

Substance P Binding to Peripheral Blood Mononuclear Leukocytes in Atopic Dermatitis

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Substance P has various immunomodulatory effects, including *in vitro* modification of lymphocyte proliferation and cytokine release. Elevated levels of substance P and increased staining of substance P-positive nerve fibres have been reported in atopic dermatitis patients. We examined fluoresceinated substance P binding to a range of lymphocyte subsets and compared the results in atopic dermatitis, non-atopic psoriasis patients and normal controls.

Fluoresceinated substance P and phycoerythrin-labelled monoclonal antibodies to CD3, CD4, CD8, CD57, CD19 and CD14 were incubated in duplicate with Ficoll-Hypaque separated peripheral blood mononuclear leukocytes. With flow cytometry the fluoresceinated substance P-positive cells were identifiable as a peak of positively fluorescent cells, and the percentages of positive cells were measured.

We have demonstrated binding of fluoresceinated substance P to all subsets examined, with significantly less binding to atopic dermatitis CD3-, CD8- and CD57-positive cells. This may affect cytokine release and hence be important in the pathogenesis of atopic dermatitis. **Key words:** flow cytometry; fluoresceinated substance P; mononuclear cell leukocyte subsets.

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Substance P (SP) is an eleven amino acid peptide that belongs to the tachykinin family. It is synthesized in the dorsal root ganglia and functions as a neurotransmitter in both the peripheral and central nervous systems. It is released by sensory nerves innervating a wide variety of peripheral tissues, including skin, gut associated lymphoid tissues, joints, upper airways and smooth muscle (1). In addition, intestinal eosinophils have been shown to contain SP-containing granules (2). SP is a potent vasodilator and intradermal injection of SP produces a wheal and flare reaction (3). The partial inhibition of this effect by antihistamines and the ability of SP to cause *in vitro* mast cell degranulation suggest that this effect is in part due to histamine release (4).

In addition to a local neurogenic inflammatory effect on skin, SP has important immunomodulatory activity. It directly stimulates interferon gamma release and T-cell proliferation in human peripheral blood lymphocytes (PBL) (5) and modulates antibody synthesis (6), guinea pig macrophage phagocytosis (7) and rabbit neutrophil function (8). In addition, it stimulates human monocyte chemotaxis (9) and cytokine release (10). SP receptors with high affinity characteristics have been identified on human T-lymphocytes (11) and B-lymphoblastoid cell lines (12), murine Peyer's patch B- and T-lymphocytes and splenic B-lymphocytes and guinea pig macrophages (7).

A previous study using fluorescence-detection flow cytome-

try recorded the binding of dichlorotriazinylamino-fluorescein-labelled SP to $21 \pm 10\%$ (mean \pm SD) of human T-lymphocytes (11). Binding to CD4 and CD8 subsets was 18% and 10%, respectively, and no binding was seen on monocytes or polymorphonuclear leukocytes.

The pathogenesis of atopic dermatitis has not been fully established but appears to involve a complex interaction between genetic susceptibility, precipitating environmental factors and disordered immune responsiveness. There is increasing evidence that neuropeptides, in particular SP, may be involved in the pathogenesis of atopic dermatitis (13–16). Exacerbations of atopic dermatitis can be provoked by stress, scratching and sweating, which may be the result of neurogenic inflammation. Several studies have demonstrated elevated cutaneous levels (16) and an increase in SP-positive nerves in lesional (13, 14) and non-lesional (15) atopic dermatitis skin. Elevated cutaneous levels of SP have been reported in atopic dermatitis (16), although others have failed to show a difference from normal controls (17).

In this study we compare mononuclear cell leukocytes (MNL) SP binding in atopic dermatitis patients with normal controls and non-atopic psoriasis patients. It is well established that the inflammatory reaction in the skin of patients with atopic dermatitis involves immune dysregulation. The immunopathology of atopic dermatitis has been identified as a delayed type hypersensitivity reaction, showing a dominance of activated T-lymphocytes and cells of the macrophage series (18). Homing of these cells and up-regulation of the inflammatory response are believed to be driven by released cytokines (19). However, an immunomodulatory role of SP in atopic dermatitis has not been established. We examined fluoresceinated SP (SP*) binding to a range of lymphocyte subsets and compare the results in atopic dermatitis patients with psoriatic patients and normal controls.

MATERIALS AND METHODS

Before this study was commenced, approval was granted by the hospital ethical committee, and all subjects gave informed consent to participation.

