

that molecules of greater than 500 daltons cannot normally pass through the skin. Moreover, according to Hurley, "Skin owes its durability to the dermis, but its chemical impermeability resides in the epidermis and almost exclusively in its dead outer layer, the stratum corneum."

[0014] Skin reactions such as allergic or atopic dermatitis are known, but induction of a systemic immune response which elicits antigen-specific immune effectors and provides a therapeutic advantage by simple application of immunogen to skin does not appear to have been taught or suggested prior to our invention.

[0015] Generally skin antigen presenting cells (APCs), and particularly Langerhans cells, are targets of sensitization agents which result in pathologies that include contact dermatitis, atopic dermatitis, eczema, and psoriasis. Contact dermatitis may be directed by Langerhans cells which phagocytize antigen, migrate to the lymph nodes, present antigen, and sensitize T cells for the intense destructive cellular response that occurs at the affected skin site (Ksipke et al., 1990). An example of atopic dermatitis is a chronic relapsing inflammatory skin disease associated with colonization of the skin with *S. aureus* and thought to be caused by *S. aureus*-derived superantigens that trigger chronic T-cell mediated skin inflammation through Langerhans cells (Herz et al., 1998; Leung, 1995; Saloga et al., 1996a). Atopic dermatitis may utilize the Langerhans cells in a similar fashion to contact dermatitis, but is identified by its inflammatory skin manifestations and the presence of Th2 cells as well as being generally associated with high levels of IgE antibody (Wang et al., 1996).

[0016] In contrast, transcutaneous immunization with cholera toxin or related ADP-ribosylating exotoxins resulted in a novel immune response with an absence of post-immunization skin findings, high levels of antigen-specific IgG antibody, the presence of all IgG subclass antibodies, and the absence of antigen-specific IgE antibody. See PCT/US97/21324 and U.S. application Ser. Nos. 60/086,196 and 09/257,188.

[0017] There is a report by Paul et al. (1995) of induction of complement-mediated lysis of antigen-sensitized liposomes using transferosomes. The transferosomes were used as a vehicle for antigen, and complement-mediated lysis of antigen-sensitized liposomes was assayed. The limit to passage through the skin by antigen was stated to be 750 daltons. Furthermore, Paul and Cevc (1995) stated that it is "impossible to immunize epicutaneously with simple peptide or protein solutions." Thus, transcutaneous immunization as described herein would not be expected to occur according to this group.

[0018] Besides the physical restriction of limiting passage through the skin of low molecular weight, passage of polypeptides was believed to be limited by chemical restrictions. Carson et al. (U.S. Pat. No. 5,679,647) stated that "it is believed that the bioavailability of peptides following transdermal or mucosal transmission is limited by the relatively high concentration of proteases in these tissues. Yet unfortunately, reliable means of delivering peptides . . . by transdermal or mucosal transmission of genes encoding for them has been unavailable."

[0019] In contrast to transcutaneous immunization, transdermal drug therapy has been understood to target the vasculature found in the dermis. For example, Moschella (1996) states, "The advantages of transdermal therapy over conventional oral administration include: 1. Avoidance of 'peak and

trough' plasma concentration profiles. 2. Avoidance of first-pass metabolism in the gastrointestinal tract and liver" (emphasis added). Thus, in the realm of drug delivery, the meaning of transdermal is to pass through the epidermis and into the dermis or lower layers to achieve adsorption into the vasculature.

[0020] Tang et al. (1997) have shown that mice in which the keratinized layer of skin and hair was removed chemically with keratinolytic agents containing calcium hydroxide are able to mount an antibody response of unknown magnitude or efficacy by adenovirus vector-encoded antigens and carcino-embryonic antigen or GM-CSF. Such a technique relied upon chemical and physical removal of the outer keratinized and lipid layer of the skin. Calcium hydroxide also acts as a skin irritant. Therefore, commercial preparations of calcium hydroxide contain emollients, aloe extract, and oils to lessen the irritant nature of the treatment and their labels advise users to test a small area of skin for irritant reactions. Chemical removal of the outer layer of the skin is not required for transcutaneous immunization, but may enhance certain aspects of it as disclosed herein.

[0021] In many cases, effective immunization that leads to protection requires help in the form of adjuvants for the coadministered antigen or plasmid and, therefore, useful immune responses require the use of an adjuvant to enhance the immune response (Stoute et al., 1997; Sasaki et al., 1998). But in PCT/US97/21324, we showed that a skin-active adjuvant was required to induce high levels of systemic and mucosal antibodies to co-administered antigens. For example, mice immunized with CT+DT induced high levels of systemic and mucosal anti-DT antibodies. Antibodies are known to correlate with protection against diphtheria. Thus, the skin-active adjuvant for transcutaneous immunization can be expected to provide 'help' in the immune response to co-administered antigen and to play a critical role in inducing a useful immune response.

[0022] Such references explain why our successful use of a molecule like cholera toxin (which is 85,000 daltons) as an antigen-adjuvant in immunization was greeted with enthusiasm and surprise by the art because such large molecules were not expected to pass through the skin and, therefore, would not have been expected to induce a strong, specific immune response.

[0023] U.S. application Ser. Nos. 08/749,164; 08/896,085; 60/086,196; and PCT/US97/21324 show that using a wide variety of ADP-ribosylating exotoxins such as, for example, cholera toxin (CT), heat-labile enterotoxin from *E. coli* (LT), *Pseudomonas* exotoxin A (ETA), and pertussis toxin (PT), can elicit a vigorous immune response to epicutaneous application which is highly reproducible. Moreover, when such skin-active adjuvants were applied along with a separate antigen (e.g., bovine serum albumin or diphtheria toxoid), systemic and mucosal antigen-specific immune responses could be elicited.

[0024] Thus bovine serum albumin (BSA), not highly immunogenic by itself when epicutaneously applied to the skin, can induce a strong immune response when placed on the skin with CT. The Langerhans cell population underlying the site of application are a preferred antigen presenting cell (APC) for activation, differentiation, and delivering antigen to the immune system. Adjuvant may act on the APC directly, or through cognate lymphocytes specifically recognizing antigen. The induction of mucosal immunity and immuno-