<tr>
<td>10</td>
<td>Niosomes</td>
<td>Enoxacin</td>
<td>Enhanced delivery across the skin</td>
<td>Fang et al.</td>
</tr>
<tr>
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<td>Niosomes</td>
<td>α-interferon</td>
<td>Enhanced delivery</td>
<td>Vyas &amp; Khar</td>
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<td>12</td>
<td>Ethosomes</td>
<td>Minoxidil</td>
<td>Enhanced skin penetration</td>
<td>Touitou et al.</td>
</tr>
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<td>Ethosomes</td>
<td>Trihexyphenidyl HCl</td>
<td>Higher entrapment and delivery to deeper layers of skin when compared with liposomes</td>
<td>Dayan &amp; Touitou</td>
</tr>
<tr>
<td>14</td>
<td>HSV amplicon</td>
<td>DNA</td>
<td>Epidermal gene transfer</td>
<td>Bo et al.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No</th>
<th>Delivery system</th>
<th>Bioactive(s)</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Liposomes</td>
<td>Triamicinolone</td>
<td>3-5 times enhanced accumulation within epidermis and dermis</td>
<td>Mezei et al.</td>
</tr>
<tr>
<td>2</td>
<td>Liposomes</td>
<td>Progesterone</td>
<td>Unsaturated fatty acid contribute to a fluidization of lipid domain within stratum corneum, results in facilitated transdermal flux of progesterone</td>
<td>Knepp et al.</td>
</tr>
<tr>
<td>3</td>
<td>Liposomes</td>
<td>Betamethasone dipropionate</td>
<td>Improved anti-inflammatory action</td>
<td>Korting et al.</td>
</tr>
<tr>
<td>4</td>
<td>Liposomes</td>
<td>Interferon-α (INF-α)</td>
<td>Improved treatment of cutaneous virus infection</td>
<td>Lieb et al.</td>
</tr>
<tr>
<td>5</td>
<td>Liposomes</td>
<td>Recombinant glycoprotein D antigen of herpes simplex virus (HSV-1)</td>
<td>Successful treatment of genital infection in guinea pigs</td>
<td>Ho et al.</td>
</tr>
<tr>
<td>6</td>
<td>Liposomes</td>
<td>Betahistine</td>
<td>Higher plasma concentration of BH</td>
<td>Ogiso et al.</td>
</tr>
<tr>
<td>7</td>
<td>Transfersomes</td>
<td>Gap junction protein (GJP)</td>
<td>Higher specific antibody titre than those elicited by subcutaneous injection of GJP in transfersome</td>
<td>Paul et al.</td>
</tr>
<tr>
<td>8</td>
<td>Transfersomes</td>
<td>Bovine Serum Albumin (BSA)</td>
<td>Facilitated penetration of antigen</td>
<td>Chen et al.</td>
</tr>
<tr>
<td>9</td>
<td>Transfersomes</td>
<td>Lidocaine</td>
<td>Antipain effectiveness of dermally applied anesthetic transfersome was similar to that of corresponding subcutaneous injection</td>
<td>Planas et al.</td>
</tr>
<tr>
<td>10</td>
<td>Niosomes</td>
<td>Enoxacin</td>
<td>Enhanced delivery across the skin</td>
<td>Fang et al.</td>
</tr>
<tr>
<td>11</td>
<td>Niosomes</td>
<td>α-interferon</td>
<td>Enhanced delivery</td>
<td>Vyas &amp; Khar</td>
</tr>
<tr>
<td>12</td>
<td>Ethosomes</td>
<td>Minoxidil</td>
<td>Enhanced skin penetration</td>
<td>Touitou et al.</td>
</tr>
<tr>
<td>13</td>
<td>Ethosomes</td>
<td>Trihexyphenidyl HCl</td>
<td>Higher entrapment and delivery to deeper layers of skin when compared with liposomes</td>
<td>Dayan &amp; Touitou</td>
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<td>14</td>
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<td>Epidermal gene transfer</td>
<td>Bo et al.</td>
</tr>
</tbody>
</table>
can induce transition from the liquid crystalline phase to the gel state.

