A Substance p Agonist Acts as an Adjuvant to Promote Hapten-Specific Skin Immunity

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Because substance p (SP) has been reported to be released from cutaneous sensory nerve endings after hapten application, we determined whether SP participates in contact hypersensitivity (CH) induction by using a SP agonist, GR73632 or δ-Aminovaleryl [Pro^9, N-Me-Leu^{10}]-substance $P_{7–11}$ and a SP antagonist, spantide I. When injected intradermally, SP agonist enhanced CH induced by conventional, but not optimal, sensitizing doses of hapten. By contrast, SP antagonist inhibited the induction of CH by optimal sensitizing doses of hapten. Moreover, SP agonist promoted CH induction and prevented tolerance when hapten was painted on skin exposed to acute, low-dose ultraviolet-B radiation. Intradermally injected SP agonist altered neither the density nor the morphology of epidermal Langerhans cells, implying that SP agonist enhanced the generation of hapten-specific immunogenic signals from the dermis. It is proposed that SP is a natural "adjuvant" that promotes the induction of CH within normal skin. Although exogenous SP agonist can prevent impaired CH and tolerance after ultraviolet-B radiation, the susceptibility of native SP to local neuropeptidases renders the neuropeptide unable to prevent the deleterious effects of ultraviolet-B radiation on cutaneous immunity. ***Key words: contact hypersensitivity/neuroimmunology/tolerance/suppression. J Invest Dermatol 112:437–442, 1999***

Exposure of murine skin to ultraviolet B radiation (UVR) causes profound changes in local (cutaneous) and systemic immunity (Kripke, 1984; Streilein, 1993). Our laboratory has used an acute, low-dose UVR protocol directed at the shaved body wall skin of mice to study the deleterious effects of UVR (Toews *et al*, 1980). When a sensitizing dose of hapten, such as dinitrofluorobenzene (DNFB), is painted on the irradiated surface immediately after the last of four consecutive daily UVR exposures, contact hypersensitivity (CH) fails to develop in certain genetically defined strains of mice, termed UVB-susceptible, but CH develops in other mice, termed UVB-resistant (Streilein and Bergstresser, 1988; Yoshikawa and Streilein, 1990; Kurimoto and Streilein, 1993). If the hapten is painted on a cutaneous site distant from the original site of UVR rather than on the exposed site, CH develops normally in UVB-susceptible mice, indicating that the primary immune defect created by UVR in this protocol is at the irradiated site (Shimizu and Streilein, 1994b). Moreover, when sensitizing doses of the hapten are painted simultaneously on UVR-exposed and unexposed skin, shortly after the last dose of UVR, normal levels of CH are induced (Shimizu and Streilein, 1994b). This result considerably strengthens the conclusion that acute, low-dose UVR impairs CH induction by a strictly local action. It has been proposed that the "strictly local" effects of acute, low-dose UVR on CH induction are mediated primarily by tumor necrosis factor (TNF)-α (Yoshikawa and Streilein, 1990; Shimizu and Streilein, 1994b).

We recently reported that calcitonin gene-related peptide (CGRP) is central to the deleterious effect on CH induction, and that CGRP acts via a TNF-α dependent mechanism in UVB-susceptible mice (Niizeki *et al*, 1997). We hypothesized that UVR stimulates nerve endings to release CGRP, which triggers dermal mast cells to secrete TNF-α (Niizeki *et al*, 1997). Although cutaneous sensory nerve endings secrete substance p (SP) simultaneously with CGRP if stimulated (reviewed in Ansel *et al*, 1996), the possible roles of SP on CH induction are presently unknown.

SP, a neuropeptide of the tachykinin family, exerts several biologic activities in the central nervous system and in peripheral organs, mainly (but not exclusively) as a neurotransmitter and neuromodulator (reviewed in Ansel *et al*, 1996). SP is also known to modulate certain parameters of the immune response, including proliferation (Payan *et al*, 1984) and IL-2 production by T cells (Calvo *et al*, 1992; Rameshwar *et al*, 1993). SP inhibits hapten-specific IgE production (Carucci *et al*, 1994). In addition, SP modifies cytokine gene expression and eicosanoid production of macrophages/monocytes (Kimball *et al*, 1988; Lotz *et al*, 1988; Laurenzi *et al*, 1990; Murris-Espin *et al*, 1995), and alters tumoricidal function (Peck, 1987), chemotaxis (Ruff *et al*, 1985), phagocytosis (Bar-Shavit *et al*, 1980), and induction of oxidative burst and thromboxane release (Hartung and Toyka, 1983) by IFN-γ activated macrophage. Recently, it was reported that a SP agonist stimulates the production of IL-12 by lipopolysaccharide-activated macrophages (Kincy-Cain and Bost, 1997).

