

Use of an autologous reaction *in vitro* to assess contributions of T and B lymphocytes to immune hyperreactivity of atopics

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Summary

The *in-vitro* proliferative reaction of peripheral blood lymphocytes (measured by [³H]thymidine incorporation) to autologous pokeweed mitogen (PWM)-induced lymphoblasts (PWM-lymphoblast-stimulated autologous mixed leucocyte reaction, PWM.AMLR) was used as a measure of immune hyperreactivity for comparison of atopic with non-atopic individuals. Accordingly, 10/24 non-atopics responded in the PWM.AMLR, and 19/19 atopics reacting to inhaled allergens responded. Autologous stimulation was associated with release of mitogenic factors from the PWM-activated stimulating cells (2/15 non-atopics, 9/15 atopics). For non-atopics, stimulation delivered by staphylococcus A (SAC)-activated cells was similar to that delivered by PWM-induced cells, while in atopics, the SAC.AMLR was never more than 50% of the PWM.AMLR, indicating a possible T cell component. Separation by panning of the stimulation cells into lymphocyte subsets supported the notion that stimulation involved a cooperation between B and T₄⁺ T cells. It is proposed that a positive PWM.AMLR is dependent upon an initial B cell activation followed by the PWM stimulus dependent upon a previous T cell activation, where atopics have more lymphocytes in an activated state than healthy non-atopics. Such a baseline priming may contribute to an innate sensitivity of atopics to environmental allergens.

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Introduction

Atopic disease has been shown to cluster within families, indicating a genetic disposition, yet such inheritance follows no identifiable rule and remains an association rather than a precise probability [1,2]. This suggests that the disposition is the result of a polygenic determination, and it remains to be shown how these genes collectively increase the sensitivity to certain antigens.

One proposal is that reduced suppressor cell activity throughout the periphery may result in immune hyperreactivity [3,4]. A second possibility is that the atopy occurs in those individuals who have at some time developed a dysregulation of immune activity in the

respiratory or gastrointestinal tracts, being areas of epithelia and associated lymphoid tissue which constitute a first line of defence against inhaled or ingested antigens [5,6]. For example, not all individuals who respond in the skin-prick test, where the epithelium is broken, develop clinical manifestations of atopy [7]. A third possibility is that once an antigen has entered the circulation, only those individuals with an intrinsic sensitivity will respond.

In a previous report [8], we have shown that peripheral blood lymphocytes which had been stimulated with pokeweed mitogen (an allergen as well as a mitogen) could induce autologous lymphocyte proliferation in just under half of a group of apparently normal randomly selected individuals. The ability of these lymphoblasts to stimulate seemed to be related to their capacity to secrete activating factors, rather than to a change in their surface antigen expression. We proposed that this activation may

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be a measure *in vitro* of an intrinsic sensitivity to an antigen not normally encountered in the peripheral circulation. As a consequence, in this report we test whether an increased proportion of atopic individuals respond to autologous lymphoblasts and whether this is a measure of an intrinsic immune hyperreactivity which contributes to the atopic state.

Subjects and methods

Subjects

A total of 24 non-atopics (13 males, 11 females, aged 25–74, median 33) and 24 atopics (14 males, 10 females, aged 5–63, median 25) participated in this study. The atopics were divided into two arbitrary groups consisting of those responding to inhaled or contact antigens (Group 1: plant/tree allergens and household dust) and those responding to injected antigens (Group 2: bee/wasp/yellow jacket stings). The Group 1 atopics manifested either alone or in combination the following symptoms: allergic rhinitis, atopic dermatitis or bronchial asthma. Most of the atopics participated in an extensive characterization including physical examination, family history and skin-prick tests to determine the allergens responsible. For a proportion of the patients, total IgE and allergen-specific IgE were determined using the PRIST and RAST assays, respectively. Peripheral blood eosinophil levels were determined in several instances. At the time of assay none were receiving hyposensitization schedules or immunosuppressive agents.

Blood samples

Blood was collected in heparinized tubes. After separation of the plasma, the cells were separated by density centrifugation over Ficoll-Hypaque (density 1.077 g/cm³, Pharmacia, Uppsala, Sweden) for 30 min at 150 × *g*. The mononuclear cell band was removed and washed three times in RPMI 1640 medium containing 100 U/ml penicillin, 100 µg/ml streptomycin and 25 mM HEPES buffer.

