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THE IMMUNOSUPPRESSIVE EFFECT OF SIDESTREAM SMOKE ON THE CONTACT HYPERSENSITIVITY RESPONSE TO THE HAPTEN DINITROFLUORBENZENE IN BALB/c MICE

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ABSTRACT. In recent years, there have been increasing reports regarding the impact of various stressors on the immune system. It is well established that epicutaneous application of haptens can induce contact hypersensitivity (CHS), a delayed-type immune response. Certain irritants such as ultraviolet B have been shown to induce CHS tolerance. In this study, using the ear swelling assay and the hapten dinitrofluorbenzene (DNFB), we produce evidence suggesting that sidestream cigarette smoke may alter the immune system of the skin of BALB/c mice in a way that results in CHS suppression. Mice were divided into three control and three dosage groups consisting of daily exposure to sidestream smoke from one filter-tip cigarette. Ear swelling of positive controls was significantly greater ($P<0.05$) than for all other groups except the pre-challenged experimental mice ($P<0.20$). Significant CHS suppression occurred in both the three-week experimental mice (80.4%; $P<0.001$), and pre-sensitized mice (18.1%; $P<0.01$).

Keywords: Immunosuppression, sidestream smoke, contact hypersensitivity, dinitrofluorbenzene (DNFB)

Immunotoxicity, which examines how various substances undermine the immune system, is a recent and burgeoning field (Bower 1999). One form of immunity that has been well established since the studies of Landsteiner & Jacob (1935) is contact hypersensitivity (CHS). CHS is a T cell-mediated immune response in the epidermis to a reactive hapten covalently coupled to cell surface proteins (Rowden et al. 1977; DiIulio et al. 1996). Hapten-specific T cells are primed by Langerhans cells (LC) which migrate from the sensitized epidermis to the skin-draining lymph nodes (Kripke et al. 1990; Wang et al. 1997). Subsequent challenges with the hapten results in cutaneous infiltration of the primed T cells and their activation to produce various cytokine mediators of CHS such as tumor necrosis factor-α (TNF-α) (Enk & Katz 1991).

During antigen priming, CD^+ T cells develop from Th0 precursor cells into either CD8 super (+) T (Th1) cells which produce inflammatory cytokines such as interleukin-2 (IL-2) and interferon-gamma (IFN-γ) or CD4 super (+) T (Th2) cells which produce anti-inflammatory cytokines such as interleukin-4 (IL-4) and interleukin-10 (IL-10) (DiIulio et al. 1996; Niizeki & Streilein 1997; Nagai et al. 2000; Simkin et al. 2000). Th1 cells are the effector cells of cell-mediated immunity such as delayed-type hypersensitivity (DTH) and CHS, whereas Th2 cells provide signals enhancing antibody production (Cher & Mosmann 1987). Differentiation of Th0 cells to the Th1 or Th2 phenotype is regulated by the cytokine environment during antigen priming. Th2 cell development requires IL-4 and Th1 cell development is promoted by interleukin-12 (IL-12) (Aragane et al. 1994; Schmitt et al. 1995).

The skin serves as a complex barrier separating the internal compartments from the external environment, including bacteria, viruses, fungi and environmental toxins (Rheins et al. 1993). The effect of skin exposure to ultraviolet light has been examined extensively. Numerous studies have focused on the effects of ultraviolet B (UVB) radiation on CHS. The induction of tolerance to CHS is a common finding (Kurimoto et al. 1994; Smith et al. 1997). Other environmental agents have also been shown to suppress CHS (Blaylock et al. 1993; Ullrich 1994; Schmitt et al. 1995; Tam et al. 1997).

One environmental irritant that the skin could encounter is sidestream smoke. Environmental tobacco smoke (ETS), in an average room of active smokers, is composed of

approximately 85% sidestream smoke (Fielding & Phenow 1988). Mainstream and sidestream smoke both contain a large number of chemical carcinogens and other toxic substances; but undiluted sidestream smoke carries many compounds, such as ammonia, benzene, carbon monoxide, and nicotine, in far greater concentrations (USDHHS 1986). In order to address the potential of this agent as a modulator of the immune system, we investigated the effect of sidestream smoke on the induction (sensitization) and elicitation (challenge) phases of CHS using the mouse ear swelling assay. Our results suggest that ear swelling is significantly suppressed by sidestream smoke in BALB/c mice, and appears to be related to the number of days of exposure prior to sensitization.