SP is a highly unstable neuropeptide when injected into living tissues, presumably because it is sensitive to indigenous neuropeptidases (Matsas *et al*, 1984). Therefore, in order to study the potential role of SP *in vivo* we chose the SP agonist (GR73632 or δ-Aminovaleryl [Pro^9, N-Me-Leu^{10}]-substance $P_{7–11}$). This molecule is quite stable when injected *in vivo*, and is a potent and selective agonist for neurokinin-1 receptors (NK-1R) (Hagan *et al*,

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Abbreviations: APC, antigen-presenting cell; CGRP, calcitonin gene-related peptide; CH, contact hypersensitivity; NK-1R, −2R, −3R, neurokinin-1, −2, −3 receptor; SP, substance p.

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1989). Here we show that signals delivered through the SP receptor (NK-1R) enhance CH induction on normal skin and overwhelm the deleterious effects of UVR on cutaneous immunity.

MATERIALS AND METHODS

Mice Adult mice, aged 8–12 wk, of C3H/HeN inbred lines were purchased from Taconic Farms (Germantown, NY). All animal procedures were approved by the institutional Animal Care and Use Committee. Experimental procedures were carried out with the animals under general anesthesia achieved by intraperitoneal injection of ketamine (12.5 mg per ml; Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (0.125 mg per ml; Phoenix Pharmaceutical, St. Joseph, MO). Each control or experimental panel consisted of five mice. Each experimental protocol was repeated at least twice with similar results.

Reagents DNFB (2, 4-dinitro-1-fluorobenzene) and oxazolone (4-ethoxymethylene-2-phenyloxazol-5-one) were purchased from Sigma (St. Louis, MO). SP agonist (GR73632 or δ-Aminovaleryl [Pro^9, $N\text{-}Me\text{-}Leu^{10}$]-substance $P_{7-11\ 11}$) and spantide I, a substance p antagonist ([$D\text{-}Arg^1$, $D\text{-}Trp^{7,9}$, Leu^{11}]-substance p) was purchased from Peninsula Lab (Belmont, CA). Peptides were stored at 1 mg per ml in distilled water at –70°C. Anti-Ia^k antibody and fluoroscein isothiocyanate-conjugated goat anti-mouse antibody were purchased from Becton Dickinson (Mountain View, CA) and CAPPEL (Durham, NC), respectively.

Intradermal (i.d.) injections Using a 1.0 ml syringe with a 30 gauge needle, the contents of the syringe were placed within the i.d. space. Successful inoculations were characterized by the appearance of a flattened swelling with defined lateral margins immediately beneath the epidermis (Niizeki and Streilein, 1997).

Identification and enumeration of epidermal Langerhans cells Epidermis was separated from dermis by incubation in ethylenediaminetetraacetic acid. Langerhans cells in these epidermal sheets were stained with monoclonal anti-Ia antibody as described previously (Bacci *et al*, 1996), and evaluated under epifluorescent microscopy. With the aid of an eyepiece with a 1 mm^2 grid, a minimum of 10 fields was counted for each sample to enumerate the number of positively stained cells and to calculate the mean number of stained cells per mm^2 epidermis.

Induction and expression of CH Twenty-five microliters of 0.5% (185 μg) or 0.004% (1.5 μg) DNFB in acetone was applied to the dry-shaved abdominal cutaneous surface of mice on day 0 (Kurimoto and Streilein, 1993; Bacci *et al*, 1997). CH was elicited on day 5 by challenging one ear of each mouse with 20 μl of 0.05% DNFB. Ear swelling was measured with an engineer's micrometer (Mitutoyo, Tokyo, Japan) 24 h and 48 h following challenge and compared with ear thickness prior to challenge.

Assessment of tolerance DNFB in acetone (185 μg) was applied to dry-shaved abdominal skin on day 0. Two weeks later, the same mice received a second epicutaneous application of DNFB in acetone (185 μg) on previously untreated, dry-shaved dorsal body wall skin (Niizeki and Streilein, 1997). The ears of these mice were challenged 5 d later and ear thicknesses were measured as described above.

UVR Shaved abdominal skin was exposed to UVB from a bank of four FS-20 fluorescent lamps with a tube-to-target distance of 46 cm as previously described (Niizeki and Streilein, 1997). These bulbs have a broad emission spectrum (250–400 nm) and high output primarily in the UVB range (290–320 nm). As measured by an IL 700 radiometer with a SEE 240 UVB photodetector (Medko Medical Instrumentarium, Finland), these lamps delivered an average flux of 1.4 per m^2 per s. Mice were exposed to UVB daily for four consecutive days (400 J per m^2 per day) on the abdominal cutaneous surface.

Statistical analysis The statistical significance of differences between means of each experimental group was determined with analysis of variance. Mean differences were considered to be significant when $p < 0.05$. Analyses were performed using StatView 512^+ (Abacus Concepts, Berkeley, CA) on a Macintosh IIci microcomputer.