**Transfer of Hydrophilic Bioactives**

For liposomal system that undergoes total dehydration, bioactive transport will cease because the hydrophilic drug is no longer in the dissolved state. Liposomes that retain constant amount of water within the bilayer following dehydration to an equilibrium state, drug transport in such case would, however, continue over extended periods of time. A major consequence of dehydration for hydrophilic drugs involves the enrichment of bioactive concentration in the aqueous phase of the bilayers leading to an enhancement in flux of bioactive into or across the skin.

**Follicular Delivery**

Intact liposomes may enter and transverse the hair follicles but their degradation into bilayer fragments seems almost certain considering the chemical environment of the sebum-filled hair follicles. Liposomes may act as initial carrier of bioactives into the hair follicles and subsequently releasing them on their degradation.

**Formulation Considerations**

Liposomes prepared by the dehydration-rehydration method penetrate deeper into the skin strata than large unilameller vesicles. Liposomes prepared from ceramides are more effective in penetration into skin than liposomes prepared from conventional phospholipids. The lamellarity, homogeneity, size, or a combination of all, which determine the overall effective liposome surface available for interaction with skin, of the different preparations influence the efficient interaction of the liposomes with the skin.

**Transfersomal Delivery**

A transfersome is the entity, which can pass spontaneously through a barrier and transport material from the application to the destination site. A transfersome crossing the skin thus mimics the behaviour of a parasite during its invasion of the host body. Such an intruder first creates a passage and then creeps through the skin barrier, with the consumption of metabolic energy, to finally distribute throughout the body. A transfersome, which has no internal source of energy, achieves the same goal by exploiting the naturally occurring energy gradient in the skin. The transepidermal water activity difference is the most obvious and probably the most important such gradient.

By the ultra deformable and self-optimizing carrier transfersome, large molecules can be brought into the body through intact permeability barriers. This permits non-invasive immunization through normal skin and gives rise to a similar or even slightly higher antibody titre than subcutaneous injection of the same immunogen formulation. The former type of immunization also results in a higher IgA/IgG ratio in the blood than the repeated immunogen injection.

A transfersome consists of natural amphiphatic compound suspended in a water based solution, sometimes containing biocompatible surfactant. Similar to liposomes, transfersomes have a lipid bilayer that surrounds an aqueous core; however, in contrast to liposomes, transfersomes contain at least one component that softens the membrane and makes skin more flexible. This allows an easy and rapid change of transfersomes shape.

The transfersomes are complex, most often, vesicular aggregate optimized to attain extremely flexible and self-regulating membrane; this makes the vesicle very deformable. Transfersomes can, therefore, cross microporous barrier very efficiently, even when available passage is much smaller than the average aggregate size. When a transfersomal formulation is applied on the skin and allowed to dry, the vesicle is attracted by intracarporal moisture into the body and penetrates the skin without compromising the protective properties of the organ.

The high and self optimizing deformalities of typical composite transfersome membrane, which are adaptable to ambient stress, allow the ultra deformable transfersomes to change its membrane composition locally and reversibly, when it is pressed against or attracted to a narrow pore. The transfersomes component that sustain strong membrane deformation preferentially accumulate, while the less adaptable molecules are diluted at sites of great stress. This dramatically lowers the energetic cost of membrane deformation and permits the resulting highly flexible particle to enter and then pass through the pores rapidly and efficiently.

An important difference between transfersomes and liposomes is the much higher hydrophilicity of the former. This forces transfersomes membrane to swell more than the conventional lipid vesicle bilayer. Higher membrane hydrophilicity and flexibility help transfersomes to avoid aggregation and thus fusion, which is observed with liposomes exposed to an osmotic stress.
Mechanism of Transfersomes Penetration

The efficacy of transpore movement of transfersomes is quite high; a suspension of transfersomes with an average diameter of 500nm can be transported through the pore 5 times smaller nearly as rapidly and efficiently as pure water. Such a high penetration capability is seen when the stress suffered by transfersomes (e.g. the flow-driving pressure) is sufficiently high. This is a key characteristic of transfersomes, with roots in the self-optimizing capability of transfersomes body or membrane. The passage of transfersomes through pores that are "too small" is nearly perfect even when their size exceeds the pore diameter by a factor of approximately 4 or even 10 (Fig. 3).

