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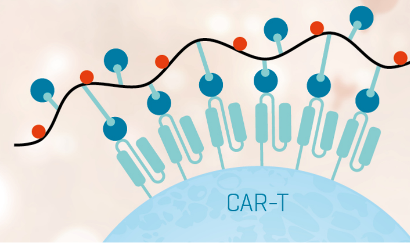

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Delayed-type hypersensitivity initiation by early-acting cells that are antigen mismatched or MHC incompatible with late-acting, delayed-type hypersensitivity effector T cells.

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DELAYED-TYPE HYPERSENSITIVITY INITIATION BY EARLY-ACTING CELLS THAT ARE ANTIGEN MISMATCHED OR MHC INCOMPATIBLE WITH LATE-ACTING, DELAYED-TYPE HYPERSENSITIVITY EFFECTOR T CELLS[1]

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The elicitation of delayed-type hypersensitivity (DTH) responses in mice is mediated by the sequential activities of two different Ag-specific, Thy-1$^+$ cells. A required early phase of elicitation is due to DTH-initiating Thy-1$^+$ cells that are CD3$^-$ and sIg$^-$ and produce Ag-specific factors that act like IgE antibodies in that they sensitize the tissues, so that after local challenge with Ag there is release of the vasoactive amine serotonin. Released serotonin locally recruits and activates CD4$^+$ Th-1 classical DTH effector T cells that secrete lymphokines that attract and activate a nonspecific perivascular infiltrate of circulating, bone marrow-derived leukocytes. The current study used isolated subpopulations of DTH-initiating and DTH-effector T cells to determine whether the two phases of the elicitation of DTH were entirely separate. The contact sensitivity model of DTH was used. Early-acting DTH-initiating cells, and late-acting DTH-effector T cells were either from oxazolone (OX)-immune or picryl chloride (PCl)-immune CBA or BALB/c donors and were transferred to CBA or BALB/c recipients. The results showed that DTH-initiation could be mediated by polyclonal DTH-initiating cells that were Ag mismatched or MHC incompatible with late-acting DTH effector T cells. In fact DTH-initiating cells could be both Ag mismatched and MHC incompatible with late-acting T cells. In addition, potential interactions between different cell populations were ruled out by showing that DTH-initiation could be mediated by a DTH-initiating clone that was Ag or MHC mismatched with the late-acting DTH-effector T cells. Thus, the OX-specific BALB/c clone could initiate DTH for PCl-specific CBA cells in CBA recipients if the recipients were challenged with both OX and PCl, but not when they were challenged with OX or PCl alone. We suggest, at least for the elicitation of DTH reactions in mice, that a more comprehensive description of these responses should accommodate the fact that there are early and late phase responses that each begin with Ag specificity and end with non-specific humoral factors. Inasmuch as the two Thy-1$^+$ cells of DTH can be of different Ag specificity, this suggests that some forms of delayed and chronic inflammation, might be initiated by an immediate hypersensitivity-like immune reactivity to one set of Ag, and could be prolonged and perpetuated by delayed reactivity to another set of Ag.

DTH[4] reactions are in vivo immune inflammatory responses that are due to extravascular recruitment of effector T cells. DTH reactions are mediated by recirculating CD4$^+$, Ag/MHC-class II-restricted, Th-1 T cells that leave the vessels, enter the extravascular tissues and then locally recruit and activate various non-specific effector cells, such as macrophages (1, 2). Work from our laboratory in the contact sensitivity model of DTH has indicated that the elicitation of these responses in immunized mice is actually due to the sequential activities of two different Thy-1$^+$ Ag-specific cells (3–5). According to our formulation, an early-acting, DTH-initiating cell in the lymphoid organs of sensitized mice, produces an Ag-specific factor (6, 7) that circulates systemically (8) and sensitizes the peripheral extravascular tissues for local release of serotonin, a vasoactive amine (9–12). Release of serotonin occurs within 2 h after challenge of the local tissue with specific Ag and results in an early 2-h skin swelling component of DTH (3–5, 12). Released serotonin binds to serotonin-2 receptors on the surrounding post-capillary venules, leading to vascular activation and the formation of gaps between endothelial cells (11, 13). This aids in the local recruitment of circulating, CD4$^+$ T cells that mediate the late, classical, 24- to 48-h DTH response (3–5, 12, 14, 15). An additional function of serotonin is to activate the recruited DTH effector T cells via serotonin-2 receptors (16). The recruited Th-1 cells then interact via their $\alpha\beta$ TCR with Ag/MHC-class II complexes on the surface of local APC. This results in release of lymphokines, such as IFN-γ, IL-2, and lymphotoxin (17, 18), that recruit and subsequently activate an infiltrate of bone marrow-derived non-specific effector leukocytes that constitute the perivascular infiltrate that characterizes DTH responses (1).

Recent studies have shown that the early-acting DTH-

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[4] Abbreviations used in this paper: DTH, delayed-type hypersensitivity; PCl, picryl chloride (TNP-chloride); OX, oxazolone; sIg, surface Ig.

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initiating cell is quite different from the classical, late-acting, Th-1 DTH-effector T cell, which is strictly thymic dependent and has the phenotype: Thy-1^+, Lyt-1^+, $CD4^+$, $CD8^-$, $CD3^+$, and is IL-$2R^+$ (2, 5). In contrast, the Ag-specific DTH-initiating cell can mature extrathymically, and thus can be induced by immunization of athymic nude mice (19, 20), and has a unique "primitive" phenotype for an antigen-specific cell of: Thy-1^+, Lyt-1^+, $CD4^-$, $CD8^-$, $CD3^-$, sIg^-, $B220^+$, IL-$2R^-$ and IL-$3R^+$ (5, 20).