RESULTS

SP agonist fails to alter epidermal Langerhans cells We have reported that CGRP, a neuropeptide present in c-type nerve terminii of the epidermis, can decrease the density of epidermal Langerhans cells via a TNF-α-dependent mechanism when CGRP is injected intradermally (Niizeki *et al*, 1997). Because SP is coexpressed with CGRP in cutaneous sensory nerves (reviewed in Ansel *et al*, 1996), we first wished to know whether SP can also alter the density of epidermal Langerhans cells. Rather than using SP itself, which is highly labile, we turned to a substance p agonist that bears a striking resemblance to native SP, and binds the SP receptor (NK-1R) with high affinity (Hagan *et al*, 1989). The dose of SP agonist used in the following experiments was chosen according to the experience reported by Yano *et al* (1989). These investigators reported that five nanomoles of SP is capable of evoking ear swelling when injected intrapinnally into mice (Yano *et al*, 1989).

Table I. SP agonist fails to alter the density of epidermal Ia^+ cells[a]

	Number of Ia^+ cells ± SEM per mm^2
PBS	699.2 ± 37
SP agonist	739 ± 48
UVB + PBS	550.4 ± 35*
UVB + SP agonist	500.8 ± 31**

[a]C3H/HeN mice received intradermal injections of PBS or SP agonist (5 nmol) on dry-shaved abdominal skin. In some mice, 400 J UVB per m^2 of radiation was delivered to the injection site within 30 min. Two hours after the injections, epidermal sheets were prepared from injected sites (26). Asterisks indicate values significantly lower than PBS (positive control): *$p < 0.05$, **$p < 0.001$.

C3H/HeN mice received i.d. injections of 5 nmol of SP agonist [or phosphate-buffered saline (PBS) as positive control] into shaved abdominal skin. Two hours later, biopsies were taken and epidermal sheets were prepared as described previously (Bacci *et al*, 1996). Cells positive for Ia antigens were enumerated with 10 mm grid. The epidermis of SP agonist-treated mice displayed a similar density of Ia^+ Langerhans cells as compared with epidermis after PBS injection (**Table I**). These data suggest that SP agonist has no effect on the density of epidermal Langerhans cells. In companion experiments SP agonist failed to reverse the deleterious effects of UVR on Langerhans cells density (**Table I**). These findings are consistent with our previous report that a CGRP antagonist is able to prevent Langerhans cells depletion by UVR, even though both SP and CGRP are presumed to be released from cutaneous nerve endings after UVR (Niizeki *et al*, 1997).

SP agonist augments the induction of CH We next wished to determine whether SP agonist can augment the induction of CH when hapten is painted on normal skin. Panels of C3H/HeN mice received i.d. injections of 5 nmol of SP agonist. Positive control mice received i.d. injections of PBS. Within 30 min, a conventional sensitizing dose of DNFB (185 μg in acetone) was carefully applied directly on the skin overlying the injection sites. Five days later, the ear pinnae of these mice, along with unsensitized negative controls, were challenged with dilute DNFB (15 μg in acetone). The ear swelling responses at 24 h (in one of two experiments) are presented in **Fig 1(*A*)**. The intensity of ear swelling measured in ears of mice that received i.d. injection of SP agonist was significantly greater than that of positive controls (only PBS injected). These results indicate that 5 nmol of SP agonist augments CH induction. A dose response experiment was conducted in which different doses of SP agonist (0.05, 0.5, and 5 nmol) were injected prior to application of DNFB (185 μg). Only the 5 nmol dose of SP agonist was capable of enhancing CH induction (data not shown). In a subsequent set of experiments, SP agonist was injected i.d. into C3H/HeN mice and thereafter an optimal sensitizing dose of DNFB (1.5 μg) was applied to the site. Control mice received i.d. injections of PBS. When the ears of these mice were challenged with dilute DNFB 5 d later, the intensity of ear swelling was virtually identical in both groups of mice (**Fig 1*B***). These findings reveal that SP agonist is capable of augmenting the intensity of CH induction if hapten is applied at conventional sensitizing doses, but not if optimal sensitizing doses

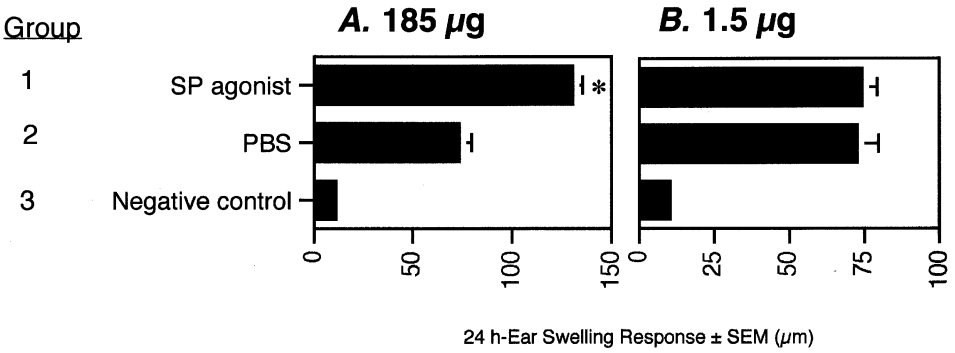

Figure 1. SP agonist can enhance CH induction. C3H/HeN mice received epicutaneous application of DNFB (*A*, 185 μg; *B*, 1.5 μg) to abdominal sites previously injected with SP agonist (5 nmol). Five days later, their ears were challenged with DNFB (15 μg). Ear swelling responses at 24 h are presented as mean ± SEM (μm). Mean values significantly higher than positive control: *$p < 0.00001$.