METHODS

Mice.—Female BALB/c mice were purchased from Harlan Sprague Dawley (Indianapolis, Indiana) at six weeks of age and used at 8–12 weeks of age. They were maintained in an environment controlled for temperature and light and allowed free access to food and water.

Ear swelling protocol.—The method of Phanuphak et al. (1974) as modified by Tamak et al. (1981) was used to quantify the ear swelling response to contact allergen (Table 1). Mice were randomized into 6 groups (3 control and 3 experimental) ranging in sample size from 18–30. Five of the six groups, including nonsmoke-exposed positive control mice (PC), were sensitized by topical application of 20 μl of 0.5% of the hapten dinitrofluorobenzene (DNFB) (Sigma, St. Louis, Missouri) in a 4:1 acetone/olive oil mixture onto their shaved abdominal surface on day 0 and day 1. Negative control (NC) mice were mock-sensitized with DNFB solvent. On day 5, five of the six groups were challenged with 20 μl of 0.2% DNFB, distributed equally, on both sides of the right ear. Background control (BC) mice were mock-challenged with DNFB solvent. After 24, 48, and 72 h, ear thickness measurements of all mice were made with an engineer's micrometer (Fisher Scientific, Burr Ridge, Illinois); and the CHS response to DNFB was assessed by subtracting post-challenge from pre-sensitized values.

Smoking procedure.—One of the three experimental groups was exposed to sidestream smoke for three weeks prior to sensitization through 72 h post-challenge. This group was designated as 3-week experimentals (3WE). A second group was exposed to sidestream smoke from day 0 (prior to sensitization) through 72 h post-challenge, and was designated as pre-sensitized experimentals (PSE). A third group was exposed to sidestream smoke from day 5 (prior to challenge) through 72 h post-challenge, and was designated as pre-challenged experimentals (PCE). Smoke exposure occurred in acrylic plastic (Plexiglas™) chambers (25.5 × 30.5 × 21.0 cm) containing six airholes 6.5 mm in diameter on two opposite sides. Mice received a 40 min exposure to sidestream smoke from one commercial brand filter-tip cigarette each day of their respective smoking regimen. The cigarettes employed contained tar and nicotine contents of 15.1 mg and 0.13 mg, respectively (Federal Trade Commission 1997).

Assessment of hyporesponsiveness.—The degree of hyporesponsiveness was assessed by calculating the percent suppression of CHS according to the formula below reported by Sauder et al. 1981.

$$\% \text{ Suppression} = \{1 - [(\text{Experimentals} - \text{Negative controls}) \div (\text{Positive controls} - \text{Negative controls})]\} \times 100$$

Values for experimentals, positive controls, and negative controls represent differences in ear swelling (mm × 10^{-2}) produced by the above ear swelling protocol.

Statistical analysis.—Mean differences in ear swelling were analyzed using ANOVA and Tukey Multiple Comparison tests (Zar 1999). The Student *t* test was used to assess the degree of reactivity. A *P* value of less than 0.05 was considered significant.

RESULTS

Six groups of BALB/c mice were compared to determine the effect of sidestream smoke on CHS (Table 1). All groups, except negative control mice, were sensitized with 20 μl of 0.5% DNFB on shaved abdomens on days 0 and 1. All groups, except background control mice, were challenged on the right ear with 20 μl of 0.2% DNFB on day 5. Ear pinna

Table 1.—Protocol used to determine the effects of sidestream smoke on DNFB hapten-induced contact hypersensitivity of BALB/c mice. [1]3-week experimental (3WE) mice were exposed to sidestream smoke from 3 weeks prior to sensitization (Day 0) to 72 h post-challenge (Day 8). [2]Pre-sensitized experimental (PSE) mice were exposed to sidestream smoke from day of sensitization (Day 0) to 72 h post-challenge (Day 8). [3]Pre-challenged experimental (PCE) mice were exposessd to sidestream smoke from day of right ear challenge (Day 5) to 72 h post-challenge (Day 8). [4]Negative control (NC) mice were mock sensitized with DNFB solvent of 4 parts acetone:1 part olive oil. [5]Background control (BC) mice were mock challenged with DNFB solvent of 4 parts acetone:1 part olive oil.