Inasmuch as it is now quite clear that the DTH-initiating cell and the recruited DTH-effector T cells are very different, we sought to determine whether the sequential phases of DTH that are mediated by these two distinct cells are entirely separate. In experiments performed previously, the nominal antigen specificity of the two DTH cells had always been the same; raising the possibility that the two phases of DTH required shared Ag specificity. It was also possible that the two DTH cells might require MHC matching. Although we had shown that the Ag-specific, Ag-binding factor produced by DTH-initiating cells was not MHC-restricted in mediation of an early 2-h skin swelling response (7), and that DTH-initiating cells could produce such factors in an allogeneic environment (4), all experiments to date on DTH initiation itself; i.e., actual recruitment of MHC-class II-restricted DTH effector T cells to mediate the late aspects of DTH, had always been performed in a syngeneic system in which the two DTH cells and the environment were MHC matched.

The current study shows that elicitation of the two phases of DTH in adoptively immunized mice are completely separate and can be mediated by Ag or MHC mismatched cells, or by cells that are both Ag mismatched and MHC incompatible.

MATERIALS AND METHODS

Mice. Female BALB/cJ and male CBA/J mice, 5 to 7 wk old were obtained from The Jackson Laboratory, Bar Harbor, ME. All mice were rested for at least 1 wk in an air-filtered enclosure before use and were sensitized at 8 to 14 wk of age to serve as donors of immune cells, or were used as cell recipients at 8 to 10 wk.

Reagents. PCl (TNP), (Chemtronics, Swannonoa, NC), recrystallized from methanol/H_2O before use, and OX (Gallard-Schlesinger, Carle Place, NY), were protected from light in a dessicator during storage at room temperature.

Anti-B220 mAb (RA3-3A1/16.1) (21) was obtained from the American Type Culture Collection, Rockville, MD. This monoclonal recognizes a determinant encoded by the A exon (CD45RA) of the T200 glycoprotein family (22) of leukocyte common antigens (CD45), that is present on DTH-initiating cells, but is not present on late-acting DTH T cells (5). Low tox rabbit C was obtained from Pel-Freez, Brown Deer, WI.

Immunization for induction of contact sensitivity effector cells. Mice were contact sensitized by painting with 0.15 ml of a 5% solution of PCl or 3% OX in an ethanol/acetone mixture (4:1 v/v) on all four paws and the skin of the clipped abdomen.

Adoptive transfer of contact sensitivity. One or 4 days after sensitization with PCl or OX, mice were killed by cervical dislocation, and their spleens and peripheral lymph nodes were removed. Cell suspensions were prepared by gentle teasing. After washing, 5 to 7 $\times 10^7$ mixed spleen and lymph node cells were transferred by i.v. injection via the retroorbital plexus to individual naive recipients.

Isolated early-acting DTH-initiating cells or late-acting DTH effector T cells. One-day OX-immune or PCl immune cells from contact sensitized donors were used as a source of isolated DTH-initiating cells (3, 4, 12). To obtain isolated late-acting DTH effector T cells (5), 4-day OX-immune or PCl-immune cells were incubated in vitro with anti-B220-containing hybridoma supernatant at 10^7 cells/ml for 45 min on a rotator at 22°C. Then, antibody-coated cells were washed twice and incubated for 45 min in a 37°C waterbath with a 1/75 dilution of C at 10^7 cells/ml. The cells were washed twice, resuspended, and then 5 to 7 $\times 10^7$ cell equivalents were injected i.v. in a 1-ml volume into naive syngeneic or allogeneic mice via the retroorbital plexus. Cells from sensitized animals that were treated just with C served as positive controls.

Measurement of contact sensitivity DTH responses. Recipients of contact sensitivity effector cells were challenged 18 to 24 h after cell transfer by topical application of 1 drop (27-gauge needle) of either 0.8% PCl or 0.8% OX, or a 1:1 mixture of 0.8% PCl and 0.8% OX in olive oil to both sides of both ears. Duplicate measurements of ear thickness were made bilaterally with an engineer's micrometer (Mitutoyo, Paramus, NJ) before challenge and at 2 and 24 h after challenge. The increment in ear thickness was expressed as the mean ± SE in units of 10^{-3} cm. In each experiment there were four to five mice per group and the ears of a separate group of nonimmunized controls were challenged and measured similarly to adoptively immunized mice. The background swelling response in this control group usually averaged about 1 U at 2 h and 2 U at 24 h and was subtracted from the swelling response of experimental animal groups. Thus, the results are presented as the net increase in ear swelling.

