

Stimulation of early eosinophil progenitors by a heat stable alveolar macrophage product from ovalbumin-sensitized and non-sensitized guinea pigs

M. I. C. GASPAR ELSAS^{1*}, P. XAVIER ELSAS[†], D. JOSEPH[‡], N. HAVET[‡], L. A. ADELINO DA SILVA^{*}, D. R. SALGADO[†], B. SALIOU[§] and B. B. VARGAFTIG[‡]

**Instituto Fernandes Figueira, FIOCRUZ, †Instituto de Microbiologia, UFRJ, Rio de Janeiro, Brazil, ‡Unité de Pharmacologie Cellulaire/Unité Associée Institut Pasteur-INSERM 285 and §Unité des Venins, Institut Pasteur, Paris, France*

Summary

Background Alveolar macrophages (AM) may participate in brochopulmonary hyper-reactivity by secreting cytokines that recruit mature eosinophils, or induce eosinophil production from recruited circulating progenitors.

Objective To define whether AM products can contribute to lung eosinophil production in immunized guinea pigs (GP), by analysing the effect of AM culture supernatants (AM-SN) on *in vitro* eosinophilopoiesis.

Methods Liquid and semi-solid bone marrow (BM) cultures were seeded with SN from 95% pure AM exposed to LPS.

Results AM-SN increased very significantly the long-term viability, cell proliferation and eosinophil production in liquid culture and supported formation of eosinophil-bearing mixed colonies, by acting on progenitors depleted of mature eosinophils. The effect on eosinophil production was not duplicated by natural or recombinant sources of GM-CSF (which nevertheless supported GM colony formation by GP BM), not by rhIL-8 (which was active on GP cells) and was not due to residual LPS. FPLC separation of active AM SN yielded a peak of apparent m.w. 43 kDa, active on both liquid and semi-solid cultures. The active moiety was heat- and trypsin-resistant. Neutralizing monoclonal antibodies to hGM-CSF, mGM-CSF, hIL-3 and mIL-3 failed to deplete the activity in AM-SN. Ovalbumin immunization induced its production by AM even without LPS challenge.

Conclusions The lack of T lymphocytes among factor-producing AM, the properties of the active material, the inability of GM-CSF to reproduce these effects, and the failure of MoAbs to GM-CSF and to IL-3 to neutralize the activity indicate it is not due to the major eosinopoietic factors GM-CSF, IL-3 or IL-5.

Keywords: eosinophil, bone marrow, alveolar macrophage

Clinical and Experimental Allergy, Vol. 27, pp. 208–217. Submitted 31 August 1995; revised 1 July 1996; accepted 1 August 1996.

Introduction

Lung eosinophilia in asthma [1–5] may involve short-lived, *in situ* secretion of eosinophilopoietic [6–8,9] cytokines (IL-5,

GM-CSF, IL-3 [2,3,5]) by antigen-stimulated T lymphocytes, with far-ranging effects on bone marrow (BM) eosinophil progenitors. Alternatively, it may involve stimulation of BM cells by absorbed, circulating antigen and/or by recirculating, activated T lymphocytes [10]. However, ovalbumin-immune guinea pigs (GP) present antigen-induced bronchopulmonary hyperreactivity, associated with long-lasting over-responsiveness to inflammatory mediators, and with chronic eosinophil

Correspondence: Maria Ignez C. Gaspar Elsas, Departamento de Pesquisa, Instituto Fernandes Figueira, FIOCRUZ, Av. Rui Barbosa 716, CEP 22.250-020, Praia do Flamengo, Rio de Janeiro, Brazil.

and CD4⁺-lymphocyte infiltration, even without antigen re-exposure [11,4].

Since circulating haemopoietic progenitors [12,13] can be recruited into inflammatory sites, and since alveolar macrophages (AM), a long-lived and abundant inflammatory cell population, can respond to the stimuli that induce bronchopulmonary hyperreactivity in GP [14] by secreting cytokines that both recruit eosinophils [15], and modulate *in situ* cell proliferation in repair processes [16,17], it is possible that, even without antigen re-exposure, AM secrete cytokines which promote eosinophil production from recruited progenitors. We have investigated this issue in the GP model of antigen-induced asthma, by evaluating the production of eosinophilopoietic substances by AM.

Materials and methods

Animals and immunization procedures

Male and female GP, weighing 450–650 g, were provided by the Charles River, Saint Antoine and Lebeau breeding facilities, with comparable results for GP from all three breeders. Immunization procedures followed exactly those of Pretolani *et al.* [11], which have been shown to reliably induce bronchopulmonary hyperreactivity without further antigen re-exposure. Sham-immunized (two alum injections 14 days apart), twice-sensitized (injected s.c. at 14-day intervals, with ovalbumin/alum) or single-sensitized (primed with ovalbumin/alum, sham-boosted at day 14 with alum) animals [11] were used 48 h after the booster injection.

SN production

AM were collected by bronchoalveolar lavage of anaesthetized GP with sterile saline (5 × 10 ml), washed in medium, separated on Ficoll-Hypaque [28] (Pharmacia, Saint-Quentin-en-Yvelines, France), washed, counted and cultured. No change in AM-SN activity was observed after further purification of AM on metrizamide. Purity of the AM population was evaluated by immunoperoxidase staining [4] with MoAbs H159 (anti-CD3), T4F8 (Pan-macrophage), MSgp8 (class II-specific). Unless otherwise indicated, AM were cultured in RPMI 1640 medium, with penicillin-streptomycin (100 IU/ml–100 µg/ml, Pen-Strep, Gibco, Cergy-Pontoise, France), and 10% Fetal Calf Serum (FCS), for 24 h at 37°C, in 5% CO₂/95% air, plus 2 µg/ml LPS (Difco OSI, Maurepas, France, see Results for LPS dose definition). FCS-free culture SN yielded comparable results. Batches of AM-SN are pools of SN produced separately, under identical conditions, from 3–6 naive animals. GP splenocyte cultures were set up exactly as in Mishell and Shiigi [26]. SN collected at 72 h from 5 ml

flasks containing 2 × 10⁶ splenocytes/ml, cultured as above, with either 2.5 µg/ml concanavalin A (Sigma, Saint-Quentin-Fallavier, France) or 100 µg/ml ovalbumin [26], were used as a natural [6,9] source of GP GM-CSF.

