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In vitro selective expansion of allergen specific T cells from atopic patients

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SUMMARY

Peripheral blood mononuclear cells from atopic donors were stimulated *in vitro* with allergens (Rye group I or *Dermatophagoides pteronyssinus*). T cell lines were originated and maintained in long term culture using IL-2 and periodical restimulations with allergen. The lines were antigen specific (i.e. responded to the allergen used to raise them and not to other antigens) and required that the antigen was presented by autologous cells (i.e. they were restricted). The restriction elements were probably at the level of HLA-DR antigens since the proliferative response was specifically blocked by anti-HLA-DR antibodies. Surface marker analysis revealed that the lines comprised mainly cells with an helper/inducer phenotype, although cells with markers of the suppressor/cytotoxic T cells were also present. The lines could be cloned by limiting dilution and clones with the same restriction and specificity as the parental line were isolated. These studies demonstrate the possibility of obtaining a large number of allergen specific human T cells that can be used for further *in vitro* studies on the regulation of the IgE response.

INTRODUCTION

Studies on experimental animals have provided a large amount of evidence demonstrating that T cells exert a control on IgE production (reviewed by Ishizaka, 1976; Katz, 1978). Investigations on this subject have been so far limited in humans owing to the difficulties connected with the clinical investigation in general and the lack of appropriate *in vitro* methodologies.

Peripheral blood mononuclear cells (PBM) from atopic patients generally give a proliferative response when challenged with the allergen *in vitro* (Girard *et al.*, 1967; Brostoff, Greaves & Roitt, 1969; Romagnani *et al.*, 1973; Ishizaka *et al.*, 1974; Gatien, Merler & Coleten, 1975; Evans *et al.*, 1976). This finding has been taken as evidence for the presence of circulating antigen specific T cells, a concept that can be, however, challenged on the ground that the allergens could behave as polyclonal T cell activators. The capacity of PBM from non-atopic individuals to respond to allergens *in vitro* has been interpreted in support of this particular view (Buckley *et al.*, 1977),

Abbreviations: PBM = peripheral blood mononuclear cells; IL-2 = interleukin-2; Tb = T blasts; PHA-SUP = supernatant of phytohaemagglutinin activated tonsil cells; Rye I = rye grass antigen group I; DP = *Dermatophagoides pteronyssinus* extract; CA = *Candida albicans* antigens; TT = tetanus toxoid; APC = antigen presenting cells; Mab = monoclonal antibody.

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although one can also argue that allergens, being ubiquitous, may sensitize a number of individuals (May & Alberto, 1972).

A study on the properties of the proliferative response as well as on the features of the responding cells would be instrumental to clarify the issue. However, the short term culture methods generally employed are not particularly suitable for this type of investigation.

Recently the use of IL-2 has made it possible to expand antigen specific T cells *in vitro* into continuous non-malignant cell lines or clones retaining antigen specificity and function (reviewed by Ruscetti & Gallo, 1981; Paul, Srendi & Schwartz, 1981). In the present study this methodology has been used to raise allergen specific T cell lines from the peripheral blood of atopic patients. Such lines seem particularly valuable for the study of the properties of the responding cells together with the interactions involved in the response.

MATERIALS AND METHODS

Media and supernatants. The medium used throughout was RPMI 1640 (Flow Laboratories, Irvine, Scotland) supplemented with 2 mM L-glutamine, 1% non-essential aminoacids, 1% pyruvate (Flow) and 5×10^{-5} M 2-mercaptoethanol (complete RPMI). The medium contained either 10% heparinized autologous plasma or 10% fetal calf serum from selected batches (Hyclone, Utah, USA). In order to maintain the growth of antigen activated Tb, the culture medium was supplemented with a supernatant (25% vol./vol.) of phytohaemagglutinin activated tonsil cells (PHA-SUP) containing high levels of IL-2. This PHA-SUP was produced by culturing tonsil cells (4×10^6/ml) in complete RPMI containing 5% human plasma and 1 μg/ml PHA.P for 40 h. The supernatant was collected and PHA removed by affinity chromatography on insolubilized porcine thyroglobulin. IL-2 activity of each preparation was tested on IL-2-dependent human T cell clones according to Gillis *et al.* (1978).

