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The effect of dexamethasone on growth and differentiation of bone-marrow derived mucosal mast cells *in vitro*

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SUMMARY

An *in vitro* culture system was used to investigate the effects of dexamethasone on the production of mucosal mast cell (MMC) growth activity from T cells, and the proliferation and maturation of MMC in culture. The addition of dexamethasone (Dex) to cultures of lymphocytes from *Nippostrongylus brasiliensis (Nb.)*-infected rats suppressed production of MMC growth activity, as assessed by the lack of MMC growth and differentiation when supernatants of the treated lymphocyte cultures were added to normal rat bone-marrow cultures. Dexamethasone treatment of normal rat bone-marrow cultures affected the maturation of the bone-marrow derived MMC by preventing normal granule development. The ratio of neutrophils:macrophages present in the cultures was also altered. Dexamethasone did not have any detectable effect on mature MMC in culture.

INTRODUCTION

Infection of rats with the intestinal nematode *Nippostrongylus brasiliensis (Nb.)* is associated with an exponential and T-cell dependent increase in intestinal mucosal mast cells (MMC) (Miller & Jarrett, 1971; Mayrhofer, 1979; Nawa & Miller, 1979). Lymphocytes from *Nb.*-infected rats were found, after stimulation *in vitro* with worm antigen or with the T-cell mitogen concanavalin A (Con A), to release factors causing the emergence and selective proliferation of mast cells in normal rat bone-marrow cultures (Haig *et al.*, 1982). The mast cells generated under these circumstances have the appearance and staining properties of MMC (Haig *et al.*, 1982). Biochemically, the cultured mast cells exclusively contain and secrete rat mast-cell protease II (RMCPII) (Haig *et al.*, 1982; 1983; McMenamin *et al.*, 1987). Further *in vitro* experiments with tissues from athymic rats showed that the thymus-dependency of MMC proliferation is based on the production of an appropriate growth factor(s) by antigen- or Con A-activated T cells of the helper subset (Haig *et al.*, 1983; McMenamin, Jarrett & Sanderson, 1985).

Physiologically, glucocorticoids are both immunosuppressive and anti-inflammatory, although the cellular and molecular basis for these effects are poorly understood. The effect of corticosteroids on *in vitro* mast-cell growth was of interest in this study because cortisone or prednisolone treatment of *Nb.*-infected rats leads to a marked reduction in MMC numbers (Jarrett *et al.*, 1967; King *et al.*, 1985). The effect of steroids on T cells was also studied because MMC hyperplasia *in vivo* is so highly T-cell dependent (Mayrhofer, 1979; Nawa & Miller, 1979). Furthermore, dexamethasone exerts an inhibitory effect on lymphokine production by T lymphocytes *in vitro* (Gillis, Crabtree & Smith, 1979a, b; Kelso & Munck, 1984).

Experiments were designed, therefore, to investigate the activity of dexamethasone in the culture system using a dual approach: firstly to determine the influence of dexamethasone on the production of MMC growth activity, and secondly to investigate its effect on the growth and differentiation of the cultured mast cells.

Abbreviations: CM, conditioned medium; Con A, concanavalin A; Dex, dexamethasone; IMLN, immune mesenteric lymph node; MMC, mucosal mast cell; NBM, normal bone-marrow; RMCPII, rat mast cell protease II.

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MATERIALS AND METHODS

Animals

F344 rats originally obtained from Olac Ltd (Bicester, Oxon) were subsequently bred from inbred colonies at the University of Glasgow Veterinary School.

Propagation of Nippostrongylus brasiliensis (Nb.)

The techniques for the culture of the nematode parasite and the infection of rats have been described elsewhere (McMenamin *et al.*, 1985). The standard infective dose was 4000 larvae per rat.

Conditioned medium (CM)

Immune mesenteric lymph node cells (IMLN) excised 14 days after infection with *Nb.* were plated in 25 cm^2 or 80 cm^2 tissue culture flasks (Nunc, Paisley, Renfrewshire) at 4×10^6 cells/ml in Iscove's serumless medium (Gibco, Paisley, Renfrewshire) (Iscove & Melchers, 1978), and stimulated with 2 μg/ml Con A (Miles, Stoke Poges, Slough, Berks). The supernatants were harvested after 48 hr, sterilized by filtration and stored at −20°. In addition to this, for Experiment 1, dexamethasone (Sigma, Poole, Dorset) was added to the IMLN cultures at 10^{-6} M, 10^{-7} M or 10^{-8} M with or without Con A. The cultures were harvested after 48 hr and the supernatants dialysed against three changes of PBS over 48 hr to remove the dexamethasone. The supernatants were then checked for MMC growth stimulatory activity using the bone-marrow assay.

