Involvement of Granulocytes and the Adhesion Receptors Intercellular Adhesion Molecule-1 and Lymphocyte Function-Associated Antigen-1 in Tissue Inflammation Induced by Th2-Type Helper Cells

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We reported recently that subcutaneously injected, anti–CD3ε-pulsed polyclonal Th2 cells mediate interleukin-4–dependent local tissue inflammation. Because a prominent polymorphonuclear infiltrate was observed in the lesions at the time of maximal tissue swelling, we investigated the involvement of polymorphonuclear leukocytes and their adhesion molecules lymphocyte function-associated antigen-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1) in Th2-cell–mediated inflammation. Pretreatment of recipient mice with a depleting monoclonal antibody to neutrophils or with blocking antibodies to LFA-1 or to ICAM-1 completely abrogated tissue swelling in Th2-cell–mediated inflammation. Granulocyte infiltration at 6 h was also inhibited by the antibodies to neutrophils and to ICAM-1, but not by that to LFA-1. Tissue swelling mediated by Th1 cells had different kinetics and was not prevented by administration of anti-neutrophil antibody: maximal edema formation occurred at 24–48 h, when the predominant cellular infiltrate was mononuclear. Because the Th1-cell–induced infiltrate at 6 h also consisted of granulocytes but was not associated with pronounced edema, the mere presence of infiltrating polymorphonuclear leukocytes seems not to be sufficient to induce edema. Because edema but not granulocyte infiltration was inhibited by anti–LFA-1 and because anti–LFA-1 antibodies are known to inhibit several functions of neutrophils, our results suggest that, in inflammation mediated by Th2 cells, granulocytes induce edema through their activation and/or degranulation. *Key words: polymorphonuclear leukocytes/interleukin-4/delayed-type hypersensitivity/extravasation/activation. J Invest Dermatol 104:350–354, 1995*

Since the first description in 1986 of two mutually exclusive subsets among murine T-helper cell clones [1], termed Th1 (mainly interleukin-2 [IL-2] and interferon-γ producers) and Th2 (mainly IL-4, IL-5, IL-6, and IL-10 producers), the relations between the lymphokine patterns secreted by these cells and their divergent functional activities have attracted much interest. Recent models [2] tend to view Th1 cells as responsible for inflammatory reactions *in vivo*, whereas Th2 cells are thought to be mainly responsible for the induction of humoral immune responses.

Th1-like cells have been shown to induce inflammatory reactions with characteristics of delayed-type hypersensitivity (DTH) [3,4]. Th2 cells were initially thought to be incapable of mediating tissue inflammation [3], but were subsequently shown to induce IL-4–dependent inflammatory reactions when injected into the ears or footpads of naive recipient mice [4] (subsequently referred to as DTH2, in contrast to classic DTH mediated by Th1 cells and termed here DTH1, [5]). The histologic pattern of tissue inflammation induced by Th1 and Th2 cells was not distinguishable [4]. At 6 h after Th-cell injection, polymorphonuclear leukocytes (PMN) were the predominant cell type in the cellular infiltrate, whereas mononuclear cells prevailed at 48 h [4]. However, the kinetics of the swelling reactions induced by Th1 and Th2 cells were different: Th1 cells induced maximal swelling at 24 to 48 h (when the mononuclear cell infiltrate predominated); in contrast, Th2-cell injection caused maximal edema formation at 6 h (when PMN constituted the cellular infiltrate; **Table I**). This finding suggested that PMN may be responsible for the swelling reaction induced by Th2 cells. Because Th1 cells also induced prominent PMN infiltration at 6 h but with little edema, it seemed that the mere presence of PMN in the tissue was not sufficient to induce frank edema.

We therefore investigated the role of PMN and their adhesion receptors intercellular adhesion molecule-1 (ICAM-1) (CD54; [6]) and (leukocyte function-associated antigen-1 (LFA-1) ($\alpha_L\beta_2$ integrin; CD11a/CD18 [7]) in DTH2 reactions. To this end, we used efficient antibody-mediated depletion of neutrophils *in vivo* or

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Abbreviations: DTH1, inflammatory responses mediated by Th1 cells; DTH2, inflammatory responses mediated by Th2 cells.