Antigens. Rye grass antigen group I (Rye I) was kindly provided by the National Institute of Allergy and Infectious Diseases (Bethesda, Maryland, USA). A *Dermatophagoides pteronyssinus* (DP) extract was prepared from mite bodies (Allergon AB, Sweden) by potter homogenization and stirring overnight at 4°C in 0·02 M phosphate buffer pH 7·2. The extract was centrifuged and the supernatant fractionated on a Sephadex G-100 column as suggested by Chapman & Platts-Mills (1978). The allergen activity of the various fractions was measured by an indirect RAST as detailed elsewhere (Romagnani *et al.*, 1976). The most active fractions which were eluted in a range of molecular weight between 10,000 and 25,000 were pooled, dialysed against distilled water and liophilized. The protein content was determined by the Lowry method using ovalbumin as a standard.

Tetanus toxoid (TT) was obtained from the Massachusetts Biological Laboratories, USA. Cytoplasmic protein extracts from *Candida albicans* (CA) were obtained from the NIBSC, London, UK.

Production of allergen specific human T cell lines. Blood was obtained from four healthy subjects and five atopic patients suffering from allergic rhinitis and/or bronchial asthma. Two of these patients were sensitive to Rye I and four to DP allergens, as demonstrated by positive skin tests and RAST. The patients were out of any treatment at the time of study. PBM obtained by Ficoll-Hypaque density centrifugation were stimulated *in vitro* with Rye I or DP. Three million PBM were cultured in 2 ml complete RPMI containing 10% autologous heparinized plasma in flat bottom 24 well tissue culture plates. The cultures were stimulated with 10 μg/ml DP or 5 μg/ml Rye I. These antigen concentrations were selected on the basis of previous titration experiments (Romagnani *et al.*, 1973). In some tests PBM were pulsed with antigen (50 μg/ml) at 37°C for 2 h, washed three times and cultured as described above. After 5–6 days the cultures were inspected microscopically and thymidine incorporation was measured by a 6 h pulse on replica cultures carried out in microplates. The blast cells developed following allergen stimulation were isolated by centrifugation on discontinuous Percoll gradients (Pharmacia, Uppsala, Sweden) and cultured at a concentration of 10^5 Tb/ml in RPMI supplemented with 25% PHA-SUP as a source of IL-2 (Lanzavecchia, Ferrarini & Celada, 1982).

Cell cloning. Tb were seeded at 1 cell/well in 96 well U bottom microplates in the presence of antigen, 3×10^4 autologous irradiated PBM (2,500 rad from a ^{60}Co source) and 30% PHA-SUP. After 2–3 weeks cell growth was detected microscopically and the growing wells were expanded further using antigen, irradiated PBM and IL-2 until a sufficient number of T cells were available.

Proliferative response to antigens or mitogens. Tb were harvested from IL-2-dependent cultures and washed three times. Ten thousand Tb were cultured in flat bottom microplates in 0·2 ml RPMI-FCS in the presence or absence of irradiated or mitomycin-C treated antigen presenting cells (APC). As a source of APC either 10^5 PBM or 2×10^4 adherent cells were used with comparable results. The cultures were stimulated with either DP (10 μg/ml), Rye I (5 μg/ml), TT (20 μg/ml), CA (1/50 final dilution) or PHA (1 μg/ml). After 40 h 0·5 μCi of tritiated thymidine (specific activity 5 Ci/mM) were added, the cultures harvested after an additional 6 h and the radioactivity incorporated counted by liquid scintillation. In some experiments Tb were cultured with autologous irradiated PBM and allergen in the presence of anti-HLA antibodies (Lanzavecchia *et al.*, 1982). The following antibodies were used: rabbit anti-human β_2 microglobulin (Dako, Copenhagen, Denmark), rabbit anti-human Ia (Winchester *et al.*, 1976), monoclonal antibodies (Mab) 2/9 and D1.12 anti-HLA-DR (Tosi *et al.*, 1981) and Mab W6/32 anti-HLA-A, -B, -C (Seralab, Crawley Down, UK).

Cell surface marker analysis. The rosette methods to detect receptors for sheep erythrocytes (E) and the immunofluorescent techniques for surface immunoglobulins and the determinants defined by monoclonal antibodies were detailed previously (Lanzavecchia *et al.*, 1982).

RESULTS

PBM from five atopic patients and four healthy donors were cultured with optimal concentrations of DP, of Rye I or with medium alone (control). After 5 days cell proliferation was evaluated by thymidine incorporation and microscopic examination. Four out of five atopic patients gave a significant proliferative response to the allergen responsible for sensitization (stimulation index > 10) and showed clusters of large blast cells. Only one normal donor gave a significant proliferative response to DP.

