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Psychological Stress Exerts an Adjuvant Effect on Skin Dendritic Cell Functions In Vivo

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Psychological stress affects the pathophysiology of infectious, inflammatory, and autoimmune diseases. However, the mechanisms by which stress could modulate immune responses in vivo are poorly understood. In this study, we report that application of a psychological stress before immunization exerts an adjuvant effect on dendritic cell (DC), resulting in increased primary and memory Ag-specific T cell immune responses. Acute stress dramatically enhanced the skin delayed-type hypersensitivity reaction to haptens, which is mediated by CD8+ CTLs. This effect was due to increased migration of skin DCs, resulting in augmented CD8+ T cell priming in draining lymph nodes and enhanced recruitment of CD8+ T cell effectors in the skin upon challenge. This adjuvant effect of stress was mediated by norepinephrine (NE), but not corticosteroids, as demonstrated by normalization of the skin delayed-type hypersensitivity reaction and DC migratory properties following selective depletion of NE. These results suggest that release of NE by sympathetic nerve termini during a psychological stress exerts an adjuvant effect on DC by promoting enhanced migration to lymph nodes, resulting in increased Ag-specific T cell responses. Our findings may open new ways in the treatment of inflammatory diseases, e.g., psoriasis, allergic contact dermatitis, and atopic dermatitis. The Journal of Immunology, 2003, 171: 4073–4080.

Psychological stress is known to contribute to the pathophysiology of many inflammatory, autoimmune, and allergic diseases (1). The perception of stress by an individual induces activation signals to the CNS with two major consequences: 1) the neuroendocrine response via the hypothalamic-pituitary-adrenal axis and release of adrenal steroid hormones such as corticosterone (CORT), and 2) the autonomous nervous system response, resulting in the local release of catecholamines, especially norepinephrine (NE), by sympathetic nerve termini (2, 3).

Glucocorticoids similar to catecholamines have been reported to influence the immune system in some contradictory ways. Although the immune suppressive action of CORT and NE was first described, an increasing number of studies have now demonstrated the pleiotropic immunomodulatory effects of these neuropeptides. Indeed, CORT as NE could modulate T cell responses (4, 5) by controlling their survival, the Th1/Th2 balance, or the cytotoxic activity of CTL, and could influence dendritic cell (DCs) maturation (6–9) and migration (10, 11). However, their relative contribution in the outcome of T cell-mediated responses in vivo, at a level that can be achieved during stress, is not well understood.

In this respect, numerous studies have reported conflicting results on the effect of psychological stress on immune cells and T cell responses, with suppressive or enhancing effects depending on the experimental protocol used (2, 12). Dhabhar and McEwen (13, 14) recently provided a rational explanation for these discrepancies by demonstrating that the effect of stress on Ag-specific skin inflammation was drastically different depending on its duration. Whereas chronic stress suppressed immunity and delayed-type hypersensitivity (DTH) reactions through the production of high dose of CORT, acute stress was responsible for the enhancement of DTH reactions via the release of both CORT and NE. This later observation is particularly relevant to human skin inflammatory diseases, such as psoriasis and atopic or allergic contact dermatitis, in which onset, progression, and severity are known to be influenced by stress (15, 16). However, the precise immune mechanisms, which could be responsible for the stress-induced enhancement of skin DTH reactions, remain unknown.

Contact hypersensitivity (CHS) is the most widely used model of Ag-specific T cell-mediated DTH reaction that, in humans, manifests as an inflammatory skin disease referred to as contact dermatitis. CHS develops after epicutaneous exposure to haptens and is mediated by hapten-specific CD8+ T cells and down-regulated by regulatory CD4+ T cells (17). Haptens are low m.w. chemicals able to bind to self Ags and endowed of proinflammatory properties. During sensitization of the epidermis, the reactive hapten is taken up by immature skin DC that migrates from the skin to the paracortical area of draining lymph nodes (LNs), where they prime hapten-specific T cells (18, 19). A subsequent skin challenge with the same hapten leads to the rapid recruitment of IFN-γ-producing CD8+ CTL in the skin, where they induce inflammation through skin cell apoptosis (20–22).

In this study, CHS was used as a model to investigate the mechanisms by which stress could enhance skin DTH reactions. We...
show that an acute restraints stress strongly enhanced the inflammatory reaction of CHS by increasing DC migration to draining LNs, resulting in enhanced CD8⁺ T cell responses and skin inflammation. This adjuvant effect of stress was dependent on release of NE by the sympathetic nervous system. These findings provide new insights into the role of the CNS in the onset of immune responses.

Materials and Methods

Mice

Female 6- to 8-wk-old BALB/c mice were purchased from Ifla-Credo (L’Arbresle, France). Animals were left to acclimate for 1 wk before entering the study. Five mice were used per group. Mice were housed individually and were provided food and water ad libidum.