Bone-marrow assay

The assay was essentially as described previously (Haig *et al.*, 1983). Briefly, bone-marrow cells at $2{\cdot}5 \times 10^5$ cells/ml in Iscove's medium supplemented with 20% horse serum (Flow Laboratories, Irvine, Ayrshire) were cultured in 24-well tissue culture plates (Linbro, Flow Laboratories) in a humidified incubator at 37° flushed with 5% CO_2 in air. CM was added to the cultures at 25%. The cells were harvested after 7 days, and total and differential cell counts were performed on cell smears stained with Leishman. Dexamethasone was added to the cultures at 10^{-6} M, 10^{-7} or 10^{-8} M with or without CM. The cultures were harvested after 7 days, and total and differential cell counts carried out as above.

Bulk bone-marrow cultures were prepared by incubating 10-ml aliquots of 5×10^5 cells/ml with 25% CM in 25 cm^2 tissue culture flasks. The cultures were restimulated and refed after 7 days and thereafter as required. Three-week-old normal rat bone-marrow cultures that contained >90% mast cells were treated with dexamethasone at 10^{-6} M, 10^{-7} M or 10^{-8} M for 24 and 48 hr. After this time, the cells were harvested, and total and differential cell counts were performed as previously described.

RMCPII extraction and analysis

On harvesting, cultured bone-marrow cells were pelleted by centrifugation and extracted with 0·15 M KCl (Woodbury & Miller, 1982). The bone-marrow cell extracts and bone-marrow culture supernatants were assayed for RMCPII content using the following ELISA method.

Plastic 96-well plates (Costar, NBL) were incubated overnight with affinity-purified sheep anti-RMCPII at 1 μg/ml in carbonate/bicarbonate buffer at 4° in a humidified box. A stock solution of RMCPII was prepared and dilutions of 10–80 ng/ml prepared from it in PBS/0·1% Tween 20/2% FCS. Appropriate dilutions of the test samples were also prepared. The plates were washed three times in buffer (PBS/0·1% Tween 20/2% FCS). One-hundred and fifty microlitres of standard and test samples were added to the plate and incubated for 1·5–2 hr at 37° in a humidified box. The plates were again washed three times. One-hundred and fifty microlitres of sheep anti-RMCPII conjugated to horseradish peroxidase were added at a 1:2000 dilution to the plates, and they were further incubated for 1·5–2 hr at 37° in a humidified container. The plates were washed five times with the washing buffer as before. Wells were then incubated for 10 min at 37° with O-phenylenediamine dihydrochloride (Sigma Ltd) in phosphate/citrate buffer (pH 5·0). The reaction was stopped by adding 50 μl of 12·5% sulphuric acid per well. The OD_{492} was read by a Titertek Multiscan micro-elisa plate reader. A standard curve was drawn and the test sample values calculated from this curve. Four dilutions were prepared per sample, and the mean of the four values calculated from the standard curve was expressed in μg/ml.

Table 1. Effect of dexamethasone (Dex) on the production of MMC growth activity by IMLN cells, stimulated by Con A, and assayed using normal bone-marrow (NBM) cultures

	Bone-marrow counts								
	Total cells $\times 10^4$/ml			Mast cells $\times 10^4$/ml			RMCPII content μg/ml (pg/cell)		
Conditioned medium added to NBM cultures	Experiment a	b	c	Experiment a	b	c	Experiment a	b	c
IMLN cells + 10^{-8} M Dex + Con A	12	10	8	1·68	0·9	0·7	0·68 (40)	0·28 (31)	0·2 (28)
IMLN cells + 10^{-7} M Dex + Con A	10	8	8	0	0	0	0·16	0·11	0·10
IMLN cells + 10^{-6} M Dex + Con A	1	2	1	0	0	0	0·01	0·03	0·02
IMLN cells + 10^{-8} M Dex alone	3	4	3	0	0	0	0·01	0·02	0·01
IMLN cells + 10^{-7} M Dex alone	2	2	1	0	0	0	0·02	0·02	0·01
IMLN cells + 10^{-6} M Dex alone	1	1	1	0	0	0	0·02	0·03	0·01
IMLN cells alone	2	1	1	0	0	0	0·02	0·02	0·01
IMLN cells + 2 μg/ml Con A	60	64	56	27·0	28·2	29·1	6·46 (24)	6·33 (22)	7·57 (26)
Medium alone	<1	<1	<1	0	0	0	0·01	0·02	0·01

