EOSINOPHIL PRODUCTION

A thesis submitted for the degree of

Doctor of Philosophy

of the University of Oxford

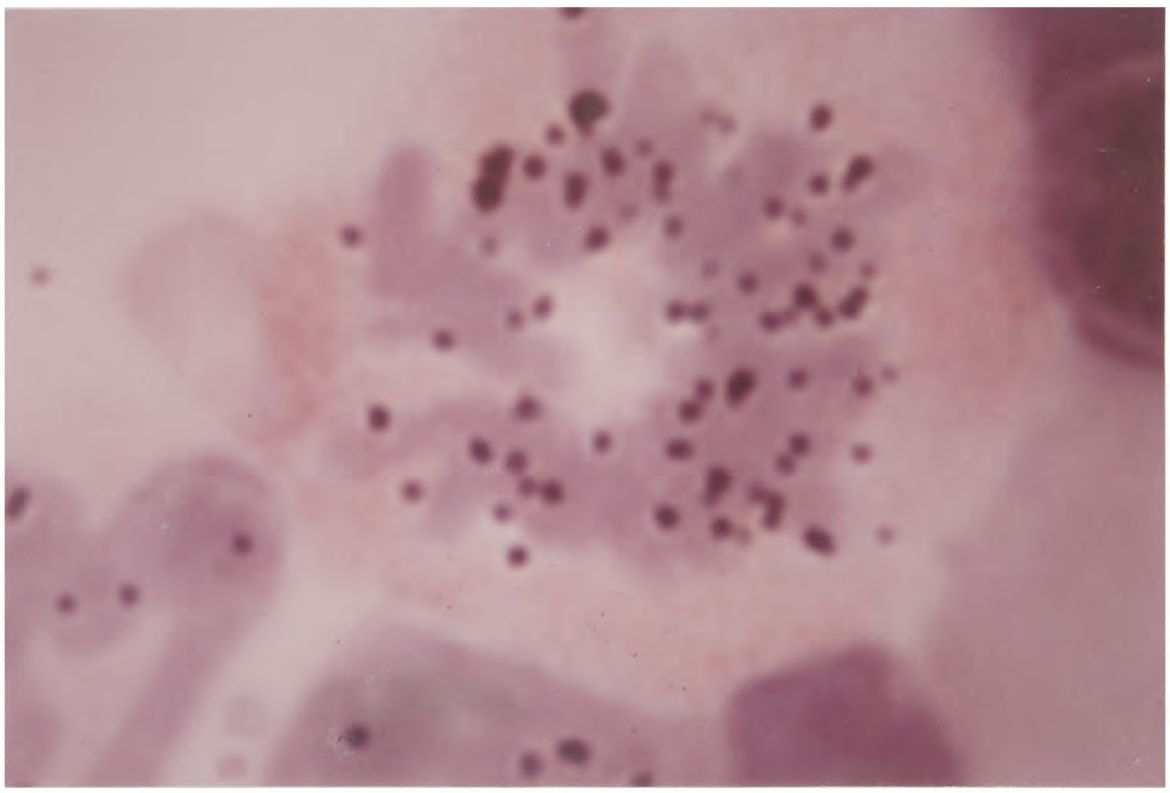
by

Christopher J. F. Spry

Exeter College, Oxford

Nuffield Department of Clinical Medicine,
The Radcliffe Infirmary, Oxford

Michaelmas term
1970



Frontispiece. A labelled eosinophil mitosis in the bone marrow of a normal rat 30 h after injection of 3H-thymidine. Autoradiograph, stained with haematoxylin and Biebrich scarlet. x 4,000.

CONTENTS

EOSINOPHIL PRODUCTION

ABSTRACT

Eosinophil granulocytes are found in all vertebrates and they are usually associated with parasitic and immunological diseases. Much of the previous work on eosinophils has concentrated on their distribution and variations in number in blood and tissues. This has led to many suggestions as to their possible functions. Few experiments have been done to study the way in which they are produced, and little quantitative information is available either about the kinetics of eosinophil production or the ways in which they can be mobilised. Even less is known about these processes under pathological states. Without this knowledge it has not been possible to distinguish factors altering eosinophil production from those influencing their turnover. For these reasons, the first part of this thesis describes experiments which were designed to measure the kinetics of eosinophil production, mobilisation and turnover in normal and stimulated rats.

