

Quantitative aspects of the T cell proliferation response to antigen P₁ from *D. pteronyssinus*: suppression by added histamine and limited effects of basophil depletion

FRANCES C. RAWLE,*† T. A. E. PLATTS-MILLS† & E. B. MITCHELL* *Division of Immunological Medicine, Clinical Research Centre, Harrow, UK and †Division of Allergy & Clinical Immunology, Department of Internal Medicine, University of Virginia, Charlottesville, Virginia, USA

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SUMMARY

We have recently shown that peripheral blood mononuclear cells from patients sensitive to the house dust mite, *D. pteronyssinus*, will proliferate *in vitro* in response to the purified major allergen, antigen P₁. Such cell populations, separated on Ficoll gradients, were shown to contain basophils, and had an average histamine content of 12 ng/10⁶ cells. Incubation with antigen P₁ resulted in the release of histamine, and histamine is known to activate T suppressor cells. In the present experiments we observed up to 80% inhibition of proliferation (mean 50–60%) with histamine added at 3.3×10^{-7} – 3.3×10^{-5} M. Cultures of T cells supplemented with irradiated non-T cells, that had been depleted of cells bearing surface membrane IgG, IgM and IgE, contained on average 63% less histamine than unseparated cultures. However, no consistent difference in the proliferative response to antigen P₁ was observed. Depletion of histamine by pre-incubation of the cells with antigen P₁ at 10^{-3} – 10^{-4} µg/ml followed by washing of the cells before culture also produced no significant change in the proliferative response. Passage of cell population over nylon wool resulted in depletion of basophils, as well as other cell types, and generally led to a decrease in proliferation. We conclude that release of mediators from basophils in cell cultures does not markedly affect the magnitude of the proliferative response to antigen P₁. The varied responses seen with cells from different individuals are likely to reflect differences in the numbers of circulating allergen sensitized T cells.

Keywords T lymphocyte proliferation *D. pteronyssinus* allergy basophils histamine inhibition

INTRODUCTION

There have been many reports describing *in vitro* proliferative responses to crude or purified allergen preparations using peripheral blood mononuclear cells (PBMC) from allergic patients (Brostoff, Greaves & Roitt, 1969; Maini *et al.*, 1971; Gatien, Merler & Colten, 1975; Black & Marsh, 1980; Hiratani *et al.*, 1981). The possible effects of allergen-induced basophil degranulation, and consequent mediator release into the cultures, on the *in vitro* proliferative response to allergens have

Present address: ICRF Human Tumour Immunology Group, University College Hospital Medical School, University Street, London WC1E 6JJ, UK.

Correspondence: Dr E. B. Mitchell, Division of Immunological Medicine, Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3 UJ, UK.

not been resolved. Many authors assume that PBMC preparations obtained by the standard technique of centrifugation over a Ficoll gradient are depleted of all granulocytes. However, when Böyum first described the separation technique (1968), he clearly showed that while eosinophils and neutrophils were found predominantly in the pellet, basophils remained with the mononuclear cells at the interface. In allergen stimulated cultures, these basophils will degranulate, releasing histamine and other mediators into the medium. In theory, these mediators could either enhance or inhibit proliferative responses to allergens. Histamine has been shown to activate T suppressor cells, in some cases at concentrations as low as 5 ng/ml ($\approx 5 \times 10^{-8}$ M) (Rocklin & Haberek-Davidson, 1981; Strannegard & Strannegard, 1977; Wang & Zweiman, 1978). There are also reports that *in vitro* lymphocyte proliferative responses in allergic patients may be particularly sensitive to histamine-induced suppression, although this is not a consistent finding (Strannegard & Strannegard, 1977; Brostoff, Pack & Lydyard, 1980; Beer *et al.*, 1982). Recently, Hofstetter, Fasano & Ottesen (1983) have reported that *in vitro* lymphocyte proliferation in response to Schistosoma antigens increases when cell culture populations are depleted of basophils. Thus, it is important to determine whether the presence of basophils has any effect on the *in vitro* proliferative responses in allergen stimulated cultures, and, in particular, whether histamine inhibition may be responsible for the fact that some workers have found *in vitro* proliferative responses to allergens to be poor and unreliable.