Subjects

The details of the patients are shown in Table I. The atopic dermatitis patients were otherwise well and fulfilled the criteria of Hanifin & Rajka (20), with moderate to severe atopic dermatitis on no systemic therapy. The patients were scored on a standard grading system based on the extent of dermatitis, and the course and the intensity of itch (21). A score summation of 3–4 is equivalent to mild, 4.5–7.5 moderate and 8–9 severe dermatitis. Our patients had moderate atopic dermatitis, with scores of 5–7. Topical steroids were avoided during the week before venepuncture. Normal controls and psoriasis patients had no personal or family history of atopy and were prick test-negative to a battery of nine common allergens. The psoriasis patients had mild to moderate psoriasis on no systemic therapy.

Table I. Details of the patients with atopic dermatitis (AD), normal controls (NC) and psoriatic patients (Ps)

| | Age (years) Mean (range) | Sex M:F |
|------------|-----------------------------|------------|
| AD n=12 | 32 (18-51) | 7:5 |
| NC n=11 | 38 (24-47) | 4:7 |
| Ps n=6 | 37 (23-50) | 4:2 |

Mononuclear cell (MNL) separation

Venous blood was taken between 09.00-10.00, and MNL were isolated by Ficoll-Hypaque separation. The blood was diluted with an equal volume of RPMI 1640 and gently laid on Ficoll-Hypaque separation medium. This was centrifuged at 350 g, and the MNL and plasma were removed by gentle suction. The MNL were diluted 1:1 with PBS centrifuged at 300 g, resuspended, centrifuged at 250 g, and finally resuspended in PBS. The MNL were counted using a haematocytometer, and the cells were diluted to 5×10^6 cells/ml with PBS.

Preparation of SP (A kind gift from Dr C J Dean, Department of Immunology, Institute of Cancer Research, Sutton, Surrey)*

Five milligrams of dichlorotriazinylamino-fluorescein (Sigma) and 1 mg SP (Sigma) were dissolved in 0.2 M sodium carbonate and 0.15 M sodium chloride (pH 9.0) and rotated together at 37°C for 2 h. After centrifugation the conjugated material was applied to a Bio-Gel P2 gel filtration column, and fractions were collected and read at 280 nm and 490 nm. The fractions with a fluorescein/protein ratio between 1.0-1.5 were pooled.

Direct labelling

Fifty microlitres of MNL were incubated with phycoerythrin (PE)-labelled monoclonal antibodies (Table II) to CD3, CD4, CD8, CD57, CD19 and CD14 (10 µl) (Sigma) for 20 min. The number of subjects examined for each MNL subset are shown in Table III. The cells were washed, and 10 µl SP* diluted 1/4 in PBS was added. After 20 min the cells were washed with PBS and 1% paraformaldehyde in PBS added. Control experiments using dichlorotriazinylamino-fluorescein (10 µl diluted 1/4 in PBS) instead of SP*, and comparing PE labelling with each monoclonal antibody with and without SP*, were performed.

Flow cytometry

In a dual immunofluorescence procedure, the various subsets of MNL were each stained in separate tubes with appropriate monoclonal antibodies conjugated to the red/orange dye PE, and those binding SP* were identified with the green dye fluorescein (FITC). Data was acquired using a FACScan flow cytometer (Becton-Dickinson) by standard methods, and cell populations were selected by gating from dual parameter displays of PE fluorescence against side scatter. The

Table III. Mean percentage (and SD) SP*-positive cells found in each MNL subset in the three groups of subjects (AD=atopic dermatitis, NC=normal controls and Ps=psoriatic patients)

| | | CD3 | CD4 | CD8 | CD57 | CD19 | CD14 |
|----|------|------------|-------|-------------|--------|------------|--------|
| AD | Mean | 16 | 15 | 16 | 11 | 37 | 8 |
| | SD | 6.1 | 4.7 | 6.2 | 3.7 | 13.0 | 2.2 |
| | | (n=12) | (n=6) | (n=12) | (n=12) | (n=12) | (n=10) |
| NC | Mean | 30 | 17 | 28 | 19 | 43 | 11 |
| | SD | 7.1 | 6.7 | 8.0 | 8.5 | 7.6 | 3.5 |
| | | (n=11) | (n=7) | (n=10) | (n=10) | (n=10) | (n=8) |
| Ps | Mean | 27 | 15 | 29 | 18 | 33 | 9 |
| | SD | 17.1 | 5.3 | 9.1 | 5.5 | 7.6 | 4.8 |
| | | (All n=6) | | | | | |
| | | $p < 0.01$ | | $p < 0.001$ | | $p < 0.01$ | |

The *p* values are the results of analysis of variance comparing the three groups of subjects.

SP*-positive FITC fluorescence of the gated population for each subset was presented as a histogram. The negative control was used to set a delineating marker, and the cells staining with SP* were recorded as a percentage (Fig. 1).

Statistical analysis

Analysis of variance (ANOVA) was used to compare the three groups of subjects. Differences between pairs of groups were tested using the Scheffé's test.