Derivation of OX-specific DTH-initiating cell clone WP-3.27 (20). Nu/nu athymic mice on a BALB/c background were contact sensitized and boosted with OX weekly for 12 wk. Two wk before death they were inoculated intradermally with 2 $\times 10^6$ live WEHI-3 cells as a source of IL-3 to expand DTH-initiating cells. After a final OX boost the day before death, lymphoid cells were harvested and passed over nylon wool columns. The nylon non-adherent, Thy-1^+-enriched cells were treated with anti-CD4 and anti-CD8 antibodies and C. The Thy-1^+, double-negative ($CD4^-$, $CD8^-$)-enriched cells were then adhered to culture dishes with two mAb against two different determinants present on DTH-initiating cells: i.e., 14–30 and B220 (Ly-5) (5). Adherent cells were transformed with Moloney murine leukemia virus and lines were expanded in IL-3. The lines had DTH-initiating activity and produced antigen-binding factors that mediated Ag-specific DTH initiation (20). For cloning by FACS, double-positive Thy-1^+ and $B220^+$ cells were sorted at a density of one cell per well that contained irradiated syngeneic splenic feeders + IL-3. Clones were expanded, analyzed for phenotype by FACS and tested in vivo for early and DTH-initiating activity. Clone WP-3.27 had early DTH-initiating activity, and produced an Ag-binding factor with Ag-specific DTH-initiating activity (20). The phenotype of this clone was Thy-1^+, Lyt-1^+, $CD4^-$, $CD8^-$, $CD3\epsilon^-$, sIg^-, $B220^+$, IL-$2R^-$, and IL-$3R^+$ (20).

Cloned cells were cultured at 5 $\times 10^5$/ml in: DMEM, 10% nu-serum, (Collaborative Research Inc., Bedford, MA) 10 mM HEPES, 2 mM glutamine, and antibiotics. Cloned cells were washed twice and injected i.v. at a dose of 4 to 5 $\times 10^6$ cells/recipient.

RESULTS

DTH-initiating cells are removed by in vitro treatment with anti-B220 mAb and complement; and are restored by 1 day immune cells. We showed previously (5) that DTH-initiating cells express B220, the high molecular mass (220 kDa) determinant encoded by the A exon of the T200 glycoprotein family of leukocyte common Ag (CD45RA) (22, 23). B220 is usually found on resting B cells (5, 22), and anti-B220 is commonly used to deplete B cells. However, B220 has been noted on T cells in several instances (24–27), but late-acting DTH effector T cells do not express B220 determinants of CD45 (5). Figure 1 shows that DTH-initiating cells were removed with anti-B220 plus C, and were reconstituted by 1-day immune cells from contact sensitized donors. Pooled lymph node and spleen cells from 4-day sensitized mice that were treated just with C transferred early (2 h) and late (24 h) components of DTH (group A). Treatment with anti-B220 + C seemingly depleted cells mediating both components of DTH (group B). However, addition of isolated early cell activity present in 1-day immune cells (group C) to 4-day cells treated with anti-B220 + C, allowed expression of the remaining late DTH activity in the 4-day immune cells (group D). It was concluded that treatment with anti-B220 + C depleted DTH-initiating cells, leaving isolated late-acting DTH effector T cells that alone could not mediate 24 h DTH. However, these re-

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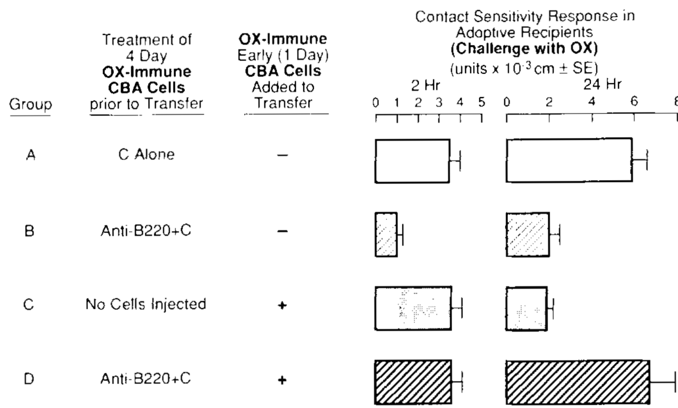

Figure 1. DTH-initiating cells are removed by treatment with anti-B220 mAb plus C, and are restored by 1-day immune cells. CBA mice were contact sensitized by topical application of 3% OX and 4 days later lymph node and spleen cells were harvested and treated in vitro with anti-B220 mAb, and subsequently with rabbit C. These cells (6.5×10^7), that potentially contained an isolated population of OX-immune, late-acting, DTH effector T cells, were transferred alone (group B), or together (group D) with 6.5×10^7 1-day OX-immune CBA cells (an isolated population of early-acting cells) to naive CBA recipients that were challenged on the ears 24 h later with 0.8% OX in olive oil. Four-day OX-immune cells, that were treated with complement alone (group A), or 1-day OX-immune cells alone (group C), were transferred to separate groups of recipients that were challenged similarly to serve as positive controls. Ear thickness was measured with an engineers micrometer before challenge, and at 2 and 24 h. The results of two separate experiments with 5 mice/group were pooled. In each experiment, a separate group of negative control mice were challenged similarly and their background ear swelling responses at 2 and 24 h were subtracted from those of the experimental groups to provide the net ear swelling that is shown.

