Low Zone Tolerance to Contact Allergens in Mice: A Functional Role for CD8+ T Helper Type 2 Cells

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Summary

Normal skin is permeable to low molecular hydrophobic substances, including allergenic chemicals. Whereas such foreign matter appears to enter the skin naturally, it rarely induces contact hypersensitivity. This suggests that immunological tolerance would be the normal state of affairs. In search of a suitable model, we painted picryl chloride or oxazolone once or repeatedly on normal skin of BALB/c or C57B1/6 mice and found subsensitizing doses to be tolerogenic. The most effective doses in inducing tolerance were doses between those at the point of inflection from no responses to threshold sensitivity. But even doses three orders of magnitude lower than these suppressed subsequent sensitization if applied repeatedly. C57B1/6 mice (low responders) were consistently easier to make tolerant than BALB/c mice (high responders). The tolerant state established by a single painting was found to be fully developed at 48 h after initiation and long-lasting (> 14 d). It could be adoptively transferred by intravenous injection of total spleen cells (SC), lymph node cells (LNC), or purified T cells and shown to be hapten specific. Pretreatment with cyclophosphamide (Cy) prevented tolerization. The T cells capable of transferring suppressive activity were found to be generated irrespective of the dose applied. On day 2 after painting, tolerance could be transferred with LNC from both tolerant and sensitized animals. On day 5, however, only cells from tolerant donors transferred tolerance. But by action of Cy, suppression was shown to be part of every sensitization, although masked. Production of hapten-specific antibodies was suppressed as well. Through depletion by monoclonal antibody in vitro the T suppressor cells were shown to be tolerant to the murine CD8+ subset (Lyt2+). Upon restimulation in vitro by haptenized and irradiated normal SC, LNC from tolerant donors produced predominantly interleukin (IL)-4, IL-5, and IL-10. In contrast, LNC from sensitized donors produced preferentially IL-2 and interferon-γ. Thus we demonstrate that painting subsensitizing doses of contact sensitizers on normal murine skin generates CD8+ Th2-like cells that give rise to hapten-specific tolerance. The model may have broader significance and apply to other species, including humans.

Animal models of contact hypersensitivity (CHS)1 to haptenic molecules (1) have contributed greatly to understanding the mechanisms that govern allergic contact dermatitis. This major skin disease causes considerable distress to affected patients and, in the form of occupational contact dermatitis, immense costs for society (2). Even now, the allergen-specific hypersensitive state, once established, cannot be reversed clinically, making it necessary to avoid forever the causative agent.

1Abbreviations used in this paper: CHS, contact hypersensitivity; Cy, cyclophosphamide; LNC, lymph node cells; Ox, oxazolone; PCL, picryl chloride; SC, spleen cells; TNBS, 2,4,6-trinitrobenzenesulphonic acid.

This paper is dedicated to Dr. Merrill W. Chase on the occasion of his 90th birthday.

Parts of this work were presented at the annual meeting of the “Arbeitgemeinschaft für Dermatologische Forschung” in Würzburg, Germany on 18–20 November 1994 (1995. Arch. Dermatol. Res. 287:397).

However, it is known that entrance through the skin of haptenic molecules generates not only T lymphocytes effecting inflammatory symptoms in sensitized skin but induces as well T cells that exert anti-inflammatory effects (3). These latter cells generally have been termed suppressor cells (4), and upon selective stimulation they mediate a state of hapten-specific tolerance (5–8). They were shown to negatively regulate the immune response in an unsuspected complex manner with several sets of T cells involved, each having characteristic surface markers and producing specific factors (9). It was proposed that in all murine systems, T cell suppression proceeds by a single major pathway involving the sequential interactions of three T cell subsets that have been termed Tₛ₁, Tₛ₂, and Tₛ₃ (10).

More recently, the T suppressor cascade as outlined above has been replaced by a simpler model of suppression (11) based on the finding that not only CD4+ but also CD8+ T lymphocytes separate on appropriate stimulation
Materials and Methods

Animals. BALB/c mice and C57Bl/6 mice were purchased from Charles River (Sulzfeld, Germany). Animals were maintained in a temperature- and light-controlled environment with free access to food and water. During experiments, animals of both sexes at 8–12 wk of age were housed singly in transparent Makrolan cages. Experimental groups consisted of five or more animals matched for age and sex. All mice in each experiment, including both normal and positively sensitized controls, were randomized in advance with respect to cage position in the room.

Chemicals. Picryl chloride (PCI; 2,4,6-trinitro-1-chlorobenzene), oxazolone (Ox; 4-ethoxy-methylene-2-phenyl-oxazolone), TNBS (2,4,6-trinitrobenzenesulfonic acid), and Cy monohydrate were all purchased from Sigma (Deisenhofen, Germany).