BM cultures

GP were sacrificed with sodium pentobarbital i.p., and BM cells were flushed out from femur fragments with Iscove's modified Dulbecco's medium (IMDM, Sigma), Pen-Strep and 10% FCS. For liquid culture, cells were seeded at 10⁶/ml per well in 24-well clusters, with or without cytokines. For semi-solid cultures, cells were plated in 0.36% agar (Difco) in IMDM with Pen-Strep and 10% FCS, at 2 × 10⁵/35 mm dish, at least in triplicate. For recombinant cytokines (rhGM-CSF, rmGM-CSF and rhIL-5 from Schering-Plough, Lys-Lez-Lannoy, France), preliminary experiments were carried out to define the active dose range on GP cells, based on the ability of rhIL-5 to stimulate *in vitro* mature GP peritoneal eosinophils (plateau effect at 250 ng/ml final [18]) and the ability of rhGM-CSF to stimulate colony formation (and *in vitro* survival, see below) by BM cells (100 ng/ml, in two experiments, induced respectively 41.3 ± 4.1 and 37.3 ± 4.5 colonies/dish, means ± SD of triplicates). rmGM-CSF supported no colonies at 1–100 ng/ml, being used at 100 ng/ml in liquid culture. rhIL-8 (Pharmingen, San Diego, USA) was used at 0.01–1 ng/ml, a concentration range which strongly supported neutrophil survival in liquid culture.

Splenocyte-SN were used at 10% (v/v), a dilution that was shown in preliminary experiments to support colony formation by BM from naive GP (in four experiments, means ± SD of triplicates/experiment, range 16 ± 9 to 50.3 ± 7 colonies/dish). Cells after liquid culture were resuspended and cytocentrifuge preparations were stained with May-Grunwald-Giemsa before counting mature eosinophils. Parallel staining of the cytocentrifuge smears for eosinophil peroxidase (EPO [19,20]) detected GP eosinophils, not neutrophils, as reported for GP [21] and murine [22, 23] but not human [24] BM, as shown by the lack of EPO⁺ cells in BM liquid cultures stimulated by rhGM-CSF or rhIL-8 (which contained >50% neutrophils). Peroxidase in lysates [23] of GP eosinophils purified from bronchoalveolar lavage fluid on metrizamide [18] resisted 8 mM cyanide, in contrast to lysates of metrizamide-purified neutrophils (D. Joseph, unpublished results). Colonies [25] were scored at day 7 and colony type was identified on agar layers after drying at 50°C, mounting on microscope slides and staining with May-Grunwald Giemsa.

Density gradient separation

Isotonic (100%) Percoll in PBS was diluted to 75%, 60% and 40% (respectively 2, 3 and 2 ml/gradient). BM cells

(1 femur equivalent/two gradients) were separated (20 min, $g \times 100$, 20°C). Cells at the three interfaces (layers I–III from top to bottom) were collected, washed, counted (total cells and eosinophils) and cultured.

Survival and cell proliferation assays

For dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) reduction assays according to Mossman [45], which quantify mitochondrial reductase activity as a measure of cell viability, BM cells in Iscove's medium, with Pen-Strep and 10% FCS, were seeded at $2 \times 10^5/200 \mu\text{l}$ per well in 96-well clusters, plus or minus cytokines, and cultured at 37°C, for 7–9 days, with addition of $10 \mu\text{l}$ of 5 mg/ml MTT (Sigma) in PBS per well for the last 6 h, before dissolving crystals in $100 \mu\text{l}$ acidified isopropanol and determination of OD₆₃₀. Tritiated thymidine incorporation by BM cells at day 4 was detected by pulsing triplicate cultures with $1 \mu\text{Ci}$ ³HTdR for 18 h [26]. Demonstration of increased cell numbers was made by a modification of Aggarwal's method [27], by staining with crystal violet (0.5% in methanol/water, 1:1 vol/vol) for 5 min, centrifuging the plates, washing once in PBS, redissolving the stain and reading of OD₅₉₅.

Characterization of the active moiety

To assess the heat stability of the active moiety in AM-SN (or rhGM-CSF), 1 ml samples in Eppendorff tubes were dipped for different periods of time in boiling water, before microcentrifugation, cooling, and assay on GP BM. Apparent m.w. was assessed by FPLC on a Superose 8 column calibrated for 8–200 kDa, using pooled, FCS-free AM-SN (previously concentrated and dialysed against PBS on an Amicon YM-10 membrane). Separation was carried out in PBS, and the individual 1 ml fractions were collected before addition to each of $100 \mu\text{l}$ FCS, filtration (0.22 μm) and assay. Controls included medium, PBS and unconcentrated/unseparated AM-SN, kept under identical conditions to the chromatographed sample.

Trypsin-TPCK (Sigma, 1 mg/ml) digestion of FCS-free AM-SN (or of 500 $\mu\text{g/ml}$ rhGM-CSF) followed each of the following protocols: (a) [T + F] + I = trypsin and factor incubated 15 min at 37°C before addition of excess highly purified inhibitor (SBTI, Sigma), or (b) [T + I] + F = AM-SN incubated as above with a preformed mixture of trypsin and trypsin inhibitor. (c) F(DIL) = AM-SN diluted in medium to the same extent as in (a) and (b), activity of digested AM-SN and rhGM-CSF being assayed by colony formation.

Neutralization was carried out by mixing 360 μl of AM-SN (or spleen cell-SN) with neutralizing monoclonal antibodies (nMoAbs; Genzyme) to hGM-CSF (ref. GM-213,

4 μg , four times the amount that totally neutralizes 50 U hGM-CSF), to mGM-CSF (ref. 1723-01, 4 μg), hIL-3 (ref. BL-3m, 25 μg) and mL-3 (ref. 1623-01, 1 μg), and completing the volume to 400 μl with FCS before incubation for 1 h at 37°C. After serial 2-fold dilution of the mixture, duplicate 100 μl samples of each dilution were assayed in liquid or semi-solid culture. Polymyxin B (Sigma) was preincubated with AM-SN for 30 min at 37°C before addition to the cultures, to a final concentration of 10% for AM-SN and 5 $\mu\text{g/ml}$ Polymyxin B in the culture.

Statistical analysis

Statistical analysis was carried out with the help of the Primer of Biostatistics software (Version 1.0, McGraw-Hill). The significance of the differences between the means of experimental and control samples within each experiment was determined by Student's *t*-test for groups of unequal sizes, that of differences between groups of several animals submitted to different immunization protocols by the Mann–Whitney test with correction for different group sizes [29]. One-way ANOVA was used to define whether results from different experiments involving the same treatment (medium, AM-SN) were homogeneous.