The blasts from seven positive cultures were isolated on a discontinuous Percoll gradient and expanded in medium supplemented with 25% PHA-SUP as a source of IL-2. Under these conditions the cells proliferated rapidly with a doubling time of 2–3 days and had to be divided to maintain the cell concentration between 10^5 and 10^6 cells/ml. After 2–4 weeks in IL-2 the T cell lines were restimulated with the specific allergen in the presence of autologous irradiated PBM as a source of APC. This restimulation was necessary because the lines became progressively refractory to IL-2 after 4–6 weeks in culture. Five out of the seven lines initially obtained were able to proliferate in response to the specific antigen and were selected for further studies. All of these lines were from atopic individuals. The other two lines (one from a normal and one from an atopic donor) became unresponsive to the specific allergen and, therefore, were rapidly lost. The selected lines were maintained growing in IL-2 for periods variable from 6 to 9 months with periodical antigen restimulations. Aliquots were stored frozen and thawed when required. Fig. 1 shows the progressive enrichment for the allergen induced proliferative response obtained in one line by repeated restimulations with the specific allergen (DP). Similar results were obtained with the other lines. Interestingly, the slope of the curve obtained by plotting the number of Tb against cell proliferation is of approximately 1, indicating that within the lines a single cell type (maybe the antigen specific Tb) was limiting (see for comparison the pattern observed with clone CP2 obtained from the bulk line).

The allergen-induced T cell lines were tested for their capacity to proliferate in response to the inducing allergen or to other unrelated antigens. Ten thousand Tb were cultured with optimal concentrations of each antigen in the presence of 10^5 autologous irradiated PBM and in the absence of exogenous IL-2. Thymidine incorporation was measured after 40 h. As evident from Table 1 only the allergen originally used to raise the line but not unrelated antigens such as CA or TT (to which, however, the donors were responsive) induced proliferation. All of the lines were induced to proliferate by PHA (data not shown).

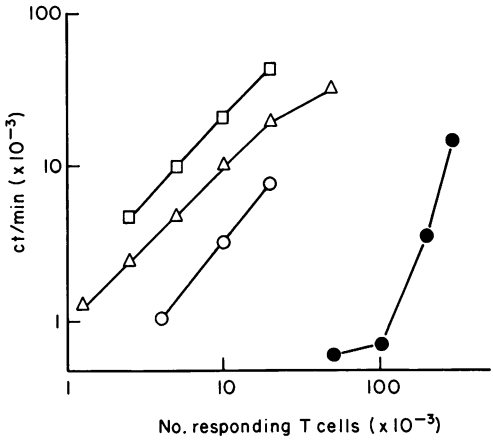

Fig. 1. Enrichment for allergen-induced proliferative response of T cells in long term cultures. Graded numbers of T cells were stimulated with DP in the presence of 2×10^4 irradiated adherent cells. Peripheral blood T cells (●); cultured Tb grown in IL-2 after one (○) or two (△) *in vitro* stimulations with DP and clone CP-2 (□) obtained from the same line. Thymidine incorporation was measured on day 2 except for peripheral blood T cells (day 5).

The restriction of allergen recognition by Tb was next investigated. Ten thousand Tb were stimulated with the specific allergen in the presence of autologous or allogeneic irradiated adherent cells as a source of APC. Proliferation was observed only in the presence of autologous but not of allogeneic APC irrespective of whether APC were from atopic or healthy donors (Table 2). By contrast the proliferative response induced by PHA did not require APC to be autologous (i.e. was unrestricted). The same Table also shows that 2×10^4 adherent cells were able to present allergen to

Table 1. Antigen specificity in the proliferative response of allergen-induced human T cell lines

Cells in culture*		^{3}H-thymidine incorporated (ct/min) in cultures stimulated with:†			
Tb	APC	DP	Rye I	CA	TT
A anti-DP	A	6,770	100	80	nd
B anti-DP	B	10,150	330	120	nd
C anti-DP	C	15,960	120	180	220
A anti-Rye I	A	130	7,700	340	100
D anti-Rye I	D	110	18,140	140	110
A anti-CA‡	A	420	250	25,650	360
C anti-TT‡	C	250	420	480	28,800

* Ten thousand Tb were cultured with 10^5 autologous irradiated PBM as a source of APC in the presence of the specific allergen or of unrelated (control) antigens.