Many patients with atopic dermatitis, asthma or perennial rhinitis have positive skin prick tests to *D. pteronyssinus*. Most of these patients have both immediate skin test reactivity and serum IgE antibody to the major allergen, antigen P₁ (Chapman *et al.*, 1983). We have recently reported that peripheral blood T cells from these patients will proliferate *in vitro* when stimulated with antigen P₁ (Rawle, Mitchell & Platts-Mills, 1984). In the present series of experiments, we have confirmed that added histamine can reduce the proliferative responses to antigen P₁ *in vitro*. However, we have shown that depletion of basophils before culture, or depletion of histamine by pre-incubation with the allergen, does not result in any consistent increase in the proliferative response to this allergen. The results suggest that allergen induced basophil degranulation is unlikely to have a major effect on T cell proliferation in PBMC cultures and is not responsible for the great differences in proliferative responses seen in different *D. pteronyssinus* sensitive patients.

MATERIALS AND METHODS

Subjects. Patients presenting with rhinitis, asthma or atopic dermatitis were assessed for sensitivity to *D. pteronyssinus* by clinical history and skin prick testing (Bencard, UK). All patients studies here were also included in our previous study on PBMC proliferative responses (Rawle *et al.*, 1984). Non-allergic controls were laboratory personnel with no history of allergic disease and negative skin prick tests to *D. pteronyssinus*.

Cell separation techniques. Heparinized venous blood was diluted 1:1 with phosphate-buffered saline and PBMC were separated by the standard technique using Ficoll-Paque (Pharmacia) or Ficoll-Triosil (sp. gr. 1.077) (Böyum 1968). T cells were purified from PBMC by rosetting with neuraminidase treated sheep red cells (SRBC) and centrifugation over Ficoll-Metrizoate (sp. gr. 1.090). Non-T cells were further separated into surface membrane immunoglobulin positive (SmIg⁺) and negative (SmIg⁻) populations by rosetting with ox red blood cells (OxRBC) coated with specifically purified goat anti-human immunoglobulin antibodies (De Gast & Platts-Mills, 1979). The technique was modified by using OxRBC coated with anti-IgE mixed in a ratio of 1:2 with those coated with anti-IgM and anti-IgG. Non-T and non-T SmIg⁻ populations were added at 20% and 10%, respectively, to purified T cells. These 'antigen presenting cells' were either irradiated at 2,000 rad using a ⁶⁰Co source or treated with Mitomycin C (25 µg/ml) for 30 min before they were added to T cells. Nylon wool columns were prepared by packing 3 g acid washed nylon wool (Fenwal Laboratories, Deerfield, Illinois, USA) into 35 ml plastic syringes and sterilized by autoclaving. These were equilibrated in culture medium containing 10% serum at 37°C before use; cells were incubated on the column for 1 h at 37°C before eluting the non-adherent cells with culture medium.

Cell culture. All cell populations were cultured at 1×10^6 per ml in RPMI 1640 Dutch Modification medium (Flow Labs, UK) containing added L-glutamine (2 mM), penicillin (100 u/ml), streptomycin (100 μ g/ml) and 10% pooled human AB+ serum. Cells were cultured in flat bottomed microtitre plates (Sterilin, UK) at 250 μ l per well, and incubated at 37°C in a humidified atmosphere with 5% CO₂. Unless stated otherwise, culture time was 7 days. Cultures were pulsed with ³H-thymidine (2 Ci/mMol, Amersham, UK) at 1 μ Ci/well 6 h before harvest, and ³H-thymidine incorporation was determined by liquid scintillation counting. Results are expressed as the geometric mean of triplicate cultures (GM).

Antigen P_1 was prepared from *D. pteronyssinus* culture by a modification of the technique described previously (Chapman & Platts-Mills, 1980), involving a 4 hr aqueous extraction, 50% saturated ammonium sulphate precipitation, Pevikon block electrophoresis and gel filtration on Sephadex G100 (Pharmacia). The preparation was stored freeze dried and diluted in culture medium as required.