Reagents

The 2,4-dinitro-fluorobenzene (DNFB) was diluted in acetone:olive oil (4:1). FITC was diluted in acetone: dibutylphthalate (1:1). Dinitrobenzene-sulfonic acid (DNBS) and tri-nitrobenzenesulfonic acid (TNBS) were used for in vitro studies (all from Sigma-Aldrich, St. Louis, MO). Abs used in in vivo experiments comprised anti-CD4 and anti-CD8 mAbs, produced respectively by the hybridoma H35.17.2, kindly provided by G. Milon (Institut Pasteur, Paris, France), and the hybridoma GK 1.5 purchased from American Type Culture Collection (Manassas, VA).

Contact hypersensitivity (CHS)

Mice were sensitized by epicutaneous application of 25 μl of 0.2% DNFB or 1% FITC onto 2 cm² of fur-shaved ventral skin. Five days later, animals were challenged by application of 5 μl of 0.2% DNFB or 1% FITC onto each side of the right ear, while the same volume of vehicle was applied on both sides of the contralateral ear. Ear thickness measurements were taken for each ear before hapten application with a spring-loaded micrometer (J15; Blet SA, Lyon, France). Measurements were repeated once daily after challenge. Ear swelling was calculated by subtracting the initial value from the value recorded on the corresponding day, and further subtracting any swelling recorded for the vehicle-control ear from the swelling recorded for the hapten-challenged ear.

Stress

Two types of psychological stresses were used, a restraint and a desequilibrium stress, which have been used before as model of painless stress (14, 23). Restraint stress was administered by placing animals (without squeezing or compression) inWell-ventilated Plexiglas restrainers, whereas acute desequilibrium stress was done by placing the animals on a plate shaker at a speed of 10 oscillations/min. Both types of stresses were administered for 2 h and 30 min before the sensitization phase of CHS. Preliminary experiments have defined this protocol as the most effective. No effect of stress was noted when it was applied 1 day before sensitization or when it was lasting less than 1 h at time of sensitization. These procedures induce a stress that is largely psychological in nature because of the perception of confinement (restraint) or unbalance (desequilibrium) on part of the animal. Restrain and unbalance activate the autonomic nervous systems and the hypothalamic-pituitary-adrenal axis and result in the activation of adrenergic and adrenal steroid receptors throughout the body (14).

Ab depletion of CD4⁺ and CD8⁺ T cells in vivo

Mice were given i.p. injections of 200 μl 1/10 diluted anti-CD4 or anti-CD8 mAb on days -1, 0, +1, and +4 of skin sensitization. Cell depletion was assessed at days +1 and +4 by staining for CD4 and CD8 molecules on PBMCs recovered from the retro-orbital plexus. In all cases, specific depletion exceeded 95% on both days.

Chemical sympathectomy

Chemical sympathectomy was performed at 7 wk of age using the neurotoxin 6 hydroxy-dopamine (6-OHDA), which reversibly destroys the peripheral sympathetic nerve terminals, as previously described (24), and specifically depletes peripheral tissues in NE (25). Mice received three i.p. injections (100 μl) of 200 mg/kg 6-OHDA (Sigma-Aldrich) in PBS containing 0.01% ascorbate, every other day (days -6, -4, and -2 before sensitization), while control mice received vehicle injections on the same injection schedule.

RU486 treatment

The glucocorticoid receptor antagonist RU486 was administered, as previously described (26). Briefly, mice received a single i.p. injection (100 μl) of 6 mg/kg of RU486 dissolved in polyethylene glycol 400 (PEG; USB, Cleveland, OH) or of the vehicle on the back, 1 h before application of stress.

Analysis of the T cell responses

In vitro secondary T cell proliferation

Auxillary and inguinal LNs were harvested 5 days after DNFB sensitization, and CD8⁺ T cells were purified through positive selection using mini MACS columns (Miltenyi Biotech, Paris, France). The resulting cell suspensions contained >95% viable CD8⁺ T cells. In vivo hapten-primed T cells (2.5 x 10⁶ cells/well) were cocultured for 3 days at 37°C in 96-well plates with 10⁴ irradiated syngenic spleen cells from naive mice that were either DNBS or TNBS derivatized, as described below, or left untreated. The proliferative responses were assessed on day 3 of culture by [3H]thymidine incorporation (1 μCi/well) for the last 12 h of culture. The results are expressed as proliferation index calculated as follows: (cpm in cultured T cells + DNBS-treated spleen cells)/(cpm in cultured T cells + untreated spleen cells).