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Table I. Differences Between Swelling Reactions and Local Cellular Infiltration Mediated by Th1 and Th2 Cells

Inflammation Mediated by	Time After Injection of Th Cells			
	6 h		48 h	
	Swelling	Infiltrate	Swelling	Infiltrate
Th1 cells	(−)	PMN	+++	Mononuclear
Th2 cells	+++	PMN	(−)	Mononuclear

treated recipient mice with monoclonal antibodies (MoAb) to the adhesion molecules ICAM-1 and LFA-1 before Th-cell administration. In this report, we show that, in mice depleted of circulating neutrophils, tissue edema and PMN infiltration were inhibited at the site of deposition of activated Th2 (but not Th1) cells. Furthermore, *in vivo* administration of a MoAb to ICAM-1 completely inhibited edema formation and PMN infiltration induced by Th2 cells. Finally, administration of a MoAb to LFA-1 inhibited edema formation induced by Th2 cells, but not PMN extravasation.

MATERIALS AND METHODS

Animals and Reagents Female BALB/c mice, 4–6 weeks of age, were purchased from IFFA-Credo (Arbresle, France). Cells were cultured in Iscove's modified Dulbecco's medium supplemented with 1% modified Eagle's medium nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10% heat-inactivated fetal bovine serum (all from Gibco, Grand Island, NY); 1 mM sodium pyruvate (Fluka, Buchs, Switzerland); and 2×10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, MO). Concanavalin A was purchased from P-L Biochemicals (Milwaukee, IL). MoAbs to CD3ε (145-2C11 [8]), CD11a (H35-89.9 [9]), CD54 (YN1/1.7 [10]), and neutrophils (NIMP-R14; [11]) were used as described. Fluorescein-conjugated goat $F(ab')_2$ anti-mouse Ig was from Tago (Burlingame, CA).

Preparation and Culture of Polyclonal Th1 and Th2 Cells $CD4^+$ T cells with a Th1- or a Th2-like phenotype were prepared as described [12,13]. Briefly, activation of $CD4^+$ T cells in 3-d cultures with 3000 rad–irradiated splenocytes in the presence of concanavalin A (2 μg/ml) was followed by expansion for 11 d in medium containing 50 U/ml recombinant human IL-2 (Eurocetus, Amsterdam, The Netherlands), in the presence (Th2) or the absence (Th1) of concanavalin A (1 μg/ml). Cells expanded only with IL-2 secreted a Th1 lymphokine pattern upon restimulation (routinely greater than 500 U IL-2 and less than 500 U IL-4 per 10^6 cells); cells expanded with IL-2 in the presence of concanavalin A had a Th2 phenotype (less than 10 U IL-2 and greater than 10^4 U IL-4 per 10^6 cells [12,13]).

Footpad Swelling Assay At the end of the culture period, viable Th1 and Th2 cells were incubated for 1 h on ice with MoAb 145-2C11 (undiluted hybridoma supernatant), washed extensively in phosphate-buffered saline (PBS), and injected subcutaneously into the hind footpads of recipient animals, as described [4]. Unless otherwise stated, antibodies were given to the recipient mice intraperitoneally 2 h before injection of Th cells. The thickness of each paw was measured blinded with a precision micrometer immediately before and at various times after injection. Swelling was defined as the difference between paw thickness after injection and thickness before injection. Results are shown as mean swelling ± SD (n = 5 mice). The Wilcoxon–Mann-Whitney rank sum test was used for statistical analysis.

Histologic Examination Histology was obtained from the ears because the inflammatory infiltrate was very focal and thus difficult to find in the footpads. Anti–CD3ε-pulsed Th1 or Th2 cells (5×10^4 in 10 μl PBS) were injected subcutaneously into the center of the ears of recipient mice. Six or 48 h later, the mice were killed and the ears were fixed in 10% formalin, embedded in paraffin, and processed for histologic analysis [4]. Sections from six different depths of the center region of each ear were mounted, stained with hematoxylin and eosin, and examined blinded by an independent observer (two to three mice per experiment; at least two experiments per antibody). Semiquantitative analysis was based on an arbitrary 0–3 scale, with 0 representing no visible leukocytic infiltration and 3 indicating pronounced, dense infiltration. Injection of non-pulsed Th cells or PBS alone resulted in no detectable infiltration [4].

