Knowledge about the nature of lymphocytes infiltrating atopic dermatitis skin is restricted to allergen-specific T cells. We investigated the proliferative capacities of T lymphocytes cultured in an antigen-independent way from biopsies of atopic dermatitis skin. When compared with peripheral blood mononuclear cells (PBMC) from healthy donors or atopic dermatitis patients, the skin-homing lymphocytes proliferated more vigorously in response to stimulation with anti-CD3 antibodies (1 μg/ml), reflecting their high response capacity. When stimulated with phytohemagglutinin (10 μg/ml) or staphylococcal enterotoxin A (0.1 μg/ml) the skin-homing lymphocytes achieved significantly lower proliferation levels than PBMC. In contrast to normal and atopic PBMC the skin-homing lymphocytes did not respond to tuberculin purified protein derivative (10 μg/ml). In the mixed lymphocyte reaction the skin-homing lymphocytes did not stimulate autologous PBMC to proliferate. We conclude that skin-homing lymphocytes have more pronounced immune deviations than PBMC in patients with atopic dermatitis. They represent a valuable approach for further investigating the pathogenesis of the disease. Key words: lymphocyte transformation test; autologous mixed lymphocyte reaction; phytohemagglutinin; staphylococcal enterotoxin A.

In the present study we describe the proliferative capacities of in vitro-cultured, non-antigen-stimulated skin-homing T lymphocytes by means of the lymphocyte transformation test. The results are compared with those for atopic peripheral blood mononuclear cells (PBMC), in order to elucidate whether lymphocytes from atopic dermatitis skin have more pronounced immune deviations and could therefore be more relevant for determining the pathogenesis of the disease. In order to include different pathways of T-cell activation the cells were stimulated by monoclonal antibodies to the CD3 complex, a mitogen (phytohemagglutinin; PHA), a superantigen (staphylococcal enterotoxin A; SEA) and a conventional antigen (tuberculin purified protein derivative; PPD). The autologous mixed lymphocyte reaction was also performed to test whether skin-homing lymphocytes induce a proliferative response in lymphocytes from peripheral blood.

MATERIAL AND METHODS

Patients

Skin biopsies were obtained from 15 consecutive adult patients aged 18–54 years (median age 27 years) with a flare-up of moderate-to-severe atopic dermatitis. Systemic treatment including UV light had not been given and the biopsied area had not been treated topically within the previous week. All but one of the patients had high IgE levels (>800 kU/l) and type I allergies. Two control groups were enrolled for peripheral blood samples: 11 patients with atopic dermatitis (age 19–54, median age 29 years) and 22 healthy volunteers (age 18–64, median age 26 years). All subjects gave their informed consent and the study was approved by the local ethics committee.

Preparation of cells

PBMC were isolated from fresh heparinized blood by density gradient centrifugation (Lymphoprep; Nycomed Pharma) and washed three times in Hank’s balanced salt solution at room temperature. Cultures of skin-homing lymphocytes were established as described elsewhere (13). Briefly, a 4-mm punch biopsy was taken from atopic dermatitis lesional skin and placed into a medium comprising 90% RPMI 1640 with glutamax (GibcoBRL) and 10% human AB serum, supplemented with antibiotics and interleukins 2 and 4. Lymphocytes cultured from skin biopsies were used for the lymphocyte transformation test after being cultured for 19–122 days (mean 32 days). Five cell cultures were used for mixed lymphocyte reactions after a culture period of 18–60 days (mean 35 days). Prior to use the in vitro-cultured skin-homing lymphocytes were washed three times in Hank’s solution and seeded into medium lacking cytokines.

In order to obtain monocytes by the plastic adherence technique, PBMC were incubated in the medium described previously in cell...
culture flasks (Nunc) for 45 min at 37 °C. The non-adherent cells were discarded and the flasks rinsed thoroughly with Hank's solution containing 10% human AB serum. Thereafter Hank's solution with 2% serum was added to the flasks which were left on ice for 30 min. The monocytes were detached from the plastic by vigorous shaking, washed once and seeded to medium (RPMI 1640, antibiotics and 2% human AB serum; 4 °C). Only viable cells were counted, as estimated by the trypan blue dye exclusion test.