RESULTS

An example of the histograms for each lymphocyte subset is shown in Figs. 1 and 2. The shape of the histogram varies with the different lymphocyte subsets tested, depending on the percentage of SP-positive cells present. Thus the CD19-positive cells show a larger second peak compared with the other examples shown, as there was a greater percentage of SP-positive cells within this lymphocyte subset. There was no difference in the shape of the peak of fluorescence between the three groups of subjects. The results showing the percentage of SP*-positive cells in each MNL subset are in Table III. When the three groups of subjects were compared, the binding of SP* to CD3-, CD8- and CD57-positive cells was significantly lower in the atopic dermatitis patients compared to the psori-

Table II. Phycoerythrin (PE) monoclonal antibodies used

| PE monoclonal ab | Cell type |
|------------------|---------------------|
| CD3 | Pan T-cell |
| CD4 | Helper T-cell |
| CD8 | Suppressor T-cell |
| CD57 | Natural killer cell |
| CD19 | B-cell |
| CD14 | Monocyte |

(All supplied by Sigma, UK, and 10 µl added to 50 µl of MNL).

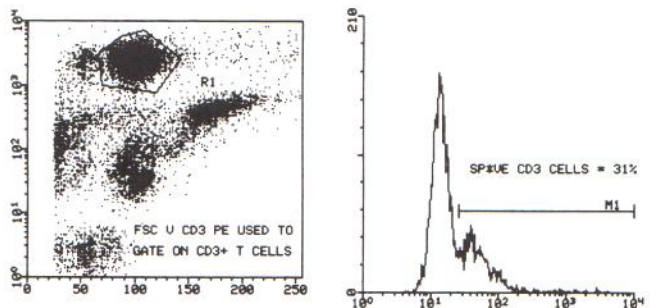


Fig. 1. The cells identified by the PE-conjugated CD3 monoclonal antibody are gated, and the FITC fluorescence of those cells is displayed in a histogram. The SP*-positive cells are seen as a peak of positively fluorescent cells and the percentage of positive cells is measured using the marker M1.

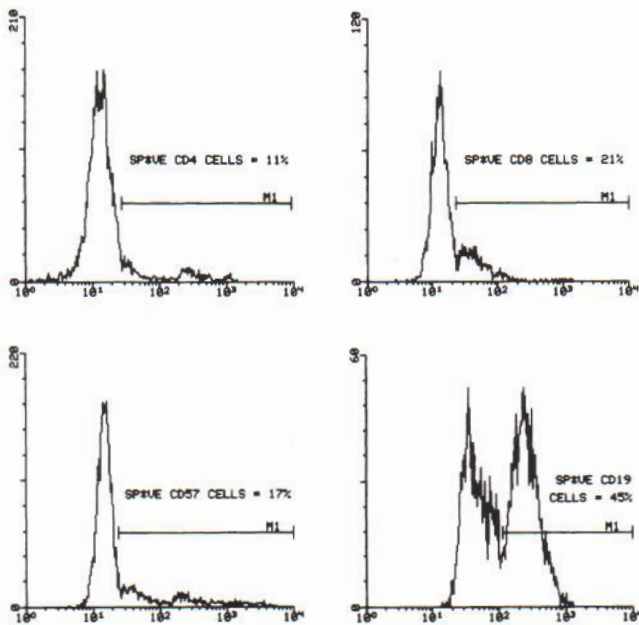


Fig. 2. Typical histograms displaying the FITC fluorescence of the CD4-, CD8-, CD57- and CD19-positive cells. The percentage of SP*-positive cells is indicated by the marker M1.

asis patients and normal controls ($p < 0.01$, < 0.001 , < 0.01), with no significant difference seen in the other cell subsets. There was no significant difference between the psoriasis patients and normal controls. When two groups of subjects were compared, the binding of SP* to CD3-, CD8- and CD57-positive cells was significantly lower in the atopic dermatitis patients compared to the normal controls ($p < 0.01$, < 0.01 , < 0.01). The binding of SP* to CD3- and CD57-positive cells was non-significantly lower in the atopic dermatitis compared with psoriasis patients ($p = 0.08$, $p = 0.07$), while binding to CD8-positive cells was significantly lower in the atopic patients ($p < 0.01$). The control experiment using dichlorotriazinylamino-fluorescein instead of SP* showed no specific binding, and the percentage of each PE-positive lymphocyte subset was not affected by the addition of SP*.