RESULTS

Because the 6-h PMN infiltration in Th2-cell–injected animals was accompanied by strong swelling **(Figs 1, 2*a*)**, we investigated the role of PMN in edema formation induced by Th2 cells, first by

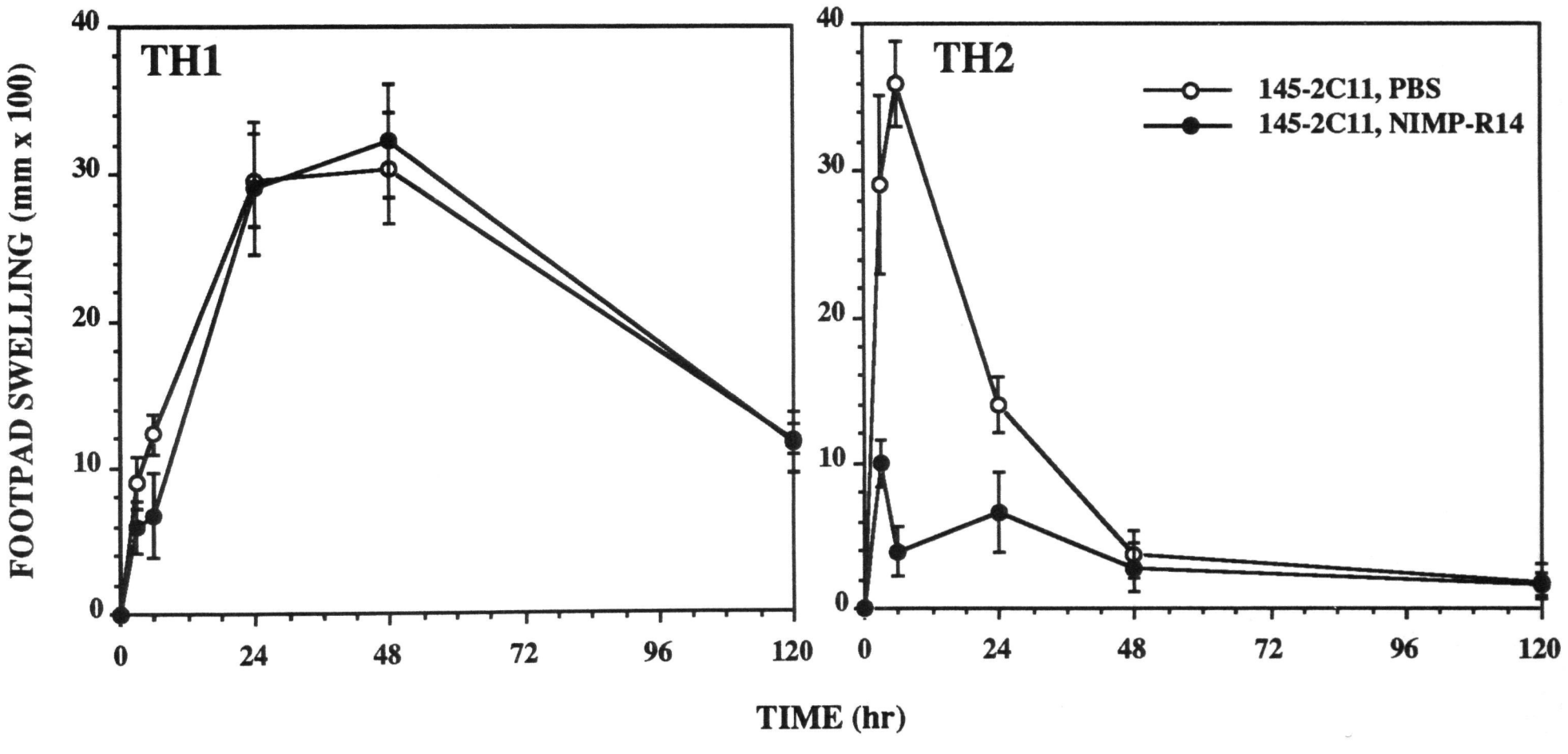

Figure 1. *In vivo* depletion of neutrophils abrogates Th2-mediated footpad swelling responses. Naive, healthy BALB/c mice received a single intraperitoneal injection of MoAb NIMP-R14 (1 mg/animal; *solid symbols*) or PBS (*open symbols*). Two hours later, the paws of each animal were injected with 2.5×10^5 syngenic anti–CD3ε-pulsed polyclonal Th1 or Th2 cells, as indicated, in 50 μl PBS. Footpad swelling was monitored over time. Data are shown as mean ± SD, n = 5 animals/group.

