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Surface phenotype of T cells producing growth of mucosal mast cells in normal rat bone marrow culture

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Summary. We have previously shown that lymphocytes from *Nippostrongylus brasiliensis* infected rats, when stimulated with antigen or concanavalin A (Con A) release factors which are comparable with murine IL-3. On addition of these factors to rat bone marrow cultures, mast cells with the morphological and biochemical properties of mucosal mast cells (MMC) proliferate and mature. Here, we use this system, along with monoclonal antibodies against rat T cells and the fluorescence-activated cell sorter (FACS), to isolate the subset of T cells responsible for the production of this MMC growth factor.

Lymphocytes from *N. brasiliensis* infected rats were separated on the FACs into populations with and without the antigens defined by OX19, W3/25 and OX8 monoclonal antibodies; these antibodies label all T cells, T-helper cells and T-cytotoxic/suppressor cells, respectively. The resultant subsets were cultured *in vitro* with Con A. The supernatants were tested for the ability to induce MMC growth and differentiation in liquid cultures of normal rat bone marrow. The phenotype of the T cells producing this factor was established as being $OX19^+$, $W3/25^+$ and $OX8^-$.

INTRODUCTION

The lymphocytes of *Nippostrongylus brasiliensis* (Nb) infected rats were found, on restimulation *in vitro* with worm antigen or 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 mucosal mast cells (MMC), and contain and secrete large quantities of the rat mast cell protease RMCPII (Haig *et al.*, 1982). Further experiments with the tissues of athymic rats showed that the thymus-dependency of MMC proliferation is based on the production of an appropriate growth factor by antigen- or Con A-activated T cells (Haig *et al.*, 1983).

Studies in the mouse showed that the T cells which selectively induce morphologically normal mouse mast cell clones to proliferate have the phenotype Ly $1^+2^-3^-$ (Nabel *et al.*, 1981). The mouse mast cells which are generated from haemopoietic tissues have some of the features of rat MMC and may be of a similar cell type (reviewed in Jarrett & Haig, 1984).

In the rat, monoclonal antibodies which recognize T-cell associated antigens include OX19 which labels all T cells (Dallman, Mason & Webb, 1982), and W3/25 (Williams, Galfré & Milstein, 1977) and OX-8 (Brideau *et al.*, 1980) which label non-overlapping T-cell subpopulations. The latter can be used to dissociate the rat helper (W3/25) and cytotoxic/suppressor (OX8) T-cell subsets.

Lymphocytes were separated in the fluorescence-

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activated cell sorter into populations with or without the antigens defined by OX19, W3/25 and OX8 monoclonal antibodies, and the resultant subsets were cultured *in vitro* with Con A. The supernatants were tested for their ability to induce MMC growth and differentiation from normal rat bone marrow in liquid culture. The data indicate that the rat lymphocyte subpopulation involved in MMC growth factor production is labelled by OX19 and W3/25 monoclonal antibodies, but not by OX8.

MATERIALS AND METHODS

Animals and parasite

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

Propagation of N. brasiliensis *(Nb)*

Faeces from culture rats infected by the subcutaneous injection of 400 infective Nb larvae were collected on absorbent paper between Days 7 and 9 of the infection. The faecal pellets were washed under running tap water for 2–3 min, soaked in sufficient water to cover the pellets for at least 2 hr, and were then gently mixed to form a thick paste. Granular charcoal (10–18 mesh; BDH Chemicals, Poole, Dorset) was added until the mixture was almost dry, but still bound together (approximately one volume of faeces to two volumes of charcoal). Moist filter paper (Whatman No. 1) was put in the centre of a petri-dish, and enough faeces–charcoal mixture was added to cover about 70% of the paper. The petri-dishes were stored covered in a moist box in a humidified incubator at 27° for 5–7 days.

The larvae were harvested by gently layering the faeces–charcoal mixture onto a piece of filter paper (K-dex, Kleenaroll Ltd, London), placed in a fine sieve (300 mesh Endecott) on a Baermann Funnel, and larvae collected by sedimentation.

Monoclonal and fluorescent antibodies

The mouse anti-rat monoclonal antibodies OX19 (Dallman *et al.*, 1982), W3/25 (Williams *et al.*, 1977) and OX8 (Brideau *et al.*, 1980) were a generous gift of D. W. Mason (MRC Unit for Cellular Immunology, Sir William Dunn School of Pathology, University of Oxford). Mouse IgG was kindly provided by A. Williamson, Department of Biochemistry, University of Glasgow. Fluorescent goat anti-mouse IgG was prepared according to the method of Barclay & Hyden (1978). Briefly, goat anti-mouse IgG was passed through a rat globulin immunosorbent column to remove reactivity for rat IgG. The antiserum was then purified by immunosorption to mouse IgG, and after elution was subsequently digested with pepsin according to Nisonoff *et al.*, (1960). The purified $F(ab')_2$ fragments were isolated by gel filtration and AcA 44 and conjugated with fluorescein isothyocyanate by the method of Barclay & Hyden (1978) to obtain fluorescein $F(ab')_2$ molar ratios of between 2 and 4. Batches of this antibody were stored at $-20°$ and each batch was titrated to obtain optimal immunofluorescence before use.

