High Frequency of IL-4–Producing CD4+ Allergen-Specific T Lymphocytes in Atopic Dermatitis Lesional Skin

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In atopic dermatitis (AD) hypersensitivity reactions to allergens are commonly observed and are assumed to make a major contribution in the pathomechanism of the disease. It may be expected that allergen-reactive Th cells play a central role in these reactions. In the present study the occurrence and function of allergen-specific T lymphocytes in dermal inflammatory lesions were studied. To this aim panels of randomly cloned CD4+ T cells from lesional skin biopsies of two housedust mite Dermatophagoides pteronyssinus (Dp)-allergic AD patients were screened for reactivity with Dp allergens. The results were compared with similar tests for Dp reactivity of T-lymphocyte clones (TLC) from the peripheral blood of these patients. In the panels of TLC generated from lesional skin (S-TLC), a considerable number of TLC appeared to be Dp-specific, 47% (n = 17) and 10% (n = 29), respectively. In the panels from the peripheral blood, the percentages of Dp-specific TLC were only 0% (n = 22) and 3% (n = 34), suggesting accumulation or expansion of these T cells in lesional skin.

The function of these TLC was studied by assaying the secretion of IL-4 and IFN-γ, which have been shown to be produced in aberrant ratios by Dp-specific TLC from the peripheral blood of AD patients (Wierenga et al: J Immunol 144:4651, 1990). All Dp-specific S-TLC produced IL-4 in combination with no or low levels of IFN-γ, whereas many of the non-Dp-specific S-TLC and blood-derived TLC (B-TLC) were observed to produce high levels of IFN-γ without significant amounts of IL-4. A functional consequence of these cytokine profiles was demonstrated by the finding that TLC producing substantial amounts of IL-4 enhanced expression of the low-affinity Fc receptor for IgE (CD23) on antigen-presenting cells to a greater extent than did IFN-γ-producing TLC. J Invest Dermatol 97:389 – 394, 1991

A high percentage of atopic dermatitis (AD) patients show elevated serum IgE levels [1] and positive intracutaneous skin test reactions to one or more environmental antigens [2]. The elevated IgE levels mainly represent IgE antibodies specific to the allergens concerned, as can be demonstrated by radioallergosorbent tests (RAST) [3]. Recently, we presented evidence that elevated serum levels of allergen-specific IgE in atopic patients are associated with an aberrant function of allergen-specific CD4+ T lymphocytes [4]. Allergen-specific TLC prepared from the peripheral blood of atopic donors, including an AD patient, were found to produce substantial amounts of IL-4 but no or only minimal levels of IFN-γ, whereas allergen-specific TLC from a non-atopic control individual and non-allergen-specific TLC from the AD patient produced IFN-γ without significant quantities of IL-4. Human IgE synthesis is known to be induced by IL-4 and suppressed by IFN-γ [5] and indeed only the IL-4—producing TLC from the atopic donors were able to induce in vitro IgE production. However, high levels of IgE probably only contribute to part of the processes involved in the pathomechanism of AD. The histologic appearance of the lesional skin is characterized by dermal perivascular infiltrates of mononuclear cells, mainly consisting of CD4+ T lymphocytes [6,7]. The additional accumulation of potential antigen-presenting CD1a+ Langerhans cells (LC) and the high expression of HLA-DR on the infiltrating CD4+ T cells [8] indicate an active local immune reactivity in the lesional skin. Strikingly, dermal infiltrates hardly contain any B cells, suggesting that IgE synthesis does not occur locally in the skin. These observations prompted us to investigate the possibility that dermatosis in AD is associated with a local intradermal cellular immune reaction of CD4+ T lymphocytes that specifically respond to allergens.

As most allergic AD patients are hyperreactive to housedust mite Dp [9,10], study of the role of Dp-specific T cells in causing and maintaining skin lesions seems to be of particular relevance [11]. Therefore, we have focused our study on the analysis of the frequency and function of Dp-specific CD4+ T cells in cellular infiltrates. For this purpose T cells from biopsies of AD lesional skin were randomly cloned and tested in vitro for Dp-specificity and lymphokine production. The results were compared with results from a similar study with randomly cloned T cells from autologous peripheral blood.
In view of our previous finding that IL-4/IFN-γ production ratios by DP-specific peripheral blood TCL of atopic patients are elevated [4], analysis of secretion of these two lymphokines by DP-specific TCL from lesional skin seemed to be of particular interest. Moreover, besides involvement in regulation of IgE synthesis, IL-4 and IFN-γ may be involved in other AD-associated processes. For instance, LC from AD patients were shown to express CD23, a low-affinity Fc receptor for IgE, and to bind IgE [12,13], particularly in clinically involved skin. Inasmuch as IL-4 was shown in vitro to induce expression of CD23 on LC [14], local production of IL-4 in lesional skin may be relevant in this respect. IFN-γ may also play an important role in the pathomechanism of AD, as experimental intradermal injection of IFN-γ in skin from healthy individuals was found to induce immunopathologic changes characteristic for inflammatory dermatoses [15] such as AD.