Histamine was added to cultures in the form of histamine acid phosphate. This was obtained as a 1 mg/ml solution sterile ampoules, diluted in culture medium as required, and added to cell suspensions before antigen P_1 . Percentage inhibition by histamine was calculated as:

$$1 - \frac{\text{GM ct/min with histamine}}{\text{GM ct/min without histamine}} \times 100.$$

Pre-incubation with antigen P_1 . Cells were resuspended in culture medium and incubated for 1 h at 37°C with antigen P_1 at 10^{-3} or 10^{-4} μ g/ml. The concentration used was the minimum required to induce > 30% histamine release, determined in previous studies on the same patients. PPD was added at 10^{-3} or 10^{-4} μ g/ml. Cells were washed twice before culture. 'Pre-incubation controls' were incubated with antigen but not washed before culture.

Histamine assay and differential cell counts. The total histamine content of cell populations was determined using the technique described by Lichtenstein & Osler (1964). Cells were lysed by incubation at 100°C for 10 min; histamine was extracted by serial partition between aqueous and organic solvents and assayed in a fluorescence assay using *o*-phthalaldehyde. Standard solutions of histamine dihydrochloride were freshly prepared for each assay. Differential cell counts were carried out on 'cytoprep' slides fixed in methanol and stained with May-Grunwald-Giemsa, at a magnification of $\times 630$ using an oil immersion lens.

RESULTS

Basophil content of cell populations

PBMC populations obtained by density gradient centrifugation over Ficoll-Paque or Ficoll-Triosil contained significant amounts of histamine, and intact basophils were seen on cytoprep slides (Table 1). Contrary to the experience of some other workers, defibrination of the blood using wooden sticks before separation did not appear to result in a significant reduction in the numbers of basophils or in the total histamine content of PBMC populations (Table 1). We have previously shown that low doses of antigen P_1 (10^{-3} – 10^{-6} μ g/ml) can trigger release of up to 70% of the total histamine from leucocytes (Mitchell *et al.*, 1982). In two experiments, incubation (1 h at 37°C) with antigen P_1 at 1 μ g/ml, which is in the concentration range used to stimulate proliferation, led to release of 58% and 65% of total cellular histamine. T cells purified by SRBC rosetting still contain measurable levels of histamine, but the amount was at least five-fold less than in PBMC populations. In keeping with this, non-T cell populations contained more histamine than the PBMC. The monocyte enriched population obtained by depleting non-T cells of cells bearing IgM, IgG and IgE on their surfaces contained fewer basophils and, on average, approximately 30% less histamine than the non-T fractions. In five experiments where the histamine content of all cell populations was measured, populations of T cells with 10% SmIg⁺ cells contained on average 63% less histamine than parallel PBMC populations.

Table 1. Mean histamine content of cell populations from rosette separations and differential cell counts from a representative experiment

Population	Mean* histamine content (range) (ng/10 ⁶ cells)	Basophils	Eosinophils	Neutrophils	Lymphocytes	Monocytes
PBMC (heparin)	12.3 (4.0–34.5)	4 (0.7%)	0	1 (0.2%)	476 (81%)	108 (18.3%)
PBMC (defibrinated)	—	4 (0.8%)†	0	5 (1%)	454 (86%)	64 (12%)
T cells	2.2 (0.29–5.9)	0 (<0.2%)	1 (0.2%)	0	596 (>99%)	2 (0.3%)
Non-T	41.0 (9.5–80.6)	9 (1.9%)	5 (1.1%)	3 (0.6%)	144 (30%)	315 (66%)
Non-T, SmIg ⁻	29.8 (2.3–150)	1 (0.3%)	3 (0.8%)	2 (0.5%)	50 (12.9%)	333 (86%)
Non-T, SmIg ⁺	—	7 (1.5%)	1 (0.2%)	4 (0.8%)	374 (79%)	90 (19%)
T+10% SmIg ⁻	4.8 (0.49–17.0)					

* Means of 12–15 experiments.

† The total histamine content in this experiment was 5% lower in cells from defibrinated blood compared to heparinized blood.

Proliferative response of separated cells to antigen P₁

Despite the marked reduction in the histamine content, cultures of T+10% SmIg⁻ cells showed little if any increase in the proliferative response to antigen P₁ compared with parallel PBMC cultures (Figs 1 & 2). Cultures of T+20% non-T cells contained considerably greater amounts of histamine than T+10% non-T SmIg⁻ cultures, but showed very similar proliferative responses (Fig. 1). This indicates that any small increase in proliferation observed in separated cell cultures were unlikely to be a result of basophil depletion. As reported previously, purified T cells without antigen presenting cells did not proliferate with antigen P₁ (Fig. 1).