IFN-γ ELISPOT assay

Cell suspensions were recovered from axillary and inguinal LNs harvested on day 5 after DNFB sensitization. The number of IFN-γ-producing LN cells was determined using an ELISPOT assay. In brief, 96-well nitrocellulose plates (MAHA 45; Millipore, Bedford, MA) were coated overnight at 4°C with anti-IFN-γ Ab (clone R46A2) and blocked with PBS/1% BSA for 2 h at 37°C. The plates were washed three times with PBS/Tween 0.1% before use. LN cells were incubated overnight at 37°C in different numbers in the presence of 0.4 mM of DNBS (or TNBS). Plates were washed three times with PBS/Tween 0.1% and incubated 2 h at room temperature with a biotinylated anti-IFN-γ Ab (clone XMGI.2) and then extensively washed. IFN-γ spot-forming cells (SFCs) were developed using streptavidin-alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN), incubated for 2 h at room temperature, and extensively washed before adding the substrate (5-bromo-4-chloro-3-indolyl-phosphate; Sigma-Aldrich). The number of IFN-γ SFCs present in each well was counted using a Carl Zeiss vision ELISPOT (Zeiss SASS, Le Pecq, France), and the results were expressed as IFN-γ SFCs/10⁶ cells.

Extraction and RT-PCR analysis of CD8 and IFN-γ mRNA

Ear samples were collected from sensitized or unsensitized mice at different times after challenge and frozen in liquid nitrogen. The detection of RNA was conducted as described in details elsewhere (21). In brief, total RNA was extracted using RNAXEL kit (Eurobio, Les Ulis, France). After DNAse I treatment, 1 μg of total RNA was reverse transcribed using poly(dt15) primers and Superscript II RT (90 min, 37°C; Life Technologies, Cergy Pontoise, France). The amount of RNA to be used for detection was normalized using the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) as reference. The CDNA obtained was amplified using different sets of primers: for HPRT (5'-CTG GTC ATT AGT AGT CAG GGG GC-3'; 3'-primer, 5'-TCA GCA AGC TGG CAA CCT TAA CCA-3'); for CD8 (5'-primer, 5'-AGG AGT CTC TTG GCT CCT CC-3'; 3'-primer, 5'-TCA CAG GCG AAG TAC CAA GGG AGC AC-3'; 3'-primer, 5'-GCT CTA AGA CCA TGA AGC CT-3'; 3'-primer, 5'-AAA GAG ATA ATC TGG CTC TGC-3'). The amplifications were conducted with 29 cycles for HPRT and 32 cycles for IFN-γ and CD8 (1 min at 94°C, 1 min 30 s at 60°C, 2 min at 72°C). The PCR products were analyzed on 1.5% agarose gel.

DC generation from bone marrow (BM) cultures

DC were generated in vitro from BM progenitors with GM-CSF, as described (27). Immature DC were collected at day 7 of culture, analyzed by flow cytometry, and used for in vivo immunization. Cells showing a typical dendritic morphology were always >90%, and expressed the DC markers CD11c and MHC class II Ags.

Ag pulsing of APCs

Cell pellets (7-day cultured BM-DC or irradiated splenocytes) were resuspended in RPMI 1640 without FCS containing DBNBS (50 μg/ml) (pH8) or TNBS (2 mM) (pH8) and incubated at 37°C for 15 min. For immunization experiments, 10⁶ DNBS- or TNBS-treated BM-DCs or control BM-DCs from BALB/c mice were injected s.c. in 200 μl of saline into naïve BALB/c recipient mice. Stress was applied immediately before or 30 min after BM-DC injections.
In vivo Langerhans cells (LC)/DC migration assay
To follow the migration of LC/DC from the skin to draining LNs, 1% FITC (25 μl) was applied onto 2 cm² of fur-shaved ventral skin. To follow the migration of BM-DCs in draining LNs during immunization experiments, DNBS-treated BM-DCs were labeled with CFSE, according to manufacturer’s protocol (Molecular Probes, Eugene, OR). Briefly, DCs were incubated 15 min at 37°C in 10 μM CFSE in RPMI 2% SVF. DCs were washed, counted, and injected in both hind footpads (5 × 10⁵ per footpad). Mice were killed at either 24 h after FITC skin painting or 48 h after BM-DC injections, and single cell suspensions were prepared from draining axillary and inguinal LNs or popliteal LNs, respectively. Cells were collected; washed; labeled with PE-conjugated anti-CD86 (clone 6L1) or biotin-conjugated anti-CD11c (HL3) mAbs and the secondary reagent streptavidin-conjugated CyChrome (all from BD Biosciences, Le Pont de Claix, France); and analyzed by flow cytometry. Cells triple stained for FITC (or CFSE), PE, and CyChrome were quantified as migrating DCs.