† Data represent the mean of triplicate determinations.

‡ TT and CA specific lines were obtained as described elsewhere (Lanzavacchia *et al.*, 1982).

nd = not done.

Table 2. Restriction of the allergen-induced proliferation response

T cell line	Stimulation	^{3}H-thymidine incorporation† (ct/min) in the presence of APC from donor*				
		A	B	C	D	none
A anti-DP	DP	8,890	320	500	300	50
	PHA	15,500	16,200	16,250	15,110	150
B anti-DP	DP	620	11,680	1,050	nd	250
	PHA	24,760	25,120	23,100	nd	450
C anti-DP	DP	770	260	12,100	nd	110
A anti-Rye I	Rye I	7,730	320	nd	250	100
D anti-Rye I	Rye I	560	420	nd	15,250	80

* Ten thousand Tb from the T cell line indicated were stimulated with the specific allergen or with PHA in the presence of 2×10^4 adherent cells from the same or unrelated donor as a source of APC.

† ^{3}H-thymidine incorporation was measured after 40 h. Data represent mean of triplicate determinations.

nd = not done.

Tb as effectively as 10^5 PBM, thus ruling out that other T cells present within the irradiated PBM might contribute to the allergen induced proliferative response observed.

To investigate whether HLA-DR antigens were involved in the process of antigen presentation, Tb were stimulated with the specific allergen, PHA or IL-2 in the presence of anti-HLA-DR antibodies or anti-HLA-A, -B, -C antibodies (control). The results of one out of five experiments carried out with identical results are shown in Table 3. Anti-HLA-DR inhibited completely the response to the allergen, but not the response to PHA or IL-2. By contrast anti-HLA-A, -B, -C or anti-β_2 microglobulin failed to inhibit any proliferative response.

In order to dissect out a possible heterogeneity within the T cell lines and to obtain more defined cellular reagents, clones were isolated from one DP specific line by limiting dilution. Twelve out of the 96 wells where the Tb were seeded at 1 cell/well showed cell growth. The clones were expanded in PHA-SUP and seven of them gave a number of cells for further testing. Table 4 shows that two of the clones could proliferate strongly in response to the antigen in the absence of exogenous IL-2, three other clones gave a much lower, although significant response, while two failed to respond.

Table 3. Inhibition of the allergen-induced proliferative response by anti-HLA-DR antibodies

Antibody added*	Specific for	^{3}H-thymidine incorporated (ct/min) in cultures stimulated by†		
		DP+APC	PHA+APC	IL-2
—	—	16,450	31,660	12,520
Rabbit antiserum	HLA-DR	120	32,120	13,750
Mab 2/9 (20 μg/ml)	HLA-DR	3,910	31,700	13,080
Mab D1-12 (20 μg/ml)	HLA-DR	1,350	31,000	13,990
Rabbit antiserum	β_2-microglobulin	15,370	32,830	12,900
Mab W6/32 (50 μg/ml)	HLA-A, -B, -C	18,860	31,770	13,300

* The antibody indicated was added at the initiation of cultures.

† Ten thousand Tb (line A anti-DP) were stimulated with DP or PHA in the presence of autologous irradiated APC or with IL-2 alone. Thymidine incorporation was measured as in Table 1.

Table 4. Proliferative response of T cell clones derived from a DP-induced T cell line

		^{3}H-thymidine incorporated (ct/min) in cultures stimulated with*	
Tb from clone	APC	DP	—
CD 1	C	12,700	200
CD 1	A	150	180
CD 2	C	13,900	250
CD 3	C	3,400	50
CD 4	C	2,800	30
CD 5	C	3,800	140
CD 6	C	150	100
CD 7	C	250	120

* Culture conditions as in Table 1.

T cell lines and clones were examined for their surface phenotype using conventional markers as well as a panel of monoclonal antibodies. The data reported in Table 5 show that the vast majority (>98%) of the cells within the lines were T cells as defined by the presence of E receptors and of the antigens detected by the OKT3 and Leu 1a monoclonal antibodies. Most of the cells were also HLA-DR positive as expected for activated T cells. Interestingly enough, the lines showed some heterogeneity in that a variable proportion (5–35%) of the cells were OKT8$^+$, Leu 2a$^+$, while the remaining of them (60–90%) were OKT4$^+$, Leu 3a$^+$. By contrast the clones had the expected homogeneous surface markers and all of them showed the helper–inducer phenotype (OKT4$^+$ Leu 3a$^+$).

