

Short communication

Staphylococcal enterotoxin induced IL-5 stimulation as a cofactor in the pathogenesis of atopic disease: the hygiene hypothesis in reverse?

Background: The incidence of *Staphylococcus aureus* (*S. aureus*) colonization on the skin of patients with atopic eczema/dermatitis syndrome (AEDS) is approximately 90% and a variety of evidence implicates epidermal staphylococcal infection as a pathogenic factor in atopic dermatitis. However, the mechanism(s) underlying the effects of this organism in the disease process are unclear. The cellular responses of AEDS sufferers and asymptomatic atopic individuals to bacterial superantigens (SAg) were investigated in an attempt to elucidate the role of staphylococcal enterotoxin B (SEB) in atopic disease.

Methods: Peripheral blood mononuclear cells (PBMC) were isolated from normal nonatopic adults, asymptomatic atopic individuals, patients with active AEDS and patients with active allergic asthma. The cells were cultured for 24 or 96 h with house dust mite (HDM), SEB and phytohaemagglutinin (PHA), and the supernatants were assayed for cytokine levels.

Results: Staphylococcal enterotoxin B selectively stimulates the production of interleukin (IL)-5 in AEDS sufferers but not in asymptomatic atopics or nonatopics. Additionally, we observed comparable susceptibility to the IL-5-stimulatory effects of SEB in allergic asthmatics.

Conclusions: Given the central role of IL-5-driven eosinophilia in progression from mild atopy to severe disease, these findings provide a plausible mechanism for the AEDS-promoting effects of staphylococcal SAg. Staphylococcal enterotoxin B may also have a similar role in atopic respiratory disease.

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Prevalence rates of both atopic eczema/dermatitis syndrome (AEDS) and atopic asthma have increased markedly in developed countries over the last 20 years, and one explanation advanced to explain these changes is the 'hygiene hypothesis' (1). In broad terms this posits that decreased contact with the immunostimulatory microbial environment in the increasingly sanitized First World has resulted in selective weakening of the T helper (Th)1 arm of the immune system. This has led to the development of T-cell memory containing excessive contributions from the Th2 end of the cytokine spectrum, which is normally counterbalanced by the Th1 component of these responses. Atopic eczema/dermatitis and atopic asthma are the two most commonly cited potential examples of this process in action (1, 2). Both are chronic inflammatory diseases in which the T-cell responses which trigger disease exacerbations display a characteristic bias towards production of Th2 cytokines such as interleukin (IL)-4 and IL-5, which is in turn associated with increased immunoglobulin E (IgE) production, eosinophilia and mast cell activation.

However, recent studies suggest that AEDS pathogenesis is more complex, and may involve contribution(s)

from additional environmental stimuli. Notably, it appears increasingly likely that toxins released by skin-dwelling bacteria play a significant role in the disease (3–6). In particular, observations to date suggest that the skin of AEDS patients has an increased susceptibility to colonization by *Staphylococcus aureus*. Indeed, the skin of more than 90% of children with AEDS has been found to be colonized by this organism and a substantial proportion of isolates produce superantigens (SAg) such as staphylococcal enterotoxin B (SEB) (7). Bacterial SAg has been demonstrated to exert potent immunomodulatory effects in a variety of experimental animal models, and to induce inflammatory reactions in humans when applied directly to the skin (8). In man, bacterial SAg stimulates T cells bearing selected T-cell receptor (TCR) β -chain variable regions, and the recent finding that bronchoalveolar lavage (BAL) fluids of severe asthmatics contains elevated numbers of activated V β 8⁺ T cells (9), suggests SAg may also play a role in this disease.

The present study sought to elucidate the modulatory effects of the SAg SEB on production of T-cell cytokines in peripheral blood mononuclear cells (PBMC) from

subjects with AEDS, and to contrast these with other atopics and nonatopic controls.

Materials and methods

Patients and controls

Adult laboratory volunteers were screened by skin prick test (SPT) and groups of nonatopics ($n = 11$) and asymptomatic atopics [house dust mite (HDM) responsive; $n = 11$] were selected. Two groups of volunteers with active atopic disease were recruited through the Immunology Department at Fremantle Hospital, comprising immunoglobulin E (IgE)-associated AEDS patients diagnosed on the set out by Hanifin (10), with a severity rating of SCORAD > 50 (11) ($n = 17$; mean age 33 years; range 11–58), and IgE-mediated allergic asthmatics with current moderate-severe disease with forced expiratory volume (FEV) 1 of less than 80% predicted and reversible airflow limitation ($n = 6$; mean age 38 years; range 19–54). Ten of 17 AEDS patients were sensitized to HDM as judged by HDM-specific IgE; IgE to SEB was not ascertained. All the asthmatics were sensitized to HDM.