Mitogen immobilization

Pokeweed mitogen (PWM, 10 µg/ml in water; Sigma, St Louis, MO, U.S.A.) was incubated on horizontally placed 25 cm² tissue culture flasks (Nunc, Roskilde, Denmark) overnight at 37°C. Glutaraldehyde (0.125% final concentration; Merck, Darmstadt, FRG) was added to each plate and incubated for 15 min at room temperature, followed by three washes in phosphate-buffered saline (PBS). The plates were stored at 4°C until use, when

they were washed a further two times. Control plates consisted of glutaraldehyde-treated flasks not incubated with mitogen.

Separation of leucocyte subsets

Cells derived from control or PWM-coated plates (depleted of macrophages which had adhered to the flasks during the 48-hr incubation) were incubated for 1 hr on 10-cm diameter sterile plastic Petri dishes (Nunc, Roskilde, Denmark) that had been coated overnight with anti-T4 (CD4), anti-T8 (CD8), or anti-B murine monoclonal antibodies (1/100 dilutions of ascites in 0.05 M carbonate-bicarbonate buffer, pH 9.6; ascites of clones ABL4 (anti-T4), BL13 (anti-B) and BL15 (anti-T8) were generously provided by Dr M. Feldmann, Charing Cross Sunley Research Centre, London, U.K.). After incubation, non-adherent cells were rinsed off the plates and adherent cells were removed by gentle scraping with a rubber tip. The separated T cells were checked for binding of fluorescinated anti-T4 and anti-T8 antibodies (OKT4, OKT8; Ortho Diagnostics, Raritan, NJ, U.S.A.) and were generally found to be >90% T4⁺ or T8⁺, respectively. The B cells were checked by their ability to bind fluorescinated goat anti-human IgM (Sigma, St Louis, MO, U.S.A.) and were generally found to be greater than 80% surface IgM⁺. The adherent cells were then washed in RPMI-1640 and resuspended alone or in combination to a final concentration of 5 × 10⁵/ml for use as stimulators in the autologous mixed leucocyte reaction (AMLR).

Cell treatments

Peripheral blood mononuclear cells (PBMC) were incubated on the PWM-treated or control flasks (5 ml at 5 × 10⁵/ml) in RPMI-1640 and 10% autologous plasma for 2 days at 37°C. For stimulation with staphylococcus A, whole organisms (SAC; 10% formalinized Cowan Strain 1; Teva Pharmaceuticals, Jerusalem, Israel) were added to a final concentration of 0.1% for 48 hr to cells incubated on control flasks as prepared above. After incubation, the culture supernatants were separated and stored at 4°C. The immobilization of PWM on plastic prevented carry-over of the mitogen onto cells or into the supernatants [8]. The cells from the plate were inactivated by 50 Gy of X-radiation delivered by a cobalt source (0.5 Gy/sec; Gammacell, Atomic Energy of Canada, Ontario, Canada) and were used as stimulators in the AMLR.

AMLR culture

All reactions were performed in volumes of 220 µl of RPMI-1640 containing 10% autologous heparinized

plasma within round-bottomed 96-well microtitre plates (Nunc, Roskilde, Denmark). For the AMLR, the reaction contained 5×10^4 responder cells and 5×10^4 stimulator cells. For the mitogen stimulation, the reaction contained 5×10^4 responder cells and $0.1 \mu\text{g/ml}$ PWM or $10 \mu\text{g/ml}$ PHA (Difco, Detroit, MI, U.S.A.). The 2-day supernatant reactions consisted of 5×10^4 responders and a $1/2$ dilution of the supernatant. Reactions were performed in triplicate or quadruplicate and the plates were incubated in a 50% CO_2 incubator at 37°C for 5 days, after which $1 \mu\text{Ci}$ of $[^3\text{H}]$ thymidine (Nuclear Research Center, Negev, Israel) was added to each well followed by a further incubation for 18 hr. The cultures were harvested and the cell-incorporated tritium determined by β -scintillation counting. Results are expressed either as counts per minute (c.p.m.) or as a stimulation index equivalent to:

$$\text{SI} = \frac{(\text{c.p.m. AMLR} - \text{c.p.m. stimulators alone})}{(\text{c.p.m. responders alone})}$$

Statistics

Unless otherwise stated, all means and standard deviations were compared using the two-tailed Student's *t*-test. All tests were performed using the EPISTAT statistical software package (distributed by Dr Tracy Gustafson, Little Rock, AR, U.S.A.), which tested automatically the variance for fit to a normal distribution and informed the user if data could not be used for parametric analysis.