Results

Characterization of the liquid culture assay

Naive GP BM cells, cultured in medium alone, lost viability over a 13-day culture period. AM-SN increased viability up to day 13, as did SN from Concanavalin A- or ovalbumin-activated splenocytes. This viability-promoting effect of AM-SN was confirmed in MTT assays (Table 1, section a). rhGM-CSF also strongly supported BM cell survival, confirming that it is active on GP cells.

One-week-old BM cultures established in the presence of AM-SN or rhGM-CSF contained an adherent stroma, to which haemopoietic cells were loosely attached, and from which single cell suspensions for haemocytometer counting could not be obtained without damage as assessed by morphology. We did, however, directly demonstrate that BM cells proliferated in these cultures. As shown in Table 1, section b, AM-SN induced very significant increases in tritiated thymidine incorporation by BM cells relative to controls, with maximal labelling at day 4. DNA replication did result in net growth, as shown in Table 1, section c, since AM-SN induced significant increases in total cell numbers, as revealed by crystal violet staining, by day 7. This shows that cultures established with AM-SN were not only viable, but proliferating, and presented a net increase in cell numbers by day 7.

Table 1. Effect of AM-SN on BM cells in liquid culture

(a) Maintenance of cellular viability up to day 13					
	Exp. I		Exp. II		
	OD ₆₃₀	<i>P</i>	OD ₆₃₀	<i>P</i>	
Control	0.199 ± 0.024		0.110 ± 0.006		
AM-SN #1	0.399 ± 0.014	<0.001	0.372 ± 0.025	<0.001	
AM-SN #2	0.477 ± 0.016	<0.001	0.393 ± 0.01	<0.001	
AM-SN #3	0.434 ± 0.02	<0.001	0.485 ± 0.182	<0.001	
AM-SN #4	0.415 ± 0.04	<0.001	0.364 ± 0.03	<0.001	
rhGM-CSF, 100 ng/ml	0.881 ± 0.137	<0.001	1.001 ± 0.09	<0.001	
(b) Tritiated thymidine incorporation at day 4					
	Exp. I	cpm, mean ± SD (<i>n</i> = 3)			
		Exp. II	Exp. III		
Control	238 ± 27	324 ± 387	1501 ± 318		
AM-SN	1358 ± 106	1995 ± 322	3005 ± 305		
<i>P</i>	<0.001	<0.005	<0.001		
(c) Increase in cell numbers at day 6					
	Exp. I		Exp. II		
Control	1.937 ± 0.378		0.677 ± 0.148		
AM-SN #5	3.251 ± 0.330	<i>P</i> < 0.005	1.224 ± 0.479	<i>P</i> < 0.01	
AM-SN #6	3375 ± 93	<i>P</i> < 0.005			

Data are, for section (a), mean ± SD of OD₆₃₀ readings, for section (b), mean ± SD of cpm, and for section (c), mean ± SD of OD₅₉₅ readings (at least triplicates for all assays).

Effect of AM-SN on eosinophils

We evaluated the effect of AM-SN on eosinophil numbers in liquid culture. Due to the development of a haemopoietic stromal layer, counts of single cell suspensions were not representative of the entire culture. Furthermore, such counts would necessarily depend on calculations based both on total cell counts and on differential counts of cytocentrifuge smears, presenting therefore the cumulative errors of two different measurements. However, vigorous resuspension of the cultures, followed by quick cytocentrifugation, staining and differential counts, allowed sampling of both haemopoietic and stromal cells, in a way which was representative of the entire culture. In this case, the percentage of eosinophils was a reliable estimate, because it was a direct measurement, because cell and granule morphology could be evaluated in detail, and because it did not exclude the eosinophils associated with the stromal cell clumps. Since both cell proliferation and net population increase were present in these cultures, an increased percentage of eosinophils necessarily reflects absolute increases in eosinophil numbers.

Cultures of naïve GP BM in medium displayed few eosinophils. In contrast, AM-SN-stimulated cultures

Table 2. Effect of AM-SN on eosinophil numbers in liquid culture

Exp	Control		AM-SN		
	% Eosinophils	<i>n</i>	% Eosinophils	<i>n</i>	<i>P</i>
(a) In individual experiments					
I	0.83 ± 1.64	12	9 ± 3.9	4	<0.001
II	0 ± 0	5	9 ± 2.3	6	<0.001
III	0.67 ± 1.15	3	14.33 ± 4.93	3	<0.01
(b) For the entire series of experiments					
	0.31 ± 0.37		10.07 ± 2.73		<0.001

Data are mean ± SD of determinations in naïve BM cultures in medium (control) or with LPS-induced AM-SN. For section (a), *n* = number of replicates. For section (b), data were derived from experiments with nine different BM, using seven batches of AM-SN produced in identical conditions, two of which were assayed on two different BM each. For the calculation of the mean values for control and AM-SN-treated cultures in the series, each experiment contributed the average of 2–12 replicate cultures. As a consequence, data originally derived from a total of 36 control and 26 AM-SN-treated individual cultures distributed among the nine experiments were used in this calculation.