Cell cultures

For initial experiments venous blood was collected and diluted 1 : 1 in RPMI 1640 (GibcoBRL, Auckland, New Zealand) containing 20 IU/ml heparin (David Bull Laboratories, Musgrave, Victoria, Australia). Peripheral blood mononuclear cells were isolated by density centrifugation over a lymphoprep gradient (GibcoBRL) and washed three times in RPMI 1640 containing 2% heat-inactivated foetal calf serum (FCS), before being resuspended at 1×10^6 /ml in culture medium (RPMI containing 10% heat-inactivated FCS). The cells were then cultured in medium alone or in the presence of SEB (Sigma, St Louis, MO, USA) at 200 ng/ml, HDM (CSL Ltd, Melbourne, Australia) at 10 µg/ml, or the mitogenic stimulant phytohaemagglutinin HA16 (PHA) (Murex Biotech Ltd, Dartford, UK) at 1 µg/ml, in a humidified atmosphere of 5% CO₂ at 37°C. After 24 or 96 h, the supernatants were collected and stored at –20°C for cytokine determination by enzyme-linked immunosorbent assay (ELISA). Excess PBMC were cryopreserved for further experiments. Results from previous studies in our laboratory have shown that this cryopreservation procedure does not alter the responses to allergens made by these cells. For the resting experiments, the cells were cultured in medium alone for 7 days, then washed and counted and cultured for 24 or 96 h as above.

Cytokine determination by ELISA

Interleukin-4, IL-5 and interferon (IFN)-γ protein levels in the culture supernatants were established using a standard ELISA technique. The paired antibodies for each cytokine were obtained from BD Pharmingen (San Diego, CA) and used at concentrations which were determined to give the greatest sensitivity. The capture antibody for IL-4 was clone 8D4-8 and its biotinylated pair was MP4-25D2. For IL-5, the capture antibody clone was TRFK5 and its pair was JES1-5A10. For IFN-γ, the capture clone was NIB42, its pair being clone 4S.B3. The samples were routinely diluted 1 : 2 in high performance ELISA (HPE) buffer (PeliKine Compact™ CLB, Amsterdam, The Netherlands), but for the IFN-γ the high levels of protein required dilutions of up to 1 : 500. The standard curves were generated using a series of dilutions of recombinant human cytokines, supplied by BD Pharmingen, and the assays were

read on a Bio-Tek microplate reader. Sensitivities were routine between 5 and 10 pg/ml for all cytokines tested.

Results

With the increasing body of evidence to suggest a role for bacterial SAg in AEDS, a panel of cell cultures was set up to determine the cytokine profiles of PBMC stimulated with SAg. The donors fell into one of the three groups, normal healthy nonatopic controls, asymptomatic individuals with an atopic phenotype and a group consisting of patients with active AEDS. Figure 1 shows the *in vitro* cytokine responses to culture medium alone, HDM, SEB and the mitogenic stimulant PHA from these three groups. Culture supernatants were harvested for cytokine analysis by ELISA at time points which gave peak cytokine production as determined in preliminary experiments. There was no difference in the SEB-induced IL-4 response between the three groups, with the levels detected ranging from 53 to 68 pg/ml. The responses seen are similar in magnitude to the mitogen-induced response. In contrast to the IL-4 data, more IFN-γ was detected to SEB than PHA (Fig. 1C). However, there was no difference in the levels of this cytokine produced by any of the groups. The level of IFN-γ produced in response to SEB ranged from 10 000 to above 100 000 pg/ml.

The most interesting observation was seen in the IL-5 responses (Fig. 1B). Here the IL-5 produced by the PBMC from the AEDS group in response to each of the treatments was dramatically higher than that produced by either the normal control group or the asymptomatic atopic group. The differences observed were significant with *P*-values of 0.0036 for SEB, 0.0133 for HDM and 0.0083 for PHA, when compared with the asymptomatic atopics. Clearly the PBMC responses of adults with active AEDS responded differently *in vitro* from the PBMC from the normal and atopic groups. This difference is marked by a large and selective increase in the secretion of the Th2-type cytokine IL-5.