	Treatment					
Test group	Day 0	Day 1	Day 5	Day 6	Day 7	Day 8
Positive Control (PC)	Sens 1 (0.5% DNFB)	Sens 2 (0.5% DNFB)	Challenge (0.2% DNFB)	24 h EAr Measurement	48 h Ear Measurement	72 h Ear Measurement
3-week Experimental[1] (3WE)	Sens 1 (0.5% DNFB)	Sens 2 (0.5% DNFB)	Challenge (0.2% DNFB)	24 h Ear Measurement	48 h Ear Measurement	72 h Ear Measurement
PS Experimental[2] (PSE)	Sens 1 (0.5% DNFB)	Sens 2 (0.5% DNFB)	Challenge (0.2% DNFB)	24 h Ear Measurement	48 h Ear Measurement	72 h Ear Measurement
PC Experimental[3] (PCE)	Sens 1 (0.5% DNFB)	Sens 2 (0.5% DNFB)	Challenge (0.2% DNFB)	24 h Ear Measurement	48 h Ear Mesurement	72 h Ear Measurement
Negative Control[4] (NC)	Mock Sens 1 (DNFB solvent)	Mock Sens 2 (DNFB solvent)	Challenge (0.2% DNFB)	24 h Ear Measurement	48 h Ear Measurement	72 h Ear Measurement
Background Control[5] (BC)	Sens 1 (0.5% DNFB)	Sens 2 (0.5% DNFB)	Mock Challenge (DNFB solvent)	24 h Ear Measurement	48 h Ear Measurement	72 h Ear Measurement

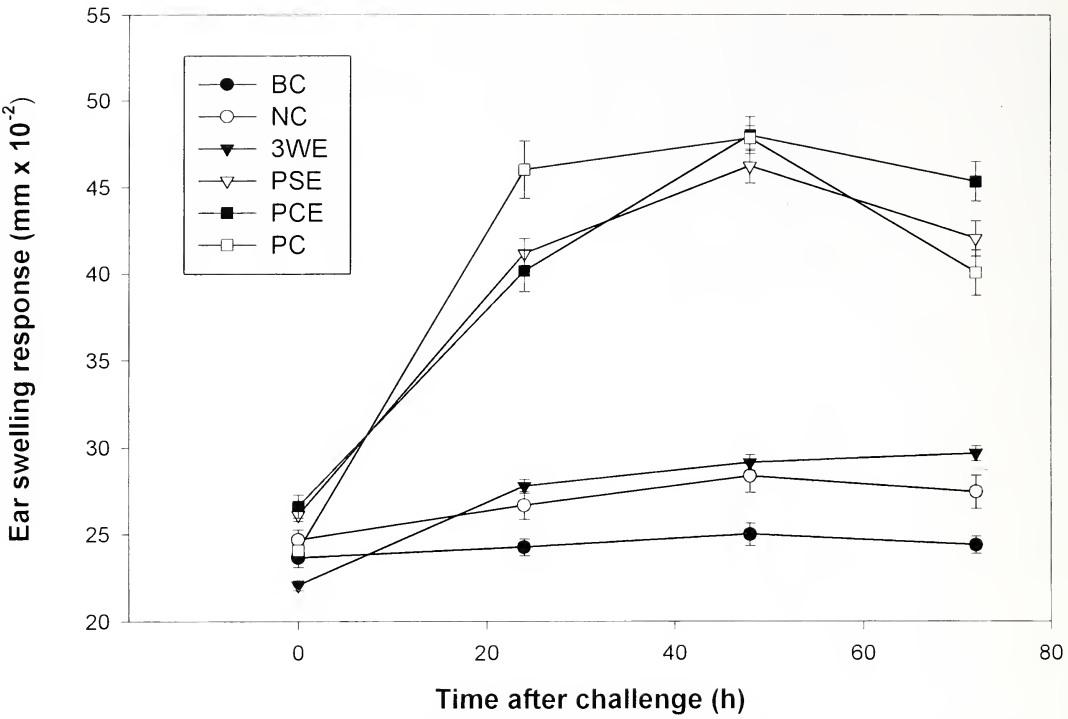

Figure 1.—Effect of sidestream smoke on contact hypersensitivity response of BALB/c mice to DNFB. Values are means ± SEM. Maximum swelling for all test groups was 48 h post-challenge. BC = background controls (n = 20), NC = negative controls (n = 21), 3WE = smoke-exposed mice from three weeks presensitization to 48 h postchallenge (n = 18), PSE = smoke-exposed mice from day of sensitization to 48 h postchallenge (n = 28), PCE = smoke-exposed mice from day of challenge to 48 h postchallenge (n = 21), PC = positive controls (n = 18).

thickness measurements for each group were recorded on day 6 (24 h post-challenge), day 7 (48 h post-challenge), and day 8 (72 h post-challenge).