Rats were given single injections of 10,000 Trichinella spiralis larvae and the extent of labelling of eosinophils in bone marrow and blood was followed on autoradiographs in these stimulated rats,

and in normal rats. In stimulated rats the bone marrow content of eosinophils increased and this resulted in a peripheral blood eosinophilia 2 - 10 days later.

Three sequential steps in rat eosinophil development in marrow were defined: primitive dividing eosinophils, more mature dividing eosinophils and non-proliferating eosinophils. The diameter of eosinophils was greatest just before they divided, and there was no relationship between eosinophil size and their maturity. Other experiments supported the concept that eosinophils leave the marrow and mature in the spleen before release into the blood.

The percentage of eosinophils in bone marrow showed no diurnal variation in normal rats. In rats given larvae intravenously the percentage of marrow eosinophils increased 23 h after stimulation. The peripheral blood eosinophil count rose 1 - 2 days later, and was related in parallel to the bone marrow level. The eosinophil mitotic index rose by a factor of 2.6, and there appeared to be a maximum number of eosinophil mitoses 40 h after stimulation. The proportions of primitive and more mature dividing eosinophils did not alter during the first 3 days, showing that all the eosinophils in the proliferative compartment were stimulated equally.

The eosinophil cell cycle parameters in normal and stimulated rats were calculated from measurements of the proportion of eosinophil mitoses labelled at intervals after single injections of 3H-thymidine. In normal rats there was a wide variation of eosinophil cell cycle times with a median of 30 h. In stimulated rats this was reduced to a median of 9 h with a narrow distribution of cell cycle times. The post-mitotic rest period (T_{G_1}) and D.N.A. synthesis period (T_S) were shortened. It was calculated that the earliest recognisable eosinophils divided a total of 4 - 5 times in normal rats, and in stimulated rats eosinophils divided a total of 9 - 10 times. Despite the additional number of divisions in the stimulated rats, marrow transit times were unaffected as the cell cycle times were shorter in the stimulated rats. It was concluded that increased eosinophil production came about by additional divisions in the proliferative compartment, associated with shortening of cell cycle times.

Eosinophil production rates were calculated from measurements of the number and labelling indices of eosinophils in weighed quantities of bone marrow combined with the known eosinophil cell cycle times. Production rates increased by a factor of 28, from 0.5×10^3 to 14.4×10^3 new eosinophils/mg bone marrow/h.

Eosinophil kinetics in the blood were studied by giving single injections of 3H-thymidine to normal or stimulated rats, and the number of labelled eosinophils in blood was measured subsequently. The interval between injections of 3H-thymidine and the appearance of labelled eosinophils in the blood (emergence time) was 40 h in normal rats and 17 h in stimulated rats, but neutrophil emergence time was not altered. This demonstrated that different factors regulated the emergence times of these two types of granulocyte.

An eosinophil releasing factor was sought in plasma removed 12 - 24 h after the injection of larvae. Six hours after injection of 2 ml plasma the eosinophil count in the blood of normal recipient rats doubled. The eosinophil releasing effect of plasma was not removed by dialysis and was stable at - 20°C. This eosinophil releasing effect was not associated with a neutrophil leucocytosis. Damaged eosinophils and homogenates of lungs containing larvae injected 16 h previously did not induce eosinophil release. Many stimuli which induce an eosinophilia 3 - 6 h after injection may act by producing 'eosinophil releasing factor' in blood as was demonstrated with larvae.