Recent data suggests that there may be a correlation between bacterial carriage rates in the nasal mucosa and allergic rhinitis (12). If this were the case there may also be a difference in the way T cells from asthmatic patients respond to SEB *in vitro*. In order to address this, PBMC were isolated from patients with active asthma and cultured concurrently with PBMC from six additional AEDS patients. They were again stimulated with SEB, HDM and PHA. From the data displayed in Fig. 2, it was clear that the pattern of response of the PBMC to all three stimuli did not differ between the asthmatics and the AEDS patients. This was true for the three cytokines measured. Therefore, it appears that there is a phenotypic difference in the IL-5-producing cells from the individuals with active atopic disease compared with the asymptomatic atopics and the normal controls.

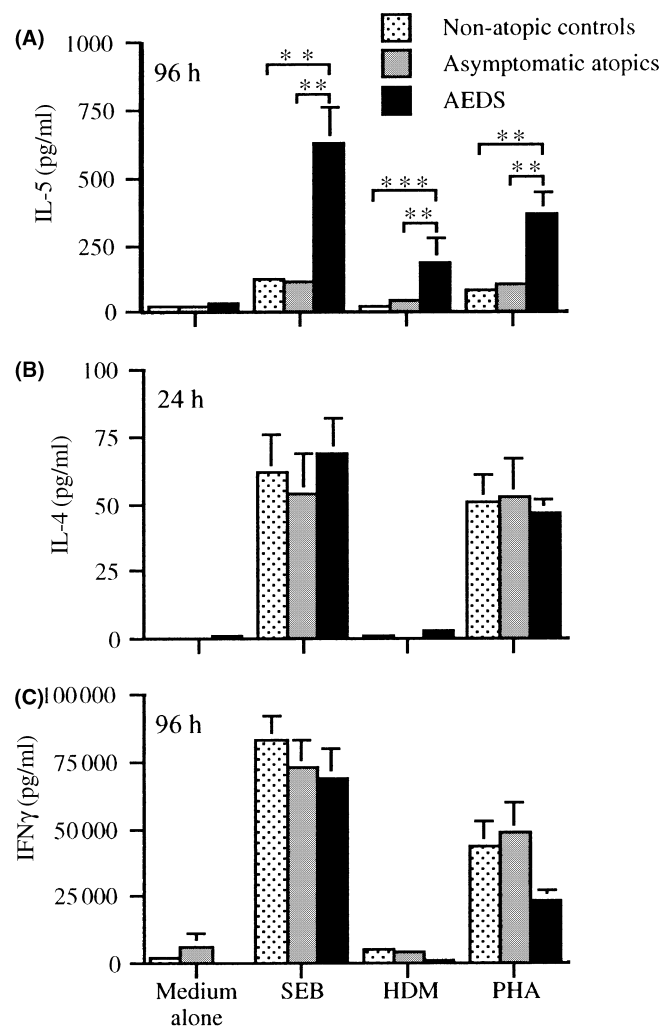


Figure 1. PBMC from patients with active atopic eczema/dermatitis syndrome (AEDS) secrete increased levels of interleukin-5 (IL-5) in response to staphylococcal enterotoxin B (SEB). (A) IL-5 responses from nonatopic controls ($n = 11$), asymptomatic atopsics ($n = 9$), AEDS patients ($n = 11$). (B) IL-4 responses, from nonatopic controls ($n = 11$), asymptomatic atopsics ($n = 9$), AEDS patients ($n = 11$). (C) IFN- γ responses ($n = 11$ for all groups). Data presented as mean + standard error of the mean. P -values were generated using the unpaired t -test as the data were normally distributed. Where no P -value is shown (here and in subsequent figures) the differences within groups were not significant. ** $P < 0.05$; *** $P < 0.005$.

Circulating T cells from patients with active atopic disease are likely to be in a higher activation state relative to those found at the periphery in normal subjects and atopic subjects who are symptom free. Therefore, it is possible that an encounter with SEB could trigger the secretion of large amounts IL-5 in the patient groups, as a direct result of preactivation. If this hypothesis was correct then 'resting' the cells prior to *in vitro* stimulation would allow them to return to an inactive state, and the expected outcome of SEB stimulation postrest would be a reduced IL-5 response equivalent to the level seen in the