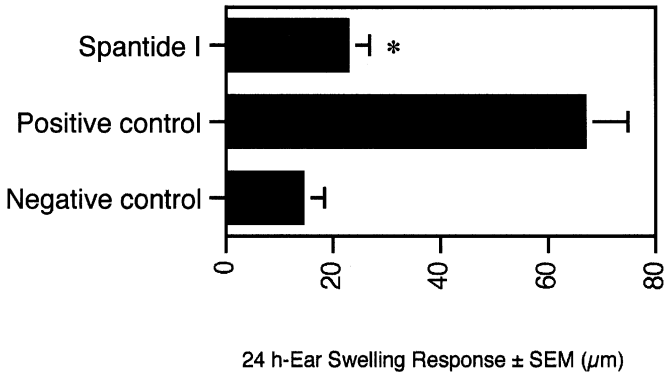

Figure 2. Spantide I (SP antagonist) impairs CH induction. C3H/HeN mice received an epicutaneous application of DNFB (1.5 μg) to abdominal skin sites into which spantide I (5 nmol) had been injected 30 min previously. Five days later, ears were challenged with DNFB (15 μg). Ear challenge and the determination of ear swelling responses are presented as described in the legend to **Fig 1**. Mean values significantly lower than positive control: *$p < 0.0005$.

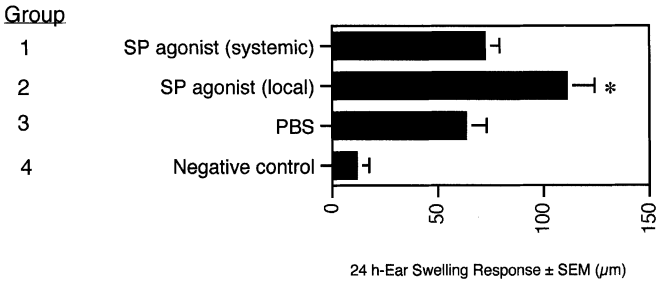

Figure 3. SP agonist enhances CH induction via a local effect. C3H/HeN mice received an i.d. injection of SP agonist (5 nmol) into abdominal skin. Within 30 min, DNFB (185 μg) was painted either on the abdominal site of injection (Group 2), or on dorsal (uninjected) skin (Group 1). Control mice received abdominal skin injection of PBS (Group 3). Ear challenge and determination of ear swelling responses are presented as described in the legend to **Fig 1**. Mean values significant higher than positive control (Group 3): *$p < 0.001$.

of hapten are applied. There is circumstantial evidence (Kurimoto and Streilein, 1993; Bacci *et al*, 1997) that optimal sensitizing doses of hapten rely almost exclusively upon epidermal antigen-presenting cell (APC), whereas conventional doses of hapten diffuse well into the dermis and actually relay primarily on dermal APC for CH induction. These findings have led us to postulate that SP augments CH induction by enhancing the activities of dermal, rather than epidermal, APC.

A SP antagonist, spantide, impairs CH induction Although 5 ng of SP agonist was incapable of enhancing CH induction with optimal sensitizing doses of hapten (**Fig 1*B***), we considered the possibility that under physiologic conditions sufficient SP is released to promote CH induction at this low level of hapten dose. To test for this possibility, we tested a SP antagonist, spantide I, for its capacity to impair CH induction when an optimal sensitizing dose of hapten was applied epicutaneously. Panels of C3H/HeN mice were subjected to intradermal injection of 5 ng spantide I (or PBS alone) before sensitization. Within 30 min, DNFB (1.5 μg) was painted on the injected site (Bacci *et al*, 1997). The ears of these mice were challenged with DNFB (15 μg) 5 d later and the ear swelling responses were measured at 24 h. The results of a representative experiment (of two) are displayed in **Fig 2.** Mice that received intradermal injections of spantide immediately prior to hapten application displayed impaired CH when compared with PBS-injected mice (positive control). This result supports the view that SP released from cutaneous nerves participates in the process by which CH is induced in mice – using either optimal or conventional sensitizing doses of hapten.