A dose response curve showed that maximum ear swelling occurred at 48 h post-challenge for all six groups (Fig. 1). Ear measurements were recorded from day 0 (pre-sensitization) through day 8 (72 h post-challenge). No significant difference in ear swelling was noted from day 0 to day 5 (pre-challenge) (Table 2). Mean ear swelling for the three control groups at 48 h was 23.77 mm $\times 10^{-2}$ (98.7%) for PC mice, 3.67 mm $\times 10^{-2}$ (14.9%) for NC mice, and 1.35 mm $\times 10^{-2}$ (5.7%) for BC mice. The three experimental groups produced a mean ear swelling of 21.38 mm $\times 10^{-2}$ (80.3%) for PCE mice, 20.11 mm $\times 10^{-2}$ (77.0%) for PSE mice, and 7.07 mm $\times 10^{-2}$ (32.0%) for 3WE mice. The increase in ear thickness of PC mice was significantly greater than that of all other groups ($P<0.01$), except for the PCE mice ($P<0.20$). Significant CHS suppression occurred in both 3WE (80.4%; $P<0.001$) and PSE mice (18.1%; $P<0.01$) (Fig. 2).

DISCUSSION

The skin has become a convenient site to study the complexities of the immune response. Because the skin is the largest and most exposed organ of the body, it comes in contact with an extensive array of environmental insults such as toxins, allergens and irritants (Rheins et al. 1993). One potential environmental irritant that the skin might encounter is that of sidestream tobacco smoke. CHS is a delayed-type immune response (Asherson & Ptak 1968) and has served as a useful model for investigating the allergen-specific immune responses of T cells and skin-associated antigen-presenting cells (APC) (Nuriya et al. 1996). In order to address whether it is possible for sidestream smoke to