In this study we present data suggesting that DP-specific CD4+ T cells are accumulated in AD lesional skin and that most of these T cells produce substantial amounts of IL-4 in combination with low levels of IFN-γ. The implications of these results for the understanding of the pathomechanism of AD will be discussed.

MATERIALS AND METHODS

Donors  TLC were prepared from two patients, aged 33 (FA1) and 27 (FA2), both suffering from a classical chronic AD. In both patients high serum titers of DP- and cat allergen-specific IgE were found by RAST. Serum from patient FA2 also contained high levels of IgE reactive with a mixture of grass pollen antigens. Total serum IgE levels were high, 15,000 IU/ml (FA2) and 20,000 IU/ml (FA1).

Antibodies and Reagents  Anti-CD4 (OKT4) and anti-CD8 (OKT8) were obtained from Ortho Pharmaceutical Corp. (Raritan, NJ), anti-CD23 (Clonab T1) from Biotest (Dreieich, FRG), peroxidase-conjugated rabbit anti-mouse antiserum from Dakopatts (Glostrup, Denmark), and FITC-conjugated F(ab′)2 fragments of rabbit anti-mouse Ig from Zymed (San Francisco, CA). Data on affinity-purified polyclonal rabbit anti-human IL-4, rat anti-human IL-4 MoAb 11B4, mouse anti-human IFN-γ MoAb MD2, and the biotinylated mouse anti-human IFN-γ MoAb MD1 are described elsewhere [16,17]. The following affinity-purified enzyme-conjugated F(ab′)2 fragments of polyclonal antiserum were used: peroxidase-conjugated goat anti-mouse Ig (Tago, Burlingame, CA), alkaline phosphatase-conjugated goat anti-biotin (Zymed Laboratories, San Francisco, CA), and peroxidase-conjugated goat anti-rat Ig (Tago). Protein extracts of DP were prepared from dephosphorylated mite cultures obtained from Commonwealth Serum Laboratories (Melbourne, Australia). The contents of the major allergens Der p I and Der p II in the extract used in this study were described previously [4]. This extract was used 1:40 final dilution. Other allergens used were protein extracts from cat dander (12.5 µg/ml) and from a mixture of grass pollen (50 µg/ml), both obtained from ARTU Biochemicals (Lelystad, The Netherlands). Further reagents used were phytohaemagglutinin (PHA, Difco, Detroit, MI), concanavalin A (Con A, Pharmacia, Uppsala, Sweden), human rIL-2 (Cetus Corp., Emeryville, CA), human rIL-4 (a generous gift of Dr. J.E. de Vries, DNAX Research Institute, Palo Alto, CA), and human rIFN-γ (Biogen, Geneva, Switzerland).

Medium and Cell Culturing  During the cloning procedure, cells were cultured in Iscove's modified Dulbecco's medium (IMDM) (GIBCO, Paisley, UK), supplemented with 10% pooled complement-inactivated normal human serum (Central Laboratory Blood Transfusion Service, Amsterdam, The Netherlands) and gentamycin (80 µg/ml). When cells were stimulated for assaying cytokine production, huma serum was replaced by 10% fetal calf serum (FCS) (Flow, Irvine, UK) and further supplemented with 35 µg/ml human transferrin (Behring, Marburg, FRG), 150 IU/ml human insulin (Novo, Copenhagen, Denmark), and 3.5 µl/ml β-mercaptoethanol (Merck, Munich, FRG). The Epstein-Barr virus-transformed human B cell line JY and the human monocytic cell line U937 were maintained in IMDM containing 5% FCS. All cultures were incubated at 37°C in a humidified atmosphere containing 5% CO2.