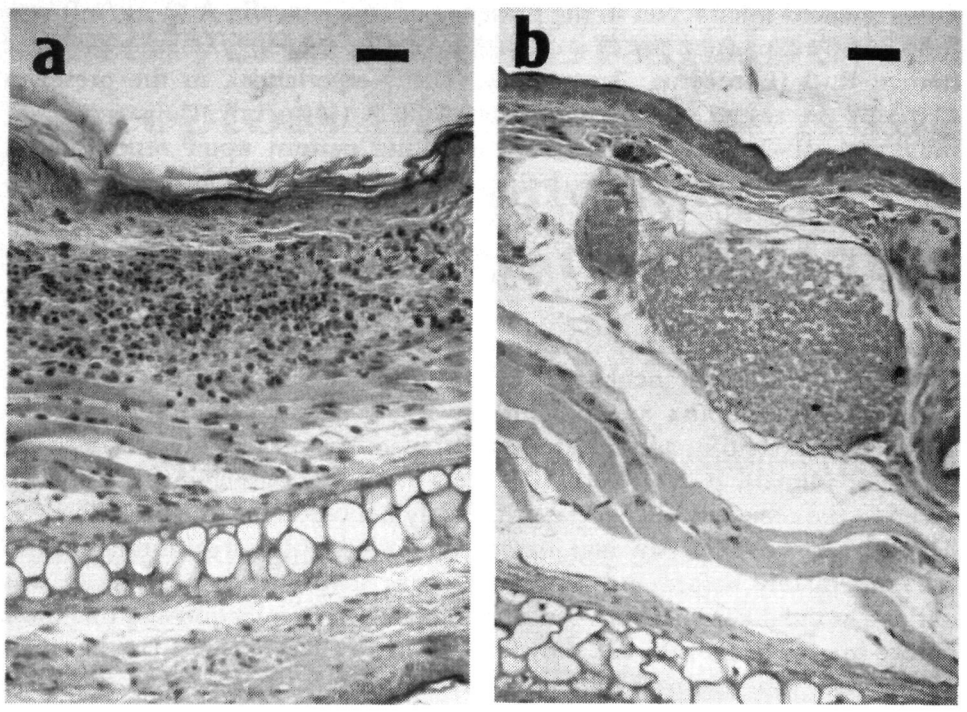

Figure 2. Treatment with MoAb NIMP-R14 abolishes PMN infiltration at the site of injection of Th2 cells. Fifty thousand 145-2C11–pulsed Th2 cells were injected into the ears of untreated mice (*a*) or of mice pre-treated 2 h previously with MoAb NIMP-R14 (*b*), as stated in the legends to **Figs 1** and **3**. The ears were obtained after 6 h and processed for histologic analysis (hematoxylin and eosin stain). *Bar*, 30 μm.