IMLN cells at 4×10^6/ml were cultured in the presence of various concentrations of dexamethasone ± Con A, and the supernatants were harvested after 48 hr. These were then used to stimulate normal rat bone-marrow cultures. The cell counts represent the mean obtained from triplicate bone-marrow cultures harvested on Day 7.

Table 2. Effect of dexamethasone (Dex) on the growth, differentiation and RMPII content of normal bone-marrow (NBM) cultures

CM and conc. of Dex added to NBM cultures	Total cells $\times 10^4$/ml	Total mast cells $\times 10^4$/ml (%)	RMCPII content of extracted cells (μg/ml)	RMCPII content of supernatant (μg/ml)
CM + 10^{-8} M Dex	30	16 (53)	3·7 (23 pg/cell)	0·6
CM 10^{-7} M Dex	28	15 (53)	3·3 (22 pg/cell)	0·6
CM 10^{-6} M Dex	25	13 (52)	2·0 (15 pg/cell)	0·5
10^{-8} M Dex	4	0	0·01	*
10^{-7} M Dex	6	0	0·02	*
10^{-6} M Dex	7	0	0·02	*
CM	59	30 (51)	8·4 (28 pg/cell)	1·5
Medium alone	1	0	< 0·01	*

Normal rat bone-marrow cells were cultured in the presence of dexamethasone ± CM. Cultures were harvested on Day 7. The results represent the mean values from triplicate cultures of a representative experiment.

* < 10 ng/ml detected.

All RMCPII standards and antibody preparations used were generous gifts from Drs Steven Gibson and John Huntley (Moredun Research Institute, Edinburgh).

RESULTS

Experiment 1

In order to determine the effect of dexamethasone on the production of mast-cell growth factor activity, IMLN cells were treated with dexamethasone during CM production (see the Materials and Methods). The CM produced was then assessed for mast-cell growth factor activity using the bone-marrow assay (see the Materials and Methods).

Dexamethasone inhibited the production of both total cell and MMC growth stimulatory activity in a dose-dependent fashion (Table 1). Total cell growth and mast-cell growth in the positive control (IMLN 14 + 2 μg/ml Con A) were, respectively, five and 16 times that of the 10^{-8} M dexamethasone + Con A-treated culture (Experiment 1a). With increasing concentration of dexamethasone, both total cell and mast-cell numbers decreased. The RMCPII content of the bone-marrow cultures also reflected the numbers of mast cells present, with the positive control again containing the highest concentration of RMCPII, and the level decreasing with increasing concentration of dexamethasone. This experiment was repeated twice with almost identical results (Experiments 1b and c).

Experiment 2

In order to assess the direct effect of dexamethasone on the growth and differentiation of mast cells, dexamethasone was added to normal rat bone-marrow cultures (see the Materials and Methods). The cultures were harvested after 7 days, and refed and restimulated with CM but without dexamethasone. The cells were then cultured for a further 7 days. Three-week-old normal rat bone-marrow cultures that contained > 90% mast cells were also treated with dexamethasone (see the Materials and Methods) to study the effect of this drug on mature cells.

Dexamethasone inhibited the total cell growth in the normal bone-marrow cultures compared to the positive control (NBM + CM), although the proportion of mast cells per culture did not vary significantly between the different doses of dexamethasone, or the positive control (Table 2).

The mast cells in the dexamethasone-treated cultures differed from the positive control culture, in that the granules in the drug-treated cells were much smaller than those of untreated cells (Fig. 1a and b). The mast cells from the treated cultures had the appearance of 4-day-old immature mast cells in comparison with the mature mast cells seen in control cultures (Haig, McMenamin & Jarrett, 1986; McMenamin, 1986). Differential cell counts revealed that, as the concentration of dexamethasone increased, the proportions of neutrophils and macrophages became inversely related (data not shown). Thus, the percentages of neutrophils and macrophages were approximately equal at 23% in the presence of 10^{-8} M dexamethasone and were, respectively, 36% and 9% in 10^{-7} M dexamethasone and 44% and 4% in 10^{-6} M dexamethasone. In the positive control the proportions of neutrophils and macrophages were 8% and 40%. This trend was seen in repeated experiments (data not shown).