Results
Enhancement of Ag-specific skin inflammation by psychological stress
Two types of psychological stress, including an immobilization and a disequilibria stress, were applied to animals on day 0, just before skin sensitization with DNFB. Control (unstressed) and stressed animals were challenged 5 days later by hapten painting on the ear. Unstressed animals developed a normal CHS reaction, which was maximal at 48 h postchallenge and decreased from day 3. In contrast, stressed animals developed a dramatically enhanced CHS response with delayed resolution (Fig. 1a). Histological analysis of challenged skin showed that skin inflammation in stressed mice was more pronounced and comprised augmented vasodilatation, dermal edema, and cellular infiltration of the dermis (Fig. 1b). Although the two types of stress gave similar increase in the CHS reaction, the restrain stress gave more pronounced effects and more reproducible results than the disequilibria stress and was thus the only stress procedure used for additional experiments.

Because CHS reaction is mediated by CD8⁺ effector T cells and down-regulated by CD4⁺ T cells (28–30), we next tested the effect of stress on CD4⁺ and CD8⁺ T cell-depleted mice (Fig. 1c). CD8⁺ T cell depletion abolished CHS in both unstressed and stressed mice (data not shown). As expected, CD4⁺ T cell depletion induced an enhancement of CHS response in unstressed mice. However, stress could further enhance the CHS in CD4⁺ T cell-depleted mice. These data show that the stress-induced increase of skin DTH is not mediated by regulatory CD4⁺ T cells, but is rather the consequence of an effect of stress mediators on the CD8⁺ effector T cell pool.

Because there is clear correlation between the number of effector CTLs at the peak of an Ag-specific immune response and the number of memory cells generated (31–33), we examined whether stress could increase the immunological memory to hapten. Stressed and control mice that had developed a CHS response by hapten painting on the right ear on day 5 (as in Fig. 1a) were both rechallenged on day 28 on the left ear with DNFB, without new stress procedure. The memory CHS reaction was significantly enhanced in previously stressed compared with control mice (Fig. 1d), demonstrating the stimulatory effect of stress on the immune system and on the hapten-specific CTL response.

Adjuvant effect of stress on the priming of Ag-specific CD8⁺ effector CTLs
CHS reaction requires the activation of hapten-specific cytotoxic CD8⁺ T lymphocytes in the draining LNs (sensitization phase) and their subsequent recruitment in the skin (elicitation phase), where they induce the inflammatory process (21). Thus, stress could act on the elicitation phase by enhancing the recruitment of effector cells in the challenged skin, or on the sensitization phase by increasing the pool of CD8⁺ effectors, or on both phases of CHS.

Study of the sensitization phase was done by analyzing the hapten-specific CD8⁺ T cell response in draining LNs on day 5 after sensitization. Purified CD8⁺ T cells from stressed mice had enhanced hapten-specific proliferative responses upon in vitro restimulation with DNBS-derivatized syngeneic cells (proliferation index: 15.2) compared with that of CD8⁺ T cells from unstressed control mice (proliferative index: 7.8) (Fig. 2a). T cell proliferation was hapten specific because no proliferation was induced upon in vitro restimulation with TNBS-derivatized spleen cells (data not shown). We next determined the frequency of DNFB-specific T cells in the draining LNs using an IFN-γ ELISPOT assay. Stressed

FIGURE 1. Psychological stress enhances the CHS reaction to DNFB. a, CHS to DNFB was analyzed in groups of five BALB/c mice either stressed by a 2.5-h immobilization procedure (■) or unstressed (○) before sensitization with 0.2% DNFB on the abdomen. Mice were challenged 5 days later by 0.2% DNFB painting on the left ear. Results are expressed as the mean ear swelling (in μm) at different time points after challenge. Results are representative of six independent experiments. b, Histological analysis of ear sections from naive mice (b1) and from either unstressed (b2) or stressed (b3) mice at 48 h postchallenge. Original magnification: ×100. c, Effect of in vivo depletion of CD4⁺ and CD8⁺ T cells on stress-induced enhancement of CHS. Anti-CD4 mAb-treated (open symbol) and PBS-treated (filled symbol) mice were subjected to restrain stress (squares) or left unstressed (circles), sensitized at day 0, and challenged at day 5. Results are expressed as the mean ear swelling at different time points after challenge (four independent experiments). d, T cell memory response. Mice either stressed (■) or unstressed (○) before sensitization at day 0 and challenged on the left ear at day 5 (as in a) were rechallenged at day 28 with 0.2% DNFB painted on the right ear and vehicle alone on the left ear. Ear swelling was assessed the 4 consecutive days. Results are representative of three independent experiments.
mice displayed a 3-fold increase in the number of IFN-γ-producing cells (mean frequency: 88 SFCs/10^5 LN cells) compared with control mice (32 SFCs/10^5 LN cells) (Fig. 2b). As previously reported in this experimental system (20, 34), IFN-γ-producing cells were entirely contained in the CD8^+ T cell subset in both stressed and control mice (data not shown). These data show that stress is able to increase the number of hapten-specific CD8^+ effectors generated by skin immunization.