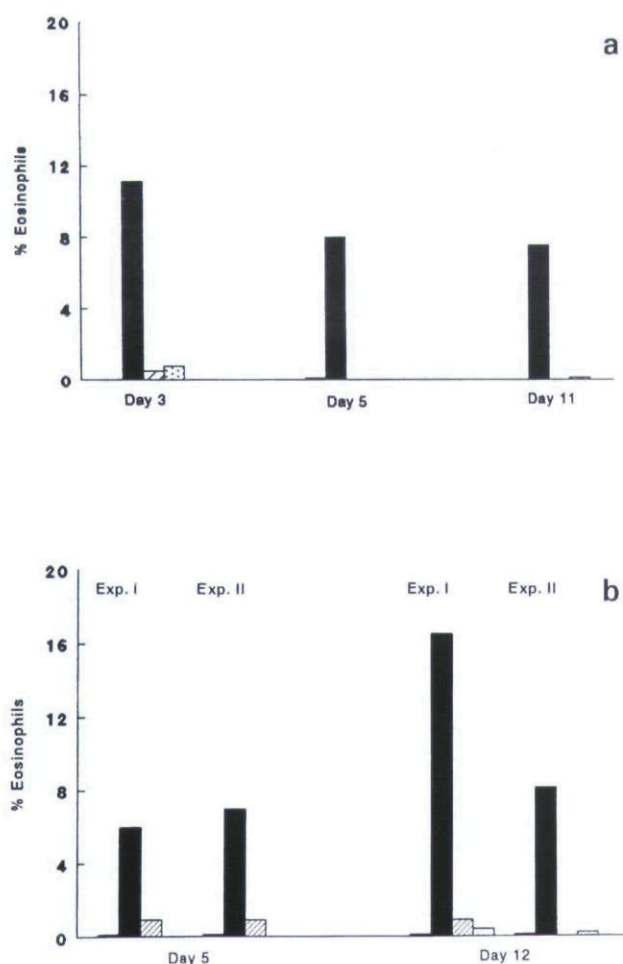


Fig. 1. Effect of AM-SN and of natural and recombinant cytokines on eosinophil production in liquid culture of naïve GP BM. (a) BM was cultured for 3, 5 or 11 days in medium (respectively 0, 0.1 and 0% eosinophils), or with LPS-induced AM-SN (■), SN of ConA-stimulated (▨) or of ovalbumin-stimulated immune splenocytes (▤). (b) BM cultured for 5 or 12 days in medium (all 0.1% eosinophils), with rhIL-5 (■), rhGM-CSF (▨), average values for days 5 and 12 respectively 0.9 and 0.45), or rmGM-CSF (▤, average values for days 5 and 12 respectively 0.9 and 0.45).

presented significantly increased eosinophil counts at day 7, as shown in Table 2, section (a), for three experiments, where a single AM-SN batch was assayed on each BM.

These results are representative of a total of nine experiments, using different BM and AM-SN. Analysis of variance for the entire series of experiments showed the data from control and from AM-SN-treated cultures to be homogeneous within each treatment. When the same batch of AM-SN was assayed on two different BM, results were also homogeneous. Highly significant differences were also found between control and AM-SN treated cultures for the entire series.

Table 3. Eosinophil production by BM after mature eosinophil depletion

(a) Depletion of mature eosinophils from BM			
Before separation	Layer II	<i>n</i>	<i>P</i>
5 ± 1	0.66 ± 1.15	16	<0.001
(b) Eosinophil production by purified progenitors			
Exp.	Control	AM-SN	<i>P</i>
I	0*	7.3*	
II	1.3 ± 0.5	8 ± 1.7	<0.005
III	1.3 ± 1.15	9.6 ± 3.0	<0.025
		6.3 ± 1.5	<0.025
		6.3 ± 0.57	<0.005
IV	0.6 ± 1.5	12.6 ± 3.0	<0.005
		8 ± 1.5	<0.005
		9.3 ± 1.1	<0.001

Data (mean ± SD) are, in section (a) % eosinophils in whole BM and in layer II, separated on Percoll in the indicated numbers of animals, and in section (b), % eosinophils (average from triplicates, except where * indicates average of duplicates) in cells from four naïve GP BM (Exps I–IV) Layer II cultures at day 6 in medium (control), or with LPS-induced AM-SN, three different AM-SN preparations being assayed in exp. III and in exp. IV.

While AM-SN increased mature eosinophil numbers from day 3 to 11 (Fig. 1a), this was not duplicated by either type of GP splenocyte SN, which were nevertheless able to support colony formation and increased survival by GP BM cells. While rhIL-5 increased eosinophil counts in liquid culture of GP BM (Fig. 1b), rhGM-CSF, which induces colony formation in GP BM, failed to similarly increase eosinophil numbers in liquid culture, although it did induce large numbers of mature and immature neutrophils. No effect was observed with rmGM-CSF.

Since the BM inoculum contained mature eosinophils, experiments were carried out to distinguish between a survival-promoting effect on mature cells [30–32] and true eosinophilopoietic activity. Percoll gradients efficiently eliminated mature eosinophils from BM before culture (Table 3, section a). Culture of the mature eosinophil-depleted progenitors in layer II with AM-SN resulted in stromal implantation, increased thymidine incorporation and crystal violet staining (not shown), and in a significantly increased number of eosinophils relative to control cultures (Table 3, section b), confirming that AM-SN was able to support true eosinophilopoiesis in eosinophil-depleted BM.

AM-SN also efficiently supported colony formation by normal and immune GP BM cells, yielding myeloid colonies

[25], identified by May-Grünwald-Giemsa staining. Eosinophils were found in and around mixed colonies (43–83% of all colonies, range, three experiments), but no pure eosinophil colonies were detected. This colony-stimulating activity rapidly titrated out and presented some high-dose inhibition. This suggests that AM-SN promote eosinophilopoiesis by stimulating primitive (mixed), rather than eosinophil-lineage-committed, myeloid progenitors

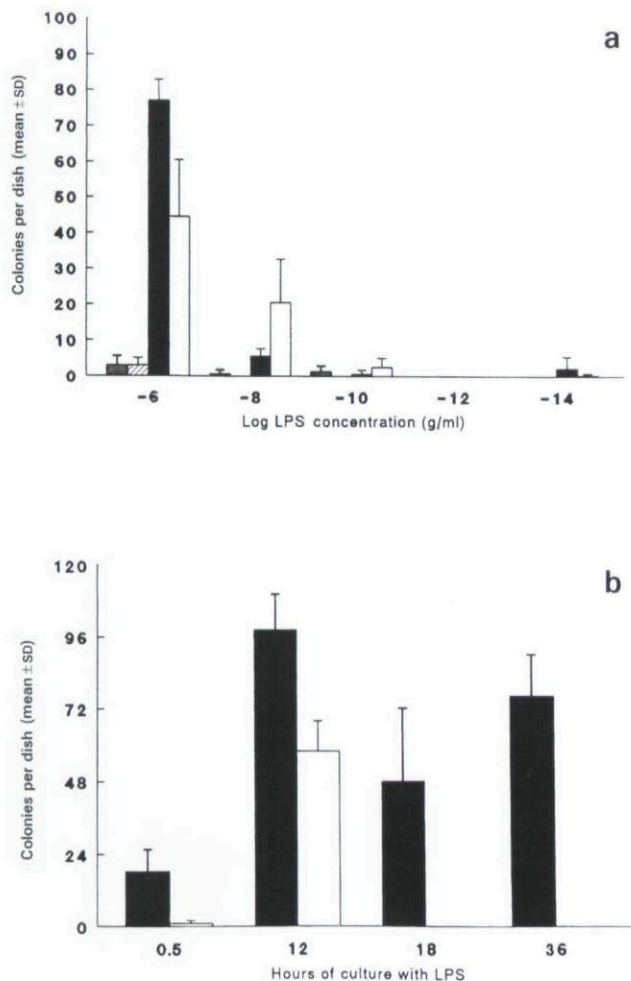


Fig. 2. Effect of LPS on colony formation by BM cells and on production of colony-stimulating activity by AM. AM were cultured with or without LPS. AM-SN or LPS were assayed in colony formation assays on naïve BM. Data are mean $\pm$ SD of triplicates from two experiments. (a) Colony formation with LPS (I (■) and II (□)) or of AM-SN induced by the same doses of LPS (respectively ■ and □ bars). (b) Colony formation with SN of AM cultured with 2 μ g/ml LPS for various periods (respectively ■ and □ bars). Panel (a) is representative of four experiments (optimal AM-SN activity always induced at 2 μ g/ml LPS), and panel (b) of five (plateau activity always reached between 12 and 36 h, detectable activity at 30 min in 3/5).