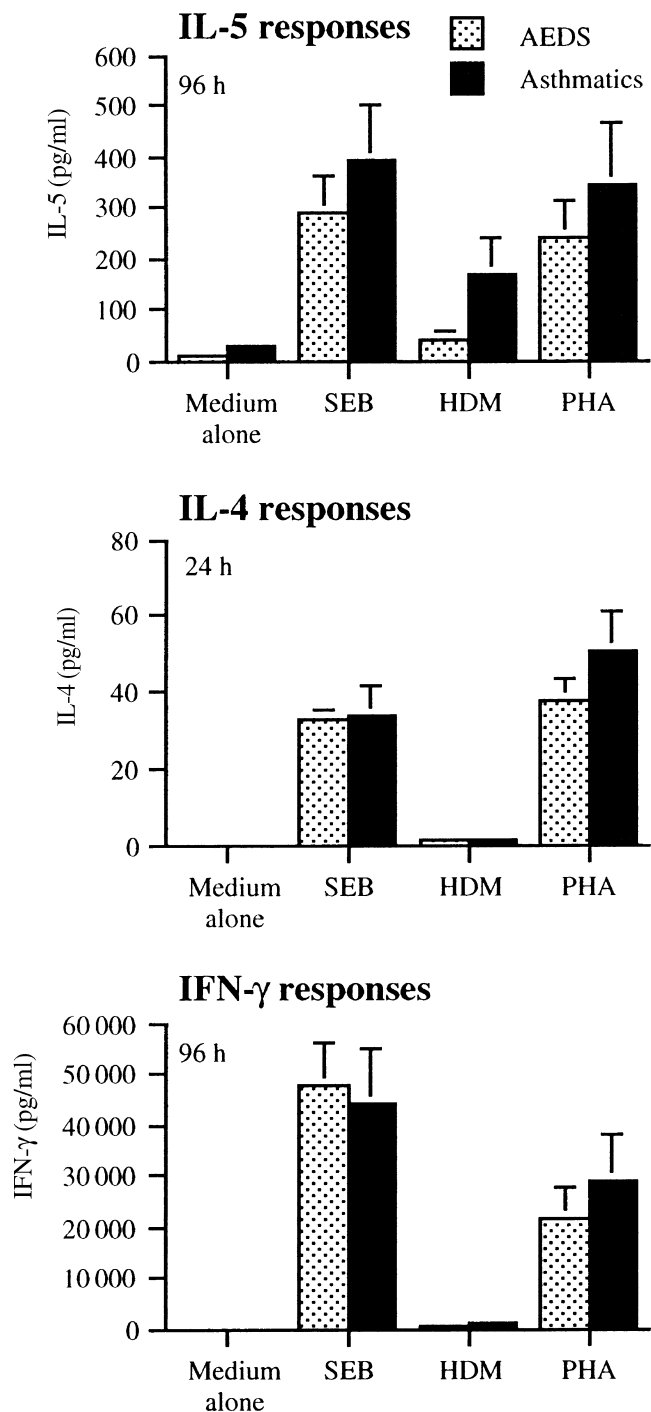


Figure 2. PBMC from asthmatic patients display the same increase in IL-5 secretion as those from AEDS patients in response to SEB. For AEDS patients, $n = 6$ and for asthmatics, $n = 5$. Data presented as mean + standard error of the mean.

asymptomatic atopic individuals. To test this hypothesis PBMC from AEDS patients were cultured in medium alone for 7 days, followed by stimulation with SEB. The result of cytokine analysis at 96 h poststimulation is shown in Fig. 3.