Results

Mononuclear cells separated from the subjects' blood

samples were incubated on plates containing immobilized PWM or on control plates for 48 hr. After this period the cells and their conditioned supernatants were separated, and the cells were used to stimulate autologous responder cells (Fig. 1). Extensive tests have shown that the immobilization procedure was strong enough to prevent carry-over of mitogen by the incubated cells [8]. For the non-atopic group, using raw radioactive counts or the transformed stimulation index, no significant difference was observed between the normal AMLR and the AMLR induced by autologous lymphoblasts (PWM.AMLR) on comparing the group responses, but individually 10/24 non-atopics generated a PWM.AMLR greater than their respective AMLR. Using Kendall's rank correlation test [9], there was a statistical association between the levels of response in the AMLR and PWM.AMLR ($P < 0.05$).

For the subjects reacting to a continuous exposure of inhaled antigens, the PWM.AMLR of every individual was significantly higher than their respective AMLR (19/19). Statistical ranking revealed a high level of correlation between the levels of the AMLR and PWM.AMLR responses for this atopic group ($P < 0.005$). In addition, the baseline AMLR of this group was significantly higher than the mean AMLR of the non-atopic group ($P < 0.005$). There was no correlation between the severity of atopic symptoms and level of response in the PWM.AMLR for this group.

The third group consisting of individuals responding to insect stings demonstrated a distinct pattern of responses. According to the c.p.m. values, both the AMLR and PWM.AMLR were high, but after transforming the raw data to stimulation indices which corrected for the high

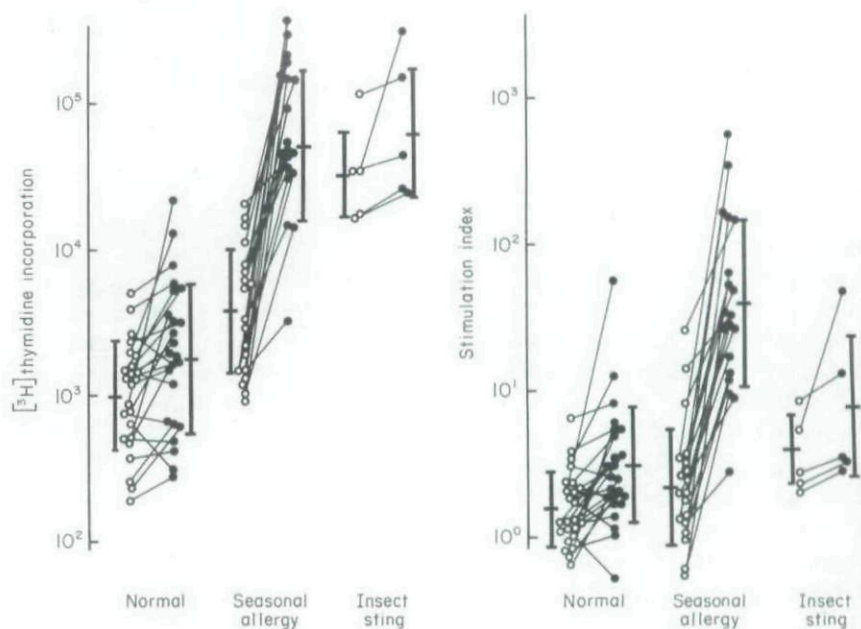


Fig. 1. Responses of atopic and non-atopic (normal) individuals in the AMLR (○) and PWM.AMLR (●). The left-hand panel presents the results as radioactive incorporation (c.p.m.), the right-hand panel presents the same results as stimulation indices. Vertical bars represents the mean $\pm$ 1 s.d.

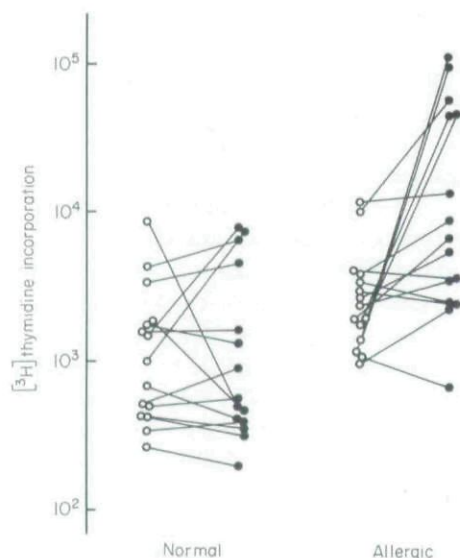


Fig. 2. Stimulating capacity of supernatants harvested from cells of non-atopics (normal, left) and atopics (right) after incubation for 48 hr on control plates (O) or immobilized PWM plates (●).

background activity, the results were not significantly different from non-atopics. Within this third group, there was no correlation between level of response in the PWM.AMLR and levels of total or allergen-specific IgE. All the atopic rhinitic subjects developed seasonal rather than perennial symptoms, nevertheless repeated testing revealed that positive responses in the PWM.AMLR remained positive to the same degree even when the subjects were asymptomatic. Non-responders in the PWM.AMLR, however, did not always remain negative, and developed strong responses when suffering from viral influenza (data not presented).