**Lymphocyte transformation test**

Triplicate cultures of $2 \times 10^5$ cells in 200 μl of medium (RPMI 1640 with glutamax supplemented with 10% human AB serum and antibiotics) were prepared in flat-bottomed 96-well plates (Nunc). For anti-CD3 stimulation the plates were coated with 100 μl mouse anti-human-CD3 monoclonal antibodies (clone CLB-T3/4; E; Research and Diagnostics) per well at a concentration of 1 μg/ml, incubated overnight at 4 °C and thereafter washed twice with phosphate-buffered saline and once with medium. The cells were also stimulated with phytohemagglutinin (PHA; Sigma; 0.1 μg/ml) or tuberculin purified protein derivative (PPD; Statens Seruminstitut, Denmark; 10 μg/ml). Upon PPD stimulation of 5 cell lines $2 \times 10^4$ (10%) autologous monocytes were added per well as antigen-presenting cells. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air for 3 days upon PHA stimulation, for 4 days upon anti-CD3 stimulation and for 5 days upon PPD and SEA stimulation. Unstimulated cultures were always prepared in parallel.

**Mixed lymphocyte reaction**

Stimulator cells ($2 \times 10^7$/ml) were treated with mitomycin C (Sigma; 30 μg/ml) in medium (RPMI 1640 with glutamax supplemented with 10% human AB serum and antibiotics) for 2 h at 37 °C and subsequently washed 3 times in Hank's solution containing 2% bovine serum albumin (Sigma). Triplicate cultures of $1 \times 10^5$ mitomycin-treated skin-homing lymphocytes (stimulators) and $1 \times 10^5$ autologous or allogeneic PBMC (responders) were mixed in the 96-well plates in 200 μl of medium as described above and incubated for 6 days. Mitomycin-treated autologous and allogeneic PBMC served as negative and positive controls, respectively, under similar conditions.

\[^{3}H\]thymidine incorporation into DNA

The cells were pulse-labeled with 1 μCi \[^{3}H\]thymidine (Amersham) per well 18 h prior to harvesting using an automatic cell harvester (Skatron). Incorporated radioactive thymidine was counted in a liquid scintillation counter (Packard) and mean counts per minute (cpm) of triplicate cultures were subsequently calculated.

**Statistical analysis**

Data are expressed as mean ± SD. A p value of ≤0.05 was considered to be statistically significant. The ability of stimulants to increase proliferation in each particular cell group was analysed by the Wilcoxon signed rank test. Comparisons of groups were performed by the Mann–Whitney U test.

**RESULTS**

Unstimulated skin-homing lymphocytes proliferated similarly to PBMC except for 1 cell line out of 15 which showed a high spontaneous proliferation rate (data not shown). The time of culturing had no effect on the in vitro reactivity of skin-homing lymphocytes to the stimuli (data not shown).

Antibodies to CD3 surface antigen were able to stimulate proliferation of lymphocytes in all three groups ($p < 0.01$). PBMC from normal controls and atopics proliferated equally upon anti-CD3 stimulation ($p > 0.1$) whereas skin-homing lymphocytes responded with higher proliferation rates than PBMC from either group ($p < 0.05$; Fig. 1A).

PHA stimulated lymphocyte proliferation in all 3 groups ($p < 0.005$). There was no difference in responses to PHA stimulation between PBMC from atopics and normal controls ($p > 0.5$), but skin-homing lymphocytes proliferated less than PBMC from both groups ($p < 0.05$; Fig. 1B).

SEA was able to stimulate lymphocytes from all 3 groups ($p < 0.005$). PBMC from atopics reached lower levels of proliferation than those from normal controls ($p < 0.05$). Skin-homing lymphocytes responded to SEA even less than atopic PBMC ($p < 0.05$; Fig. 1C).

PPD increased the proliferation rate of both atopic and normal PBMC ($p < 0.005$), which responded equally well to the stimulus ($p > 0.1$). However, PPD failed to stimulate the skin-homing lymphocytes ($p > 0.05$; Fig. 1D). The presence of autologous monocytes in the culture did not improve the response of skin-homing lymphocytes to PPD stimulation (results not shown).

Results of the mixed lymphocyte reaction are shown in Fig. 2. None of the 5 skin-homing lymphocyte cultures stimulated autologous PBMC to proliferate when compared with the negative controls, whereas allogeneic PBMC induced apparent proliferation, indicating appropriate test conditions. Also, the skin-homing lymphocytes were able to stimulate allogeneic PBMC.

**DISCUSSION**

The present study describes the functional activity of the range of T lymphocytes homing to the skin of atopic dermatitis patients after the cells are cultured in an antigen-independent way. The main findings are that, in comparison with atopic PBMC, the skin-homing lymphocytes have increased reactivity to anti-CD3 stimulation, their reactivity is reduced upon PHA and SEA stimulation and they do not respond to PPD.