The dexamethasone-treated cultures that were refed and restimulated without dexamethasone yielded mast cells that had larger granules, looked more mature, and were similar in morphology to the mast cells from the untreated cultures (Fig. 2a and b). The addition of dexamethasone to mature mast cell cultures did not cause any change in cell numbers, in the RMCPII content of the cells or supernatants (Table 3), or in mast cell morphology as judged by Leishman staining of cytocentrifuge preparations.

DISCUSSION

In addition to exerting anti-inflammatory and immunosuppressive effects *in vivo*, glucocorticoids inhibit and enhance a wide range of cell types and functions *in vitro* by specific receptor-mediated mechanisms (Fahey, Guyre & Munck, 1981). The generation of MMC in culture from normal rat bone marrow is a T-cell dependent phenomenon (Haig *et al.*, 1983) and, more

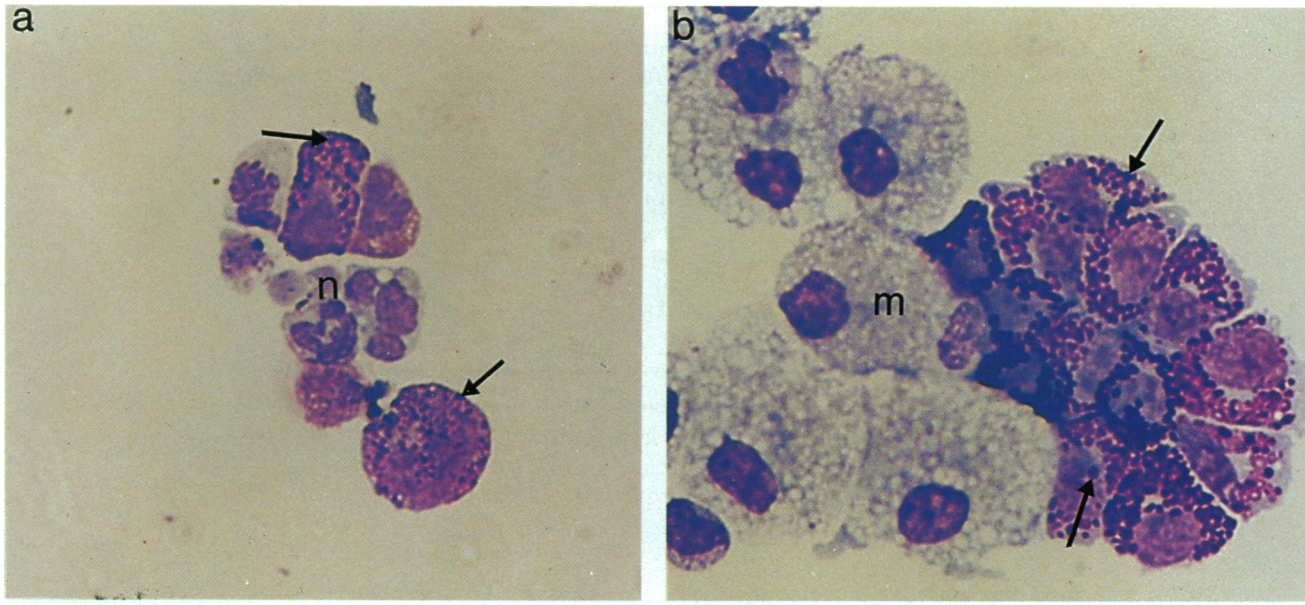

Figure 1. (a) 10^{-7} M dexamethasone-treated normal bone-marrow culture, harvested on Day 7. Note the small granules of the mast cells (arrows). Neutrophils (n) are also present. Leishman stain (magnification × 730). (b) Normal bone-marrow after 7 days in culture in the presence of CM. The granules of the mast cells are much larger than those in (a) (arrows). Note also the presence of macrophages (m). Leishman stain (magnification × 730).