Eosinophil half-life in the blood of normal rats was 6.6 h. Surprisingly, eosinophil half-life did not decrease 2 days after

injection of the stimulus at a time when many eosinophils were moving from the blood into the tissues. It was concluded that there was an increase in the size of the intravascular eosinophil pool size. Blood eosinophil half-life was lengthened 5 days after injecting larvae at the height of the eosinophilia, and it was also increased in splenectomised rats 2 days after stimulation.

In another section of the thesis, work is described which centered on the processes involved in the initiation of increased eosinophil production. Studies on rats injected with larvae proved to be very useful for studying the regulation of eosinophil production, as the tissues containing the stimulus were separate from the responding marrow. The initial response to the stimulus was studied in the tissues, lymph and blood.

First, the inflammatory reactions induced by Trichinella spiralis larvae in lung, liver and muscle were examined histologically. Mast cell degranulation was followed by mononuclear cell and eosinophil accumulation. In other experiments, mast cell degranulation was not found to be essential for eosinophil production and intravenous injections of larvae coated with peritoneal macrophages produced a smaller eosinophilia than uncoated larvae. However, in lymph nodes stimulated

by Trichinella spiralis larvae, large numbers of dividing large pyroninophilic lymphocytes were found which were labelled with 3H-thymidine. These lymphocytes were considered to be the progenitors of the thoracic duct lymphocytes which have previously been shown to induce eosinophils production.

A study was made of the distribution and fate of large pyroninophilic lymphocytes derived from the thoracic duct of rats with oral trichinosis. Large pyroninophilic lymphocytes, which were labelled with tritiated nucleic acid precursors, were distributed in a similar way in normal and stimulated rats. The majority of transferred labelled large pyroninophilic lymphocytes were found in the small intestine, spleen and lymph nodes, where they divided after 24 h. At 48 h they had the morphology of plasma cells. Only a few labelled large lymphocytes reached the bone marrow. When ^{51}Chromium labelled lymphocytes were injected, less than 0.5% of the injected activity was found in each femur 22 h later. These results showed that if large pyroninophilic lymphocytes initiate eosinophil production by short range processes, then these lymphocytes may be a small subpopulation of the cells normally found in thoracic duct lymph.

In related work, presented in the appendix, experiments were done to determine whether thoracic duct lymph, draining from rats with unilateral pyelonephritis, would stimulate neutrophil production when transferred to normal rats. No effect was found. This result, which agrees with other recently reported experiments, is evidence that neutrophil production is not initiated by lymphocytes.

It was concluded that in rats given Trichinella spiralis larvae intravenously, alterations in the numbers of eosinophils in the blood take place in several ways; either through changes in the distribution of mature eosinophils, or by variations in eosinophil production. The measurements which were done on each of these processes has shown that they are regulated by separate mechanisms, each with a distinct time course involving both humoral and cellular mediators. In conditions associated with an eosinophilia these mechanisms act in unison to provide eosinophils in increased numbers in response to the stimulus.

This work has shown that regulation of eosinophil production and blood eosinophil levels takes place by a number of inter-related processes, in ways which are as finely balanced as those known to control neutrophil or erythrocyte production and distribution.

TERMINOLOGY

Eosinophil leucocytes (eosinophils).

These were defined as leucocytes that contained many refractile, cytoplasmic granules which stained strongly with eosin or Biebrich scarlet at an alkaline pH.

Eosinophilia

This word is used in its original sense: "By eosinophilia we understand an increase only of the polynuclear eosinophil cells in the blood" (Ehrlich & Lazarus, 1900).

Large pyroninophilic lymphocytes

These were lymphoid cells which had a diameter larger than 10 μ when the cells had been prepared by centrifuging at 22 g for 5 min with a Cytocentrifuge. The cytoplasm of these cells was strongly pyroninophilic when stained with methyl-green pyronin.

'Stimulated' lymphocytes

These were defined as large pyroninophilic lymphocytes found in the thoracic duct lymph of rats which had been given oral trichinosis 3-5 days previously.

'Labelled' cells

These were defined as cells which were found in autoradiographs to have 4 or more silver grains over their nuclei. Slides were not used where background counts showed that 2 or more grains could commonly occur by chance over the cells on the slide.