Production and properties of the active AM-SN

AM from the cultures used to produce SN were respectively 96.77% and 95.18% positive for class II antigens and for a macrophage-specific marker (0.5% and 0.6% in the negative controls). CD3⁺ cells were <0.5%. Bronchoalveolar lavage fluid cells were 95% macrophages, 2% neutrophils, 1% eosinophils and 1% lymphocytes, plus variable contamination by epithelial cells and mast cells, all of which, with the exception of lymphocytes, were eliminated by centrifugation on Ficoll-Hypaque [28] and on metrizamide [18], neither of which affected production of active AM-SN.

LPS itself, required for induction of active AM-SN, had no colony-stimulating activity for GP BM (Fig. 2a). LPS did not promote survival or proliferation of GP BM cells in liquid culture. Its effect on AM to induce secretion of the active moiety was early (30 min) but not immediate (Fig. 2b), with plateau levels seen at 12–36 h. Polymixin B neutralization did not eliminate the colony-stimulating activity of AM-SN.

AM-SN were separated on a Superose 8. Activity was recovered in the excluded volume, presumably representing aggregates, and in a 43 kDa peak, which was active in both semi-solid (Fig. 3a) and liquid culture (Fig. 3b), suggesting that both assays detect the same material. Dipping 1 ml AM-SN samples in boiling water (5 or 10 min) failed to destroy the activity. After 30 min at 100°C, 30% of the initial

Table 4. Effect of rhIL-8 on GP BM

(a) Colony formation:			
	Exp. I	Exp. II	Exp. III
Control	0 $\pm$ 0	0 $\pm$ 0	1 $\pm$ 1
AM-SN	30.3 $\pm$ 4.5	45 $\pm$ 4.3	35 $\pm$ 4.5
IL-8, 1 ng/ml	0 $\pm$ 0	0 $\pm$ 0	6.6 $\pm$ 2.58
IL-8, 0.1 ng/ml	7.3 $\pm$ 2.5	0 $\pm$ 0	1.6 $\pm$ 0.57
IL-8, 0.01 ng/ml	0 $\pm$ 0	0 $\pm$ 0	3 $\pm$ 1
(b) Liquid culture:			
	Exp. I	Exp. II	Exp. II
	Eosinophils	Neutrophils	Eosinophils
Control	0.66 $\pm$ 0.57	0 $\pm$ 0	0 $\pm$ 0
AM-SN	8.6 $\pm$ 3.05	ND	9.3 $\pm$ 2.08
IL-8, 1 ng/ml	1.3 $\pm$ 0.57	78.8 $\pm$ 8.08	0 $\pm$ 0
IL-8, 0.1 ng/ml	0.33 $\pm$ 0.57	64 $\pm$ 3.6	0 $\pm$ 0
IL-8, 0.01 ng/ml	0.66 $\pm$ 0.57	61*	0 $\pm$ 0

Data are, for section (a), mean $\pm$ SD of colony numbers in triplicates established in medium alone (control) or with rhIL-8, or with AM-SN, and for section (b), mean $\pm$ SD of % eosinophils or % neutrophils in triplicates (except where * indicates the average of duplicates). ND = not determined. Differences between AM-SN-treated cultures and controls were significant (section (a), P < 0.001, section (b), P < 0.01).

colony-stimulating activity remained (Fig. 4a). Trypsinization failed to destroy the colony-stimulating activity of FCS-free AM-SN (Fig. 4b), although it did inactivate rhGM-CSF, and even increased AM-SN activity in one experiment.

MoAb to hGM-CSF (or to mGM-CSF, to hIL-3 and to mIL-3), failed to abolish the ability of AM-SN to support eosinophilopoiesis in both assays, but partially neutralized the colony-stimulating activity of splenocyte-SN.

Relationship to IL-8

Some properties of AM-SN resembled those of IL-8 [34]. However, rhIL-8 had weak and inconsistent colony-stimulating activity, unlike crude AM-SN (Table 4, section a). rhIL-8 did not induce eosinophil differentiation in liquid culture (Table 4, section b), but supported the survival/differentiation of neutrophils for up to 7 days, indicating that it is active on GP.