To test the hypothesis that activation by PWM caused cells to release lymphocyte proliferation-inducing factors, the supernatants from the 48-hr cultures on control and immobilized-PWM plates were used to stimulate autologous responding cells (Fig. 2). Of the non-atopics, the supernatants from the PWM plates of 2/15 subjects generated proliferation greater than the supernatants from control plates, while for the atopics (inhaled allergens) 9/15 generated such a response indicating enhanced production or release of an activating factor ($P < 0.025$).

Staphylococcus A (SAC, a T cell independent B cell mitogen [10]) was used to induce lymphoblasts, and these cells were then compared with unstimulated cells or PWM-activated cells for their abilities to stimulate autologous responder cells (SAC.AMLR, AMLR and PWM.AMLR, respectively; Fig. 3). SAC-activated cells were as efficient as PWM-induced cells for four normal

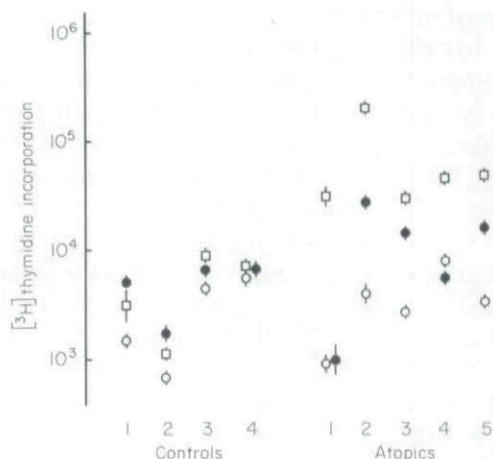


Fig. 3. Comparison of the responses of autologous cells of atopics and non-atopics (controls) to unstimulated cells (AMLR, O), staphylococcus A-activated cells (SAC.AMLR, ●), and to PWM-activated cells (PWM.AMLR, □). Results presented as mean radioactive incorporation (c.p.m.) $\pm$ s.e.m.

non-atopics, while for five atopics with allergic rhinitis, the non-SAC.AMLR did not approach the level of the PWM.AMLR.

Separating the stimulators into T4⁺ T cells, T8⁺ T cells and B cells and testing their ability to stimulate either alone or in combination revealed that T4⁺ T cells and B cells together generated the best stimulating activity (Table 1). The separations of the unactivated controls and PWM-activated cells by panning resulted in variances in the background reactivity which were independent of the PWM stimulation, but which were consistent for each antibody used for panning. Accordingly, the results are

Table 1. Ability of lymphocyte subsets alone or in combination to stimulate autologous cells of two atopic individuals

Stimulators	Ratio of PWM.AMLR/AMLR	
	Experiment 1	Experiment 2
Unseparated	11.57 [†]	2.24 [†]
T8 ⁺ T cells	1.66	0.80
T4 ⁺ T cells	1.11	0.70
B cells	0.94	1.04
T4+B	9.92 [†]	2.52*
T8+B	1.02	0.66
T4+T8	0.81	0.90

Due to variations in background (see text) the results are presented as the ratio of the PWM.AMLR to the AMLR. Statistical differences between PWM.AMLR and AMLR are indicated: * $P < 0.05$; [†] $P < 0.01$; [‡] $P < 0.005$.

presented as the ratio of the AMLR using PWM-activated cells to the AMLR using unactivated control cells.

Discussion

The purpose of this study was to determine whether atopic individuals are more hyperreactive than their non-atopic counterparts using an *in-vitro* estimate of apparent immunological hyperreactivity, the reaction to autologous lymphoblasts. We demonstrated that the peripheral blood lymphocytes of 19/19 (100%) atopics with clinical allergy and positive skin-prick tests to inhaled antigens reacted to autologous PWM-induced lymphoblasts, while 10/24 non-atopics responded to a similar stimulus. The proportion of non-atopic controls responding compares well with the 45–50% previously observed for a random selection from a healthy population [8].