SP agonist promotes CH induction by a local effect We wished next to know whether SP agonist acted locally or systemically to augment induction of CH. Panels of C3H/HeN mice received i.d. injection of 5 nmol SP agonist into abdominal skin (Group 2), or dorsal body wall skin (Group 1). Positive control mice received i.d. injections of PBS on abdominal skin (Group 3). Within 30 min, DNFB (185 μg) was applied to abdominal skin of all three groups of mice. Five days later, the ear pinnae were challenged with 15 μg DNFB. The results of a representative experiment (one of two) are presented in **Fig 3**. Mice that first encountered DNFB via SP agonist-injected skin displayed enhanced CH, whereas mice that first encountered DNFB via uninjected skin (these mice received SP agonist injections into dorsal skin) displayed CH comparable in intensity to positive controls. Thus, the capacity of SP to enhance CH induction is expressed locally, at the site of SP agonist injection, not at a distance (i.e., systemically).

SP agonist prevents UVR-impaired CH Because SP agonist can augment CH induction in normal, unperturbed skin, we wondered whether SP agonist might also be capable of preventing the impaired CH that follows exposure to acute, low-dose UVR. To test this possibility, panels of C3H/HeN mice received four consecutive daily dose of acute, low-dose UVR. Immediately after the last exposure, mice received i.d. injections of 5 nmol of SP agonist or PBS (UVR controls). Within 30 min, DNFB (185 μg in acetone) was applied directly on the skin overlying the injection sites. Five days later, the ear pinnae of these mice, along with unsensitized negative controls, were challenged with dilute DNFB (15 μg in acetone). The 24 h ear swelling responses of C3H/HeN mice (in one of two experiments) are presented in **Fig 4**. The intensity of ear swelling measured in ears of mice that received i.d. injection of PBS after UVR was significantly less than that of positive control (no UVR exposure); however, mice that received i.d. injections of SP agonist after UVR displayed ear swelling responses virtually identical to positive controls. Thus, SP can prevent impaired induction of CH after acute, low-dose UVR.

SP agonist can prevent induction of UVR-dependent tolerance We next examined the possibility that 5 nmol of i.d.-injected SP agonist could prevent the development of hapten-specific tolerance as has been reported for UVR (Toews *et al*, 1980;

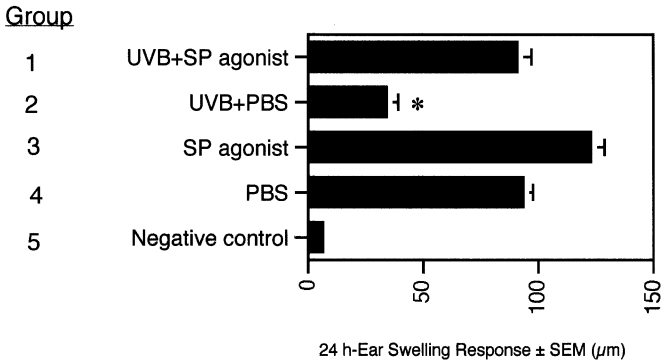

Figure 4. SP agonist reverses the deleterious effect of UVR on CH induction. Abdominal skin of C3H/HeN mice received exposure to 400 J UVR per m^2 daily for four consecutive days. Immediately after the last exposure, SP agonist was injected intradermally into the exposed sites. Within 30 min after injections, DNFB (185 μg) was applied to the injected surface. Ear challenge and the determination of ear swelling responses are presented as described in the legend to **Fig 1**. Mean values significantly lower than positive control (Group 4): *$p < 0.00001$.

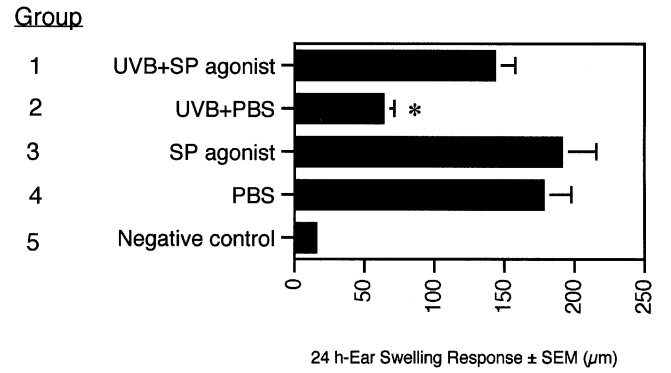

Figure 5. SP agonist prevents induction of tolerance after UVR. Abdominal skin of C3H/HeN mice received exposure to 400 J UVR per m^2 daily for four consecutive days. Within 30 min of the last exposure, SP agonist was injected intradermally into the exposed sites. Immediately after injections, DNFB (185 μg) was applied to the injected sites. Fourteen days later, a second dose of DNFB (185 μg) was painted on previously untreated skin sites (dorsal). Ear challenge and the determination of ear swelling responses are presented as described in the legend to **Fig 1**. Mean values significantly lower than positive control: *$p < 0.001$.