Study of the elicitation phase was performed by analyzing the recruitment of IFN-γ-producing CD8^+ T cells in the challenged ear skin of sensitized stressed and control mice. We have previously shown that recruitment of CD8^+ effector T cells could be followed by RT-PCR analysis for CD8 and IFN-γ mRNA obtained from ear skin at different times after challenge (20, 21). CD8 and IFN-γ mRNAs were neither found in skin of naïve mice nor in ears of challenged, but unsensitized mice. In contrast, in sensitized mice, up-regulation of both CD8 and IFN-γ mRNA occurred rapidly after challenge and was strongly expressed at 24 h postchallenge (Fig. 2c). Stressed mice exhibited a higher and faster up-regulation of both CD8 and IFN-γ mRNA compared with control mice, suggesting that more CD8^+ T cells have infiltrated the challenged sites in the former mice. Quantification of PCR products estimated a 3- to 4-fold increase in specific mRNAs in stressed vs control mice at 24 and 48 h postchallenge (Fig. 2d).

Taken together, our results suggest that stress delivers an adjuvant signal that potentiates priming of specific CD8^+ effectors in LNs leading to increased numbers of T cells recruited in the challenged skin for initiation of CHS.

**DC, but not T cells, are the targets of stress-induced enhancement of CHS responses**

DC are required for the induction of CHS reaction (35, 36) and for efficient CTL responses in vivo (37, 38). Thus, stress-induced enhancement of CHS reaction and CD8^+ CTL priming could be due either to a direct effect on T cells or to an indirect effect on the Ag-presenting skin DC. To discriminate between these two possibilities, we set up a series of transfer experiments in which CHS was induced in stressed and control mice that were immunized by s.c. transfer of haptenated bone marrow-derived DC (hap-DC). CHS induced by hap-DC immunization in unstressed mice was comparable in magnitude and kinetics to CHS induced by topical DNFB sensitization (Fig. 3a), confirming previous observations (39, 40). Interestingly, the influence of stress on CHS varied depending on its timing with respect to hap-DC transfer. No change in the CHS response was noted when stress was given to recipient mice before the immunization by hap-DC, suggesting that T cell precursors are not sensible to stress. Conversely, CHS was enhanced when stress was delivered to recipient mice after hap-DC injections. These transfer experiments demonstrate that integration of the stress signal requires the presence of hap-DC, suggesting that DC and not T cells are the targets of stress.

**Stress increases DC migration from the skin to draining LNs**

Because DC need to migrate from the skin to draining LNs to induce T cell priming and CHS response (41–43), we next examined whether stress affected the migratory properties of DC. To this end, hap-DC were labeled with CFSE and injected into the hind footpads of control and stressed mice. Migration of hap-DC was assessed as the number of CFSE^+ cells in the popliteal draining LN, 48 h after injection. Fig. 3, b and c, shows that stress applied immediately after hap-DC injection is responsible for a 3-fold increase in the number of CFSE^+ cells in draining LNs, as compared with either stress applied before hap-DC injection (data not shown) or with the normal migration of hap-DC in unstressed mice. These data confirm that DC are the targets of stress in our experimental system.

Next, FITC painting experiments were conducted in stressed and unstressed mice to follow the rate of migrating skin DC, as previously described (18). We first verified that FITC could induce a CHS response that was enhanced by stress (Fig. 3d). Then FITC was applied onto the ventral skin of naïve stressed and control mice, and the number of FITC^+ DC (defined as FITC^+/CD86^+ / CD11c^+ cells) in draining LNs was analyzed 24 h later (Fig. 3e). Stressed mice exhibited a 3-fold increase in the number of FITC^+ DC cells recovered from skin-draining LNs, compared with control mice (Fig. 3, e and f), demonstrating that stress can increase the hapten-induced migration of skin DC. However, FITC^+ DC
was injected into mice 1 h prior to the application of stress. Although RU486 increased the inflammatory response in unstressed control mice, it was unable to block the stimulatory effect of stress on CHS, indicating that glucocorticoids and CORT are not involved in the adjuvant effect of stress (Fig. 4a).