The AMLR using normal B cells and macrophages as stimulators has been studied previously in atopic patients [11,12]. In allergic rhinitis, the AMLR appears to be relatively low [11], while it may be elevated in asthmatics [12]. A comparison of our technique, however, with the regular AMLR may not be valid, since the mechanisms appear to differ. Unlike the AMLR, the ability to stimulate in the PWM.AMLR is independent of HLA-D/DR expression or expression of any other surface antigen, and instead may be associated with secretion of a proliferation-inducing factor [8]. Indeed, in this study, the culture medium harvested after a 48-hr incubation on immobilized PWM was more likely to contain a stimulating factor if the cells incubated were of atopic rather than non-atopic origin. A similar release of stimulating factor was observed [13] during the response of atopics' lymphocytes to grass pollen allergens. In this report, however, the allergen itself was present in the supernatants assayed, and the presence of a stimulating factor was inferred from the finding of a greater stimulation than could be accounted for by the presence of the allergen/mitogen alone. Immobilization of the mitogen on a plastic surface for the assay of the PWM.AMLR eliminates the problem of carry-over and we have already demonstrated that the stimulating activity cannot be attributed to contaminating PWM nor to IL-1 or IL-2 [8].

Lymphoblasts generated after stimulation with immobilized phytohaemagglutinin (PHA) using our conditions are not capable of stimulating autologous responder cells [8]. Since PHA is a T cell mitogen, and PWM is a T-dependent B cell mitogen, activated B cells may serve an important function in generating the PWM.AMLR, a concept supported by the observations of Crow and Kunkel who found activated but not resting B cells were capable of stimulating in an AMLR [14].

We certainly found that SAC-activated cells (a T-independent process) could stimulate autologous responders, and the mean level of stimulation was higher among atopics than among non-atopics, again implying a stimulation by activated B cells. Nevertheless, the SAC.AMLR did not approach the level delivered in the atopic PWM.AMLR which suggested a role for T cells functionally more active than those present in the non-atopic cultures. Separation of PWM-activated stimulating cells demonstrated that the T cell activity was associated with the T4⁺ fraction. Accordingly, we propose that the PWM.AMLR is dependent upon an activation of B cells prior to their exposure to PWM as well as a similar previous activation of T helper cells. It is possible that B cell stimulating factor (IL-4, BSF-1) of T cell origin is mediating the process and may be the soluble stimulating activity we have observed to be released during the 48 hr activation with PWM, particularly as BSF works in cooperation with SAC or anti-IgM to induce B cell proliferation [15].

An intriguing proposal is that the priming or activation of B and T cells is a parallel rather than sequential process. A recent report has shown that deficient immunoglobulin production *in vitro* by B lymphocytes of hypo- γ -globulinaemic children may be restored in some instances by adding a PWM-stimulated T cell-derived factor and pre-activating the B cells with staphylococcus A, while either procedure alone was ineffective [16]. With respect to the responders of atopics, it may be that allergen-activated B cells but not resting B cells are responding in a T cell-dependent fashion to the PWM, while in the controls resting B cells predominate. Consequently, we are now investigating the role of B cell activation in determining the level of an autologous response to PWM-induced lymphoblasts.

In no way may the PWM.AMLR be considered a specific test for atopic reactivity. Firstly, the reaction does not correlate with IgE levels or with the severity of atopic symptoms. In addition, strong PWM.AMLR reactions are developed by lymphocytes of individuals suffering from viral influenza. We would suggest instead that the reaction measures a degree of activation of B and T cells which lends them to react more strongly to allergenic stimuli. Some degree of support for this notion of predisposition is given by the observation that three non-atopics used in this study, all of whom generated a positive PWM.AMLR, later presented themselves at the Allergy clinic for skin-prick testing and were all found to develop strong responses alone or in combination to grass allergens, household dust and mite allergens, or mouse allergens, yet the three individuals remain asymptomatic.

Thus, the PWM in this instance might be considered as a substitute for the allergen itself, delivering a stimulus

independent of environmental conditions or the need to cross epithelial barriers. The responses to autologous PWM-induced lymphoblasts of individuals allergic to insect stings suggests that the cells were already so activated by allergen (i.e. the baseline AMLR) that they were refractory to further activation by PWM, the allergic stimulus being more acute and considerably stronger than that delivered in general to the seasonal atopics. The clear corollary, which we have begun to study in this report, is that the PWM.AMLR may prove a useful addition to the techniques already available for dissecting the mechanics of the atopic response.

Acknowledgments

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