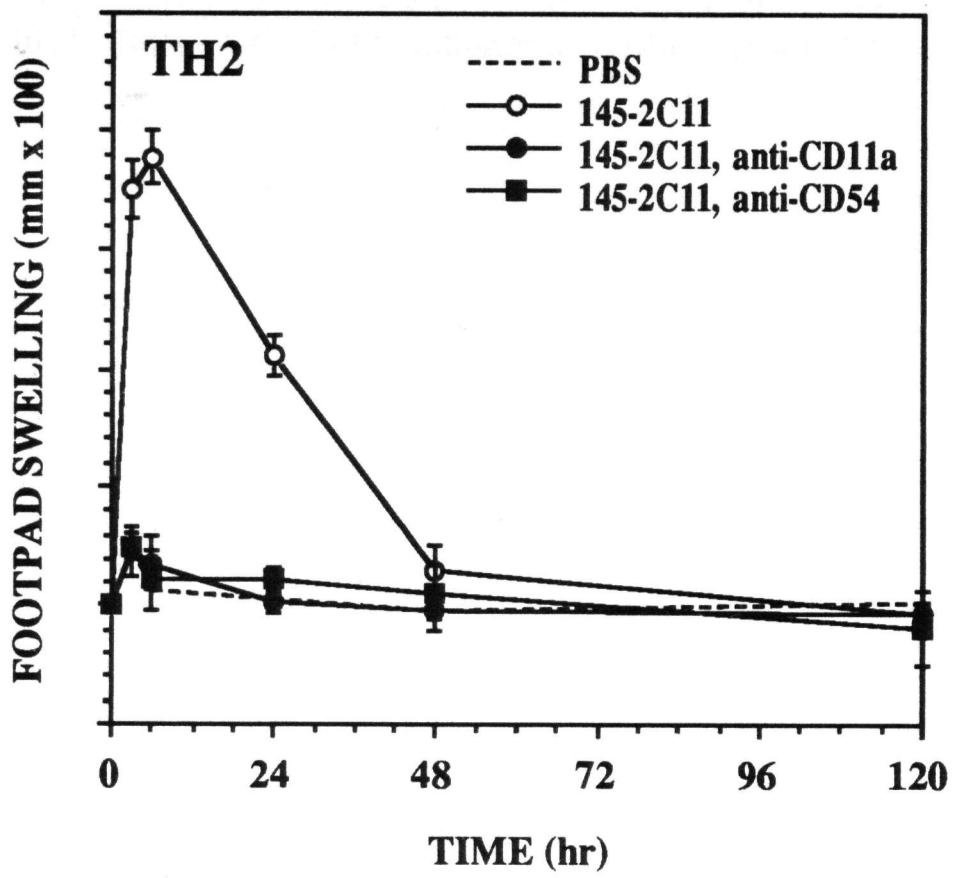

Figure 3. MoAbs to LFA-1 and to ICAM-1 abrogate Th2-mediated footpad swelling. Naive, healthy BALB/c mice received a single intraperitoneal injection of PBS (*open circles*), of MoAb to CD11a (H35-89.9; *solid circles*), or of MoAb to CD54 (YN1/1.7; *solid squares*; both MoAbs 1 mg/animal). Two hours later, the paws of each animal were injected with PBS (*dotted lines*) or with 2.5×10^5 syngenic anti–CD3ϵ-pulsed Th2 cells in 50 μl PBS. Footpad swelling was monitored over time. Data are shown as mean ± SD, n = 5 animals/group.

depleting the recipient mice with a MoAb cytotoxic to neutrophils. We injected recipient animals intraperitoneally with 1 mg of purified MoAb NIMP-R14 to an as yet uncharacterized neutrophil surface antigen [11]; such a treatment has been shown to deplete circulating neutrophils for 5 d or longer and to decrease strongly the lipopolysaccharide-induced local neutrophil infiltration [14]. Two hours later, the paws and ears of NIMP-R14–treated and control animals were injected subcutaneously with 2.5×10^5 anti–CD3ϵ-pulsed polyclonal Th1 or Th2 cells, and the ensuing swelling reactions were monitored over time. As shown in **Fig 1**, NIMP-R14 had no effect on Th1-mediated swelling (mean ± SD, 106.3 ± 12.5% of maximal swelling at 48 h), whereas it strongly inhibited the response with Th2 cells (10.8 ± 4.7% of maximal swelling remaining at 6 h; $p < 0.005$). A second experiment showed very similar results. NIMP-R14 also strongly inhibited the PMN extravasation and lesional infiltration seen 6 h after the injection of either Th2 cells (from grade 2 to grade 0; **Fig 2*b***) or Th1 cells (not shown). This indicates that PMN were required for tissue swelling induced by Th2 cells. However, the lack of prominent swelling despite a PMN infiltrate as observed in Th1-cell–induced reactions indicates that the mere presence of PMN in the tissue was not sufficient to induce edema. It is therefore possible that the PMN in infiltrates caused by Th2 cells were activated to induce edema, whereas they may not be activated in DTH1 reactions at 6 h.

Because LFA-1 and ICAM-1 are well known to be involved in leukocyte–endothelial-cell interaction and leukocyte extravasation, we next assessed the involvement of these adhesion molecules in DTH2. When recipient mice received a single intraperitoneal injection of either MoAb H35-89.9 to LFA-1 [9] or MoAb YN1/1.7 to ICAM-1 ([10]; ascites fluid diluted in PBS; 1 mg MoAb per animal) 2 h before the injection of Th2 cells, the swelling response was completely abrogated **(Fig 3)**. Anti–LFA-1 inhibited maximal swelling at 6 h by 90.5 ± 6.1%, and anti–ICAM-1 by 94.2 ± 6.9% (mean ± SD; in both cases, $p < 0.005$). Similar results were obtained from three (YN1/1.7) and two (H35-89.9) independent experiments. In addition, Th2-mediated PMN infiltration at 6 h **(Fig 4*a*)** was completely abrogated by administration of MoAb YN1/1.7 (from grade 3 to grade 0; **Fig 4*b***), which does not deplete circulating PMN [14]. It is interesting that although the swelling response induced by Th2 was completely blocked by MoAb H35-89.9, the tissue infiltration by PMN at 6 h was not prevented by administration of this antibody **(Fig 4*c*)**.