One could argue that the proliferative capacities of the skin-homing lymphocytes become reduced during prolonged in vitro culture, but we did not observe any correlation between the age of cultures and cell reactivity. Also, the strong proliferation upon anti-CD3 stimulation clearly excludes this presumption. Our data indicate that skin-homing lymphocytes have a facilitated signal transduction via the CD3 molecular complex, i.e. downstream from the T cell antigen receptor. Interestingly, a similar proliferation pattern was found on monocyte-depleted blood lymphocytes from atopic dermatitis patients by Glinski et al. (20).

We demonstrated that stimulation of the skin-homing lymphocytes with PHA, a non-physiological mitogen, gives a markedly lower proliferation compared with both PBMC groups. This finding is difficult to interpret as the exact mode of action of PHA is not known. PBMC from atopic patients gave similar results to those from healthy controls, thus confirming previous observations (21, 22).

*Staphylococcus aureus* colonization on the skin of atopic dermatitis patients is extremely common (23, 24), but its contribution to the pathogenesis of the disease is poorly
understood. Several studies have suggested that staphylococcal enterotoxins can exacerbate atopic dermatitis (25–27). We found that PBMC from atopic dermatitis patients achieved significantly lower proliferation levels in response to SEA than normal PBMC, as also shown by König et al. (28). Although the skin-homing lymphocytes were able to respond to SEA, they proliferated even less than atopic PBMC. Nevertheless, the response may be sufficient to exacerbate and maintain dermatitis in atopic skin and does not exclude the possible role of staphylococcal enterotoxins in this. It is well known that by means of its superantigenic properties SEA can stimulate a wide range of T cells (29). However, in vivo exposure to staphylococcal superantigens is reported to induce anergy of the superantigen-reactive T-cell clones in mice (30, 31). Also, Yoshino et al. (32) showed recently that a majority of PBMC from patients with severe atopic dermatitis, with suppressed in vitro proliferative responses to staphylococcal enterotoxin B, express an early apoptosis cell marker APO2.7. Thus, mechanisms of anergy and/or apoptosis ensuing from substantial exposure of the immune system to the exotoxins can lead to the reduced response to SEA of lymphocytes from atopic dermatitis skin. This in turn can result in an inability of patients with atopic dermatitis to control skin colonization by this microorganism.

Our results describing normal response of PBMC from atopic dermatitis patients to PPD are in concordance with previous studies (21, 33). Contrary to atopic PBMC, the skin-homing lymphocytes from the same subjects were not able to respond to PPD, a finding that did not change after addition of autologous monocytes as antigen-presenting cells in the cultures. The non-responsiveness cannot simply be explained as a phenomenon acquired during in vitro culture, as we found that T cells obtained under similar conditions from a biopsy of a positive tuberculin skin test of a non-atopic person responded well to PPD (unpublished observation). Elliott & Hanifin (34) described that PBMC from atopic dermatitis patients have normal in vitro responsiveness to certain microbial antigens while showing absence of cutaneous delayed hypersensitivity to the same antigens. As PBMC from atopic individuals proliferate normally upon

Fig. 1. Proliferation rates of lymphocytes from peripheral blood of healthy, non-atopic persons (Control PBMC), and atopic dermatitis patients (Atopic PBMC), and of skin-homing lymphocytes (SHL) from atopic dermatitis skin biopsies following in vitro culture. The proliferative response of skin-homing lymphocytes is augmented upon anti-CD3 stimulation (A), decreased upon phytohemagglutinin (PHA) and staphylococcal enterotoxin A (SEA) stimulation (B and C, respectively) and absent upon tuberculin purified protein derivative (PPD) stimulation (D). Error bars are ±SD from mean.

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PPD stimulation, the nonresponsiveness of the skin-homing lymphocytes seems instead to be caused by a lack of tuberculin-reactive cells in the skin infiltrate or a downregulation of their function. This is supported by the findings of diminished tuberculin skin reaction occurring during exacerbation of atopic dermatitis (35) and an inverse association between tuberculin skin responses and atopy (36).

In vitro cultured activated T cells, expressing HLA-DR, are known to induce proliferation of autologous lymphocytes with suppressor activity (36–39). The phenomenon is thought to reflect an immunoregulatory mechanism occurring in vivo. We observed that skin-homing T lymphocytes did not stimulate autologous blood lymphocytes, although they express HLA-DR (19). This could indicate impaired immune surveillance mechanisms towards skin-homing lymphocytes.

The present results provide evidence that T cells grown in vitro from atopic dermatitis lesional skin have more pronounced immune deviations than atopic blood lymphocytes. They offer a valuable approach to further investigating the pathogenesis of the disease.

ACKNOWLEDGEMENT

A. V. was supported by scholarships from the Danish Rectors’ Conference and the Danish Health Research Council, grant no. 9600822 (Aarhus University—Novo Nordisk Centre for Research in Growth and Regeneration).

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T cells from atopic dermatitis skin