Experimental Sensitization and Tolerization. The right ear was used for sensitization and the left ear was used for challenge. When more than one application for sensitization was desired, allergens were applied on clipped areas of abdomen or dorsum as required. Both allergens were dissolved in olive oil/acetone (1:5); solutions were prepared immediately before using. Volumes of 15 µl each of PCI or of Ox were applied once epicutaneously by means of a pipette (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany). The single doses applied in this manner ranged from 7.50 µg down to 0.00045 µg PCI and from 1.00 µg down to 0.0075 µg Ox. From dose–response curves drawn thereof, standard doses in 15 µl for obtaining sensitization were found to be 450 µg PCI and 750 µg Ox, whereas standard doses to secure tolerization were 4.5 µg PCI and 7.5 µg Ox, respectively.

Double Sensitization. Standard doses of both PCI and Ox were applied on clipped abdominal areas of the right and left side, thus keeping both ears available for concurrent contact testing.

Assessment of CHS. On day 5 after the sensitizing application, 45 µg PCI or 30 µg Ox dissolved in 15 µl of the solvent were placed on the dorsum of the left ear. Ear thickness was measured both at 24 and 48 h with an engineer’s micrometer (Oditest, Kroeplin, Schüchtern, Germany) and compared with ear thickness before challenge. All measurements were read blindly by K. Steinbrink.

Assessment of Tolerance. Single applications of low doses (4.5 µg PCI, 7.5 µg Ox, and lower doses) which in previous sensitization experiments had been found to be subimmunogenic, were 7 d later directly followed by standard sensitization (450 µg PCI or 750 µg Ox) on the abdominal wall; tolerance was assessed by just one final contact test on the left ear.

Treatment with Cy. Mice were injected intraperitoneally with Cy at a dose of 200 mg/kg in PBS 2 d before application of a sensitizing or tolerizing dose or of the solvent.

Culture Media. RPMI-1640 was supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10% heat-inactivated FCS (all from Gibco, Eggenstein, Germany), 5 × 10⁻³ M 2-ME, and 1 µg/ml indomethacin (both from Sigma).

Preparation of LNC, Spleen Cells, and Purified T Cells. 5 d after sensitization or 2 d after the last painting of a tolerizing dose, respectively, mice were killed by ether euthanasia and spleens or lymph nodes were taken. LNC were obtained either from auricular, inguinal, or mesenteric lymph nodes. Single cell suspensions of LNC or spleen cells (SC) were prepared under aseptic conditions by mechanical disaggregation and passed through a sterile nylon gauze. Cells were washed three times and resuspended in RPMI-1640 complete. Viability was noted to be ≥95%, as determined by trypan blue exclusion. Purified T cells were prepared using the method of Julius et al. (29). Briefly, LNC (10⁷/ml) were incubated in a nylon wool column for 1 h at 37°C. After two washings with medium, T cells were pelleted by centrifugation and viability was determined by trypan blue exclusion. Enrichment of T cells (>95.5%) was examined by FACS® analysis (Becton Dickinson, Heidelberg, Germany).

Cell Transfer Experiments. 2 d after the last painting of a tolerizing dose, LNC or SC suspensions were obtained as described above. For adoptive transfer, 10⁷ viable LNC or SC in 0.5 ml RPMI-1640 were injected intravenously into naive syngeneic
mice. For depletion of subpopulations, cells were incubated with anti-L3T4 mAb (antimurine CD4, clone GK 1.5) or anti-Lyt2 mAb (antimurine CD8, clone 53.6; both from Becton Dickinson) at a final dilution of 1:100 for 1 h at 4°C. The cells were washed three times with RPMI to remove FCS and incubated with magnetobeads coated with sheep anti-rat IgG (DynaAl, Hamburg, Germany) for 1 h at 4°C. Thereafter, tubes were placed in a magnet and the residual cells collected. The negative-depleted cells were washed twice by centrifugation, and 10^7 cells were injected intravenously into recipients. The L3T4 and Lyt2^-neg-ative populations were determined by FACS® analysis and shown to contain <0.5% positive cells. 1 h after injection, animals were sensitized and 5 d later, they were ear challenged.

Preparation of Hapten-modified SC Spleens of untreated mice were excised and disaggregated into single cell suspensions. Erythrocytes were lysed and the remaining cells washed three times in HBSS (Gibco). 10^7 cells/ml were resuspended in HBSS containing 10 mM TNBS and incubated for 10 min at 37°C. Cells were washed three times with RPMI-1640 complete to remove free hapten. Alternatively, cells were oxazolonated by adding 10 µl of a 0.5% ethanolic solution of Ox to the SC (10^7/ml PBS) at 37°C for 10 min. Thereafter, cells were washed three times and irradiated (2,500 rad).