One naturally occurring transdermal gradient is osmotic gradient. Such a gradient is created by the difference in the total water concentration between the skin surface and the skin interior. When a lipid suspension is placed on the skin surface and partly dehydrated by water evaporation loss, the lipid vesicles feel this gradient and try to escape complete drying by moving along this gradient. They can only achieve this if they are sufficiently deformable to pass through the narrow pores in the skin. Less deformable vesicle like standard liposomes, are confined to the skin surface where they dehydrate completely and fuse46.

Lipid hydrophilicity leads to xenophobia, the tendency to avoid dry surrounding and causes carriers sitting near or at the skin surface to resist to dehydration in order to remain maximally swollen. Transfersomes near the skin surface, thus, try to follow the local hydration gradient and thereby get into deeper and better hydrated skin strata. This causes transfersomes carrier to retract from the relatively dry skin surface and to get into more humid region in the deeper skin layers47.

Distribution of Transfersomes in the Body and Kinetics of its Penetrations

After having penetrated through the outermost skin layer, transfersomes reach the deeper skin stratum, the so-called dermis. From this latter skin region, they are normally washed out via lymph into blood circulation and throughout body. If applied and exposed to suitable conditions, transfersomes can thus reach all such body tissues that are accessible to the subcutaneously injected liposomes46.

Transfersome penetration through the skin is very efficient but preceded by a lag time. Such time delay must originate partly from the need for the excess water evaporation from the skin surface, before and actual start of transfersome penetration.

The kinetics of therapeutic action is a function of velocity of transdermal carrier penetration and the speed of drug distribution and action. The precise reach as well as kinetics of transfersome penetration through the intact skin is affected by skin characteristics, carrier characteristics, applied dose, and application condition and form.

Transfersomal Application in Topical Antigen Delivery

Transfersomes have good encapsulation and sufficient driving capability to be used as carrier of the water-soluble antigen. Antigen carrier must fulfil two criteria to bring an appreciable amount of antigen from skin surface into the more deeply located skin strata. Antigen carrier should respond to or create a gradient that drives the antigen-carrier complex from the skin surface into the skin interior. Secondly, antigen carrier should be able to pass the skin barrier without uncontrollably losing too much of the enclosed therapeutic material 36.

Transfersomes fulfil these two basic requirements and thus are used as potential carrier system for non-invasive transcutaneous immunization of mice with water-soluble bovine serum albumin (molecular mass = 64 kDa) or the aggregates of the integral membrane gap junction protein (GJP, molecular mass ≥ 170 kDa). Transfersomes and cholera toxin facilitate the penetration of co-administered antigen through the stratum corneum and increase the effectiveness of non-invasive immunization. These delivery vehicles are useful in delivery of simple antigens (BSA and

Fig. 3—Penetration of transfersome through the pore in stratum corneum
DT) to experimental animals, however, their utility in delivering a broad range of complex vaccines remains to be determined\(^1\).

**Niosomal Delivery**

Niosomes are non-ionic surfactant based unilamellar or multilamellar vesicles in which aqueous solution of solute is entirely closed by a membrane. They are used as an alternative to liposomes because of their cheapness and greater stability. Colloidal particle such as liposomes and their analogues (niosomes) and small crystals can target the hair follicle. Generally, particles larger than 10 µm remain on the skin surface, at 3-10 µm they concentrate in the follicles and at less than 3µm they penetrate follicles and the stratum corneum alike. Follicles have efficient mechanism for inducing immune responses to proteins\(^27,48\). Niosomes can be used as vehicle for the TI because surfactant in formulation could act as permeation enhancers\(^37\), or at optimum size they may concentrate on the hair follicles. Liposomes and niosomes can become useful delivery systems for transcutaneous immunization because of their ability to modulate antigen transfer\(^45\). Niosomes have application in topical and transdermal delivery of both hydrophilic and hydrophobic agents. Niosomes have been used as carriers to encapsulate lidocaine, estradiol, cyclosporin, erythromycin, α-interferon, and plasmid DNA for the human interleukin-1 receptor for topical and transdermal delivery\(^38\).