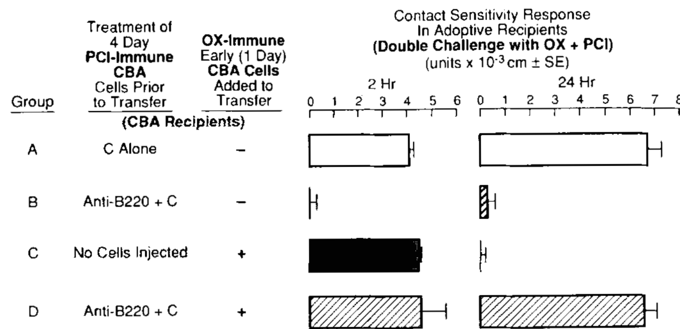

Figure 2. DTH-initiation: early and late cells can be of different Ag specificities. CBA mice were contact sensitized with 5% PCl and 4 days later lymph node and spleen cells were harvested and treated in vitro with anti-B220 + C to obtain an isolated population of PCl-immune, late-acting DTH T cells. These cells (6.5×10^7) were transferred alone (group B), or together with 6.5×10^7 cells from 1-day OX-immune CBA donors (an isolated population of early-acting cells) (group D), to naive CBA recipients that were challenged on the ears with *a mixture* of 0.8% PCl and 0.8% OX 1:1 in olive oil, 18 to 24 h later. Four-day PCl-immune cells that were treated with complement alone (group A), or 1-day OX-immune cells alone (group C) were transferred to separate groups of recipients that were challenged similarly to serve as positive controls. Ear thickness was measured with an engineers micrometer prior to challenge, and at 2 and 24 h in the experimental groups shown, and in a separate group of negative control mice that were challenged similarly.

maining cells could mediate 24 h DTH when DTH-initiating activity was reconstituted with 1-day immune cells that contained isolated DTH-initiating cells.

DTH initiation: early and late cells can be of different antigen specificities. We tested whether isolated late-acting DTH T cells of one Ag specificity could be recruited by early-acting DTH-initiating cells of another Ag specificity. Figure 2, group A shows that 4-day PCl-immune cells treated with C alone as a positive control transferred the ability to elicit the early, 2 h, initiating phase of DTH, and also the late, 24 h, classical effector phase of DTH. In contrast, group B shows that treatment of this population with anti-B220 + C abolished transfer of the ability to elicit both phases of DTH. Figure 1 shows that in an Ag-matched system the B220 population contained isolated late-acting DTH T cells, because the ability of this population to transfer late, classical DTH could be restored by adding back an isolated source of DTH-initiating cells (Fig 1, group D). Figure 2, group D shows that OX-immune early cells were able to recruit PCl-immune late-acting cells left after anti-B220 + C treatment to enable the full elicitation of DTH in mice that were doubly challenged on the ears with a mixture of PCl + OX. One-day OX-immune cells, when transferred alone, just mediated an early, 2-h ear swelling response (group C). It was concluded that early DTH-initiating cells and late-acting DTH-effector T cells could be of different Ag specificities. When these cell populations were transferred together to naive recipients they led to full expression DTH in mice challenged simultaneously with both Ag.

DTH initiation: early and late cells can be MHC incompatible. Figure 3 show similar experiments that sought to determine whether the early and late cells of DTH could be MHC incompatible. Group A in Figure 3*A* shows that 4-day PCl-immune CBA cells, that were treated with C alone, transferred the ability to elicit 2- and 24-h components of DTH in CBA recipients that were challenged with PCl. Group B again shows that treatment with anti-B220 + C eliminated early cell activity and therefore abolished transfer of both components of DTH. Group C shows that 1 day PCl-immune BALB/c cells,

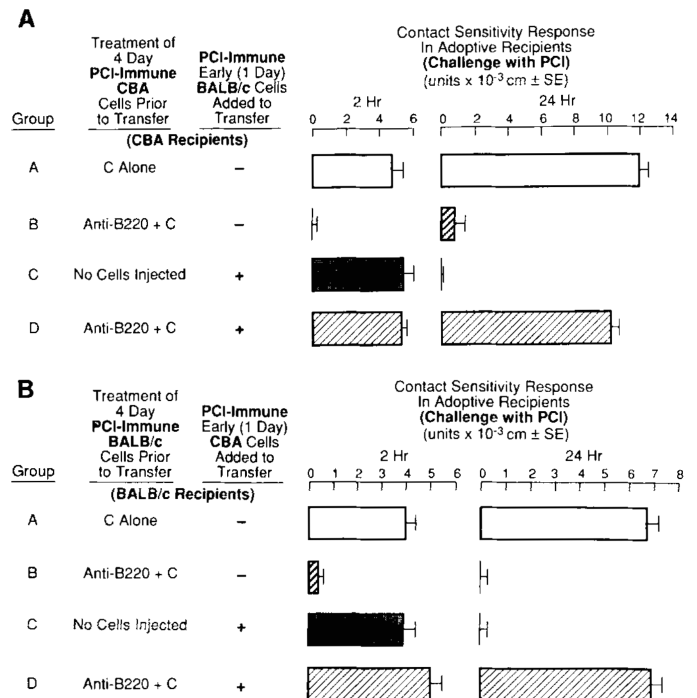

Figure 3. DTH-initiation: early and late cells can be MHC incompatible. Four-day PCl-immune CBA cells (*A*), or BALB/c cells (*B*), were treated in vitro with complement alone as positive controls (group A), or with anti-B220 + C to isolate late-acting DTH T cells. These latter cells were transferred alone (group B) or together with an isolated population of early-acting, 1-day, PCl-immune BALB/c cells (*A*) or CBA cells (*B*) to naive CBA recipients (*A*) or to BALB/c recipients (*B*) that were skin challenged with PCl the next day. Four-day PCl-immune CBA cells (*A*) or BALB/c cells (*B*) treated with complement alone (group A), or 1-day PCl-immune BALB/c cells (*A*, group C) or CBA cells (*B*, group C) were transferred as positive controls. Ear thickness was measured before challenge and at 2 and 24 h in these groups and in a group of negative controls.