DISCUSSION

We have examined the binding of SP* to free SP receptors on monocytes and a range of lymphocyte subsets. There is only one previous study that examined fluoresceinated SP binding to human MNL (11). In this study T-lymphocytes were separated from PBL by rosetting with sheep erythrocytes, and the monocytes by adherence. Using flow cytometry, dichlorotriazinylamino-fluorescein-labelled SP was found to bind to $21 \pm 10\%$ (mean \pm SD) of human T-lymphocytes in 6 normal subjects. This binding increased to $35 \pm 8\%$ in PHA-stimulated lymphocytes in the 2 subjects examined. In 3 patients dual immunofluorescent staining, using PE-labelled monoclonal antibodies to helper and suppressor T-lymphocytes, showed that fluorescent-SP bound to $18 \pm 5\%$ of CD4 and $10 \pm 3\%$ CD8 T-cells. These results are similar to our findings, except that the CD8 binding was less than any group of subjects in our study.

SP stimulates chemotaxis (9) and cytokine release in mono-

cytes (10). However, Payan et al. (11) failed to demonstrate monocyte binding of SP*, whereas we showed a small population of SP*-positive monocytes (atopic dermatitis 8%; normal controls 11%; psoriasis 9%). The failure of Payan et al. to demonstrate monocyte binding of SP may be because monocyte adherence was used to separate the cells and methods of preparation can influence antigen binding and function of monocytes. SP has been shown to affect antibody production (6), and we have demonstrated strong binding of SP* to CD19-positive B-cells.

We have demonstrated significantly less binding of SP* to cells bearing surface CD3, CD8 and CD57 in atopic dermatitis patients compared to normal controls. Three possible explanations for this finding are that in these subsets there are i) fewer receptors, ii) more bound SP, iii) an increase in SP-positive cells within the skin and therefore fewer in the peripheral blood: i) It is possible that the MNL SP-receptors may be down-regulated because of chronic exposure of the neuropeptide found *in vivo*. Repeated stimulation with SP on canine tracheal epithelium results in typical tachyphylaxis (22). Furthermore, autologous desensitization experiments have suggested that pretreatment with SP reduced the number of binding sites, but not the binding affinity of [125 I]SP on IM-9 B-lymphoblasts (23). Increased SP in blood and synovial fluid of patients with rheumatoid arthritis has been reported (24, 25). One such study demonstrated that peripheral blood MNL from controls but not rheumatoid arthritis patients showed enhanced proliferation with SP (25). The authors suggested that this could be due to desensitization of lymphocyte receptors. Furthermore, on analyzing the binding properties of SP to rheumatoid lymphocytes, they showed that the receptor numbers were slightly decreased but the affinity remained the same.

An alternative explanation of fewer available or lower affinity SP receptors on CD3-, CD8- and CD57-positive cells may be a reduction in the number of these cells in peripheral blood in atopic dermatitis patients. Reduced numbers of peripheral blood CD8 (26, 27) and NK cells (28), and reduced NK cell activity (29) have been reported in atopic dermatitis. SP has been shown to have selective modulation of natural killer activity of murine intraepithelial leukocytes (30); however, the effect of SP specifically on CD8-positive cells has not been previously examined. SP stimulates the proliferation of lymphocytes (5), and the reduced numbers of NK and CD8 cells that have been reported in atopic dermatitis may be secondary to reduced stimulation by SP. However, around 40% of CD57-positive cells co-express CD8 (31), and the reduced binding of SP* to CD57-positive cells, and also to the pan-T-cell marker CD3-positive cells, may be due to reduced CD8 binding.

ii) Increased binding of SP to MNL subsets in atopic dermatitis would result in fewer available receptors for *in vitro* binding of SP*.

iii) Active recruitment of SP-positive CD8 and CD57 cells into the skin of atopic dermatitis patients may be occurring, resulting in reduced levels of these cells in the peripheral blood. As no significant difference was seen between the psoriasis patients and normal controls, such recruitment would be unique to atopic patients and could not be explained simply by the presence of skin inflammation.

As outlined above, the reduced binding of SP* to CD3-, CD57- and CD8-positive cells may be due to decreased binding to the CD8 subset in atopic dermatitis. CD4-positive cells can

be divided into Th1 and Th2 subtypes, based on their cytokine profile released on activation – Th1 preferentially producing IFN- γ , IL-2 and TNF- β and Th2 producing IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (32). Evidence suggests that CD8-positive T-cells show similar heterogeneity, and in normals peripheral blood CD8-positive T-cell clones tended to show a Th1 type phenotype (33). Diminished IFN- γ release is a well recognised finding in atopic dermatitis (34, 35). The reduced SP binding found in our atopic dermatitis patients may result in reduced stimulation of CD8 cells by SP and in part explain the diminished IFN- γ production seen in atopic dermatitis patients.

In conclusion, this study is the first to show SP binding to NK cells, B-lymphocytes and monocytes. It supports a previous study (11) showing binding of SP to helper and suppressor T-cells. Finally, atopic patients showed a significant reduction in SP binding in CD8-, CD3- and CD57-positive cells, which may be important in the pathogenesis of atopic dermatitis.

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