**Ethosomal Delivery**

Ethosomes are lipid vesicles with high content of ethanol. They can penetrate the skin and enhance compound delivery both to deep skin strata and systemically because ethanol fluidizes both lipid and bilayers of the stratum corneum intercellular lipid. The soft, malleable vesicles then penetrate the disorganized lipid bilayers\(^27\). The ethosomal system consists of phospholipids, ethanol and water. Ethosomal system has been much more efficient at delivering a fluorescent probe to the skin in terms of quantity and depth than either liposomes or hydroalcoholic solution. Ethosome had a high entrapment capacity for molecule of various lyophilicities\(^51\).

In comparison to liposomes prepared in the absence of ethanol, the phospholipid in ethosomes is packed less tightly and the membrane has permeability for cations\(^46\). Permeation enhancement from ethosomes is greater than ethanol alone and this indicates that there may be some kind of synergistic mechanism between ethanol, vesicles and skin lipids. The ethosomal lipids are in a more fluid state than liposomes from the same components without ethanol.

**Mechanism of Penetration**

When ethosomal carriers are applied to the skin, a number of concomitant processes may take place involving the stratum corneum and pilosebaceous pathways. Ethanol in the ethosome disturbs the organization of the stratum corneum lipid bilayer and enhances its lipid fluidity. The flexible ethosome vesicle can then penetrate the disturbed stratum corneum bilayers and can even forge a pathway through the skin by virtue of their particulate nature. The release of entrapped molecule in the deeper layers of the skin and its transdermal absorption may be the result of fusion of ethosome with skin lipids and drug release at various points along the penetration pathways. Ethosomal penetration may also involve pilosebaceous pathways as they may be trapped in follicles\(^39\). Thus, ethosomes can be used as potential carrier for TI but, at the same time, effect of ethanol on the entrapped antigen must also be kept in mind.

**Delivery through Viral Vectors**

Viral vectors can be utilized for epidermal transfer of the DNA or other suitable antigen. Viral vectors include adenovirus vector, RSVE and HSV amplicon vector. The epidermal gene transfer by means of HSV amplicon vector produces necrosis and cytotoxicity and hence, they cannot be applied repeatedly or for longer period of contact\(^1\). Reconstituted viral vectors or virosomes have also been utilized for intracellular targeting of encapsulated DNA/antigen. The RSVE can be applied topically for efficient gene or antigen transfer. RSVE constitute two types of proteins, namely haemagglutinin (HN) and fusion (F) proteins, which determine the fusogenicity\(^49\). The RSVE can be effective as cell microinjection vector to transfer genes (DNA) or antigen to APCs for eliciting immune response through topical route. They can serve as an efficient immunoadjuvant.

**Topical Genetic Immunization**

Genetic immunization, a novel vaccination approach is based on the fact that DNA starts expressing proteins when it enters the cells. The DNA administered through topical route expresses itself in
the immunocompetent cells of the skin. These proteins are exogenous to the body and thus, undergo processing and presentation by means of both MHC class I and MHC II receptors. The MHC class I activates CD8 T cells and thus, cellular immunity; while, MHC class II activates CD4 T cells and hence, production of antibodies (humoral immunity). Chitosan-based nanoparticles have shown their potential for topical genetic immunization.

Another non-invasive approach is the targeting of naked DNA to the hair follicles. Topical application of aqueous solution of naked DNA encoding HBsAg induces an immune response. The topically applied naked DNA can be applied to restricted area of abdominal skin (previously treated with depilatory agent to remove hair) to increase antibody titres. This approach indicates that follicles can be targeted for topical delivery of naked DNA.

Conclusions

Traditionally, the skin immune system has been perceived as an environment for immunopathology but, in recent years, the potential for exploitation of skin for the purpose of vaccination has received a great deal of attention. TI appears to offer a new method for the delivery of vaccines with special advantages over parenteral multiple boosting immunization as well as multivalent vaccine delivery. TI appears to induce potent, functional immune responses coupled with significant advantages for vaccine delivery compared to parenteral vaccination.

The Langerhans cells present in the epidermis are very efficient antigen presenting cells, which create the immunologic competence to the skin. This immunologic competence is reflected from the fact that very small amount of antigen is required to initiate immune response. Clinical study is warranted to prove the effectiveness of their delivery.

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