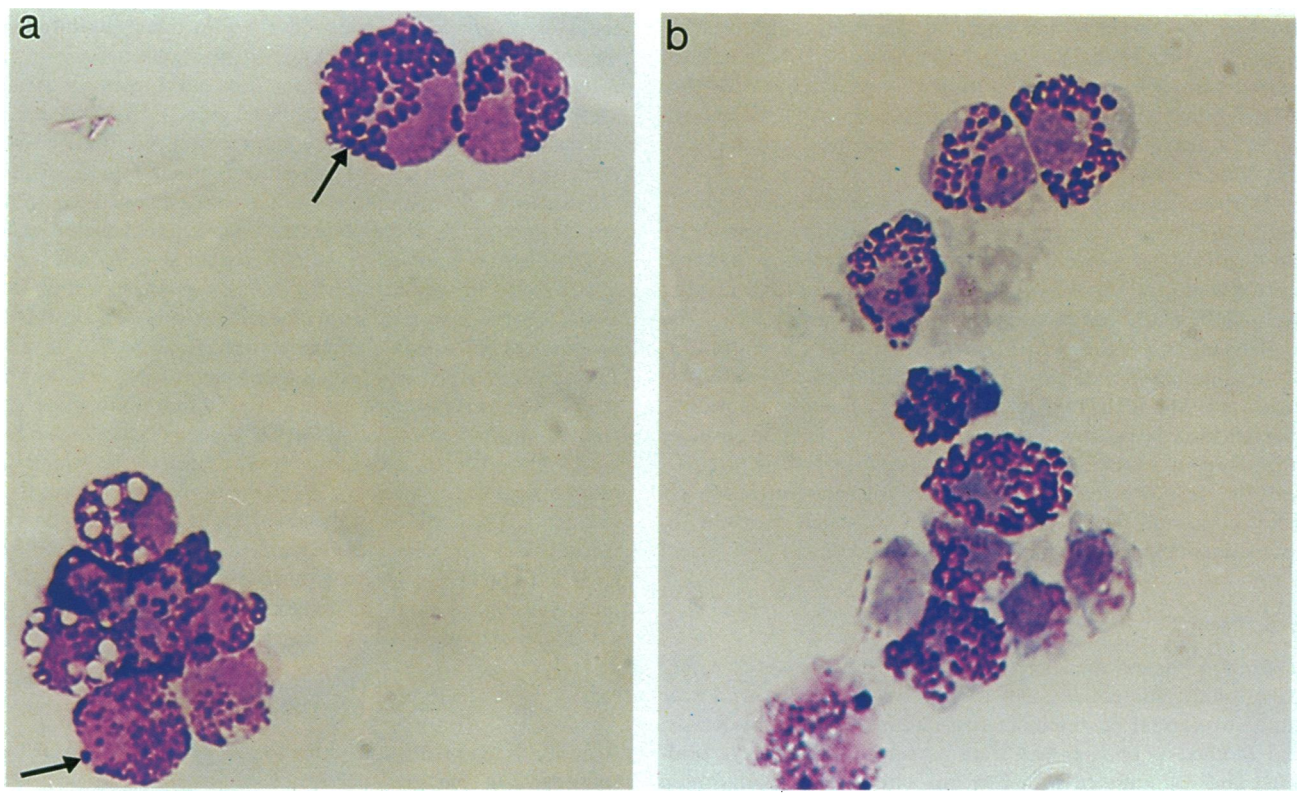

Figure 2. (a) Seven-day-old 10^{-7} M dexamethasone-treated culture, washed, refed, restimulated and cultured for a further 7 days. The cells are much more heavily granulated, and the granules themselves are now much bigger (arrows) than in Fig. 1a. These cells form >90% of culture. Leishman stain (magnification × 730). (b) Seven-day-old normal bone-marrow culture, refed, restimulated and cultured for a further 7 days. The mast cells form >90% of the culture. Leishman stain (magnification × 730).

Table 3. Effect of dexamethasone (Dex) on 3-week-old normal rat bone-marrow cultures (NBM)

Conc. of Dex	Total cell no. $\times 10^4$	Mast cell no. $\times 10^4$	RMPII content cell extract (μg/ml)	RMCPII content cell supernatant (μg/ml)
Before treatment	50	49·5	14·85 (30 pg/cell)	2·41
24-hr treatment				
10^{-8} M Dex	52	51·2	14·33 (28 pg/cell)	2·23
10^{-7} M Dex	49	48·4	14·51 (30 pg/cell)	2·53
10^{-6} M Dex	50	49·4	15·82 (32 pg/cell)	2·56
48-hr treatment				
10^{-8} M Dex	50	49·5	14·35 (30 pg/cell)	2·32
10^{-7} M Dex	47	46·5	13·96 (30 pg/cell)	2·26
10^{-6} M Dex	49	48·8	14·14 (29 pg/cell)	2·29