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mediated 2-h reactions in allogeneic CBA recipients. Group D shows that these allogeneic early-acting cells were able to recruit isolated, late-acting, CBA DTH effector T cells that remained after treatment with anti-B220 + C; to therefore mediate DTH-initiation and allow full expression of DTH in CBA recipients. Figure 3B shows a similar experiment performed in BALB/c recipients in which 1-day PCl-immune CBA cells mediated DTH-initiation for isolated late-acting PCl-immune BALB/c cells in BALB/c recipients.

Figure 4 shows that the ability of MHC mismatched early cells to initiate DTH was not a consequence of mere transfer of allogenic cells. As shown in Figure 4, allogeneic early cells (group C) required specific Ag challenge to mediate DTH initiation that was equivalent to syngeneic early-acting cells of the same Ag specificity (group B). In contrast, allogenic early cells of a different Ag specificity did not work (group D). It was concluded that DTH initiation could be mediated by early-acting cells that were MHC incompatible with the late DTH effector T cells and were acting in an MHC incompatible environment. However, late DTH T effector cells required MHC matching with the environment (Fig. 4, group B vs group E), because they are MHC class II restricted (4).

DTH initiation: early and late cells can be both Ag mismatched and MHC incompatible. These experiments suggested that the early and late phases of DTH were separate because the early-acting and late-acting cells could be Ag mismatched or MHC incompatible. If the two phases are completely separate then it should be possible to elicit DTH with cells that are both Ag mismatched and MHC incompatible. Figure 5 shows an experiment designed to test this point. Four-day PCl-immune CBA cells were depleted of early-DTH activity by treatment with anti-B220 + C. These cells failed to elicit early or late DTH in CBA recipients challenged with OX or with PCl or with OX + PCl (groups A, B, and C). One-day OX-immune cells from CBA or BALB/c donors were used as a source of early DTH activity that was elicited in CBA or BALB/c recipients by challenge with OX, (groups D and

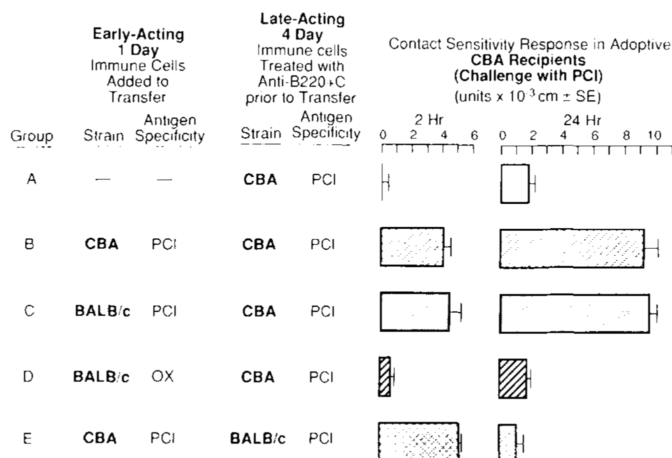

Figure 4. DTH initiation: MHC mismatched early cells require specific Ag challenge; whereas MHC mismatched late cells do not work with specific Ag challenge. Four-day PCl-immune CBA cells (groups A, B, C, and D), or 4-day PCl-immune BALB/c cells (group E), were treated in vitro with anti-B220 + C to obtain isolated late-acting DTH T cells. These cells were transferred alone (group A), or together with an isolated population of early-acting, 1-day PCl-immune CBA cells (groups B and E), or 1-day PCl-immune BALB/c cells (group C), or 1 day OX-immune BALB/c cells (group D), to naive CBA recipients that were skin challenged with PCl the next day.

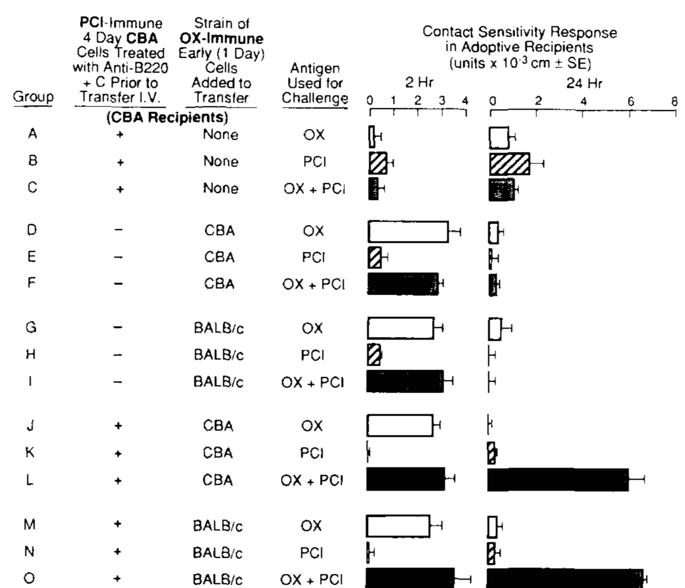

Figure 5. DTH-initiation: early and late cells can be of different Ag specificities and can be MHC incompatible. Four-day PCl-immune CBA cells were treated with anti-B220 + C to isolate late-acting DTH T cells. These latter cells were transferred alone (groups A, B, and C), or together with an isolated early-acting population of OX-immune CBA cells (groups J, K, and L), or BALB/c cells (groups M, N, and O) to CBA recipients that were skin challenged the next day with either OX alone, PCl alone, or a mixture of OX + PCl. The isolated, early-acting, OX-immune, BALB/c, or CBA cells were transferred alone to CBA recipients that were skin challenged the next day with either: OX alone (groups D and G), or PCl alone (groups E and H), or a mixture of OX + PCl (groups F and I). Ear thickness was measured before challenge and at 2 and 24 h in these groups, and in a separate group of negative controls that were challenged either with OX, PCl, or with OX + PCl to provide background swelling responses that were subtracted. The results of two separate experiments with five mice per group were pooled.