The implication of the sympathetic nervous system was next examined by in vivo NE depletion. Peripheral sympathetic nerve terminals containing NE were reversibly destroyed before DNFB sensitization by chemical sympathectomy using the neurotoxin 6-OHDA. This procedure was previously reported to induce 90% reduction in NE release for up to 9 wk after the last injection (24). Unstressed 6-OHDA-treated control animals developed a normal CHS to DNFB. Alternatively, 6-OHDA treatment strongly reduced the stress-induced increase in CHS and resulted in a CHS response that was similar to that obtained in PBS-treated unstressed mice (Fig. 4b).

To confirm whether treatment with 6-OHDA affected the migratory properties of skin DC in control and stressed mice, FITC painting experiments were conducted as described above. As shown in Fig. 4c, stress increased the migration of skin DC to draining LNs in PBS-treated mice. In contrast, 6-OHDA treatment abolished the effect of stress on DC migration by reducing the number of FITC-positive DC in the skin-draining LNs from $48.10^3$

**FIGURE 3.** Stress-induced increase in the migratory rate of skin DCs to draining LNs. a, DNBS-pulsed BM-derived DCs (hap-DCs) were injected s.c. ($1 \times 10^5$) in the back skin of naive control unstressed mice (○), and in mice that were stressed before (●) or after (■) hap-DC transfer. Five days later, the CHS response was measured, as described in Fig. 1. b and c, CFSE-labeled hap-DCs were injected ($5 \times 10^5$) s.c. into both hind footpads of naive mice. Stress was applied 30 min after the injection, and the percentage of CFSE<sup>+</sup> cells was analyzed 48 h later by FACS. b, Dot-plot analysis of double staining for CD11c<sup>+</sup> and CFSE<sup>+</sup> cells. c, Number of CFSE<sup>+</sup>CD11c<sup>+</sup> cells in draining LNs (mean ± SD of four mice per group). Data are representative of one experiment of three. The difference between the two groups was statistically significant (t test, $p = 0.002$). d, CHS response to FITC in unstressed mice (○) or in mice stressed before FITC sensitization (■). The results are expressed as ear swelling. Data are representative of two experiments. e and f, FACS analysis of skin DC migration to LNs at 24 h after skin painting with FITC. Mice were unstressed or stressed before FITC painting. Results show dot-plot analysis of FITC<sup>+</sup>CD11c<sup>+</sup> cells (e) and absolute numbers of FITC<sup>+</sup>CD86<sup>+</sup>CD11c<sup>+</sup> cells (f) (mean ± SD of five mice per group). Data are representative of one of five experiments. The difference in the two groups was statistically significant (t test, $p < 0.001$).

**FIGURE 4.** NE mediates the stress-induced enhancement of CHS and of DC migration. a and b, CHS reaction. a, Mice were injected s.c., 1 h before stress, with RU486 (open symbol) or vehicle alone (filled symbol). Stressed (squares) and unstressed (circles) mice were sensitized with DNFB and challenged 5 days later. b, Mice received 3 i.p. injections of 6-OHDA (open symbol) or vehicle only (filled symbol) and stressed 2 days after the last injection. Stressed (squares) and unstressed (circles) mice were sensitized with DNFB and challenged 5 days later. Results of one representative experiment of three are shown and are expressed as the mean ear swelling at different time points after challenge. c, FACS analysis of skin DC migration to LNs at 24 h after skin painting with FITC in unstressed (○) or stressed (■) mice that were PBS treated or 6-OHDA treated. FITC painting was applied 30 min after stress application. Results show the absolute numbers of FITC<sup>+</sup>CD86<sup>+</sup>CD11c<sup>+</sup> LN cells (mean ± SD of seven mice per group). Similar results were obtained in two other independent experiments. * , The difference in the two groups was statistically significant (t test, $p < 0.0001$).

**NE as a potent mediator of stress**

Glucocorticoids produced under control of the hypothalamic-pituitary-adrenal axis, and catecholamines released by the sympathetic nervous system are the two main mediators of acute stress (3). To evaluate the effect of glucocorticoids and CORT in our model, mifepristone (RU486), an inhibitor of glucocorticoid receptors, phenotype was similar in both stressed and control mice with strong expression of MHC class II, CD80, and CD86 molecules (data not shown).

Glucocorticoids produced under control of the hypothalamic-pituitary-adrenal axis, and catecholamines released by the sympathetic nervous system are the two main mediators of acute stress (3). To evaluate the effect of glucocorticoids and CORT in our model, mifepristone (RU486), an inhibitor of glucocorticoid receptors,
to $16.10^3$. These results demonstrate that NE is responsible for the increased migratory rate of hapten-presenting DCs in stressed mice. They also indicate that activation of the sympathetic nervous system and NE release by peripheral nerve endings play a key role in the adjuvant effect of stress on the induction of the CHS reaction and CTL responses during a psychological stress.