In DTH2, IL-4 is required within the first 2 h after injection of T cells [4]. To determine the sequence of IL-4 and ICAM-1 requirements, we injected recipient mice with 1 mg of YN1/1.7 at various times before or after Th2 injection. Efficient blocking of the swelling response was observed when MoAbs were injected 2 h before Th2 cells or when they were injected simultaneously **(Fig 5)**. When YN1/1.7 was injected 2 h after T cells, incomplete but still significant blocking was observed. When the MoAb was given 4 h after Th2 cells, no significant inhibition of the footpad swelling response was observed. Thus, ICAM-1 is required within the first 4 h, whereas IL-4 acts only within the first 2 h [4]. These findings are compatible with a sequential dependence of DTH2 first on IL-4 and then on ICAM-1.

DISCUSSION

The injection of activated Th2 cells into the skin results in local edema and PMN infiltration. The degree of infiltration with PMN varied among independent experiments (compare **Figs 2*a*** and **4*a***), whereas the swelling responses varied much less. The extent of leukocyte infiltration within the same experiment and condition, however, was constant. The precise localization of the infiltrate within the thickness of the ear had no influence on the results.

These experiments demonstrate clearly that Th2-mediated tissue swelling is dependent on PMN. PMN may induce swelling by infiltrating the site of Th2-cell deposition. Animals treated with anti–ICAM-1 and injected with Th2 cells exhibited neither swelling nor PMN infiltration. It is thus conceivable that the MoAb to ICAM-1 interfered with one of the steps required for tissue infiltration by PMN. ICAM-1 expressed on endothelium is a potential target of the antibody used. It is unlikely that MoAb YN1/1.7 blocked DTH2 by interfering with *in vivo* IL-4 synthesis by the injected Th2 cells, for the following reasons: 1) even very high doses (10 μg/ml or greater) of anti–ICAM-1 only partially (by 30% or less) inhibited IL-4 release from Th2 cells activated *in vitro* (data not shown); and 2) anti–ICAM-1 still inhibited DTH2 when injected at a time point (2 h after Th2 cells) at which *in vivo*