In 11 experiments on nine *D. pteronyssinus* sensitive patients who gave a positive proliferative response, separated cells showed higher proliferation in eight cases and lower proliferation in three cases (Fig. 2). In some experiments, (as in Fig. 1) background proliferation was reduced in separated

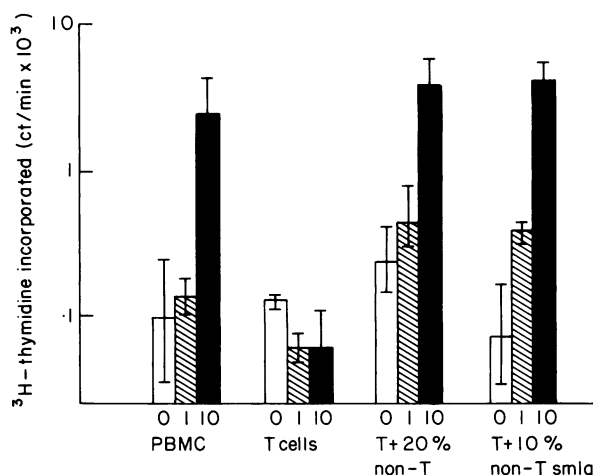


Fig. 1. Comparison of proliferation responses in PBMC and cell populations separated by rosetting. T+10% non-T SmIg⁻ to cultures are more extensively depleted of basophils than the T+20% non-T cultures. □ = unstimulated control. ■ = 1 µg/ml antigen P₁. ▨ = 10 µg/ml antigen P₁. Values are geometric mean of triplicate cultures, with bars representing the range.

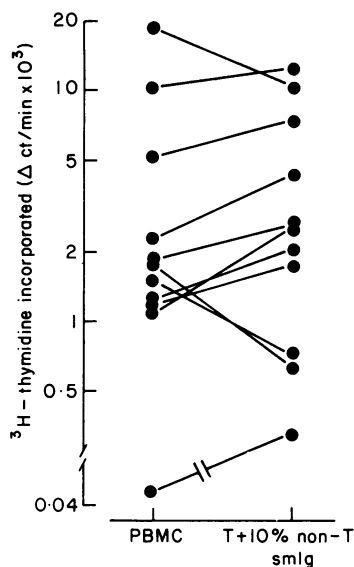


Fig. 2. Comparison of proliferation in response to 10 $\mu\text{g/ml}$ antigen P_1 in cultures of PBMC and cells depleted of basophils by rosetting. Figures are specific difference ct/min ^3H -thymidine incorporated = (GM stimulated cultures – GM control cultures).

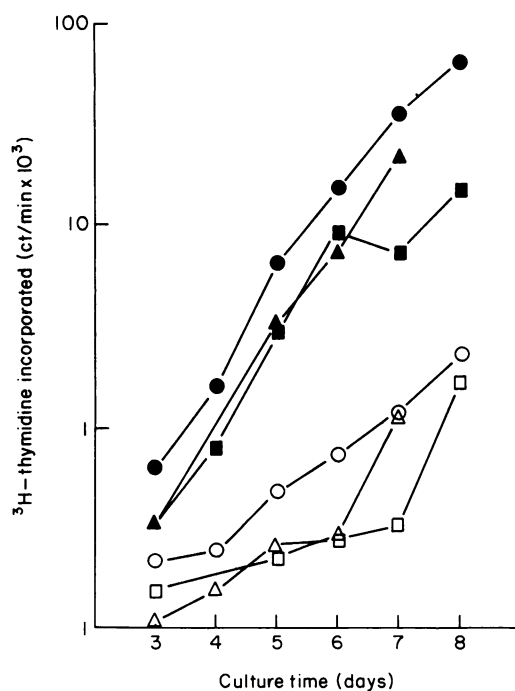


Fig. 3. Inhibition of proliferation of PBMC cells by histamine. ■, ▲, ● = cultures stimulated with 10 $\mu\text{g/ml}$ antigen P_1 . □, Δ, ○ = unstimulated cultures. ●, ○ = no histamine. ▲, Δ = 3.3×10^{-7} M histamine. ■, □ = 3.3×10^{-5} M histamine.

cell cultures leading to an increase in the calculated stimulation index. In experiments on non-atopic subjects, neither PBMC (15 subjects) nor separated cells (two subjects) showed any proliferative response to antigen P_1 . In cases where PBMC cultures from allergic subjects showed a poor proliferative response to antigen P_1 , there was no significant increase in stimulation in separated cell cultures.