Table 5. Cell surface markers of allergen specific T cell lines and clones

Cells from:	Percentage of positive cells for					
	E-RFC*	OKT3/Leu 1a†	OKT4/Leu 3a‡	OKT8/Leu 2a§	HLA-DR	SIg
Line A anti-DP	>95	>95	60–70	30–35	>90	0
Line C anti-DP	>95	>95	90	5–10	>90	0
Line D anti-Rye I	>95	>95	65–70	25–30	>90	0
Clone CD 1	>95	>95	>95	0	>95	nd
Clone CD 2	>95	>95	>95	0	>95	nd
Clone CD 5	>95	>95	>95	0	>95	nd

* Cells forming rosettes with neuraminidase treated sheep erythrocytes.
† Identify all circulating T cells.
‡ Identify the subset containing the helper T cells.
§ Identify the subset containing the suppressor/cytotoxic T cells.

DISCUSSION

The present paper demonstrates that antigen specific T cell lines can be raised from atopic patients by stimulating their peripheral blood with the appropriate allergen and by expanding the Tb so obtained with IL-2. If restimulated with the antigen at intervals, the lines can be maintained in culture for long periods. Moreover antigen stimulation selects for the specific Tb whose concentration increases progressively in the growing cell line.

The lines were restricted in that they only recognized antigen together with autologous APC.

Such restriction was presumably at the level of the recognition of HLA-DR related structures on the APC, since anti-HLA-DR antibodies inhibited selectively the proliferative response to the allergen but not that to PHA or to IL-2. These findings are in agreement with our previous data on human T cell lines specific for TT or CA antigens (Lanzavecchia *et al.*, 1982). It is of interest, in this connection, that the Tb responding to soluble antigens differ from alloreactive Tb in that the response to IL-2 of the latter cells is inhibitable by anti-DR antibodies (Moretta, Accolla & Cerottini, 1982). Another argument in favour of the hypothesis that HLA-DR antigens on APC were the molecules responsible for the restriction was that one line responded to DP in the presence of autologous APC and also in the presence of APC from an unrelated individual sharing certain HLA-DR determinants (unpublished data).

Taken together, the above findings demonstrate that atopic individuals have a number of circulating allergen specific T cells. Presumably, these cells are not the only responding cells when PBM are stimulated with the allergen *in vitro*, but become the predominant population displaying all the features of antigen specific T cells under highly selecting conditions (Kurnick *et al.*, 1979; Fischer, Beverly & Feldmann, 1981; Lanzavecchia *et al.*, 1982). Surface marker analysis of the lines showed that they comprise an heterogeneous cell population and that, together with a majority of cells displaying an helper T cell phenotype, other cells, possibly representing suppressor or cytotoxic lymphocytes, were also present. Interestingly, preliminary studies on the capacity of the lines of inducing a polyclonal activation of B cells have demonstrated that they have helper activity, a finding that is consistent with that of their predominant cell surface phenotype.

The cloning experiments have so far yielded only clones with helper phenotype. This finding may be related to the predominance of the helper cells in the original lines or due to some selection operated by the cloning procedures. Whatever the reason may be, it seems, however, possible to obtain cells with different functions by more extensive cloning experiments, especially if cell fractionation procedures are also used. The enrichment for antigen specific T cells observed in the lines represents a powerful tool for those studies on the fine specificity of human T cells for allergens (Ishizaka *et al.*, 1974).

Recently some investigators including some of us have reported methods to study IgE production *in vitro*. These studies have demonstrated that PBM from atopic patients produce IgE molecules and IgE antibodies spontaneously (Fiser & Buckley, 1979; Saxon, Morrow & Stevens, 1980; Romagnani *et al.*, 1980). By contrast, there is considerable disagreement as to whether PBM from normal or atopic individuals can be induced to produce IgE following stimulation with polyclonal activators or specific antigens (Fiser & Buckley, 1979; Saxon & Stevens, 1979; Romagnani *et al.*, 1980; Nonaka *et al.*, 1981). These discrepancies are a clear demonstration of the complexity related to the techniques available. The use of allergen specific T cell clones may represent a new experimental approach to the problem.

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