Cell preparation, labelling and fluorescent antibody cell sorting FACS

Rats were infected with 4000 Nb larvae, and the mesenteric lymph nodes were removed on Day 14 after infection. Two nodes in 10 ml of Hanks' (HBSS) (Gibco, Paisley, Strathclyde) and 10% horse serum (HS) (Flow Laboratories, Irvine, Ayrshire) were decanted into sterile stomacher bags and stomached in a Colworth stomacher (Seward Laboratories, Bury St Edmonds, Suffolk) for 20 seconds. The cell suspension was filtered through a four-fold thickness of lens tissue and washed twice in HBSS + 10% HS + 2-mercaptoethanol (Sigma, Poole, Dorset) + penicillin/streptomycin (Flow). A suspension of 10^8 cells/ml was incubated with 2·5–5·0 μl/ml of the monoclonal antibody (50–100 μl/10^7 cells) for 30 min at 4°. After three washes in HBSS + 10% normal rat serum (NRS) + 2 mM NaN_3, the cells were further incubated for 30 min at 4° with FITC-labelled goat anti-mouse IgG (1 : 10 or 1 : 20 dilution, depending on the monoclonal being used). The cells were washed again three times in HBSS + 10% NRS + 2 mM NaN_3 and maintained on ice prior to sorting. Separation was carried out on a fluorescence-activated cell sorter (FACS IV: Becton-Dickinson, Sunnyvale, CA). Labelled lymphocytes were analysed, and sorted fractions collected on ice in Iscove's serum-free medium (Gibco). Cells were sorted at an average rate of 2000–2500 per second. Appropriate forward angle light scatter gating excluded dead cells. Under these conditons, the average recovery (of viable cells) was 58% and the purity 94–97%.

Cell cultures

The FACS-sorted cells were centrifuged and adjusted to 4×10^6/ml in Iscove's serumless medium and added to feeder cells. To prepare these, spleens were taken from normal syngeneic rats and irradiated with 3000

rads from a ^{60}Co source. The spleens were stomached as for the MLN cells, washed, adjusted and plated at 2×10^2 cells/well in 96-well tissue culture plates (Nunc, Paisley, Strathclyde). The FACS-sorted cells at 4×10^5/well and Con A (Sigma) at 2 μg/ml were added, and the mixture was incubated for 48 hr in a humidified incubator at 37° flushed with 5% CO_2. The cell-free supernatants were harvested and tested for their capacity to stimulate the growth of MMC in normal rat bone marrow.

A pool of two rat bone marrows was used for each assay. The bone marrow cells were prepared in Iscove's medium + 20% HS adjusted to a concentration of 5×10^5 cells/ml. One hundred μl of cells were added to 96-well flat-bottomed tissue culture plates (Nunc) to which were added 50 μl of FACS-sorted cell supernatants and 50 μl of Iscove's medium + 20% HS. The supernatants were tested in triplicate. The cells incubated at 37° in a humidified incubator flushed with 5% CO_2, were harvested after 7 days, and total cell counts were performed. Cytocentrifuge preparations were made in a Cytospin 2 (Shandon Southern Ltd, Runcorn, Cheshire) and the slides were stained with Leishman. Differential cell counts were performed on the cell smears, at least 500 cells being counted per slide.

Table 1. Mast cell growth in bone marrow cultures stimulated with CM* from (a) OX19, (b) W3/25 and (c) OX8-labelled IMLN

Source of CM		Total cells† ($\times 10^{-4}$)	Mast cells† ($\times 10^{-4}$)
(a)	Unfractionated IMLN cells	48	26·8
	OX19-positive IMLN cells	70	42·8
	OX19-negative IMLN cells	8	0·8
	Medium alone	4	0·4
(b)	Unfractionated IMLN cells	56	23·5
	W3/25-positive IMLN cells	52	21·8
	W3/25-negative IMLN cells	8	0·7
	Medium alone	3	0·1
(c)	Unfractionated IMLN cells	60	25·2
	OX8-positive IMLN cells	4	0·1
	OX8-negative IMLN cells	64	31·4
	Medium alone	4	0·4

* The IMLN cells at 4×10^6/ml were stimulated with 2 μg/ml Con A and the supernatants harvested after 48 hr. These were then used to stimulate normal rat bone marrow cultures.