G), or with OX + PCl (groups F and I), but not by challenge with PCl alone (groups E and H).

When these OX-immune early CBA or BALB/c cells were mixed with PCl-immune, isolated, late-DTH CBA cells, and the recipients were challenged with OX, then just the early OX-specific activity was elicited (groups J and M). When recipients of these early and late cell mixtures were challenged with PCl then neither component of DTH was elicited (groups K and N). In contrast, recipients of these cell mixtures, that were challenged simultaneously with a mixture of OX and PCl, elicited early and late components of DTH (groups L and O).

In the experiment shown in Figure 5, groups K and N demonstrate that mixing and transfer of isolated syngeneic late-acting T cells with isolated early-acting cells, but challenge of recipients only with the Ag specificity of the late cells is insufficient to elicit late DTH. Groups L and O show that challenge with the two different Ag specificities of both the early syngeneic or allogeneic cells and the late syngeneic cells is required for full expression of DTH. Group O shows that the early and the late cells can be both Ag mismatched and MHC incompatible, and that full DTH was expressed when both Ag were used for elicitation, and the late-acting DTH cells were MHC matched with the recipients. Taken together these results confirm the concept that two cell types act in an obligate sequence to mediate elicitation of DTH; and establish that these interdependent phases are completely separate.

DTH initiation: mediation by OX-specific clone 3.27 cells that can recruit OX-immune or PCl-immune late

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DTH T cells. The experiments above were performed with polyclonal DTH-initiating cells from actively sensitized mice and showed that the early and late phases of DTH were completely separate. Because polyclonal DTH-initiating cells were used, it was possible that the results depended on interactions between different cell subsets in this mixed population. Therefore experiments were performed to see if similar results could be obtained with a DTH-initiating cell clone. This OX-specific clone (WP-3.27) was derived from OX contact sensitivity immunized and boosted nude mice on a BALB/c background (20). Figure 6, group C shows that clone 3.27 mediated elicitation of the early 2-h component of DTH in OX-challenged allogenic CBA mice. Group D shows the DTH-initiating activity of the clone that allowed expression of late, OX-specific DTH activity that remained in 4-day OX-immune CBA cells, that were treated with anti-B220 + C, and were injected with the clone into CBA recipients. Thus, an MHC-incompatible DTH-initiating clone can recruit isolated, late-acting DTH T cells to reconstitute the full expression of DTH.

The experiment shown in Figure 7 tested whether the DTH-initiating clone could be both MHC incompatible and Ag mismatched with the isolated late-acting DTH effector T cells. Groups A and B show that 4-day PCl-immune CBA cells treated with C alone transferred both early and late activities to CBA recipients challenged with PCl, or with a mixture of PCl + OX. Groups C and D show that elimination of early cell activity by treatment with anti-B220 + C abolished the ability of residual late-acting T cells to elicit of 24-h DTH. Group H shows that when the OX-specific, early-acting, nu/nu-BALB/c clone 3.27 was mixed with the PCl-specific, late DTH T cells from CBA mice, and the CBA recipients of this mixture were challenged with both PCl and OX, that full expression of DTH was restored. Importantly, the DTH-initiating clone acted in a strictly Ag-specific manner because group G shows that a mixture of the allogeneic OX-specific clone and PCl-specific late DTH T cells syngeneic to the CBA recipients, led to elicitation of neither early nor late aspects of DTH when the recipients were challenged with PCl alone. It was concluded that an Ag-specific DTH-initiating clone could complement isolated, late-acting, DTH effector T cells that were MHC incompatible and Ag mismatched.

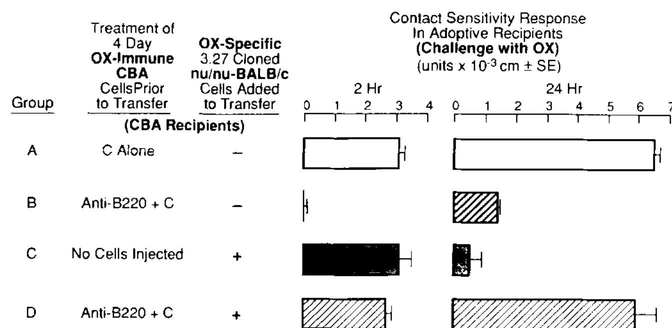

Figure 6. DTH-initiation: mediation by OX-specific 3.27 clone cells that complement OX-immune late DTH T cells in mice challenged with OX. Four-day OX-immune CBA cells were treated with C alone as positive controls (group A) or with anti-B220 + C to isolate late-acting DTH T cells. These latter cells were transferred alone (group B), or together with the OX-specific nu/nu-BALB/c DTH-initiating clone WP-3.27 (group D), to CBA recipients that were skin challenged with OX. Ear thickness was measured before challenge and at 2 and 24 h in these groups and in negative controls that were just challenged with OX to provide background swelling responses that were subtracted. Mice in group C received clone cells alone.