Histamine inhibition

Histamine acid phosphate added at 0.1–10 $\mu\text{g/ml}$ (3.3×10^{-7} – $3.3 \times 10^{-5}\text{M}$) inhibited proliferation in both unstimulated and antigen P_1 stimulated PBMC cultures (Fig. 3). Up to 80% inhibition was seen, but the mean value was around 50%, and this was only slightly greater with $3.3 \times 10^{-5}\text{M}$ histamine (mean 60%) than with $3.3 \times 10^{-7}\text{M}$ (mean 50%). On average, proliferation in separated cultures was inhibited to a slightly lesser extent; mean inhibition was 32% with 10^{-6}M histamine in cultures stimulated with 10 $\mu\text{g/ml}$ P_1 ($n=9$). However, the range of results seen was very wide (0–73%) and in one experiment where inhibition was studied in parallel cultures, greater inhibition was seen with 10^{-6}M histamine in T + 10% non-T SmIg⁻ cultures (mean 64%, range 41–83%) than in PBMC cultures (mean 44%, range 5–68%).

Alternative techniques for basophil or histamine depletion

Pre-incubation of cells with $10^{-3}\text{ }\mu\text{g}$ antigen P_1/ml followed by washing to remove the mediators released was carried out on PBMC and also, in one experiment, on the non-T population prior to irradiation and addition of T cells. This procedure significantly reduced the total histamine content of the cells cultured (Table 2). In five experiments, no consistent effect of pre-incubation was observed, and in general, proliferation was not increased. Where small increases in proliferation were seen (see Table 2, Expt. 1, day 5 and Expt. 2, days 5 and 7) a very similar effect was seen with

Table 2. Effects of alternative techniques for basophil or histamine depletion

		Histamine ng/10 ⁶ cells (% depletion)	Day 5		Day 7	
			Control‡	10 µg/ml antigen P ₁ ‡	Control‡	10 µg/ml antigen P ₁ ‡
<i>Pre-incubation with Antigen P₁</i>						
Expt. 1	PBMC	21.8	210	577	455	2,425
	Pre-inc. control	—	286	849	788	2,179
	Pre-inc. + wash	13.3 (39%)	585	1,250	1,039	2,974
	PPD pre-inc. + wash	21.5 (1%)	320	1,258	1,623	2,698
Expt. 2	T + 20% non-T	8.4	319	13,546	401	5,868
	T + 20% non-T					
	Pre-inc. Control	—	255	6,412	739	4,036
	T + 20% non-T					
	Pre-inc. + wash	3.6 (57%)	315	13,616	432	12,640
	T + 20% non-T					
	PPD pre-inc.					
	+ wash	6.8 (19%)	179	14,439	699	12,610
<i>Nylon wool adherence</i>						
Expt. 3	PBMC	5.7	263	813	588	2,032
	Non-adherent Cells	1.9 (66%)	23	47	11	76*
Expt. 4	PBMC	ND	279	2,263	747	11,330
	Non-adherent Cells	ND (48%)†	104	1,817	270	21,124

* In parallel cultures, these cells gave a positive (but much reduced) proliferative response to PPD, indicating that they were not dead.

† Mean histamine depletion after passage over nylon wool in four experiments.

‡ Results are expressed as the mean ct/min ^3H -thymidine incorporation at day 5 or day 7.

cells that had been pre-incubated with PPD, which did not change the histamine content. Passage of cells over nylon wool columns resulted in a depletion of histamine in the non-adherent cells (mean 48%, $n=4$) (Table 2). In three of four experiments, non-adherent cells showed very poor proliferation in culture, not only with antigen P₁ but also with PHA and PPD. In one experiment where good proliferation was seen, there was a two-fold increase in the response to 10 $\mu\text{g/ml}$ antigen P₁ at Day 7, but this was not apparent on day 5 or at lower antigen doses (Table 2).