**Discussion**

This study demonstrates that acute psychological stress dramatically enhances Ag-specific DTH reactions by exerting an adjuvant effect on DCs mediated by the sympathetic nervous system. Using the model of CHS to hapten, we show that an acute stress applied shortly before sensitization enhances the frequency of hapten-specific CD8$^+$ effector T cells primed in draining LNs during the afferent phase of CHS and recruited to the skin at the challenged site. This effect is due to increased migration of hapten-bearing DCs from the skin to draining LNs. NE appears to be the major mediator of stress because blockade of NE production results in the abolition of the stress-induced enhancement of CHS and of skin DC migration.

The magnitude of the CHS response and of hapten-specific CD8$^+$ T cell expansion during the sensitization phase is controlled by the presence of CD4$^+$ T cells, which down-regulate CHS (21, 28, 44, 45). Thus, the stress-induced increase in CHS could have been due either to enhanced efficiency of CD8$^+$ T cell priming and/or expansion or to alteration of the CD4$^+$ T cell regulatory compartment. This latter hypothesis is unlikely inasmuch stress could enhance CHS similarly in normal mice as well as in CD4$^+$ T cell-depleted mice. Alternatively, stressed mice exhibited a 2- to 3-fold increase in the frequency of specific IFN-γ-producing CD8 effectors in draining LNs as well as higher numbers of activated CD8$^+$ T cells at the site of skin challenge. These data strongly support that increased CHS by acute stress resulted from more efficient CD8$^+$ T cell priming in a CD4$^+$ T cell-independent way.

Previous studies have reported that acute stress or neuromediators are able to influence proliferation, cytokine production, and cytotoxicity of T cells (3–5). However, our data indicate that in vivo enhancement of CD8 priming and of CHS could not be explained merely by a direct effect of stress mediators on T cells, but rather by an indirect mechanism via Ag-presenting DC. This is supported by experiments using adoptive transfer of hap-DC into naive recipients in which acute stress was applied either before or after DC transfer. When stress was applied to recipients before DC transfer, i.e., when all cell types involved in the CHS response except hapten-bearing DC could receive stress signals, there was no detectable change in either the intensity of CHS or the hapten-specific CD8$^+$ T cell responses. Conversely, both skin inflammation and CD8 T cell priming were augmented when mice were first immunized by hap-DC and subsequently exposed to stress. These data strongly indicate that hapten-bearing DC (and not T cells) are the main targets of stress mediators.

Migration of hapten-loaded skin DC to LNs is mandatory for induction of T cell priming during the afferent phase of CHS. DC migration is associated with their maturation into functional APCs expressing higher levels of MHC class II and of the costimulatory molecules CD80/CD86 (46). That DC mediate the stress-induced enhancement of skin DTH through priming of higher numbers of specific CD8$^+$ effector T cells could have been due either to an increase in the Ag-presenting functions of DC, or to enhanced numbers of skin DC able to migrate from skin to LN, or both. Experiments using either FITC painting or s.c. injection of CFSE-labeled BM-DC revealed that stress increased by 2- to 3-fold the number of LN DCs originating from skin, correlating with a mean increase of 2- to 3-fold in the magnitude of CHS and in the number of specific CD8$^+$ effectors. To correlate the migration of skin DC with maturation induced by the hapten FITC in vivo, we analyzed, on LN DC, the expression of MHC class II and of two different costimulatory molecules, CD80 and CD86. Indeed, both CD80 and CD86 were up-regulated and MHC class II molecules were expressed at high levels in FITC$^+$ LN DCs, which thus corresponded to fully mature DC. However, DC from stressed mice expressed the same level of costimulatory molecules as those of normal unstressed mice, suggesting that stress did not affect the hapten-induced maturation process of DC in vivo. Along these lines, intradermal injection of LPS in FITC-painted mice increased the number of LN DCs emigrating from the skin, but did not affect either their expression of costimulatory molecules nor their immunostimulatory properties in MLR (47). Thus, our data are consistent with recent reports showing that, besides the cytokine secretion profile and the expression of costimulatory molecules by mature DC, the abundance of Ag-presenting DC in lymphoid organs is a critical factor in the magnitude of a T cell response to Ags in vivo (48–50). Several hypotheses that are currently being tested could account for the stress-enhanced enhancement in the number of Ag-bearing LN DC: 1) increased recruitment of DC precursors from blood at the site of hapten exposure, by stress-induced augmentation of locally produced cytokines and chemokines (12); 2) increased mobilization of epidermal and dermal DC resulting from local activation by stress mediators (51); 3) increase in the life span of DC emigrants. In this respect, Josien et al. (52) have recently reported that the immunoenhancing properties of TRANCE on T cell responses were due to its ability to augment the number of skin DC in draining LNs, by increasing their survival and longevity.