Niizeki and Streilein, 1997). One way to detect UVR-dependent tolerance is to expose mice first to hapten via UVR-treated abdominal skin, and then to reimmunize the animals subsequently with a sensitizing dose of hapten painted on untreated dorsal skin. Failure of the mice to develop vigorous CH following the second immunization is evidence that tolerance has been induced. In the following experiments, panels of C3H/HeN mice received i.d. injections of SP agonist (5 nmol) or PBS only into dry-shaved abdominal skin after the last of four consecutive daily exposures of acute, low-dose UVR. DNFB (185 μg in acetone) was then painted within 30 min on the epicutaneous surface overlying the injected site. Positive control mice received i.d. injections of PBS without UVR exposures. Two weeks later, DNFB (185 μg) was again applied to freshly shaved skin surface distant from the site originally exposed to UVR. The pinnae of all mice, plus a negative control panel, were challenged 5 d later with 15 μg DNFB in acetone, and measured with an engineer's micrometer 24 h later as an indication of the development of CH. The results of a representative experiment (of two) are presented in **Fig 5**. C3H/HeN mice that first encountered DNFB via UVR-exposed skin developed feeble CH when immunized subsequently with hapten on normal skin, i.e., the mice had acquired tolerance. The group of mice that first encountered DNFB via SP agonist-injected skin after UVR, however, developed high intensity CH when compared with positive control. These data indicate that SP can prevent UVR-dependent tolerance.

SP agonist can reverse UVR-dependent tolerance In the next series of experiments, we inquired whether SP agonist can eliminate tolerance after it has been established by painting hapten on UVR-exposed skin. To state the goal of this experiment in query form: can SP agonist abrogate UVR-dependent tolerance if the agonist is injected i.d. 30 min before the *second* application of DNFB (185 μg), but 2 wk after these mice were exposed to four doses of UVR followed immediately by epicutaneous DNFB (185 μg). The results of a representative experiment (of two) are presented in **Fig 6**. C3H/HeN mice that first encountered DNFB via UVR-exposed skin developed feeble CH when immunized subsequently with hapten on normal skin (Group 2); however, the group of mice (Group 1) that first encountered DNFB via UVR-exposed skin, and then re-encountered the same hapten through skin treated with SP agonist 2 wk later developed intense CH, comparable with unirradiated positive controls. These data indicate that SP can reverse UVR-dependent tolerance by enhancing the effects of the second exposure to hapten, and this is a different consequence of SP activity from that which overcomes the local effects of UVR on CH induction at the site of irradiation. The ability of SP agonist to prevent tolerance induction after UVR resembles the capacity of IL-12 to reverse UVR-dependent tolerance (Schwarz *et al*, 1996; Riemann *et al*, 1996).

DISCUSSION

Several lines of recent inquiry have led investigators to consider a role for neuropeptides normally found in epidermal terminii of c-type nerve fibers in cutaneous immunity of the CH type (Ansel *et al*, 1996). Although Paul Langerhans originally proposed that Langerhans cells themselves were components of the nervous system (Langerhans, 1968), their origins from hematopoietic progenitors has been clearly established (Frelinger *et al*, 1979; Katz *et al*, 1979). Granstein and colleagues reawakened interest to the neural connectivity of Langerhans cells by demonstrating that c-type nerve fiber terminii containing CGRP were present on the surfaces of Langerhans cells in normal skin (Hosoi *et al*, 1993). More recently, it has been reported that CGRP was able to inhibit CH induction (Asahina *et al*, 1995), and that CGRP was released from c-type fiber terminii in rat skin exposed to UVR (Benrath *et al*, 1995). Most recently, Niizeki *et al* demonstrated that an antagonist of CGRP can reverse the deleterious effects of acute, low-dose UVR on CH induction (Niizeki *et al*, 1997). Because many nerve endings contain both CGRP and SP, and because these neuropeptides often display antagonist functional properties, it is important to know the extent to which SP can modify cutaneous immunity. Several laboratories have inquired recently into the possible role of SP on induction and expression phases of CH (Ek and Theodorsson, 1990; Gutwald *et al*, 1991; Goebeler *et al*, 1994). The results of the experiments, however, failed to prove a role for SP in the induction phase of CH. Therefore, the results of the experiments that form the basis of this report indicate definitive roles for SP during CH induction in normal skin, and potential roles during CH induction in UVR-exposed skin and in UVR-dependent tolerance.