Lymphokine ELISAs. LNC (2 X 10^5 cells/well) were incubated with 10^5 hapten-modified and irradiated (2,500 rad) SC using 96-well round-bottomed microtiter plates (Greiner, Frickenhausen, Germany). After 72 h of culture in a humidified atmosphere of 5% CO2 at 37°C, supernatants were harvested, spun at 750 g for 15 min to eliminate dead debris, and frozen at ~80°C. ELISAs for IL-2, IL-4, IL-10, and IFN-γ were performed using sandwich ELISA kits purchased from Genzyme (Ismaning, Germany). All protocols were completed according to the manufacturer's instructions. IL-5 was quantified in a two-side sandwich ELISA as described previously (30). Briefly, maxisorb plates (Nunc, Wiesbaden, Germany) were coated with 50 µl of 2 µg/ml rat anti-mouse IL-5 antibody (clone TRFK5; Dianova, Hamburg, Germany) and incubated overnight at 4°C. Subsequently, wells were blocked by adding 200 µl 10% FCS in PBS for 2 h at room temperature. Samples and standards (recombinant murine IL-5; Biermann, Bad Nauheim, Germany) were diluted in RPMI-1640 complete and incubated for 4 h at room temperature. After washing, each well received 100 µl of 1 µg/ml biotinylated rat anti-mouse IL-5 (clone TRKF4, Dianova). Plates were incubated for 45 min at room temperature followed by three washings. Streptavidin-horseradish peroxidase was added at a concentration of 1 µg/ml (100 µl) and incubated at room temperature for 30 min. Plates were subsequently washed 10 times, and 100 µl substrate (ABTS, 2,2-azino-di-3-ethylbenzthiazoline sulfonate; Boehringer, Mannheim, Germany) at a concentration of 1 mg/ml in 44 mM Na2HPO, 28 mM citric acid, and 0.003% H2O2 was added. The colored product was measured at a wavelength of 405 nm. Samples were assayed in duplicate and differences between replicate wells were uniformly <5% for IL-2, IL-5, and IFN-γ, and <10% for IL-4 and IL-10. The sensitivities for these assays are IL-2 <10 pg/ml, IL-4 <1 pg/ml, IL-5 <15 µg/ml, IL-10 <1 pg/ml, and IFN-γ <1 pg/ml.

ELISA for Anti-Ox and Anti-PCI Antibodies (According to (31)). Hapten protein conjugates were prepared for use in ELISA. Ox (60 mg in 0.5 ml methoxylethanol) was added slowly to 10 ml of a 25 mg/ml solution of BSA in 0.5 sodium bicarbonate buffer. The mixture was stirred for 3 h at room temperature and the pH maintained at 8 by addition of 1 M sodium hydroxide. Protein was desalted by Sephadex G-25 column chromatography (Phar- nocia, Freiburg, Germany), dialyzed against water, lyophilized, and stored at −20°C. For the detection of antibodies to PCI, a second conjugate was similarly prepared substituting PCI for Ox. The wells of microtiter plates (Nunc) were coated with 100 µl (10 µg) of hapten protein conjugate in PBS. After incubation for 18 h at 4°C, plates were washed three times with PBS containing 0.5% Tween 20. Subsequently, serum samples were placed on the plates using serial logarithmic dilutions and maintained for 2 h at 4°C. This was followed by the addition of goat anti-mouse IgG, IgG1, IgG2a, IgG2b, and IgG3 conjugated with peroxidase at a concentration of 1 µg/ml (Camon, Wiesbaden, Germany); bound antibodies were detected by the addition of ABTS. Antibody titers are expressed as mean OD 405 nm ± SEM.

Statistics. Statistical significance of differences between experimental groups was evaluated using the Wilcoxon-Mann-Whitney two sample test. Differences of p <0.01 were considered significant.

Results and Discussion

The Murine Model. An in vivo model was required that, besides easy handling, would permit ready detection of dose-related differences in response to experimental sensitization. This was achieved by painting various doses of hapten once onto one ear, a site whose lymphatic drainage is clearly defined. When more than one application was needed, abdominal or dorsal skin sites or both were treated in addition with hapten. The other ear was reserved for determining the attained sensitivity in a contact test.

A conspicuous dose dependency was found in the animals' reactivity to both PCI and Ox. With PCI as sensitizer, single doses in 15 µl olive oil/acetone (1:5) ranging from 1.8 X 10^-12 to 3 X 10^-5 mol were used. A somewhat narrower dose range of Ox was examined, extending from 3.5 X 10^-11 to 4 X 10^-6 mol. Dose–response curves were established first in BALB/c mice known to be an inbred strain of high responders, and subsequently in C57Bl/6 mice, an inbred strain of low responders (32, 33).