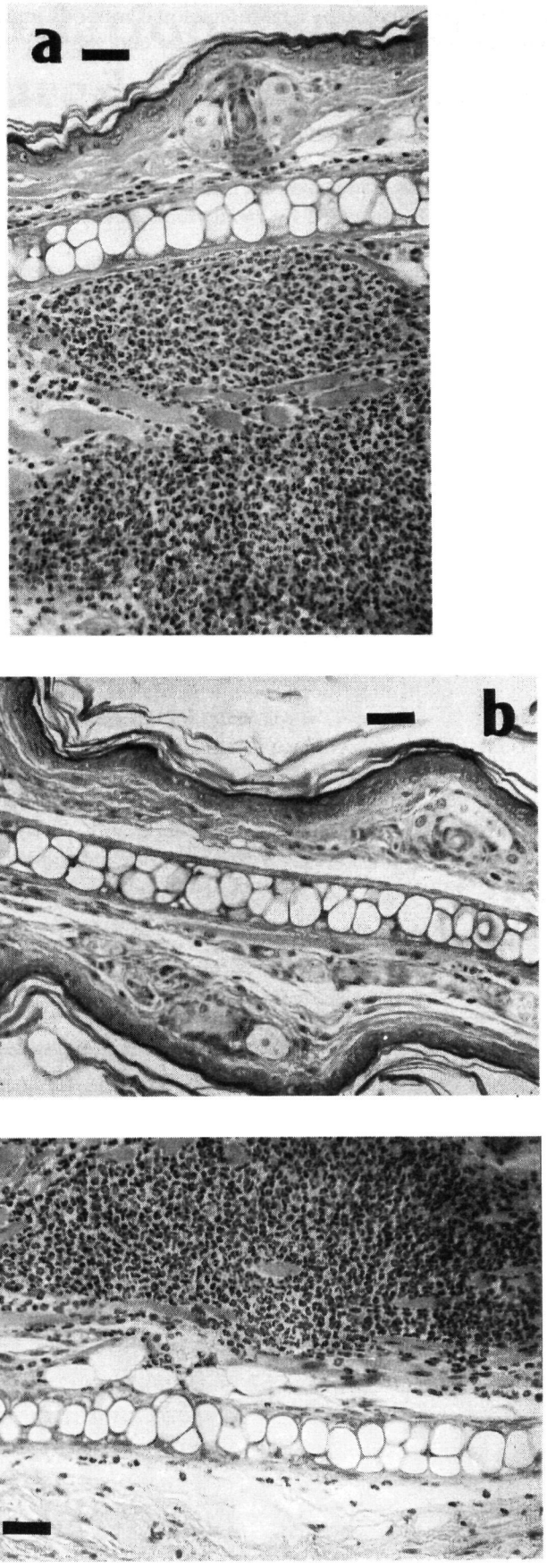

Figure 4. MoAb to ICAM-1 but not MoAb to LFA-1 abrogates PMN infiltration. Fifty thousand 145-2C11–pulsed Th2 cells were injected into the ears of untreated mice (*a*) or of mice pre-treated 2 h previously with MoAb YN1/1.7 (*b*) or H35-89.9 (*c*), as stated in the legends to **Figs 1** and **3**. The ears were obtained after 6 h and processed for histologic analysis (hematoxylin and eosin stain). *Bar*, 30 μm.

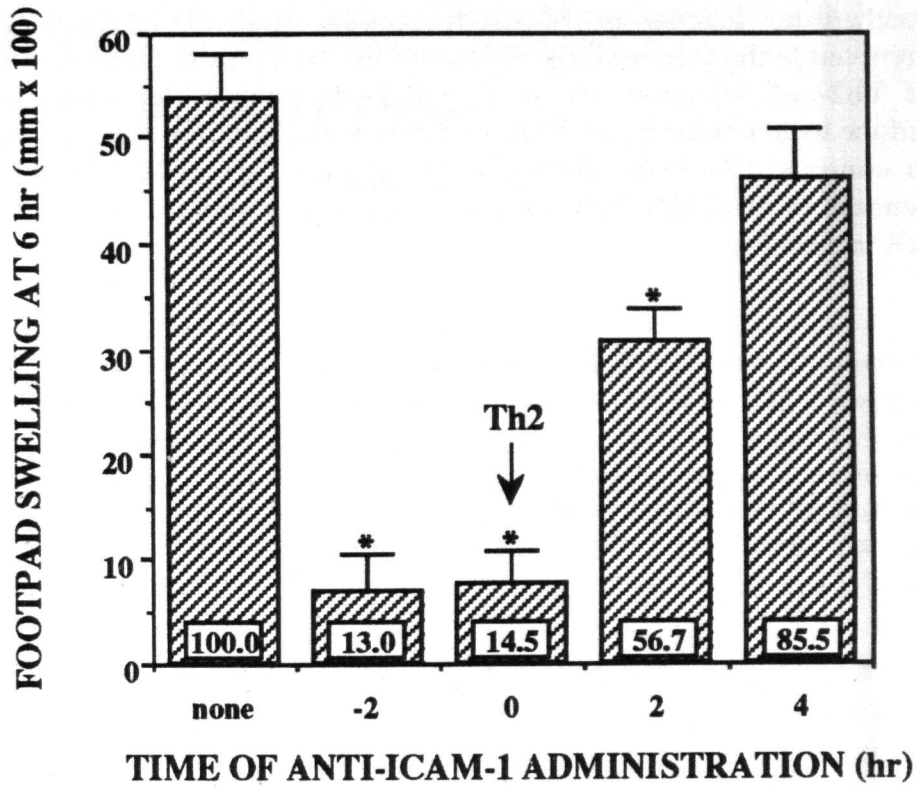

Figure 5. Effect of delayed administration of MoAb to ICAM-1. Naive, healthy BALB/c mice received a single intraperitoneal injection of MoAb YN1/1.7 (ascites fluid; 1 mg/animal) 2 h before, simultaneously with, or 2 or 4 h after the injection of 2.5×10^5 anti–CD3ϵ-pulsed Th2 cells into the hind paws. Results are shown as the swelling at 6 h as compared with maximal swelling in the absence of anti–ICAM-1 (mean ± SD, n = 5). *Asterisks* indicate significantly reduced swelling ($p < 0.05$). The *boxed figures* at the bottom of the columns indicate the percentage of remaining swelling, as compared to uninhibited swelling responses in control mice (*left column*).

neutralization of IL-4 no longer had any effect [4], supporting the view that ICAM-1 is required in a phase after IL-4 production.