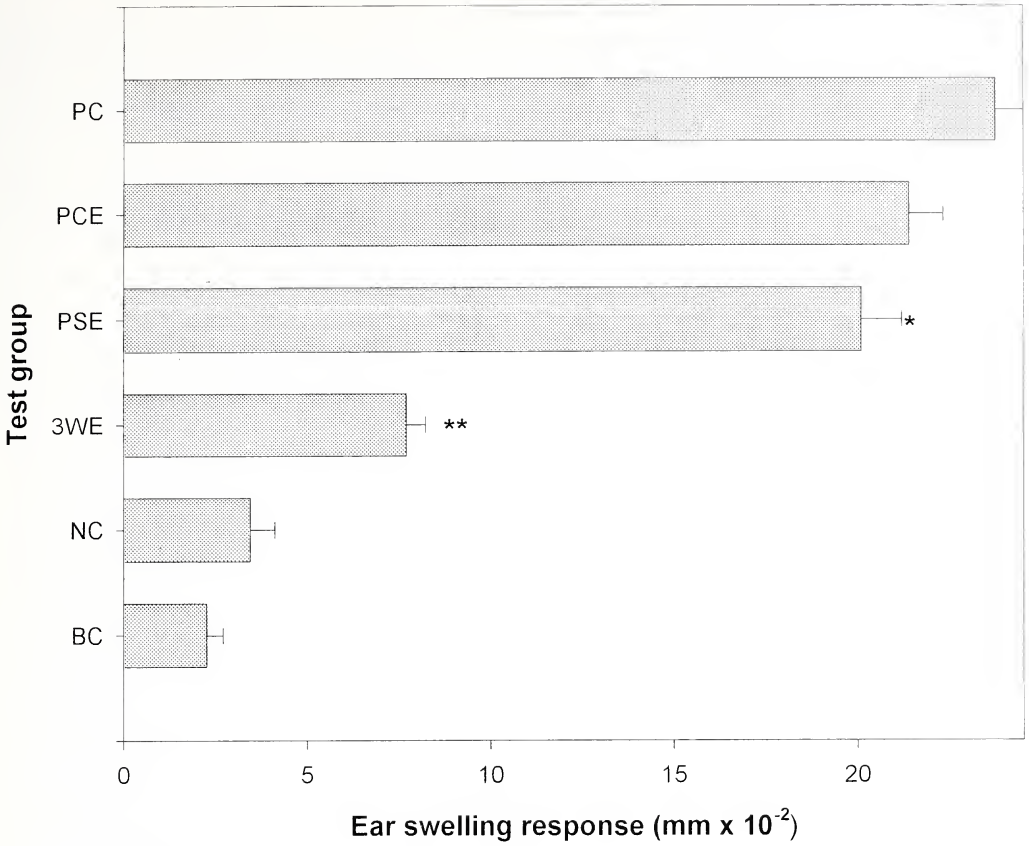

Figure 2.—Maximum ear swelling of BALB/c mice. Test groups are background controls (BC), negative controls (NC), three-week experimentals (3WE), pre-sensitized experimentals (PSE), pre-challenged experimentals (PCE), and positive controls (PC). Values are means ± SEM; n = 18–30/test group (see Table 2). Ear swelling of positive controls was significantly greater ($P<0.05$) than for all other groups, except the pre-challenged experimentals. Asterisks indicate significance of percent suppression of contact hypersensitivity (*$P<0.01$, **$P<0.001$).

impact the immune system, we examined the effect of this agent on the induction and elicitation of CHS using the mouse ear swelling assay.

There are numerous studies that have illustrated a variety of deleterious effects produced by sidestream smoke exposure on mammalian systems. In humans, these include low birth weight of offspring born to mothers exposed to sidestream smoke during pregnancy (Martin & Bracken 1986), respiratory dysfunction (White & Froeb 1980), cardiovascular ailments (Aronow 1978; Garland et al. 1985) and increased incidence of cancer (Correa et al. 1983; Wigle et al. 1987). Nonhuman animal studies have also demonstrated many negative effects of sidestream smoke exposure (Mays et al. 1997; Resnik & Marquard 1980).

In this investigation, we produce evidence that sidestream cigarette smoke may alter the immune system of the skin in a way which results in an increased unresponsiveness or tolerance to CHS. Experimental BALB/c mice were exposed to sidestream smoke for varying periods of time. One group (3WE) was exposed to sidestream smoke daily for three weeks prior to sensitization with the hapten DNFB. A second group (PSE) was exposed to sidestream smoke for the first time on the day they were sensitized (D0), whereas a third group (PCE) wasn't exposed to sidestream smoke until the day they were challenged with

Table 2.—Ear swelling response. The effects of sidestream smoke on DNFB hapten-induced contact hypersensitivity of BALB/c mice. [1]The increase in ear thickness was measured from day of sensitization (Day 0) to 72 h post-challenge (Day 8). [2]Intergroup ear measurements showed no significant differences between Day 0 (pre-sensitized) and Day 5 (pre-challenged) mice. [3]Maximum ear swelling for all groups occurred at 48 h post-challenge. The increase in ear thickness of positive control mice is significantly greater ($p<0.01$) than that of all other groups at 48 h post-challenge, except for the PC Experimental mice ($p<0.055$). [4]No significant difference from 48 h post-challenge measurement. [5]Mice exposed to sidestream smoke from day of sensitization (Day 0) to 72 h post-challenge (Day 8). [6]Mice exposed to sidestream smoke from day of challenge (Day 5) to 72 h post-challenge (Day 8).

		Increased ear thickness (mm × 10^{-2})[1]						
Test group	*n*	24 h Post-Challenge[2] (+ SEM)	Change (%)	48 h Post-challenge (+ SEM)	Change (%)	72 h Post-challenge (+ SEM)	Change (%)	% Change from 48 h
Positive Control (PC)	18	21.933 (1.34)	+ 91.1	23.766 (0.77)	+ 98.7[3]	15.993 (1.27)	+ 66.5	− 32.2
3-week Experimental (3WE)	30	5.700 (0.45)	+ 25.8	7.066 (0.45)	+ 32.0	7.600 (0.53)	+ 34.4	+ 2.4[4]
PS Experimental[5] (PSE)	28	15.036 (1.01)	+ 57.6	20.107 (1.10)	+ 77.0	15.933 (1.21)	+ 61.0	− 16.0
PC Experimental[6] (PCE)	21	13.521 (1.12)	+ 50.8	21.381 (0.94)	+ 80.3	18.721 (1.06)	+ 70.3	− 10.0
Negative Control (NC)	21	1.976 (0.57)	+ 8.0	3.665 (0.67)	+ 14.9	2.764 (0.78)	+ 11.2	− 11.2
Background Control (BC)	20	0.600 (0.44)	+ 2.5	1.350 (0.44)	+ 5.7	1.036 (0.52)	+ 3.1	− 2.6