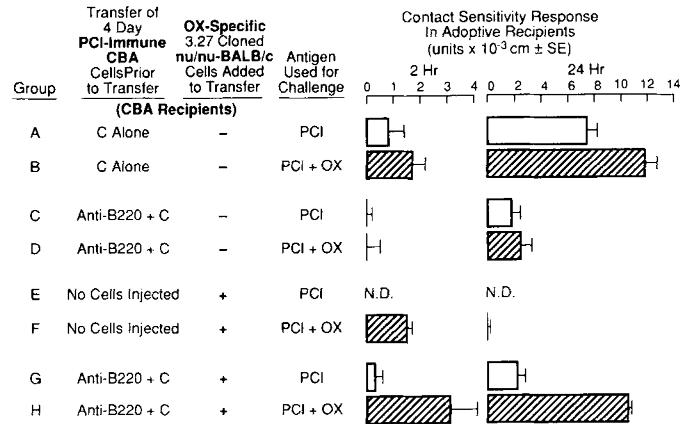

Figure 7. DTH-initiation: mediation by OX-specific clone 3.27 cells that complement PCl-immune late DTH cells in mice challenged with OX + PCl. Four-day PCl-immune CBA cells were treated with C alone as positive controls (groups A and B) or with anti-B220 + C to isolate late DTH T cells that were transferred alone (groups C and D) or together with the OX-specific, clone 3.27 (nu/nu-BALB/c cells) (groups G and H) into CBA recipients that were challenged with PCl alone, or with a mixture of PCl + OX. Ear thickness was measured before challenge and at 2 and 24 h in these groups and in separate groups of negative controls that were just challenged with PCl or with PCl + OX to provide background swelling responses that were subtracted.

DISCUSSION

Recent results confirmed our concept that two Ag-specific Thy-1$^+$ cells act in an obligate sequence to mediate the elicitation of DTH. These studies established that the two cells were quite different in phenotype (5). Early-acting DTH-initiating cells, were Thy-1$^+$, Ly-1$^+$(CD5$^+$), CD4$^-$, CD8$^-$, CD3$^-$, sIg$^-$, B220$^+$ (CD45RA$^+$), Il-2R$^-$, and Il-3R$^+$; whereas late-acting DTH-effector Th-1 T cells were Thy-1$^+$, CD5$^+$, CD4$^+$, CD8$^-$, CD3$^+$, sIg$^-$, B220$^-$, Il-2R$^+$, and Il-3R$^-$ (5, 20). The early-acting cells initiated the elicitation of DTH by prior production of Ag-specific factors that sensitized the peripheral tissues for release of the vasoactive amine serotonin, after local challenge with Ag (9–16). Released serotonin contributed to local recruitment of late-acting DTH effector T cells that locally produced cytokines following activation via surface $\alpha\beta$-TCR cross-linked by Ag/MHC complexes on local APC.

In the current study we determined whether the two sequential phases of DTH elicitation, that are mediated by these two different Ag-specific cells, were completely separate, or were linked. Thus, we investigated whether the two components needed to be of the same Ag specificity, or needed to be MHC matched. This was an important point. If it were shown that the two phases needed to be matched in Ag or MHC specificity, this would imply that the early-acting DTH-initiating factor not only was involved in the initiation of DTH via serotonin release, but also functioned in elicitation of the delayed component, mediated by CD4$^+$ Th-1 cells. Thus, further mechanisms of action for the early-acting factor would have to be postulated. However, if it were shown that the two cells could be unrelated in Ag specificity, then this would suggest that DTH, and thus some forms of delayed and chronic immune inflammation, might be initiated by an immediate hypersensitivity-like immune reactivity to one set of Ag, and could be prolonged and perpetuated by

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delayed reactivity to another set of Ag.

The differing phenotype of the two Thy-1$^+$ cells of DTH provided a way to test these possibilities. When 4-day immune cells from contact sensitized donors were treated with anti-B220, the remaining cells were known to have latent, late-DTH activity that required complementation by DTH-initiating cells (5). In addition, cells from 1-day immune animals were known to have only DTH-initiating activity (3–5, 12). Thus, we asked whether 1-day cells of a given Ag specificity could complement 4-day immune anti-B220 treated cells of a different Ag specificity. Our results showed that the two components of DTH could be of different Ag specificites. When mice received a mixture of early- and late-acting cells of two Ag specificities, then challenge had to consist of both Ag to elicit DTH. Challenge of recipients of both cell types with only the early Ag produced only the early response; challenge only with the late Ag elicited neither response, while challenge with both Ag elicited full DTH. This confirms that two Ag-specific cells act in an obligate sequence to mediate the elicitation of DTH.

Similar results were obtained in experiments investigating whether the two DTH cells required MHC matching. As expected, the late cells needed MHC matching with the recipient. However, it was found that the early DTH-initiating cell need not be MHC matched with the recipient environment nor with the late-acting DTH effector T cells. In fact, DTH could be mediated by a mixture of early- and late-acting cells that were both Ag mismatched and MHC incompatible. Therefore, the two phases were completely separate.