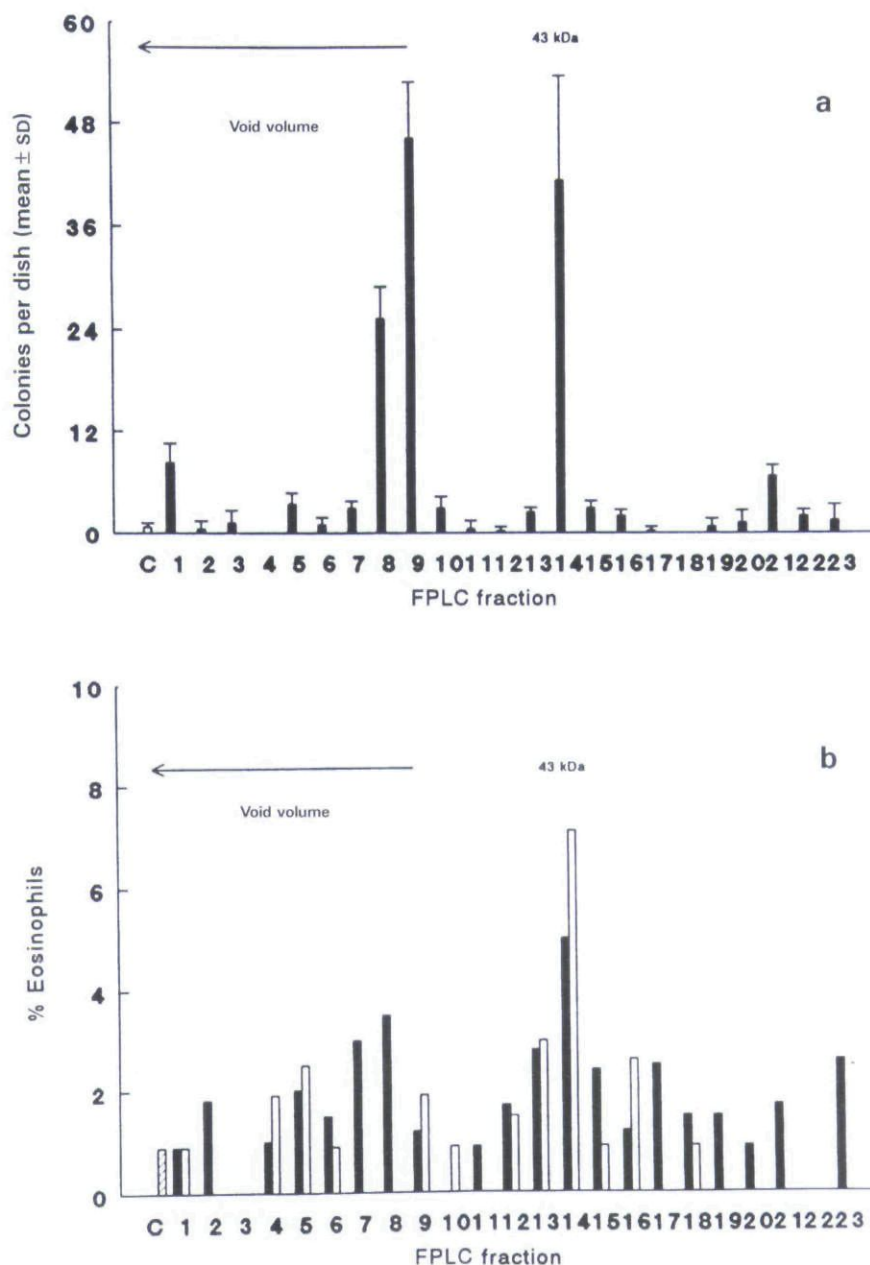


Fig. 3. FPLC separation of AM-SN. Fractions 1–9 represent the void volume. Controls (c) were cultured with medium alone. Data are: (a) mean $\pm$ SD of triplicate colony formation assays; and (b) % eosinophils at days 4 (□) and 7 (■) of liquid culture. Panel A and B are representative of four experiments in which activity was found in the same fractions.

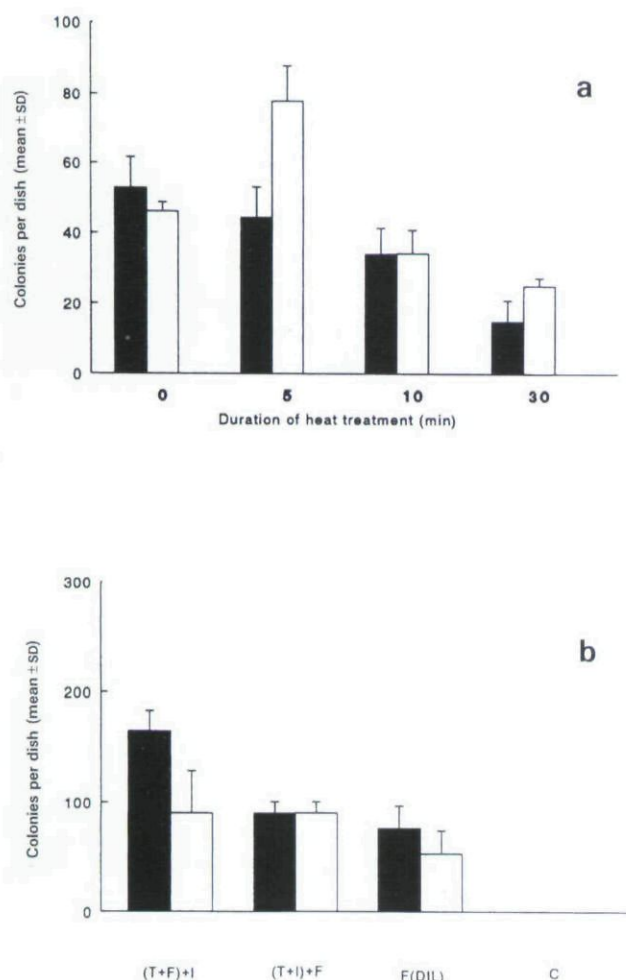


Fig. 4. Effect of heating and trypsinization on AM-SN. Two batches of AM-SN (Exps I and II, respectively ■ and □ bars) were heated (a) or digested with Trypsin (b) as described in Materials and Methods before assaying for residual colony-stimulating activity. Medium controls (c) yielded no colonies. Data are mean $\pm$ SD of triplicates. Panel (b): (F)DIL, diluted AM-SN, (T + F) + I, digested AM-SN (T + I) + F, undigested control. Panel (a) is representative of four experiments, panel (b) of four, with comparable results.

Effect of immunization

The possible effect of ovalbumin sensitization on secretion of the active moiety by AM was evaluated in AM from GP submitted to different immunization protocols, and cultured in the presence of LPS, ovalbumin or medium. As shown in Table 5, section a, comparable activity was found in SN from LPS-stimulated cultures of immune and non-immune animals, suggesting the activity is induced by LPS irrespective of immunity. Some activity was also found in ovalbumin-induced AM-SN, with no significant differences between the three groups (not shown). However, activity comparable to

Table 5. Effect of immunization on the activity of AM-SN

(a) In LPS-stimulated AM-SN								
Group	Animal							
	1	2	3	4	5	6	7	8
Twice-sensitized	5	4	11	8	6	6	6	10
Single-sensitized	10	6	13	ND	12	15	8	ND
Sham-immunized	7	2	9	11	6	17	11	ND
b) In unstimulated AM-SN								
Group	Animal							
	1	2	3	4	5	6	7	8
Twice-sensitized	3	6	8	2	0	1	2	2
Single-sensitized	0	0	ND	0	0	0	0	ND
Sham-immunized	1	0	0	1	ND	0	0	0

Data are % eosinophils at day 6 in liquid culture assayed on the same naïve BM. ND = not determined. For the differences between twice-immunized and either single-immunized or sham-sensitized animals, *P* was respectively <0.05 and <0.05 by the Mann-Whitney test for unstimulated AM-SN, but non-significant for LPS-stimulated AM-SN.

that of LPS-induced AM-SN was also found in some, but not all, SN from AM of twice-immunized, not of single-immunized or sham-sensitized animals, cultured in medium alone. These differences between twice-immunized GP and control groups were significant (Table 5, section b), suggesting that AM from twice-immunized GP release the active moiety even in the absence of allergen or LPS.