† The cell counts represent the mean number of triplicate bone marrow cultures harvested on Day 7.

RESULTS

OX19 labelling

Table 1a shows that cells which label with the monoclonal antibody OX19 are capable of producing MMC growth factor, whereas those which are negative for this marker are not. Maximal cell growth, including MMC growth, was seen in the bone marrow cultures stimulated with the conditioned medium prepared from the OX19-positive cells (i.e. the T-cell enriched fraction). The conditioned medium prepared from the OX19-negative cells did not stimulate the bone marrow above the level of the medium-alone control.

W3/25 labelling

Cells which stained positively for this monoclonal antibody also produced a factor capable of stimulating MMC growth in rat bone marrow cultures. Table 1b shows that the W3/25-positive cells gave similar MMC numbers to the unfractionated MLN cells.

OX8 labelling

Table 1c demonstrates that the factor is not produced by cells which stained positively for the monoclonal antibody OX8, the marker for the T-cytotoxic/suppressor cell. Cells which were negative for this marker produced the factor that induced MMC in normal bone marrow cultures.

Supernatants from the various combinations of positive and negative cells ± feeder cells, Con A and medium-alone controls did not give any significant MMC growth in normal rat bone marrow.

DISCUSSION

The experiments described here analysed the phenotype of the T cells which produce a factor able to stimulate MMC growth in normal rat bone marrow cultures. They demonstrate that the cells express antigens defined by the OX19 but not by the OX8 monoclonal antibodies.

Although unfractionated MLN cells produced the factor, the OX19-positive fraction which was T-cell enriched gave a much higher total cell count and a larger proportion of mast cells. The removal of the macrophages and other non-T cells had an enhancing effect on MMC growth factor production in agree-

ment with our earlier results (Haig *et al.*, 1983). Unfractionated and W3/25 positive MLN cells gave approximately the same total cell growth and numbers of mast cells, although both total and mast cell numbers were less than the numbers obtained from the cultures stimulated by the OX19-positive fraction supernatant. As W3/25 also labels macrophages (Barclay, 1981), this again supports our earlier observation (Haig *et al.*, 1983) that the inclusion of these cells has a detrimental effect on MMC growth factor production. IL-2 has been shown to be a product of W3/25-positive cells, and supports the idea that this subset plays the helper/inducer role in the rat (Cantrell, Robins & Baldwin, 1982). T-cytotoxic/suppressor cells, which are labelled by OX8 monoclonal antibody, did not produce the factor in amounts large enough to generate mast cells in normal bone marrow culture. The negatively labelled population, on the other hand, gave numbers of MMC comparable to the unfractionated MLN cells supernatant. The small increase in total cell growth was, in fact possibly caused by the removal of suppressor cells.

The factor(s) produced in this culture system would seem to be comparable with murine IL-3. Il-3 encourages the growth of a number of haemopoietic cells lines but, in particular, has mast cell growth factor activity (reviewed by Jarrett & Haig, 1984).

The murine WEHI-3b cell line, which produces IL-3 constitutively, has provided the means to purify and analyse IL-3 (Ihle, 1984) which has numerous effects on haemopoietic lines. IL-3 is normally a product of mitogen- or antigen-stimulated Thy 1^+, Lyt 1^+, 2^- T cells (Nabel *et al.*, 1981) which also produce IL-2 (Miller & Stutman, 1983).

On the basis of molecular weight, tissue distribution and functions of cells reactive with the antibodies , it appears that in the rat, man and mouse, the antigens OX19, Leu 1, T1 and Ly 1 are homologous, and that OX8, Leu 2a, T5 and Ly 2 antigens are similarly so (Mason *et al.*, 1983). The isolation of the rat T-cell subset responsible for the production of MMC growth factor was the first step in providing a source of the factor itself. However, it is now recognized that the sequential expression of genes by a subset of T cells, or even by a single clone, may alter the expression of immune function and phenotype of the cell as it differentiates. In this way, the cells may gain or lose a function in culture with time or in response to different environmental signals (Pawelec, Schneider & Wernet, 1983; Freeman *et al.*, 1983). This feature clearly limits the usefulness of cloning T cells and makes the cloning of IL-3 genes necessary instead. Recently, the cDNA for murine IL-3 has been cloned (Fung *et al.*, 1984; Yokota, 1984), and no doubt this will soon be followed by the production of large quantities of pure human, murine and rat IL-3 for an analysis of the control of haemopoiesis.

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