Acute psychological stress can modulate immune responses by triggering two main neurological pathways, the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis, resulting in the production of neuropeptides, catecholamines, and glucocorticoids (2, 3). We showed that glucocorticoids and CORT are not involved in the adjuvant effect of stress. Indeed, in vivo blockade of glucocorticoid receptors by the specific antagonist RU486 did not reverse the stress-induced enhancement of CHS. Rather, glucocorticoids exert anti-inflammatory effects, as illustrated by the observation that CHS to DNFB is enhanced in RU486-treated mice or in adrenalectomized mice (data not shown). These results are in keeping with studies showing that glucocorticoids inhibit DC maturation, reduce Th1 immune responses, and induce the selective expansion of regulatory Tr1 cells in vivo (4, 7, 53). Alternatively, the adjuvant effect of stress on the afferent phase of CHS primarily involves the sympathetic nervous system through the release of catecholamines (i.e., epinephrine and NE). Indeed, pretreatment of mice with 6-OHDA, which prevents release of NE by peripheral nerve endings, abrogated stress-induced enhancement of both skin inflammation and DC migration. Although NE released in vivo under stress conditions appears instrumental for enhanced CHS response, recent data by Seiffert et al. (9) show that injection of epinephrine i.p. or i.d. at the time of sensitization reduced CHS responses. The discrepancy between these data may be due to differences in the experimental protocols used and namely to different dose-effect and tissue distribution of catecholamine, upon i.p. or i.d. injection, as compared with its in vivo release in response to physiological stress. Indeed, it is possible that at physiological dose released under stress conditions, catecholamine exerts an adjuvant effect on skin DC and increases CHS, while pharmacological doses used for injection in normal mice moderately decrease CHS. In addition, minute amounts of NE released by stress in vivo occur in all catecholaminergic nerve termini, allowing a larger tissue distribution of catecholamine and possibly affecting a higher
number of skin DC as compared with local i.d. injection and hence with i.p. delivery. Finally, it is possible that NE is necessary, but not sufficient for the stress-induced enhancement of CHS, and that during an acute stress NE acts in concert with other mediators (neurotransmitters, cytokines, and chemokines) for the increase in CHS responses.

Catecholamines can bind to both α and β adrenergic receptors, which are differently expressed on leukocytes (5). Depending on the type of receptor to which it binds (2, 12), NE can have opposite effects on monocyte/DC function and cytokine production. Whereas stimulation of α receptors leads to cell activation and production of proinflammatory cytokines (54), β receptor agonists have anti-inflammatory effects (55). In this respect, NE was shown to block monocyte production of TNF-α, IL-1, or IL-12 (12, 56), and to inhibit APC function of skin LC, via binding to β adrenergic receptor (9). In contrast, NE via stimulation of α adrenergic receptor can augment LPS-stimulated production of TNF-α by peritoneal macrophages (57). The precise mechanism by which NE increases DC migration from skin to draining LNs is unclear at present. However, our data are reminiscent of recent studies reporting that DC migration is controlled by β1-adrenergic receptors expressed by immature DC, including both epidermal LC and BM-DC. Indeed, NE augments emigration of DC from skin explants in vitro as efficiently as the DC chemokine 6Ckine and in vivo blockade of β1-adrenergic receptors reduces DC migration to LNs in FITC painting experiments (11). It may thus be postulated that under stress condition, NE, which is released by nerve endings both in the skin (58) and in lymphatic vessels (59), could bind to adrenergic receptors on hapten-loaded maturing LC and trigger their chemotaxis from epidermis to dermis and from dermis to draining LNs via differential lymph. In addition, because NE was reported to influence the maturation of monocytes (2, 12), it is also possible that NE released in the hapten-painted skin provides additional DC activation signals, resulting in increased hapten presentation. Indeed, several recent reports demonstrate that NE binding to α1 adrenergic receptors activates monocytes through NF-κB pathway (54), and in contrast that NF-κB proteins regulate DC development, maturation, survival, and Ag-presenting functions (60, 61).

Previous studies have shown that psychological stress increases cell trafficking and vascular permeability, leading both to enhanced recruitment of blood lymphocytes to lymphoid organs (62) and high numbers of circulating blood DC (63). Our study extends this observation by demonstrating that acute stress can also mobilize more DC from skin to draining LNs, enhancing interactions between Ag-presenting DC and naive CD8 T cells. Thus, stress mediators that are released throughout the body during a psychological stress appear to prepare the immune system for mounting optimal immune responses. Our findings may help understand the mechanisms behind stress-induced worsening of several allergic and autoimmune skin diseases. Furthermore, the remarkable adjuvant properties of acute stress on DC migration and enhancement of T cell-mediated skin diseases might open new therapeutic avenues.

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References