I.d. injections of a SP agonist augmented the intensity of CH induction through the skin of normal mice. This positive effect was observed when a high dose of sensitizing hapten (DNFB, 185 μg) was applied epicutaneously, but was not observed at much lower sensitizing doses, often called optimal sensitizing doses (Kurimoto and Streilein, 1993). Kurimoto and Streilein (1993) provided strong circumstantial evidence several years ago that optimal sensitizing doses of hapten induced CH primarily through the actions of epidermal APC (Langerhans cells), whereas conventional sensitizing doses induced CH primarily with the aid of dermal APC. In fact, Bacci *et al* (1997) have recently reported that conventional sensitizing doses are so toxic that there are virtually no functionally competent Langerhans cells in the epidermis within

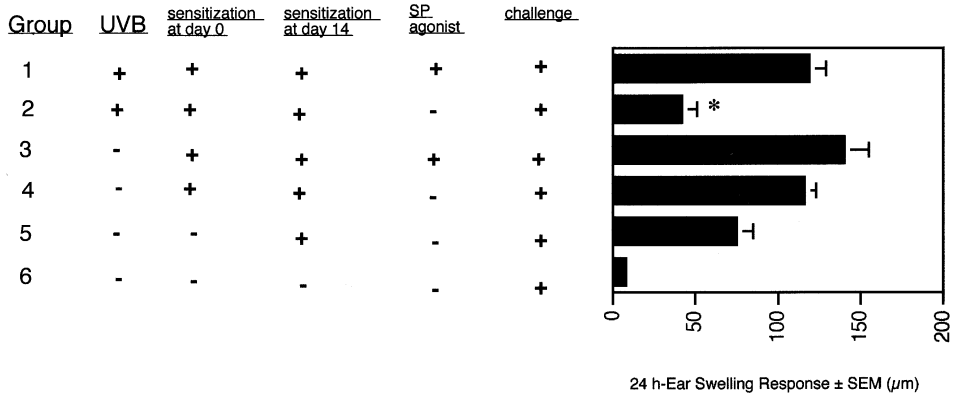

Figure 6. SP agonist reverses UVB-induced tolerance. Abdominal skin of C3H/HeN mice received exposure to 400 J UVR per m^2 daily for four consecutive days (Groups 1, 2). Immediately after the last exposure, DNFB (185 μg) was applied at the exposed sites. Fourteen days later, 5 nmol of SP agonist (Groups 1, 3) or PBS (Groups 2, 4, 5) was injected into previously untreated dorsal skin sites. Within 30 min after these injections, a second dose of DNFB (185 μg) was painted on the injected sites. Ear challenge and the determination of ear swelling responses are presented as described in the legend to **Fig 1**. Mean values significantly lower than positive control (Group 4): *$p < 0.00001$.

the first 6–12 h after hapten is applied at high dosage. This line of reasoning leads us to postulate that SP enhances CH induction by acting on dermal cells, rather than epidermal cells.

The ability of a neuropeptide of presumed epidermal origin to have a primary mode of action on a dermal cell is not unprecedented. Niizeki *et al* recently demonstrated that i.d. injection of CGRP impaired CH induction by inducing dermal mast cells to release TNF-α. These experiments were conducted with low (optimal) sensitizing doses of hapten (doses that act primarily via epidermal APC), suggesting that neuropeptides placed in the dermis can diffuse into the epidermis and modify the functional properties of Langerhans cells. To underscore this point, several reports from our laboratory over the past 7 y have amply demonstrated that i.d. injections into murine skin of cytokines (TNF-α, IL-10) and other neuropeptides (α-MSH) can influence the form and function of epideraml Langerhans cells as well as the ability of the injected skin to support CH induction and tolerance (Vermeer and Streilein, 1990; Yoshikawa and Streilein, 1990; Kurimoto and Streilein, 1992; Shimizu and Streilein, 1994a; Niizeki and Streilein, 1997). In the aggregate these findings indicate that neuropeptides and cytokines can diffuse readily across the dermal–epidermal junction and can influence susceptible bone marrow-derived cells that participate in induction and regulation of cutaneous immunity.

It is of considerable interest that SP agonist promoted CH induction when hapten was painted on skin exposed to acute, low-dose UVR. SP agonist joins several other agents that have been similarly shown to enable hapten painted on UVR-exposed skin to induce intense CH. The list of such agents includes anti-TNF-α antibody, vitamin C, and IL-12 (Yoshikawa and Streilein, 1990; Müller *et al*, 1995; Nakamura *et al*, 1997). The results of these experiments do not indicate whether SP agonist acts in a unique fashion to reverse the effects of UVR, or whether it acts through one of the agents previously described. We doubt that the effects of SP agonist are related to vitamin C because SP itself promotes reactive oxygen intermediate formation from macrophages (Hartung and Toyka, 1983; Murris-Espin *et al*, 1995), and vitamin C is believed to prevent UVR damage by inhibiting oxygen radical formation (Nakamura *et al*, 1997). Although SP resembles CGRP in its capacity to selectively activate TNF-α production from mast cells (Ansel *et al*, 1993), we favor the view that SP agonist-activated APC are resistant to TNF-α. In fact, SP is reported to induce the generation of several cytokines that have opposing effects to TNF-α and IL-1 (Lotz *et al*, 1988; Laurenzi *et al*, 1990; Ansel *et al*, 1993; Viac *et al*, 1996). Because SP agonist has been reported to induce IL-12 production by lipopolysaccharide-treated macrophages (Kincy-Cain and Bost, 1997), we currently favor the hypothesis that SP agonist promotes CH induction after UVR by promoting IL-12 production. This hypothesis is supported by the findings that injection of IL-12 alone abolishes the deleterious effects of UVR on CH induction (Schwarz *et al*, 1996), and that IL-12 supersedes the ability of IL-10 to suppress sensitization (Müller *et al*, 1995).