Three-week-old bulk cultures of normal rat bone-marrow containing 99% mast cells were treated with various concentrations of dexamethasone in the presence of CM. The cultures were harvested after 24 and 48 hr. The results represent the mean values from triplicate cultures of a representative experiment.

specifically, is dependent on a factor(s) supplied by helper T cells (McMenamin *et al.*, 1985). The present results show that MMC growth stimulatory activity is greatly reduced in those CM derived from IMLN cultures to which dexamethasone has been added. An IL-3-like activity is normally generated in rat CM which is responsible, at least in part, for the growth and differentiation of bone-marrow derived MMC (Haig, 1986). These results therefore extend previous observations by Culpepper & Lee (1985) who indicated that dexamethasone inhibited the appearance of IL-3 activity in supernatants of murine helper T-cell clones. Investigations at the molecular level revealed that dexamethasone directly inhibited transcription of the IL-3 gene (Culpepper & Lee, 1985). Dexamethasone can inhibit the production of several lymphokines that are produced in response to mitogen activation, namely IL-1 (Smith *et al.*, 1980) and IL-2 (Gillis *et al.*, 1979a, b). Thus, a common mechanism may be implicated for the inhibition of gene expression for various lymphokines in T cells by dexamethasone.

We have also demonstrated that dexamethasone affects mast-cell development in bone-marrow cultures. When dexamethasone was added to bone-marrow cultures at the time of plating, mast-cell development was impaired. Compared to untreated control cells, the granules of treated cells were much smaller and the cells generally exhibited an immature phenotype. Removal of dexamethasone from the bone-marrow cultures permitted maturation of the mast cells, which were then indistinguishable from those in control cultures. The neutral serine proteinase RMCPII is located uniquely within the granules of rat MMC and is also present within the cultured mast cells (Gibson & Miller, 1986; McMenamin *et al.*, 1987). Dexamethasone-treated cells contained less RMCPII than control cells, and also secreted less RMCPII into the culture supernatant. No effect was observed when dexamethasone was added to cultures containing mature MMC.

These present observations extend earlier findings *in vivo* that corticosteroids suppress the development of intestinal mucosal mastocytosis during nematode infection (Jarrett *et al.*, 1967; Olsen & Schiller, 1978), and provide an explanation for the findings of King *et al.* (1985), who observed that methylprednisolone treatment caused the depletion of mature MMC and of RMCPII from the jejunum of rats previously infected with *Nb.* by showing that the putative *in vitro* counterpart of rat MMC is also suppressed in its development by corticosteroids. Collating the *in vivo* and *in vitro* data, it would seem that the effects of glucocorticoids on MMC are two-fold: firstly, they inhibit the production of MMC growth factor(s) on which the *in vitro*-generated cells are absolutely dependent for their survival, growth and differentiation, and secondly, they act directly on the mast cells themselves inhibiting the normal pattern of granule formation. The hyperplasia of MMC and their persistence in the parasitized gut can be explained by the production of MMC growth factors by T cells. Treatment of these animals with glucocosteroids would cause factor production to cease or be at too low a level to sustain or stimulate and maintain the population of MMC or their precursors. In addition, dexamethasone also acts on the MMC precursor directly inhibiting development. These observations go some way to providing an explanation for the disappearance of MMC from the intestines of corticosteroid-treated rats.

The numbers of neutrophils in the cultures increased with increasing dexamethasone concentration, and the number of macrophages decreased. This is interesting as it has been observed that systemic glucocorticoid treatment induced monocytopenia (Parrillo & Fauci, 1978) and neutrophilia (Parrillo & Fauci, 1979) in humans. The mechanisms of these actions have not been elucidated, but increased release of neutrophils by the bone-marrow has been observed (Parrillo & Fauci, 1979). There are obviously many complicated regulatory circuits involved in haemopoiesis, and the effects of glucocorticoids on the different cell types have still to be unravelled.

The culture system developed in our laboratory enables us to study mast-cell development from the precursor stage through to maturity. It has allowed dissection *in vitro* of responses found *in vivo* to reveal the component parts of an integrated response to, in this case, parasite infection where dexamethasone is acting on different cell types involved in the immune response.

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