Preparation of T-Cell Clones  Skin punch biopsies of 4 mm diameter were washed, minced in sterile PBS (Ca2++ and Mg2+-free), and dispersed cells were filtrated in order to remove the tissue debris. TCL were generated from filtrated fractions using a limiting dilution culture protocol resulting in a cloning efficiency of more than 60%, as described previously [18]. Cells were diluted (0.3 cell/well) in 96-well flat-bottom microtiter plates (Costar, Cambridge, MA). To each well 1 × 106 irradiated (3000 rad) allogeneic PBMC, 1 × 104 irradiated JY cells, 20 U/ml rIL-2, and 1 µg/ml PHA were added. After about 2 weeks, proliferating clones were further expanded in 24-well culture plates (Costar). Subsequently, clones were assayed for allergen specificity in a proliferation assay. TCL from peripheral blood were generated and tested similarly.

Cell Typing  Cell typing was performed by indirect immunoperoxidase labeling on cytospin cell preparations labeled with mouse anti-CD4 or anti-CD8 MoAb using a peroxidase-conjugated rabbit anti-mouse antiserum.

T-cell Proliferation Assay  TCL cells (2 × 104/well) were washed three times in HBSS + 2% FCS and stimulated with antigen in Costar 96-well flat-bottomed culture plates, using 3000 rad-irradiated autologous PBMC (104/well) as APC. Cells were cultured for 40 h, the last 16 h in the presence of 0.3 µCi/well of [3H]Tdr (Radiochemical Centre, Amersham, UK). Incorporation of [3H]Tdr was determined by liquid scintillation spectroscopy and expressed as mean cpm of triplicate cultures. TCL were considered to be DP specific if incorporated radioactivity exceeded 1500 cpm.
Figure 2. Proliferation of S-TLC after Dp-specific stimulation. After 48 h of culture in the presence of Dp allergens and autologous monocytes, [3H]TdR was added and cells were harvested 16 h later. Open bars, negative control experiments without Dp allergen; hatched bars, experiments with Dp allergen. TLC that appeared to be Dp specific according to the proposed criteria were 1S1, 1S2, 1S4, 1S5, 1S6, 1S8, 1S10, and 1S13 from patient FA1, and 2S7, 2S18, and 2S28 from patient FA2.

and was at least 10 x the amount measured in the control cultures without Dp antigen.

Preparation of TLC Supernatants TLC cells (4 x 10^6/well) were washed three times in HBSS + 2% FCS and stimulated with PHA (1 µg/ml) in the presence of irradiated allogeneic monocytes (10^5/well) in Costar 96-well flat-bottom plates in 200 µl culture medium. Cell-free supernatants were collected after 24 h. Separate but similar cultures were tested in a T-cell proliferation assay to confirm the reactivity of the TLC.

IL-4 and IFN-γ Assays Estimation of the IL-4 and IFN-γ contents of the TLC supernatants was performed with specific solid-phase sandwich ELISA assays, as described elsewhere [16,17]. For measuring IL-4 secretion, microtitre plates (Nunc, Roskilde, Denmark) were coated with a polyclonal rabbit anti-human IL-4 antiserum. Bound IL-4 was detected with rat anti-human IL-4 MoAb 11B4 and visualized with a goat anti-rat IgG-peroxidase conjugate using 2,2′-azinobis-3-ethylbenz-thiazoline-6-sulfonic acid (1 mg/ml; Sigma, St. Louis, MO) and H2O2 (1 µl/ml) in phosphate-citrate buffer (pH 5) as a substrate. Using a similar protocol, IFN-γ production was determined with a mouse anti-human IFN-γ MoAb (MD2), a biotinylated mouse anti-human IFN-γ MoAb (MD1), and alkaline phosphatase-conjugated goat anti-biotin, using p-nitrophenyl phosphate (1 mg/ml; Sigma) in 1 M diethanolamine-HCl buffer (pH 9.8) as a substrate. As references, rIL-4 and IFN-γ were used. The sensitivity of both assays was 100 pg/ml.

Analysis of CD23 Induction TLC cells (2 x 10^6/well) were stimulated with ConA (15 µg/ml) in the presence of cells of the human monocyteic cell line U937 (1 x 10^6/well) in Costar 96-well round-bottom plates in 200 µl culture medium. Cells were collected after 3 d, washed three times, and labeled on ice for 30 min with the anti-CD23 MoAb Tu1 in HBSS containing 2% FCS and 0.1% sodium azide. Subsequently, labeled cells were washed three times and incubated on ice for 30 min with rabbit anti-mouse-FITC conjugate. Cells were washed three times again and fluorescence was analyzed on a FACS (FACSAN, Becton-Dickinson), using a 480-nm argon ion laser. For each sample 5000 cells were analyzed. The gating scale of the histogram was adjusted to keep the percentage of positive cells in the negative control samples below 1%. Based on the dotplot parameters forward and side light scatter, the large U937 cells were gated in order to prevent any contribution of fluorescence by the TLC.