Fig. 1. A and B shows that very low doses did not cause noticeable responses upon contact testing 5 d later. Increasing the dosage stepwise gave rise to inflammatory reactions on the challenged ear ranging from threshold sensitivity caused by 4.5 µg PCI or 7.5 µg Ox to full CHS secured by 450 µg PCI or 750 µg Ox. Increasing the dosage further did not intensify the ear swelling, indicating that a plateau of high responsiveness had been reached. Degrees of CHS inducible in BALB/c mice were consistently higher than those in C57Bl/6 mice but, in principle, dose dependency of responses to the two sensitizers was essentially the same. Thus, under the conditions elaborated, two chemically unrelated allergens exerted sensitizing capacity within a dose range of two orders of magnitude, covering therein a spectrum from minimal to optimal sensitization.

The Tolerogenic Effect of Subsensitizing Doses. Doses that had been found to be too low to sensitize BALB/c mice and C57Bl/6 mice adequately, both of PCI and Ox, were assumed to be nonetheless high enough to cause tolerance. Proof of efficacy in this respect came from experiments in which those animals that had not sufficiently responded to
the first treatment were subjected to effectual sensitization on second attempt. They were found to attain significantly lower sensitivities than animals pretreated solely with solvent (Fig. 2). The initial stimulus from the low dose application was followed directly 7 d later by intentional sensitization, avoiding a contact testing in between lest there be any influence, in whatever direction, upon the ongoing process.

The presence of tolerance must be proved through a subsequent attempt to sensitize. This is usually performed rigidly to be most convincing. However, it is possible for too strong a sensitizing regimen to override a tolerogenic stimulus and thus leave a tolerogenic component unrecognized. In the model presented here, sensitization was performed by a single painting of a dose of allergen found to be adequate to secure full sensitivity but not to exceed the dose required to avoid flooding the organism with the allergenic hapten.

Evidently, tolerization is precisely as dose dependent as is sensitization, although at a dose range three orders of magnitude lower. The most effective tolerizing doses were 4.5 µg PCI and 7.5 µg Ox, as seen in Fig. 1, A and B, that, at the most, had established threshold sensitivity. Still smaller doses applied to the skin, that is, 0.0045 µg PCI or 0.0075 µg

Ox, were essentially ineffectual in establishing tolerance by a single application. However, repeated application of low doses enhanced tolerance considerably. We tried several regimens, administering equal or unequal doses at various intervals, on one or on different sites; we finally determined the most effective treatment to be several applications of equal doses applied every other day on different sites. Consequently, three applications of a dose whose tolerogenic effect was insignificant when applied just once, did actually downregulate subsequent sensitization significantly when duly spaced out. Six applications (not shown) rather than three enhanced tolerance further and ten applications were still more effective (Fig. 3). Tolerance reached higher levels in C57BI/6 mice (up to 100%) than in BALB/c mice (up to 80%), owing to genetic factors (32, 33).

Figure 1. (A and B) Dose–response curves of PCI and Ox. Panels of BALB/c mice and C57BI/6 mice (five each) were painted once with designated doses of chemicals dissolved in olive oil/acetone (1:5) on the right ear (additional doses actually tested were more than those entered on the curves). After 7 d, the left ears were challenged with doses of 45 µg PCI and 30 µg Ox and ear swelling was measured 24 h later. Values presented are mean ± SEM.

Figure 2. Downregulating effect of single low doses upon subsequent sensitization. Panels of mice (five each) were painted once with designated doses of Ox on the dorsum of the right ear. After 7 d, all groups were sensitized by painting 750 µg Ox on the clipped abdominal wall. 5 d later, animals were challenged on the left ear and ear swellings measured 24 h. Values presented are mean ± SEM. With low doses of PCI applied epicutaneously, results were similar (not shown).

Figure 3. Tolerance is increased by repeated paintings of low doses every other day on different sites, for 3 or 10 such applications. 2 d after the last painting, animals were sensitized by epicutaneous application of 750 µg Ox and challenged on the left ear 5 d later. Values of ear thickness at 24 h are presented in a percentage as mean ± SEM. With PCI as tolerizing chemical, results were similar (not shown).
**Kinetics.** The kinetics underlying the development of tolerance was examined by carrying out the subsequent active sensitization after different time intervals. It was found that up to 24 h after application of a tolerizing dose, no down-regulating effect was demonstrable. At 36 h, down-regulation was apparent but not yet at a significant level. At 48 h after the tolerizing application, however, the suppressive effect was statistically significant with no further increase for up to 14 d (Table 1). When the time interval between a single tolerizing application and subsequent sensitization was prolonged to 21 d, tolerance had meanwhile faded. However, four tolerizing paintings on the right ear at weekly intervals and subsequent sensitization after another week extended the tolerant state to 5 wk (not shown). By cellular transfer technique as a tool of investigation it was confirmed that cells derived from regional (auricular) lymph nodes taken at 48 h after the tolerizing application exerted suppressive activity upon subsequent sensitization of the recipients with no further increase of efficacy later (data not shown). These results demonstrate that tolerance is established more rapidly than CHS. When sensitization was tested at 48 h, the response was practically zero.