DNFB (D5). The 3WE mice were the least responsive to CHS with only a 32% increase in ear swelling. The PSE mice showed a 77% increase in ear swelling, and the PCE mice had an increase in ear swelling of 80.3%. The degree of ear swelling of both the 3WE and PSE mice was significantly less than that of naive (PC) mice ($P<0.01$), which had an ear swelling increase of 98.7%. The ear swelling response of PCE mice was not significantly less than the PC mice ($P<0.20$). The degree of immunosuppression showed a similar pattern. The CHS response was suppressed by 80.4% in the 3WE mice ($P<0.001$) and 18.1% in the PSE mice ($P<0.01$), indicating that sidestream smoke exposure can induce immunotolerance in BALB/c mice. These results are similar to those observed for ICR mice, in which CHS of 3WE mice was suppressed by 80.8%. However, neither the PSE nor PCE mice showed significant CHS suppression (Mays & Mays 1999).

It was not the purpose of this study to determine the cause of the observed immunosuppression. However, other studies regarding CHS offer some possible explanations. For the epidermis to produce the optimal toxicologic, immunologic and biochemical barrier, each of the major cell types of this tissue, including keratinocytes (KC) and Langerhans cells (LC) must function together in a dynamic and integrated fashion (Rheins et al. 1993). Epidermal/dermal intercellular biochemical signals (e.g., interleukins, intercellular adhesion molecules, growth factors, etc.) produced by these cells provide skin with local homeostatic signals to ensure it's integrity when exposed to a variety of insults, including those leading to common inflammatory dermatoses, such as irritant CHS (Baadsgaard & Wang 1991; Murayama et al. 1997). Induction of CHS is a multistep process that begins when a highly reactive hapten (e.g., DNFB) is applied to the cutaneous surface (Kurimoto et al. 1994). The initiation of the immune response requires presentation of antigen in the context of MHC class II molecules to the appropriate T cell clones (Unanue 1984). It is possible that sidestream smoke interferes with this initial antigen-presenting process.

Another potential factor affecting immunosuppression is the role of epidermal Langerhans cells (LC). Interest in cutaneous immune reactions was stimulated in 1977 by the discovery that LC express surface markers characteristic of cells of the macrophage-monocyte lineage (Klareskog et al. 1977). LC possess Ia^+ antigens, and are generally thought to be APC of the skin (Rowden et al. 1977). It is now known that epidermal LC are critical for the induction of CHS to simple chemicals and for the induction of allogenic T cell responses (Toews et al. 1980; Shimada et al. 1987; Manome et al. 1999). Blaylock et al. (1993) showed that a 30 ng application of T-2 mycotoxin produced a 44% suppression of ear swelling in BALB/c mice. Their findings further suggested that the T-2 toxin significantly decreased both MHC class II (Ia) antigen expression on LC, and antigen presentation to T cells. Perhaps sidestream smoke also has a suppressive effect on Ia^+ expression and hapten presentation to T cells.

Sidestream smoke might also produce immunosuppression at some later stage in the immune response process. Skin LC are immature dendritic cells (DC) that form an extensive network in the epidermis, and upon exposure to noxious stimuli such as contact sensitizers (e.g., DNFB), they enter the lymphatics and migrate into T cell zones of lymph nodes to become interdigitating DC (Tang & Cyster 1999). For mature DC to function in an immunogenic manner, it is important that they rapidly interact with antigen-specific T cells in the paracortical area (Bigby et al. 1989; Bottomly 1999). One mechanism that appears to enhance encounters between DC and T cells is for DC to produce T cell attracting chemokines. DC up-regulation of macrophage-derived chemokine (MDC) has been shown to preferentially attract antigen-specific T cells, but not naive T cells (Tang & Cyster 1999). Recently, it was reported that DC exist in at least two forms. Myeloid-like cells (DC1) produce abundant IL-12 and induce a Th1 response, and lymphoid-like cells (DC2) induce a Th2 response. In addition, T helper cells may themselves regulate Th1 and Th2 responses by determining the survival of the appropriate DC subset (Rissoan et al. 1999). Th1 and Th2 responses are produced by the two subsets of T helper cells. Th1 and Th2 cells are determined by the type of cytokine/growth factor secretions in response to antigens (Simon et al. 1991; Xu et al. 1997a; Krasteva et al. 1998). Th1 cells secrete IL-2, IFN-γ, and lymphotoxin, and are primarily as-