RESULTS

Relative Importance of Atopics According to Proliferation Profiles in PBMC Two patients (FA1 and FA2) were selected for suffering from severe AD characterized by erythema and immediate hypersensitivity to allergens. Both patients showed high titers of total serum IgE. RAST indicated high levels of serum IgE specific for Dp and some other allergens. In order to obtain an impression of the relative importance of Dp-specific T cells, we have tested the proliferative capacity of PBMC in response to Dp and the other allergens that were positive in RAST and prick tests, i.e., cat and grasspollen allergens.

The specific proliferative responses to Dp were much higher than to cat or grass pollen allergens (Fig 1). This observation points to a relatively high frequency of Dp-specific T cells in the circulation of both patients and stresses that, apart from B-cell reactivity to Dp, Dp-specific T-cell reactivity is significant in both patients. Based on these findings we have further focused our study on the comparison of frequency and function of Dp-specific T cells from skin lesions and peripheral blood of the two patients.

Frequency of Dp-Specific T Cells in Lesional Skin and PBMC From both patients TLC were randomly generated by limiting dilution cultures of cells derived from lesional skin biopsies. A method leading to a high cloning efficiency was applied, using mitogenic stimulation with PHA, in the presence of allogeneic feeder cells and IL-2 [18]. The frequency of Dp-specific TLC within the two panels of CD4+ skin-derived TLC (S-TLC) was determined by testing the proliferative reactivity to Dp in a [3H]TdR incorporation assay, using irradiated autologous PBMC as APC. According to the proposed criteria, eight of 17 S-TLC from patient FA1 (47%) and three of 29 S-TLC from patient FA2 (10%) appeared to be reactive with Dp (Fig 2).

Subsequently, we investigated to what extent these results reflected the frequencies of Dp-specific T cells in the peripheral blood. To this aim, panels of random TLC from the peripheral blood (B-TLC) of both patients were prepared and screened as well. None of 22 B-TLC from FA1 (0%) and only one of 34 B-TLC from FA2 (3%) showed a significant proliferative response to Dp (Fig 3). This observation suggests that Dp-reactive T cells are accumulated in lesional skin areas.

Cytokine Production Dp- and non-Dp-specific S-TLC and B-TLC were examined for their production of IFN-γ and IL-4 upon
mitogenic stimulation with PHA, in the presence of irradiated monocytes as accessory cells. Mitogenic stimulation results in cytokine secretion profiles similar to those seen after antigen-specific stimulation (Wierenga et al, submitted for publication). Cell-free supernatants were collected after 24 h and the contents of IFN-γ and IL-4 were determined with specific ELISA. All eight Dp-specific S-TLC that were tested produced IL-4 in combination with low amounts of IFN-γ (Fig 4). Much more variable cytokine profiles were observed in the panels of non-Dp-specific S-TLC (Fig 5). Simultaneous IL-4 and IFN-γ production was detected in four of six TLC, but the production ratios varied enormously from high levels of IFN-γ without IL-4 (TLC 157) to high levels of IL-4 without substantial IFN-γ (TLC 2S12). A similar pattern was found within the panel of B-TLC (Fig 6). All B-TLC produced detectable but strongly variable amounts of IFN-γ, whereas IL-4 production by most of these TLC was minimal or even undetectable. Only two B-TLC, 1B3 from patient FA1 and 2B5 from patient FA2, showed considerable IL-4 production, of which the latter was found to be Dp-specific in the earlier experiments (Fig 2). These results suggest that the frequency of IL-4-producing T cells is relatively high in lesional skin, especially within the Dp-specific population. However, although IL-4 seems to be preferentially produced, it seems that both IL-4 and IFN-γ secretion can be expected to occur in the skin lesions.

**Effects of TLC on CD23 Induction** To investigate the significance of local cytokine production by lesional skin T lymphocytes for the induction of CD23 expression on APC, we co-cultured ConA-stimulated S-TLC with cells of the monocytic cell line U937, which is known to be able to express this surface molecule. FACS analysis revealed a strong induction of CD23 on U937 cells in case of co-culture with IL-4-producing S-TLC, irrespective of the specificity of the TLC (Fig 7). Clones producing IFN-γ without substantial IL-4 also induced some CD23 expression on U937 cells, however, to a lesser extent than the IL-4-producing TLC. The notion that clones 1S4 and 2S12 increased CD23 expression even to nearly double the amount of expression induced by an optimal con-
centration of exogenous rIL-4 may point to additional or synergistic effects of IL-4 and IFN-γ on CD23 expression.