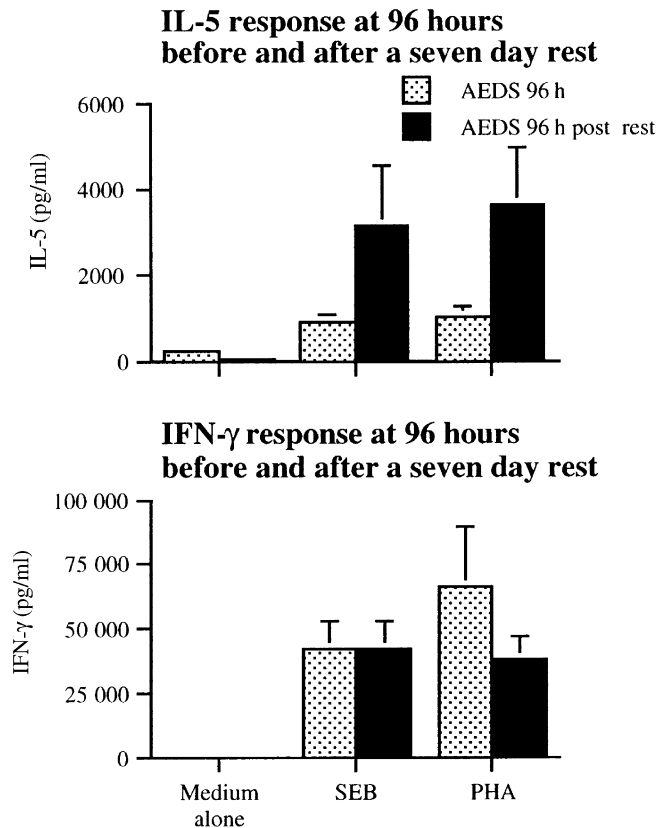


Figure 3. The increased SEB-induced IL-5 response is not because of the increased state of activation of the responding T cells. Cytokine responses to SEB and PHA by PBMC from AEDS patients ($n = 5$) stimulated on day 0 and supernatants harvested at 96 h and from PBMC which were rested for 7 days prior to stimulation. The supernatants from the rested PBMC were also harvested at 96 h poststimulation. Data are presented as mean + standard error of the mean.

It appears that the IFN- γ response of the 'rested' cells to SEB was not different from that observed in the cultures which were not rested. Additionally, the mean IL-5 responses to SEB and PHA of the rested cells were higher than those seen when the cells were stimulated without resting, but these differences did not attain statistical significance.

Discussion

An important feature of our findings is the restricted nature of the stimulatory effects of SEB on IL-5, which unlike effects on IL-4 and IFN- γ production, were found only in subjects with active atopic disease. Current surveys indicate that in the order of 45% of adults in First World countries are atopic, as judged by SPT reactivity, but only approximately a quarter of atopics develop clinically severe disease, suggesting that additional 'cofactors' are required to drive progression to

disease severity (13). In this context it is noteworthy that IL-4 production is required to stimulate production of the IgE antibody responsible for SPT reactions and is hence a common feature of all atopic responses, and in the majority of atopics this is accompanied by IFN- γ [the mixed 'Th0' response pattern (13)]. However, high level production of IL-5 and ensuing eosinophilia appears to be the specific hallmark of severe allergic disease. The finding here that susceptibility to the IL-5 stimulatory effects of SEB is restricted to atopics with active disease adds to the growing plausibility of epidermal staphylococcal infection as a potential cofactor in disease progression, particularly in AEDS where the organisms are commonly found at the site of the lesion. It is pertinent to reiterate the report on putative SEB-activated T cells in BAL fluid of asthmatics (9), and recent findings on nasal carriage of these organisms in relation to perennial allergic rhinitis (12). Collectively these data suggest that the costimulatory effects of this, and related bacterial SAg, may play a role in driving the progression of allergic disease(s) at respiratory sites and in the skin. The mechanism(s) underlying the potential allergy-promoting effects of SEB remain to be defined, but may involve the role of IL-5 in enhancing the survival of pro-inflammatory eosinophils at sites of disease, via inhibition of apoptosis (14). More basic and clinical research in this area is clearly warranted, in particular microbiologically oriented intervention studies targeted at potential control of atopy symptoms in SEB-responsive patients who can be readily identified via the relatively simple laboratory techniques demonstrated above.

Our findings suggest that susceptibility to SEB-induced IL-5 stimulation is not related to the atopic phenotype *per se*, but instead is a hallmark of active, clinically significant, atopic disease. Because of limitations in the cell numbers available for these studies it was not feasible to formally identify the cells responsible for SEB-induced IL-5 production, and this issue should be addressed in follow-up experiments. However we were able to test the possibility that *in vitro* SEB susceptibility may be related to *in vivo* preactivation of IL-5 secreting cells in the symptomatic subjects, by 'resting' their cells in culture medium for 7 days prior to SEB stimulation. However, SEB hyperresponsiveness was still observed in rested cultures, and tended to increase rather than decline. This could reflect the viability of different cell populations remaining in culture following 7 days with no stimulation, and/or changes in the balance between production and utilization or catabolism of IL-5 over the extended culture period. It should be noted that our present findings contrast with the recent report from Kondo et al. (15) which suggested a positive relationship between overall IL-5 production (as measured by circulating levels in serum) and AEDS severity. In the present study we were unable to demonstrate a similar relationship between SEB-stimulated IL-5 secretion by PBMC and AEDS severity (data not shown). This may be a reflection

of low statistical power, or of the fact that IL-5 in the two studies was measured in different compartments.