sociated with macrophage activation and delayed-type hypersensitivity. Among the interleukins secreted by Th2 cells are IL-4, IL-5, IL-6, and IL-10. These T helper cells are normally B cell activators. Both types of T cells secrete IL-3, granulocye-macrophage colony stimulating factor (GM-CSF), TNF-α, and several other induction-specific proteins (Mosmann et al. 1986; DiIulio et al. 1996). It has been shown that the Th1 product IFN-γ inhibits proliferation of Th2 clones in vitro (Gajewski & Fitch 1988), and Th2 clones produce a protein called cytokine synthesis inhibitory factor (CSIF) that inhibits the synthesis of several cytokines by Th1 clones (Fiorentino et al. 1989). Thus, sidestream smoke could have a dual immunosuppressive influence by inhibiting Th1 cytokine production (Th1 response) and stimulating the production of the Th2 cytokine CSIF.

In addition to LC-T cell interaction, keratinocytes (KC), which are the major constituents of the epidermis, are well known for their capacity to secrete a variety of cytokines and growth factors. One KC-produced cytokine is IL-12 which, until recently, was believed to be produced exclusively by macrophages and B cells (Schwarz 1995). IL-12 is known to shift T helper cell response to a Th1 cytokine profile (Xu et al. 1998) and stimulate the production of IFN-γ (Trinchieri 1994; Maguire 1995). IL-12 is required in both the sensitization and challenge phases to produce maximum CHS, and neutralization of IL-12 during sensitization induces hapten-specific tolerance (Riemann et al. 1996; Schwarz et al. 1996). Another cytokine that has received attention is IL-10. Although it was previously considered to be produced primarily by Th2 cells, evidence now indicates that KC also produce IL-10, and that KC-derived IL-10 has an important immunoregulatory function (Enk & Katz 1992). IL-10 converts LC from being potent inducers of primary immune reactions such as CHS to becoming tolerogenic APC (Enk & Katz 1995). Hapten-sensitized LC express high levels of B7-2 and lower levels of B7-1 costimulators on their surface (Xu et al. 1997b). It has been suggested that IL-10 induces tolerance by downregulating the B7 costimulators on APC (Enk et al. 1993). UV radiation is thought to produce immunosuppression by inducing KC to release IL-10, which inhibits the CHS-stimulatory cytokine IL-12 (Schmitt et al. 1995). The lack of B7 costimulators may interfere with LC-T cell interaction and thereby modulate skin cytokine profiles that leads to CHS suppression (Kondo et al. 1996). Suppressor T cell activation has also been associated with UV light-induced immunosuppression (Baadsgaard et al. 1988; Karpus & Swanborg 1989). Perhaps sidestream smoke produces immunosuppression of CHS in a manner similar to that of UV radiation.

The role of Th2 cells in CHS is not clear, but it appears that Th2-derived IL-4 intensifies CHS suppression 48 h post-challenge (Wiegmann et al. 1997). This may partially explain why our maximum percent suppression occurred at 48 h post-challenge. Mast cells are known to contribute to the induction of CHS by DNFB. This is primarily by the mediation of T lymphocyte recruitment in draining lymph nodes by the production of macrophage inflammation protein (MIP)-1 beta (Wang et al. 1998). Perhaps sidestream smoke inhibits this process by decreasing the number of mast cells or blocks their function. Nitric oxide (NO) is another factor implicated in the development of CHS. Elicitation of CHS response to DNFB is known to stimulate the enzyme nitric oxide synthase (NOS) in both KC and LC. NOS converts the amino acid arginine to NO, which contributes to the ear swelling reaction. Certain agents such as N-methyl-L-arginine (L-NMA), which is an NOS inhibitor, significantly reduced CHS induced by treatment of BALB/c mice with DNFB (Ross et al. 1998). It may be that sidestream smoke somehow interferes with NOS activity leading to immunosuppression.

The process of CHS illicitation is complex, and there are many variables that could be involved in the sidestream smoke-induced immunotolerance observed in our laboratory. Although we are aware of at least one study dealing with the effect of cigarette smoking on the immune system (Jung & Irwin 1999), to our knowledge, the work in our lab, including a preliminary investigation on ICR mice (Mays & Mays 1999) and this study on BALB/c mice, are the first investigations of the effect of sidestream smoke on immunity.

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