**Transfer of Tolerance.** Adoptive transfer of cells from tolerant BALB/c or C57Bl/6 mice into naive recipients was performed by intravenous injection of SC, LNC, or purified T cells from regional nodes (Table 2). Transfer of 10⁷ viable cells generally sufficed to significantly downregulate contact reactions in recipients subjected to active sensitization within 60 min after transfer and challenged 5 d later, as compared to controls receiving lymphoid cells from donors treated with solvent alone. Furthermore, recipients which after transfer had been double sensitized, exhibited upon contact testing significantly downregulated responses to the allergen in question but attained full CHS to the unrelated chemical. Thus, the tolerant state induced by low doses of allergen applied onto normal skin was long-lasting (>14 d) and mediated by hapten-specific suppressor cells.

Moreover, adoptive transfer experiments revealed that not only were cells derived from regional nodes effective in downregulating subsequent sensitization but also cells derived from lymph nodes draining distant skin sites and even distant mucosal areas (Fig. 4). However, the degree of transfer from the different sources was not equal, being highest in cells from regional nodes, somewhat lower in cells from inguinal nodes, and lowest in cells from mesenteric nodes, irrespective of whether the transferred cells had been harvested on day 2 or 5 after tolerization.

The same technique also showed that the application of sensitizing doses induced generation of suppressor cells, which however, were detectable only at 48 h after sensitization. Of viable cells, 10⁷ were as effective in downregulating subsequent sensitization as 10-fold as many cells (Fig. 5). In contrast, transfer of cells harvested on day 5 after sensitization showed no suppressive activity (not shown).

**Sensitivity to Cy.** The suppressor cells that were found to be present surprisingly early and that were widely distributed among peripheral lymphatic organs, were tested for sensitivity to Cy. This drug is known to act on B cells and a population of short-lived, rapidly proliferating suppressor T cells (34, 35). Both BALB/c and C57Bl/6 mice were treated with Cy (200 mg/kg, i.p.) 2 d before epicutaneous application of either tolerizing or sensitizing doses or only the solvent. Compared with animals that had not been pretreated with Cy, the tolerizing effect of low allergen

### Table 1. Kinetics of Tolerance Development after Painting of Low Doses

| Subsequent sensitization after | Ear swelling (in mm × 10⁻²) |
|-------------------------------|-----------------------------|
| 12 h                          | 19.2 ± 3.8                  |
| 24 h                          | 18.4 ± 0.8                  |
| 36 h                          | 12.2 ± 4.5                  |
| 48 h                          | 10.7 ± 2.2*                 |
| 3 d                           | 11.0 ± 2.7*                 |
| 4 d                           | 11.6 ± 3.0*                 |
| 7 d                           | 11.0 ± 3.7*                 |
| 14 d                          | 11.5 ± 2.8*                 |
| 21 d                          | 16.0 ± 4.3                  |
| Positive control              | 18.2 ± 2.9                  |

The tolerogenic dose of 7.5 μg Ox was painted on the dorsum of the right ear of panels of BALB/c mice (five each) at time intervals before sensitization ranging from 12 h to 21 d. All groups were sensitized simultaneously by painting 750 μg Ox on the clipped abdominal wall and challenged 5 d later on the left ear. Values of ear swelling measured at 24 h are presented as mean ± SEM. *p <0.01.

### Table 2. Adoptive Transfer of Tolerance

| Donor                          | Challenge with Ox | Challenge with PC1 |
|-------------------------------|-------------------|--------------------|
| Painting with Cells transferred|                   |                    |
| Solvent SC                    | 16.8 ± 4.0        | 15.9 ± 3.8         |
| 1 × 7.5 μg Ox Spleen cells     | 6.3 ± 2.1*        | 16.6 ± 2.9         |
| 3 × 7.5 μg Ox Spleen cells     | 0.9 ± 1.2*        | 15.4 ± 3.2         |
| 1 × 7.5 μg Ox LNC              | 3.2 ± 1.5*        | 16.9 ± 3.6         |
| 1 × 7.5 μg Ox Purified T cells (lymph node)| 2.3 ± 1.0* | 15.3 ± 4.0         |

10⁷ lymphoid cells from donors treated as indicated were injected intravenously into naive recipients. Recipient BALB/c mice were double sensitized within 60 min after transfer by single paintings of 750 μg Ox on the right side of the clipped abdominal wall and 450 μg PC1 on the left side. 5 d later, all groups were challenged with the two sensitizers on the right and left ear, respectively, and ear thickness was measured at 24 h. Values presented are mean ± SEM. *p <0.01.