**DISCUSSION**

Results of our experiments have shown a relatively high frequency of CD4+ huedust mite Dp-allergen–specific T cells in lesional skin infiltrates of two AD patients. Although this phenomenon coincided with high Dp-specific proliferative responses of circulating T cells, the frequency of Dp-specific T cells in the peripheral blood was significantly lower in both patients, suggesting local accumulation of Dp-specific T cells in lesional skin. This could be the consequence of selective homing of these T cells into the skin.

On the other hand, accumulation may also result from local activation and subsequent proliferation of the allergen-specific T cells. Arguments in favor of local T-cell proliferation are the fact that lesional CD4+ T cells in AD express activation markers, such as HLA-DR [8], and the finding that LC in lesional skin of AD patients express CD23. The expression of CD23 on LC is upregulated by IL-4 and to a lesser extent by IFN-γ [12], which are both produced by activated T cells.

The specificities of the non-Dp-specific lesional skin TLC remain to be determined, but it seems likely that a certain percentage of these clones is reactive to allergens other than Dp, regarding for instance the low but significant proliferative responses of circulating T cells to cat and grasspollen allergens, as seen in patient PA2.

Results from this study further suggest a substantial local IL-4 production in lesional skin areas in response to challenges with Dp allergens, as the majority of the Dp-specific S-TLC were found to produce IL-4 upon stimulation. We further showed that especially IL-4–producing S-TLC were able to induce CD23 expression on APC. These findings may explain the expression of CD23 by LC and consequently the occurrence of IgE bound to the cell surface of LC in lesional skin [12,13], which in contrast to other dermatoses is characteristic for AD [19].

A large proportion of the circulating IgE antibodies in AD patients is specific for allergens, implying also that a substantial proportion of the IgE molecules on the cell surface of LC is allergen specific. Epstein-Barr virus-transformed B cells that have bound IgE via IL-4–induced CD23 were reported to be extremely efficient antigen-trapping and -presenting cells for T cells with the same specificity [20]. It should be expected, therefore, that allergen-specific IgE-bearing LC are excellent APC for allergen-specific T cells, too. Indeed, IgE+ LC appeared to be very potent APC for in vitro Dp-induced proliferative T-cell responses, whereas IgE− LC both from AD patients and from control individuals failed to present the allergen [21].

Chronic T-cell reactivity in AD can be explained by a continuous influx of environmental allergens in AD lesional areas by rubbing and scratching. With the combination of allergen, circulating allergen-specific IgE (indicated by IgST), the demonstrated local presence of CD23-expressing LC and IL-4–producing allergen-specific TLC in lesional areas, all elements for continuous T-cell reactivity are present in AD lesional skin. Experiments are underway to further investigate this presumed interrelationship between IL-4–producing Dp-specific T cells and facilitated presentation of Dp allergens by LC.

So far, no data have been published on a causal relation between local IL-4 production and tissue damage, as observed in dermatitis in general and in AD in particular. Recent information suggests, rather, a relation between IFN-γ production and inflammatory dermatoses [15,22]. Inasmuch as most Dp-specific S-TLC were shown to produce detectable amounts of IFN-γ, this may provide a contribution to the inflammatory effect of the local CD4+ T-cell reactivity in AD. However, regarding the limited quantities of IFN-γ production by these T cells, it seems likely that other signals play a role in this process as well. Experiments in the mouse model indicated that CD4+ T cells that produce IL-4 (Th2 cells) also produce IL-5 [23]. In line with this finding are recent observations by our group demonstrating that IL-4–producing Dp-specific CD4+ TLC from the peripheral blood of AD patients also produced high levels of IL-5 and that supernatants of these TLC were able to attract and activate eosinophils (Wierenga et al, submitted for publication). Activated eosinophils are frequently seen in AD lesional skin [24] and are known to cause dramatic tissue damage. Promoted by these data, IL-5 secretion by Dp-specific S-TLC from AD patients is presently being investigated.

Conclusively, based on the relatively high frequency of allergen-specific CD4+ T lymphocytes in AD lesional skin and their predominant IL-4 production as observed in this study, it may be hypothesized that type IV allergic reactions to allergens play a key role in the local pathomechanisms of skin lesions in AD. The local production of IL-4 by infiltrating, activated T cells explains CD23 expression on LC, which enables them to bind allergen-specific IgE and to present allergen more effectively. More patients need to be tested, however, to confirm this as a general feature in atopic dermatitis lesions.

**REFERENCES**


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