It is pertinent to note recent results from a mouse model of AEDS. These studies demonstrated that animals bred in specific pathogen-free conditions did not develop discernible disease, but if they were housed in conventional conditions they developed dermatitis similar to the human atopic disease (16). This finding provides a precedent for an important role for environmental (in particular microbial) agents as cofactors in driving the progression of allergy towards clinical expression of overt disease at skin sites. Our current findings are compatible with such a model for AEDS pathogenesis, and merit more detailed

follow-up with larger patient numbers spanning a wider range of disease severity. A logical first step in this process is to determine whether *in vitro* hyperresponsiveness to SEB amongst AD patients can be correlated with skin colonization by SEB-producing strains, and these studies are in progress in our laboratories.

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References

1. STRACHAN DP. Hay fever, hygiene, and household size. *Br Med J* 1989;**299**: 1259–1260.
2. MARTINEZ FD, HOLT PG. The role of microbial burden in the aetiology of allergy and asthma. *Lancet* 1999;**354**: 12–15.
3. HERZ U, BUNIKOWSKI R, MIELKE M, RENZ H. Contribution of bacterial superantigens to atopic dermatitis. *Int Arch Allergy Immunol* 1999;**118**: 240–241.
4. BREUER K, WITTMANN M, BOSCH B, KAPP A, WERFEL T. Severe atopic dermatitis is associated with sensitization to staphylococcal enterotoxin B (SEB). *Allergy* 2000;**55**:551–555.
5. BUNIKOWSKI R, MIELKE M, SKARABIS H, HERZ U, BERGMANN RL, WAHN U, RENZ H. Prevalence and role of serum IgE antibodies to the *Staphylococcus aureus*-derived superantigens SEA and SEB in children with atopic dermatitis. *J Allergy Clin Immunol* 1999;**103**: 119–124.
6. CAMPBELL DE, KEMP AS. Proliferation and production of interferon-gamma (IFN- γ) and IL-4 in response to *Staphylococcus aureus* and staphylococcal superantigen in childhood atopic dermatitis. *Clin Exp Immunol* 1997;**107**:392–397.
7. HOEGER PH, LENZ W, BOUTONNIER A, FOURNIER JM. Staphylococcal skin colonisation in children with atopic dermatitis: prevalence, persistence, and transmission of toxigenic and nontoxigenic strains. *J Infect Dis* 1992;**165**: 1064–1068.
8. STRANGE P, SKOV L, LISBY S, NIELSEN PL, BAADSGAARD O. Staphylococcal enterotoxin B applied on intact normal and intact atopic skin induces dermatitis. *Arch Dermatol* 1996;**132**:27–33.
9. HAUKE PJ, WENZEL SE, TRUMBLE AE, SZEFLER SJ, LEUNG DY. Increased T-cell receptor $\text{v}\beta 8 +$ T cells in bronchoalveolar lavage fluid of subjects with poorly controlled asthma: a potential role for microbial superantigens. *J Allergy Clin Immunol* 1999;**104**:37–45.
10. HANIFAN JM, RAJKA G. Diagnostic features of atopic dermatitis. *Acta Dermatol-Venereol* 1980;**92**:44–47.
11. EUROPEAN TASKFORCE ON ATOPIC DERMATITIS. Severity scoring of atopic dermatitis: the SCORAD index. *Dermatology* 1993;**186**:23–31.
12. SHIOMORI T, YOSHIDA S-I, MIYAMOTO H, MAKISHIMA K. Relationship of nasal carriage of *Staphylococcus aureus* to pathogenesis of perennial allergic rhinitis. *J Allergy Clin Immunol* 2000;**105**:449–454.
13. HOLT PG, MACAUBAS C, STUMBLES PA, SLY PD. The role of allergy in the development of asthma. *Nature* 1999;**402**:B12–B17.
14. WEDI B, WIECZOREK D, STÜNKEL T, BREUER K, KAPP A. Staphylococcal exotoxins exert proinflammatory effects through inhibition of eosinophil apoptosis, increased surface antigen expression (CD11b, CD45, CD54, and CD69), and enhanced cytokine-activated oxidative burst, thereby triggering allergic inflammatory reactions. *J Allergy Clin Immunol* 2002;**109**:477–484.
15. KONDO S, YAZAWA H, JIMBOW K. Reduction of serum interleukin-5 levels reflect clinical improvement in patients with atopic dermatitis. *J Dermatol* 2001;**28**:237–243.
16. MATSUDA H, WATANABE N, GEBB GP, SPERL J, TSUDZUKI M, HIROI J, MATSUMOTO M, USHIO H, SAITO S, ASKENASE PW, RA C. Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. *Int Immunol* 1997;**9**:461–466.