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Donors painted with 7.5 μg oxazolone
2 days prior to transfer
1 x 10⁷ LNC auricular
1 x 10⁷ LNC inguinal
1 x 10⁷ LNC mesenteric
5 days prior to transfer
1 x 10⁷ LNC auricular
1 x 10⁷ LNC inguinal
1 x 10⁷ LNC mesenteric
with solvent
1 x 10⁷ LNC auricular

Figure 4. Comparison of transfer capability of LNC derived from different sources. Equal numbers of viable cells (10⁷) derived from auricular (regional), from inguinal (nonregional), and from mesenteric (mucosal) lymph nodes were adoptively transferred. The recipients were sensitized within 60 min after transfer by a single painting of 750 μg Ox on the right ear and challenged 5 d later on the left ear. Values of ear thickness measured at 24 h are presented in a percentage as mean ± SEM.

Donors painted with 750 μg oxazolone
2 days prior to transfer
LNC (1.0 x 10⁷)
LNC (2.5 x 10⁷)
LNC (5.0 x 10⁷)
LNC (10 x 10⁷)
7.5 μg Ox
750 μg Ox

Figure 5. Sensitization normally includes generation of suppressor cells. LNC (regional) were taken from donors that 2 d previously had been painted once on the right ear with 750 μg Ox, a dose that regularly induces CHS. Different numbers of viable cells ranging from 10⁷ to 10⁸ were adoptively transferred into panels of C57Bl/6 mice (six each). The recipients (hatched bar) including controls (solid bar) were sensitized within 60 min after transfer by a single painting of 750 μg Ox on the right ear and challenged 5 d later on the left ear. A single painting of a sensitizing dose generated T suppressor cells within 2 d. Increasing the number of cells transferred did not heighten the degree of tolerance. Values presented are mean ± SEM.

In a further experiment along this line, animals of both strains were treated with Cy and sensitized to Ox 2 d later. Upon challenge, the usual test dose (30 μg Ox) was compared with two log₁₀ doses farther down (Table 3). Upon contact testing it became apparent that the Cy–treated animals responded to a test dose that was 10-fold below the dose to which untreated animals used to respond. Thus, removing suppressor cells from the normal pool of hapten-specific effector cells by Cy treatment revealed suppression to be a usual constituent of sensitization as a whole.

Suppression of Antibody Production. To see whether suppressor cell activity in our model would also involve suppression of antihapten antibody production, 10 C57Bl/6 mice were rendered tolerant to Ox by painting 7.5 μg Ox 10 times every other day on different abdominal and dorsal skin sites. Similarly, 10 control animals were painted solely

Table 3. Cy Enhances Sensitivity to Contact Testing

|                  | Painted with | Challenge with Ox | Ear swelling (in mm × 10⁻²) |
|------------------|--------------|-------------------|-----------------------------|
| i.p. injection   |              |                   |                             |
| PBS              | 750 μg Ox    | 30.0              | 14.5 ± 3.4                  |
| PBS              | 750 μg Ox    | 3.0               | 2.3 ± 1.5                   |
| PBS              | 750 μg Ox    | 0.3               | 0.0 ± 0.0                   |
| Cy               | 750 μg Ox    | 30.0              | 28.6 ± 3.1                  |
| Cy               | 750 μg Ox    | 3.0               | 13.1 ± 3.4                  |
| Cy               | 750 μg Ox    | 0.3               | 2.6 ± 2.6                   |

Panels of C57Bl/c mice (six each) were treated with PBS or 200 μg/kg Cy and subsequently sensitized by painting 750 mg Ox on the right ear. Upon contact testing, Cy–treated animals exhibited a 10-fold higher sensitivity indicating that before sensitization, generation of suppressor cells was blocked.
With PCI as tolerizing agent, results were essentially the
completed of L3T4 cells, whereas the fraction depleted of Lyt2
revealed that tolerance was transferred by the fraction de-
cells did not downregulate subsequent sensitization (Fig. 8).

Transfer and elicitation 5 d later, readings of ear swelling re-
were adoptively transferred into naive animals with 10^7
beads coupled with sheep anti-rat IgG. The residual cells
suppressor cells, lymphoid cells from auricular lymph nodes
derived from these nodes were depleted in vitro by mAbs to either L3T4 or Lyt2 antigens. The antibody-coupled cells were removed by mag-
neto beads coupled with sheep anti-rat IgG. Equal numbers of viable cells
draining the site of application of a tolerizing dose were de-
controls sensitized to PC1. This was also found in the sub-
classes IgG1 (Th2 response) and IgG2a (Th1 response), as
well as in the subclasses IgG2b and IgG3 (not shown). Antibody production measured on day 12 was not significantly
different from that on day 19. Similar results were obtained
when BALB/c mice were used, although suppression was
not as complete. With PCI as tolerizing or sensitizing agent,
antibody titers measured in both strains were generally lower.