Thus, DTH can be viewed as a cascade reaction of multiple sequential steps (27), and each of the two obligate sequential phases of DTH can be viewed as a separate cascade reaction that begins with an Ag-elicited, Ag-specific step and ends with release of nonspecific humoral factors. The Ag-specific initial trigger of the early phase is mediated by an Ag-specific, non-MHC-restricted factor that is produced by a primitive, relatively thymic-independent cell with an unusual phenotype for an Ag-specific cell of: Thy-1$^+$, Lyt-1$^+$, CD4$^-$, CD8$^-$, CD3$^-$, sIg$^-$, B220$^+$, IL-2R$^-$, and IL-3R$^+$ (4, 5, 19, 20). The Ag-specific factor sensitizes mast cells (9–13, 28), and perhaps other serotonin-containing cells (28, 29) in the peripheral tissues so that serotonin is released after local Ag challenge to elicit DTH. At the end of the early phase, released, nonspecific, serotonin mediates local recruitment, and activation of Th-1 cells (9–11, 14–16). Among these recruited DTH effector T cells are a few, or perhaps even just one T cell (30), with appropriate Ag/MHC-restricted, $\alpha\beta$-TCR that bind to the surface of local APC, leading to release of cytokines as the non-specific humoral mediators of the second phase of DTH.

In the experiments using MHC mismatched allogenic DTH-initiating cells, it was possible that in vivo systemic allo-reactivity between donor and recipient cells had led to an alteration in the requirements for the two sequential Ag-specific Thy 1$^+$ cells to mediate elicitation of DTH; perhaps via release of cytokines. However, control experiments showed that transfer of allogenic cells per se did not mediate DTH-initiation nor late DTH (Fig. 4, group D). Although the transferred allogeneic DTH-initiating cells might be filtered out in the 18 to 24 h between transfer and challenge (3, 4, 12), they would still be able to release Ag-specific, DTH-initiating factor into the circulation (8), and thereby sensitize the skin for Ag-specific early reactivity. We showed previously that irradiation, which is known to impair recirculation of cells, did not impair the ability of syngeneic early-acting cells to sensitize the skin of recipients for early Ag-specific reactivity (4). With MHC-mismatched cells, as with MHC-matched cells, elicitation of DTH required the presence of: 1) both early-acting cells and late-acting cells; 2) MHC matching of the late-acting cells and the recipient; and, importantly, 3) local challenge with Ag appropriate to the specificity of both cells. This confirmed that the two Ag-specific cells act in an obligate sequence to mediate DTH, and that the two phases of DTH are separate. The MHC-matching results were obtained with optimal numbers of transferred cells because we were attempting to adoptively immunize recipients to simulate actively sensitized animals. Thus, although we have shown that allogeneic cells are competent to initiate DTH, we cannot exclude that syngeneic cells are quantitatively superior.

An important and unresolved question concerns the molecular nature of the Ag-specific factor that is produced by the early-acting cells and that mediates DTH initiation. This study confirms that the factor is Ag specific and not MHC restricted. An important experiment in this study further demonstrated the Ag specificity of the DTH-initiating clone we described recently. The clone was derived from nude mice (19, 20) and not normal mice, because DTH-initiating cells could be boosted in nudes (31) because nude mice lack CD8$^+$ Ts cells that down-regulate DTH-initiating cells (31–33). We showed previously that the OX-specific clone WP 3.27, or its Ag-binding factor, could initiate DTH for OX-specific late-acting DTH effector T cells when recipients were challenged with OX and not with PCl (20). In the current study we showed that the OX-specific clone could initiate DTH for PCl-specific late-acting DTH effector T cells if the recipients were challenged with both OX and PCl, but not when they were challenged with OX or PCl alone.

The fact that immunization induces Ag-specific DTH-initiating cells in normal mice (5, 12) and athymic nude mice (19), but not in mice with severe combined immunodeficiency (19), which lack T and B cells because they cannot join rearranged gene segments (34–37), suggests that the factor is encoded by a rearranging gene family. However, several results to date indicate that neither Ig nor TCR genes are involved. For Ig these results include: 1) the factors do not bind to anti-Ig columns (7, 8); and 2) the DTH initiating clone WP-3.27 is sIg$^-$ and does not transcribe Ig H or L chain genes (W.-R. Herzog, et al., unpublished observations). For TCR these results include: 1) polyclonal DTH-initiating cells (5) and the DTH-initiating clone (20) are surface negative for CD3ϵ; 2) the clone does not transcribe CD3δ, CD3ϵ, nor CD3ζ mRNA (20); 3) the clone does not transcribe mRNA of α or β TCR chains (W.-R. Herzog, et al., unpublished observations); and 4) for TCR-δ, the clone only transcribes an abnormally large, 3.5-kb mRNA transcript that is detected by TCR-Cδ and Jδ_1 probes, and Southern analysis with the Jδ_1 probe has failed to demonstrate that the δ TCR locus is rearranged (W.-R. Herzog, et al., unpublished observations). Taken together, these results may suggest that another set of rearranging genes besides Ig or TCR encodes the Ag-specific DTH-initiating factor.

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In conclusion, the results of this study demonstrate further that two different and independent Ag-specific Thy-1$^+$ cells act in an obligate sequence to mediate elicitation of DTH via two separate and interconnected cascades. The DTH effector T cell of the classical late cascade appears to be a CD4$^+$, TCR-$\alpha\beta^+$, Ag/MHC-restricted cell that produces a Th-1 profile of cytokines. In contrast, DTH-initiation is a completely separate and early cascade that ends with release of serotonin as the nonspecific final mediator, and is due to an Ag-specific, non-MHC restricted DTH-initiating factor that does not appear to be an Ig nor a TCR, and thus may be encoded by another rearranging gene family.

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