The mechanism(s) by which SP agonist reverses UVR-dependent tolerance are also of interest. TNF-α appears to play little if any role in tolerance after UVR, whereas there is considerable evidence to implicate IL-10 as the responsible tolerance-promoting cytokine (Niizeki and Streilein, 1997). Because we have recently found that dermal mast cells are the most important cutaneous source of IL-10 after UVR exposure that leads to tolerance, SP agonist could prevent tolerance by preventing mast cells from releasing IL-10. Alternatively, SP agonist may restore the APC defect in UVR-exposed skin, perhaps through IL-12, which would enable functionally enhanced hapten-bearing APC to supersede the skin-derived tolerance-conferring stimulus that UVR creates on dermal cells after UVR (Kurimoto *et al*, 1994).

The target cells upon which SP agonist acts in the dermis is unclear. Dermal APC seem to express NK-1R, as do mast cells. On the one hand, mast cells have the ability to produce TNF-α (Ansel *et al*, 1993). Moreover, TNF-α released from dermal mast cells is known to be a cytokine that impairs CH induction (Niizeki *et al*, 1997). On the other hand, SP-activated dermal APC may be resistant to TNF-α. This hypothesis is supported by our finding that injection of SP agonist into skin after four consecutive daily exposures of UVR rendered the skin resistant to the deleterious effects of UVR. Mice treated in this way responded to epicutaneous application of DNFB with intense CH (**Fig 4**).

The ability of SP agonist to reverse the deleterious effects of UVR on cutaneous immunity presents a paradox. Terminii of c-type nerve fibers in the epidermis contain both CGRP and SP (reviewed in Ansel *et al*, 1996), and both are released following UVR exposure (Benrath *et al*, 1995). Yet, CGRP appears to dictate the immune outcome. As mentioned previously, CGRP plays a leading role after UVR in causing mast cells to release TNF-α, which is a primary mediator of the immune deficits observed (Niizeki *et al*, 1997). As exogenous SP (SP agonist) reverses these UVR-dependent immune deficits, why doesn't SP neutralize the effects of CGRP when both are released by UVR exposure? The reason may rest with the stability of these two neuropeptides. Native SP has a very short half-life *in vivo*, presumably because it is readily degraded by indigenous neuropeptidases (Matsas *et al*, 1984). CGRP is much more stable, as is the SP agonist we used in our experiments. Thus, CGRP may dominate after UVR exposure simply because SP is rapidly degraded and unable to materially influence subsequent immune events. Most recently, Scholzen *et al* (1998) demonstrated that CH was enhanced in neuropeptidase knockout mice. It is noteworthy that this effect could be abrogated by injections of a NK-1R-specific antagonist (Scholzen *et al*, 1998). This result enables us to postulate that enhancement of signals through NK-1R could enhance CH response by blocking degradation of SP.

Our results provide evidence that SP agonist can induce the functional enhancement of APC. This interpretation is further supported by our finding that SP agonist reversed UVR-dependent tolerance when injected i.d. 2 wk after the tolerance was induced. At this time, tolerance is systemic, and hapten-specific regulatory

T cells reside widely in lymph nodes and spleen. Yet an i.d. injection of SP agonist followed by epicutaneous hapten induced vigorous CH in mice previously exposed to UVR and hapten. It is relevant that we demonstrated that SP agonist, in the dose and route injected, had no systemic effect on hapten immunity. Therefore, the finding that SP agonist abolishes *established* UVR-dependent tolerance suggests that SP may be acting locally within the skin as an "adjuvant", amplifying the immunogenic signal that arises when hapten is painted epicutaneously. We suspect that cutaneous APC may be energized by SP, which enables these cells to become the mediators of the "adjuvant" effect. Our experiments are designed to test this possibility.

Finally, our finding that SP agonist promotes CH induction contrasts with the report that SP itself fails to enhance CH induction (Gutwald *et al*, 1991). There are several possible explanations for this discrepancy. SP can bind to a range of specific receptors (NK-1R, NK-2R, NK-3R), whereas SP agonist binds only to NK-1R (Hagan *et al*, 1989). These receptors may have competing properties that would prevent SP from delivering the type of unambiguous signal that SP agonist can give. Perhaps the binding of SP agonist to NK-1R is more stable than that of native SP (Hagan *et al*, 1989), which would enable the agonist to be a more effective ligand. Finally, SP has a much shorter half-life *in vivo* than the SP agonist because the latter is resistant to neuropeptidases (Matsas *et al*, 1984). This may enable the agonist to be a more effective stimulator through time.

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