Depletion of T Cell Phenotypes. To further characterize the
suppressor cells, lymphoid cells from auricular lymph nodes
draining the site of application of a tolerizing dose were de-
plicated in vitro by mAb to either L3T4 or Lyt2 antigens.
The antibody-coupled cells were removed by magnetobeads coupled with sheep anti-rat IgG. The residual cells were adaptively transferred into naive animals with 10^7
cells each and compared to adoptive transfer of 10^7 cells of
the total cell pool. After sensitization within 60 min after
transfer and elicitation 5 d later, readings of ear swelling re-
vealed that tolerance was transferred by the fraction de-
pleted of L3T4 cells, whereas the fraction depleted of Lyt2
cells did not downregulate subsequent sensitization (Fig. 8).
With PCI as tolerizing agent, results were essentially the
same (not shown) in experiments with C57Bl/6 mice as
donors and recipients. Thus, the T cells exerting suppres-
sion in our model of low zone tolerance were shown to
express the Lyt2^ determinant. There were no hints indi-
cating that other T cells or other types of cells would par-
icipate in the suppression. The Lyt2^ subset of the mouse
is considered equivalent to CD8^ T cells in humans. These
T cells were in our model effective on immune cells, which

Figure 7. Painting subsensitizing doses of
Ox diminishes the production of anti-Ox IgG
antibodies. C57Bl/6 mice were painted
with 7.5 μg Ox or solvent 10 times (five
each). Two d after the last application, both
groups were sensitized with 750 μg Ox and
challenged 5 d later. A negative control
group (n = 5) was pretreated with solvent
and subsequently sensitized to the irrelevant
hapten PC1. Serum samples were collected
19 d after sensitization and levels of anti-Ox
antibodies were determined by ELISA. Antibody titers are ex-
pressed as mean OD 405 nm ± SEM.

Adoptive Transfer of
BALB/c

LNC

LNC + anti-L3T4

LNC + anti-Lyt2

7.5 μg Ox

solvent

* p < 0.005

Figure 8. Adoptive transfer of in vitro-depleted LNC derived from
tolerant donors. 70 BALB/c mice were tolerized by six consecutive
paintings of 7.5 μg Ox every other day on different sites, with the last
two applications painted simultaneously on both ears. After 2 d, the
mites were killed and the auricular lymph nodes of both sides taken. Cells
derived from these nodes were depleted in vitro by mAbs to either L3T4 or Lyt2 antigens, and the antibody-coupled cells were removed by mag-
neto beads coupled with sheep anti-rat IgG. Equal numbers of viable cells
(10^7) of the nondepleted original cell pool and of both fractions were
adaptively transferred into panels of naive BALB/c mice (seven each).
The recipients (hatched bars), and also tolerized and solvent-treated con-
trols (solid bars), were sensitized within 60 min after transfer by a single
painting of 750 μg Ox on the right ear and challenged 5 d later on the left
ear. With C57Bl/6 mice treated similarly with PCI, results were compara-
ble (not shown).

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is characteristic for efferent suppressors (9). They are equivalent to the third subset of the murine suppressor T cell circuit, termed Ts3 cell, that is highly sensitive to Cy, antigen specific, and expresses the Lyt2+ phenotype (9). The Ts3 type was originally described by Sy et al. (36) and designated Ts-aux (auxiliary suppressor cells) as being essential for suppression of CHS.

Patterns of Cytokine Production after In Vitro Activation. In light of the present knowledge of immunosuppression by T cells (11) it was mandatory to determine whether LNC from tolerant mice would, upon appropriate stimulation, produce a different pattern of cytokine activity than LNC from sensitized mice. Regional lymph nodes were taken from tolerized, sensitized, and solvent-treated mice of both strains, and disaggregated. LNC were then incubated with SC that had been haptenized in vitro with TNBS and subsequently irradiated. After coculturing the cells for 3 d, the supernatants were tested for Th2 cytokines as well as Th1 cytokines. It was found (Table 4) that LNC derived from tolerant mice produced predominantly IL-4, IL-5, and IL-10, but no detectable IL-2 and little IFN-γ.

Conversely, LNC from sensitized mice secreted preferentially IL-2 and IFN-γ, but little IL-4, IL-5, and IL-10. Moreover, BALB/c mice (high responders) produced significantly more IL-2 than C57Bl/6 mice (low responders) but less IL-4, IL-5, and IL-10.

We did not investigate the entire spectrum of cytokine production but rather concentrated on the critical cytokines that characterize the two patterns (37). Originally, they were discovered by Mosmann and co-workers (12, 13) in a panel of murine helper T cell clones, that is, in CD4+ T cells. Some years later it was shown that alloreactive murine CD8+ T cell clones also produce the Th1 pattern: they secrete IFN-γ and lymphotoxin similar to Th1 clones but IL-2 at significantly lower levels (14). However, synthesis of IL-4 and IL-5 could not be detected. But eventually it was reported that CD8+ T cells can be induced to produce IL-4 as well (15). When such cells were primed in vitro with immobilized antibodies to CD3 (β chain of the TCR complex) in the presence of IL-2 and IL-4 they were as active in producing IL-4 as similarly primed CD4+ T cells. Thus, naive CD8+ T cells also differentiate upon appropriate stimulation into functional subsets that act like the two helper T cell subsets.