DISCUSSION

We have demonstrated in these experiments that, despite the fact that histamine added to the cultures can inhibit proliferation, depletion of the endogenous histamine in the cell populations by a variety of techniques did not result in a consistent increase in the proliferation seen. There are several possible explanations for these results. Histamine released from basophils in the cultures might be at too low a concentration to cause significant inhibition, or alternatively, other mediators released from basophils might counteract its effects. For instance, it has been reported that heparins from various sources can stimulate or inhibit *in vitro* proliferation (Lippman & Matthews, 1977). None of the depletion techniques used were entirely specific for basophils and removal of other cell types might affect the proliferative response. However, no significant increase in proliferation was observed using three entirely different techniques for histamine depletion.

It may be that very low levels of histamine present in these cultures are, indeed, sufficient to cause some suppression and that the degree of basophil depletion achieved in these experiments was insufficient to show a marked effect. Despite experimenting with many different techniques, we were unable to develop a more satisfactory method for complete basophil depletion. Since rosetting with anti-IgM and anti-IgG coated OxRBC gives > 90% depletion of B cells (De Gast & Platts-Mills, 1979), we expected to be able to obtain equally good depletion of basophils by rosetting with anti-IgE coated OxRBC. The relatively poor depletion might reflect instability of basophil rosettes, or poor binding of anti-IgE to cell bound IgE molecules.

One might expect that after removal of the endogenous source of histamine, the remaining cells would be more sensitive to added histamine. In a few instances, where histamine inhibition in PBMC cultures was low, increased inhibition was observed in basophil depleted cultures, but in most experiments, no such increase could be demonstrated. This might be considered as further evidence that endogenous histamine does not markedly affect proliferation. Basophil degranulation occurs at doses of antigen P₁ considerably lower than those required to induce proliferation. Thus, the poor proliferation observed with cells from some patients at low doses of antigen P₁ could be attributed to histamine inhibition. But, from our data it seems unlikely that mediator release has an important effect on the proliferative response at higher antigen doses.

Our results contrast with those of Hofstetter *et al.* (1983), who reported a consistent enhancement of proliferation in response to Schistosome antigens in cells non-adherent to nylon wool. As evidence that this enhancement was due to basophil depletion, they cited the fact that it could be reversed fully by addition of histamine. However, they showed no increase in the susceptibility to histamine inhibition in the nylon wool non-adherent cells, and as they themselves point out, nylon wool adherence is known to deplete certain monocytic and T suppressor cells, which could equally explain the enhanced response. The markedly reduced proliferation we observed in three of four experiments using nylon wool non-adherent cells might reflect either loss of adherent antigen presenting cells or a reduction in the numbers of T cells capable of responding to antigen.

Despite the general lack of effect of basophil depletion observed in these experiments, inhibition by histamine released from basophils is a factor which should not be overlooked when studying the magnitude of *in vitro* proliferative responses to allergens. In particular, changes in the releasability of basophil granules, for instance after desensitization (Pruzansky & Patterson, 1967; Sadan *et al.*, 1969; Evans *et al.*, 1975) must be allowed for when interpreting any changes in the overall degree of proliferation.

In our previous study, we have shown that the cells responding to antigen P₁ in these cultures are

T cells, predominantly of the helper phenotype, and that interleukin-2 can be detected in the culture supernatants (Rawle *et al.*, 1984). Many patients sensitive to *D. pteronyssinus* showed excellent proliferative responses, in some cases as good as those seen with antigens giving classical delayed hypersensitivity reactions, e.g. PPD. However, some of the *D. pteronyssinus* sensitive patients showed poor or negative proliferative responses to antigen P₁. There was no striking difference between T cell proliferation in patients with asthma or eczema, and patients with rhinitis showed rather weaker proliferation. In no case have we observed a significant increase in the proliferative response of these 'low responders' as a result of cell separation or histamine depletion. It, therefore, appears that the wide range of proliferative responses to antigen P₁ seen in patients with immediate hypersensitivity to *D. pteronyssinus* is likely to reflect differences in the numbers of circulating sensitized T cells rather than the effects of histamine inhibition. The exact role played by these allergen sensitized T cells in the pathogenesis of allergic diseases remains to be established.

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