Discussion

We evaluated whether AM, which are long-lived, cytokine-secreting cells [15,16,19], release eosinophilopoietins. LPS-stimulated AM-SN promoted eosinophil production from eosinophil-depleted BM cells and strongly supported formation of eosinophil-containing myeloid colonies. Unlike for human and rodent BM exposed to IL-5 [7], GM-CSF [6] or IL-3 [8], no pure eosinophil colonies were found, although culture conditions were permissive for eosinophil survival and differentiation, suggesting that the active moiety in AM-SN acted on a primitive myeloid progenitor. As crude AM-SN were used, there might be other factors present, which should be expected, however, to promote, in addition to mixed colonies, pure eosinophil colony formation and to be eliminated by FPLC separation, trypsinization and heat-inactivation. The same FPLC fraction acts on both assays, suggesting progenitor stimulation underlies eosinophil differentiation in liquid culture.

T lymphocytes were <0.5% in AM cultures. Production of the active moiety required LPS, a macrophage- rather than a

T cell-stimulant. Activity was not due to: (a) contaminating granulocytes or mast cells, which were eliminated on Ficoll-Hypaque, with no decrease AM-SN activity, (b) carry-over of LPS, which did not induce colony formation nor eosinophilopoiesis in liquid culture. Other cells in BM (macrophages, monocytes, neutrophils, eosinophils, mast cells) were not led by LPS to secrete an activity replacing AM-SN, suggesting they differ from AM in this respect.

The colony-stimulating activity resisted boiling and trypsinization. While IL-3 from other species does resist boiling, GM-CSF or IL-5 do not [8]. IL-3, like IL-5, is usually associated with T lymphocytes, not AM [8,7,9]. IL-8, also produced by GP [35], is secreted by LPS-stimulated AM [36], resists heating (60 min, 70°C [34]), and regulates haemopoiesis [37,38]. IL-8 is made by [39] and acts on eosinophils, but *in vivo* selectively induces neutrophils [40]. However, IL-8 is trypsin-sensitive [34], unlike the active moiety in AM-SN. rhIL-8 acts on GP [41] but failed to stimulate colony formation and eosinophil differentiation, and to induce eosinophilia [42]. This suggests the active moiety is not IL-8. IL-8 and eotaxin [43,44] are low m.w. chemokines (respectively 8 kDa, 7–14 kDa), smaller than 43 kDa. Eotaxin, constitutively produced by GP lung [43,44] and spleen, should be present in unstimulated naïve GP AM-SN and Splenocyte-SN, which were inactive.

The following further suggests the activity is not due to GM-CSF, IL-5 or IL-3. AM-SN were obtained from antigen-free, T lymphocyte-depleted cultures. SN of concanavalin A-activated naïve, or of ovalbumin-stimulated immune splenocytes, active on BM, did not replace AM-SN. rhGM-CSF, also active on GP BM cells, did not replace AM-SN. MoAbs to hGM-CSF (and to mGM-CSF, hIL-3 and mIL-3) failed to abolish the eosinophilopoietic activity of AM-SN in liquid culture, but MoAb to hGM-CSF did neutralize GP CSF from splenocyte-SN.

While LPS elicited comparable levels of activity in AM cultures of immune and non-immune animals, spontaneously released activity, without antigen or LPS, was only found among AM-SN from twice-sensitized GP [4,11]. This suggests the booster ovalbumin injection may have affected AM *in vivo*, leading to the apparently spontaneous release of an eosinophilopoietic substance. These findings point to a possible mechanism of sustained lung eosinophilia without antigen restimulation.

Acknowledgements

We thank C. Ruffié, M.-A. Nahori, L. Alves, A. Pimentel and E. Lima for technical help, Drs M. Pretolani and J. R. Lapa e Silva for materials, Dr F. P. Camara for statistical advice and CNPq/RHAE, FINEP, PAPES/FIOCRUZ, CAPES-COFECUB and INSERM/Institut Pasteur for funding.

References

- 1 Calhoun W, Sedgwick J, Busse W. The role of eosinophils in the pathophysiology of asthma. *Annals NY Acad Sci.* 1991; 629:62–72.
- 2 Kay AB. 'Helper' (CD4⁺) T cells and eosinophils in allergy and asthma. *Amer Rev Respir Dis* 1992; 145:S22–S26.
- 3 Kroegel C, Virchow J, Walker C. Asthma. *New Engl J Med* 1993; 328:1639–1640.
- 4 Lapa e Silva J, Bachelet C, Pretolani M et al. Immunopathologic alterations in the bronchi of immunized guinea pigs. *Am J Resp Cell Mol Biol* 1993; 9:44–53.
- 5 Walker C, Kaegi M, Braun P, Blaser K. Activated T cells and eosinophilia in BAL from subjects with asthma correlated with disease severity. *J Allergy Clin Immunol* 1991; 88:935–942.
- 6 Garland J. Colony stimulating factors. In: Thomson A, ed. *The Cytokine Handbook*. New York: Academic, 1991:260–300.
- 7 Sanderson C J. Interleukin-5. In: Thomson A, ed. *The Cytokine Handbook*. New York: Academic, 1991:149–167.
- 8 Schrader JW. Interleukin-3. In: Thomson A, ed. *The Cytokine Handbook*. New York: Academic, 1991:103–18.
- 9 Howard M, Miyajima A, Coffman R. T-cell-derived cytokines and their receptors. In: Paul W, ed. *Fundamental Immunology*, 3rd. edn. New York: Raven, 1993:763–800.
- 10 Walker C, Virchow JC, Bruijnzel P, Blaser K. T cell subsets and their soluble products regulate eosinophilia in allergic and nonallergic asthma. *J Immunol* 1991; 146:1829–35.
- 11 Pretolani M, Lefort J, Vargaftig B. Active immunization induces lung hyperresponsiveness in the guinea pig. Pharmacologic modulation and triggering role of the booster injection. *Amer Rev Respir Dis* 1988; 138:1572–8.
- 12 Testa U, Pelosi E, Gabbianelli M et al. Cascade transactivation of growth factor receptors in early human hematopoiesis. *Blood* 1993; 81:1442–56.
- 13 Kawano Y, Takaue Y, Hirao A et al. Synergistic effect of recombinant interferon- γ and interleukin-3 on the growth of immature human hematopoietic progenitors. *Blood* 1991; 77:2118–21.
- 14 Bachelet M, Lagente V, Fouque F et al. Antigen-dependent activation of alveolar macrophages from ovalbumin-sensitized guinea pigs: relevance of the route of administration and the amount of antigen provided. *Clin Exp Allergy* 1990; 20:690.
- 15 Resnick M, Weller P. Mechanisms of eosinophil recruitment. *Am J Resp Cell Mol Biol* 1993; 8:349–55.
- 16 Gosset P, Tscopoulos A, Wallaert B et al. Increased secretion of TNF- α and IL-6 by alveolar macrophages consecutive to the development of the late asthmatic reaction. *J Allergy Clin Immunol* 1991; 88:561–71.
- 17 Kelley J. Cytokines of the lung. *Amer Rev Respir Dis* 1990; 141:765–88.
- 18 Coeffier E, Joseph D, Vargaftig B. Activation of guinea pig eosinophils by rhIL-5. Selective priming to PAF-Acether and interference of its antagonists. *J Immunol* 1991; 147:2595–602.
- 19 Ten R, Pease L, McKean D, Bell M, Gleich G. Molecular cloning of human EPO. Evidence for the existence of a peroxidase multigene family. *J Exp Med* 1989; 169:1757–69.
- 20 Pretolani M, Ruffié C, Lapa e Silva J et al. Antibody to VLA-4 prevents antigen-induced bronchial hyperreactivity and cellular