The sole variable in our model is the dose of hapten applied. Low doses initiate an immune response that is entirely different from that initiated by high doses, suggesting compartmentalization of functional subsets of T cells. We have found that LNC from mice tolerant to contact sensitizers produced preferentially type 2 cytokines, whereas, LNC from sensitized mice produced predominantly type 1 cytokines. It must be emphasized that the LNC derived from our tolerant mice are not exclusively composed of CD8+ suppressor cells, just as LNC from sensitized mice do not solely contain CD4+ Th1 cells (38) but include regularly suppressor cells, as shown by pretreating mice with Cy (Fig. 6). Nonetheless, a switch in cytokine production was obvious from a typical Th1 response in sensitized mice to a Th2 response in tolerant mice. However, on the B cell level such a switch was not detectable. Either the method used was not sensitive enough or antibody production to these particular antigens was indeed completely suppressed, as was delayed hypersensitivity. The differences in producing the respective cytokines among the two strains (Table 4) appear to reflect their different genetic backgrounds (32) and explain, in part, their diverse responses to tolerization and sensitization. This is the first demonstration, to our knowledge, that suppressor cells generated in low dose tolerance to contact allergens are CD8+ Th2-like T cells. Through their production and secretion of Th2 cytokines, they exert anti-inflammatory effects that counteract subsequent attempts to sensitize.

Obviously, CD8+ Th2-like cells have a different significance in tolerance to contact allergens than in infectious disease. Unresponsiveness to intracellular pathogens such as Mycobacterium leprae results in decreased resistance. In fact,

### Table 4. Cytokine Production of LNC from Tolerized and Sensitized Mice

| Painted with | IL-2 | IFN-γ | IL-4 | IL-5 | IL-10 |
|-------------|------|-------|------|------|-------|
| Solvent     | <10.0| <1.0  | <1.0 | <15.0| <1.0  |
| BALB/c 4.5 μg | 60.2 ± 10.5 | 145.0 ± 37.5 | 413.8 ± 99.6 | 532.0 ± 45.1 |
| 450 μg     | 433.1 ± 56.7 | 4304.8 ± 105.4 | 10.8 ± 9.9 | 50.5 ± 21.0 | 25.8 ± 10.1 |
| Solvent    | <10.0| <1.0  | <1.0 | <15.0| <1.0  |
| C57Bl/6 4.5 μg | 252.9 ± 56.8 | 711.5 ± 92.9 | 1415.3 ± 89.5 |
| 450 μg     | 120.9 ± 23.6 | 473.4 ± 49.6 | 12.8 ± 9.5 | 43.2 ± 28.9 | 24.6 ± 12.9 |

Values presented are mean ± SEM of two separate experiments.

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human CD8+ Th2-like cells were found to be present in skin lesions of the susceptible lepromatous form of leprosy that contain large numbers of bacilli (17). In contrast, CD4+ Th1 cells secrete IFN-γ preferentially and are the predominant cell type present in the resistant tuberculosis lesions of leprosy that contain few bacilli. In a mouse model using transgenic mice, it was shown more recently that an IL-4-dependent switch to IL-5 production in virus-specific CD8+ T cells delayed viral clearance from the murine host (21).

Low zone tolerance to contact allergens has certain features in common with inhalation and oral tolerance to non-replicating antigens. In an experimental model developed for eliciting immune responses to low doses of inhaled OVA repeated exposures to this antigen rendered rats tolerant to subsequent parental immunization with OVA. This tolerant state was selective for the IgE antibody isotype and could be transferred adoptively with CD8+ T cells. However, the cells mediating this particular state of tolerance were found to secrete upon in vitro activation high levels of IL-2 and IFN-γ, that is, type 1 cytokines (39).

Different immunologic mechanisms appear to underly oral tolerance depending on dosage and frequency of feeding (19). Low dose antigen feeding generated active suppression by T cells producing IL-4, IL-10, and TGF-β. In contrast, high dose feeding enhanced the secretion of both Th2 and Th1 cytokines. Moreover, clonal energy was found to be involved in high dose tolerance, and in Peyer’s patches antigen-reactive Th1 and Th2 cells were deleted by activation-induced apoptosis (40).

We are inclined to believe that tolerance to potential contact sensitizers might have pathophysiological importance. It could represent a fundamental mechanism dealing with small amounts of a foreign substance penetrating the skin. Such a mechanism would prevent an inflammatory response that otherwise would be inevitable unless a massive amount of that substance were to exceed the allowance. Tolerance would then be broken and contact dermatitis would result. “Contact tolerance” seems to apply to this type of peripheral hypo-responsiveness and we take the risk of proposing the term.

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