PMN infiltration of skin tissue may not always be associated with edema. Th1-cell injection results in PMN infiltration at 6 h, but there was no significant edema at this time. It is thus possible that PMN actively contribute to tissue swelling in DTH2, in contrast to DTH1. The same speculation can explain our results with anti–LFA-1 (see below).

The antibody to LFA-1 used in our experiments (H35-89.9) was clearly not able to inhibit PMN extravasation, but was efficient in preventing edema formation induced by Th2 cells. The failure to block PMN extravasation may be due to the functional redundancy of adhesion molecules for extravasation rather than to failure to block the binding of LFA-1 to its counterreceptor *in vivo*, as H35-89.9 has been shown to inhibit efficiently hemorrhagic skin necrosis elicited by lipopolysaccharide and tumor necrosis factor [14]. It has been shown that neutrophils use both LFA-1 and Mac-1 as ligands for endothelial-cell ICAM-1 to achieve adherence and endothelial transmigration [15–18]. However, it has been suggested that the use of either β2 integrin may depend on the cellular activation status: resting neutrophils bind via LFA-1 whereas chemoattracted neutrophils adhere chiefly via Mac-1 [19]. It is therefore likely that LFA-1 is not absolutely necessary for PMN adhesion and extravasation, because the interaction between Mac-1 and ICAM-1 can compensate for the blocking of LFA-1. The role of Mac-1 in DTH2 remains to be assessed. There seems to be no such functional redundance for extravasation of PMN in the case of ICAM-1, because MoAb YN1/1.7 completely blocked the extravascular accumulation of PMN in our model.

The *in vivo* blocking of LFA-1, however, efficiently inhibited tissue swelling in DTH2 **(Fig 3*b*)** without affecting PMN extravasation **(Fig 4*c*)**. It is thus likely that the interaction of LFA-1 with one of its ligands is a prerequisite for edema formation by infiltrating PMN. This is compatible with the hypothesis that PMN require activation and/or degranulation to induce edema. This concept is supported further by observations showing that LFA-1 plays a role in PMN activation and degranulation [20].

The 48-h swelling response induced by Th1 was not dependent on the 6-h PMN infiltration, as it was not blocked by MoAb to neutrophils. Thus, the 48-h swelling response caused by Th1 cells seems to depend on mononuclear cells, because they are the predominant cell type infiltrating at 48 h after injection of Th1 cells. Edema at this time may be induced by either lymphocytes or macrophages and may also require cell activation. A role for host T cells is supported by the observation that Th1 cells reduced swelling responses when injected into nude recipient mice, as compared to euthymic littermates [21]. The role of macrophages in DTH1 could be explored with methods that efficiently deplete macrophages *in vivo*.

In conclusion, our results indicate that classic DTH mediated by Th1 cells and the newly described DTH2 reaction induce leukocyte infiltration that is indistinguishable by histology but use different

mechanisms leading to edema formation. In DTH2, PMN are involved in the 6-h swelling reaction; they are recruited into the site of Th2-cell injection via an ICAM-1–dependent step and may induce local edema by an LFA-1–dependent activation mechanism. In contrast, 24–48-h edema formation in DTH1 is largely independent of 6-h PMN infiltration, but correlates with mononuclear-cell infiltration.

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ANNOUNCEMENT

"Molecular mechanisms in dermal-epidermal interactions," a symposium sponsored by the Deutsche Forschungsgemeinschaft and the Alexander-von Humboldt-Foundation, will be held in Kloster Irsee/Germany (120 km west of Munich), March 10–13, 1995, organized by E. Christophers, Kiel; T. Krieg, Cologne; and D. Roop, Houston.

The aim of this symposium is to discuss the role of cell-cell and cell-matrix connections for cellular differentiation, embryonic development, wound healing, and inborn as well as acquired diseases of the skin. The meeting will include plenary lectures, poster sessions, and workshops.

Participants will be limited to 80 to allow a detailed and profound discussion.

Additional information is available from Prof. T. Krieg. Application forms for participation should be received by January 31, 1995. Send to Prof. T. Krieg, Department of Dermatology, 50924 Köln FRG.