- infiltration in the guinea pig airways. *J Exp Med* 1994; 180:795–805.
- 21 Cheng J, Pillar J, Shirley J et al. Antigen-mediated pulmonary eosinophilia in IgG1-sensitized guinea pigs: EPO as a simple specific marker for detecting eosinophils in bronchoalveolar lavage fluid. *J Pharmacol Exp Therap* 1993; 264:922–9.
 - 22 Strath M, Warren D, Sanderson CJ. Detection of eosinophils using an EPO assay. Its use as an assay for eosinophil differentiation factors. *J Immunol Methods* 1985; 83:209–15.
 - 23 Bach M, Brashler J, Sanders M, Bienkowski M. Pitfalls in the quantitative estimation of the secretion of granule proteins by eosinophils. *J Immunol Methods* 1991; 142:243–50.
 - 24 Mlynek M, Leder L. The cytochemical demonstration of cyanide resistant peroxidase does not identify eosinophils selectively. *Blut* 1985; 51:347–53.
 - 25 Metcalf D. Haematopoietic colonies: *in vitro* cloning of normal and leukemic cells. Amsterdam: Elsevier-North Holland, 1977.
 - 26 Mishell B, Shiigi S. Selected Methods in Cellular Immunology. San Francisco: Freeman, 1980.
 - 27 Aggarwal B. Human lymphotoxin. *Methods Enzymol* 1985; 116:441–8.
 - 28 Boyum A. Ficoll hypaque method for separating mononuclear cells and granulocytes from human blood. *Scand J Clin Lab Invest* 1968; 21(Suppl. 97):77.
 - 29 Snedecor G, Cochran W. Statistical Methods 6th edn. Ames: Iowa University Press, 1978.
 - 30 Lopez A, Williamson D, Gamble J et al. rhGM-CSF stimulates *in vitro* mature human neutrophil and eosinophil function, cell surface expression and survival. *J Clin Invest* 1986; 78:1220–8.
 - 31 Rothenberg M, Owen W, Silberstein D et al. Human eosinophils have prolonged survival, enhanced functional properties and become hypodense when exposed to human IL-3. *J Clin Invest* 1988; 81:1986–92.
 - 32 Owen W, Rothenberg M, Silberstein D et al. Regulation of human eosinophil viability, density and function by GM-CSF in the presence of 3T3 fibroblasts. *J Exp Med* 1987; 166:129–41.
 - 33 Pretolani M, Lefort J, Leduc D, Vargaftig B. Effect of rhIL-5 on *in vivo* lung responsiveness to PAF of lung from actively sensitized guinea pigs. *Br J Pharmacol* 1992; 106:677–84.
 - 34 Zachariae C, Matsushima K. Interleukin-8. In: Aggarwal B, Gutterman J eds. *Human Cytokines: Handbook for Basic and Clinical Research*. Boston: Blackwell Science, 1992.
 - 35 Yoshimura T, Johnson D. cDNA cloning and expression of guinea pig neutrophil attractant protein-1 (NAP-1). *J Immunol* 1993; 151:6225–36.
 - 36 Thomassen M, Meeker D, Antal J, Connors M, Wiedemann H. Synthetic surfactant (Exosurf) inhibits endotoxin-stimulated cytokine secretion by human alveolar macrophages. *Am J Respir Cell Mol Biol* 1992; 7:257–60.
 - 37 Gewirtz A, Zhang J, Ratajczak J et al. Chemokine regulation of human megakaryocytopoiesis. *Blood* 1995; 86:2559–67.
 - 38 Laterveer L, Lindley I, Hamilton M, Willemze R, Fibbe W. Interleukin-8 induces rapid mobilization of hematopoietic stem cells with radioprotective capacity and long-term myelolymphoid repopulating ability. *Blood* 1995; 85:2269–75.
 - 39 Simon H, Yousefi S, Weber M et al. Human peripheral blood eosinophils express and release interleukin-8. *Int Arch Allergy Immunol* 1995; 107:124–6.
 - 40 Jagels M, Hugli TE. Neutrophil chemotactic factors promote leukocytosis. A common mechanism for cellular recruitment from bone marrow. *J Immunol* 1992; 148:1119–28.
 - 41 Lagente V, Pruniaux M, Junien J, Moodley I. Modulation of cytokine-induced eosinophil infiltration by phosphodiesterase inhibitors. *Am J Respir Crit Care Med* 1995; 151:1720–4.
 - 42 Xiu Q, Fujimura M, Nomura M et al. Bronchial hyper-responsiveness and airway neutrophil accumulation induced by interleukin-8 and the effect of the thromboxane A2 antagonist S-1452 in guinea-pigs. *Clin Exp Allergy* 1995; 25:51–9.
 - 43 Rothenberg M, Luster A, Lilly C, Drazen J, Leder P. Constitutive and allergen-induced expression of eotaxin mRNA in the guinea pig lung *J Exp Med* 1995; 181:1211–6.
 - 44 Jose P, Adcock I, Griffiths-Johnson D et al. Eotaxin: cloning of an eosinophil chemoattractant cytokine and increased mRNA expression in allergen-challenged guinea-pig lungs. *Biochem Biophys Res Commun* 1994; 205:788–94.
 - 45 Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; 65:55–62.

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.