ABBREVIATIONS

M	Mitosis
G_1	Post-mitotic rest phase
S	D.N.A. synthesis phase
G_2	Post-S and pre-M phase
T_C	Cell cycle time (hours)
T_S, G_1, G_2, M	Time for each phase of the cell cycle (hours)
$T_{\frac{1}{2}}$	Half-life of peripheral blood eosinophils (hours)
T_E	Emergence time. Time between injection of 3H-thymidine and the appearance of labelled cells in the blood (hours)
Kp	Marrow eosinophil production rate (new eosinophils/mg bone marrow/hour)
G_o	Eosinophils temporarily excluded from the proliferating pool of eosinophils in marrow
3H-adenosine	Tritiated adenosine
3H-thymidine	Tritiated thymidine
^{51}Cr	Radioactive chromium-51
h	Hours
Ci	Curies
D.N.A.	Desoxyribosenucleic acid
R.N.A.	Ribosenucleic acid
S.P.F.	Specific pathogen free

CHAPTER 1

HISTORICAL INTRODUCTION

The eosinophil: a distinct type of cell

Wharton Jones (1846) has been credited with the discovery of eosinophils, but Schultze (1856) was the first to describe eosinophil morphology clearly, and was the first to suggest that they were a unique cell type. Specific staining methods for eosinophils were developed by Ehrlich (1879) who submitted this work for his doctorate thesis in Berlin. He discovered that acidic dyes such as eosin stained the cell granules strongly, hence their name. More recently Biebrich scarlet has been found to be a particularly useful stain in the study of eosinophils (Spicer & Lillie, 1961; Schaefer & Fischer, 1968), and this stain was the one most commonly used in the present study.

Eosinophil granules

Eosinophil granules consist of an electron-dense bar-shaped internum (Fedorko, 1968), and an electron translucent outer part

which contains acid hydrolases and other enzymes found in lysosomes (Archer, G.T. & Hirsch, 1963 a). This means that the eosinophil granule can be looked upon as a special type of lysosome. The subject is reviewed by Archer, R.K. (1968). The internum is a crystalline structure with peroxidase activity (Archer, G.T. & Hirsch, 1963 a; Archer, G.T., Air, Jackas & Morrell, 1965; Miller, de Harven & Palade, 1966). A recent report by Presentey (1969) describes an hereditary abnormality of human eosinophils in which peroxidase is absent, but the patients were healthy. No electron micrographs of these cells have yet been published. When particles are ingested, the granules fuse with the phagosomes and release their enzymes around the particles (Archer, G.T. & Hirsch, 1963 b; Cotran & Litt, 1969). The internum may remain undissolved and can be found in the phagosome even after relatively long periods (Zucker-Franklin, 1968). In the rat, peritoneal eosinophils have been found to ingest ferritin particles without coalescence of phagosomes and eosinophil granules (Bro-Rasmussen & Egeberg, 1966). The interna can also be found lying undissolved when eosinophils have disintegrated in tissues, and they may aggregate to form Charcot Leyden crystals (Archer, G.T. & Blackwood, 1965). Buddecke, Essellier & Marti (1956) report that Charcot Leyden crystals consist of a basic zinc-containing protein. It is still not

known whether the internum is a functional constituent of eosinophils, or whether it is merely an inactive product which accumulates during the period when eosinophil granules are being synthesised.

In some parasitic infections, including Ascaris infections, some tissue eosinophils appear to contain fewer granules than normal (Gross & Schmidt, 1954), but this phenomenon has not been studied in any depth. Connell (1968) has reported finding vacuoles in the cytoplasm of up to 75% of human eosinophils in vivo in patients with allergic diseases. Ross & Klebanoff (1966) described the presence of free eosinophil granules in uterine tissue, and postulated that eosinophil granules had an unknown function outside the eosinophil. Recently, Morgan & Beeson (1970) have found that eosinophils in mouse bone marrow are no longer identifiable within a few hours of induction of a severe pyogenic infection which involved massive neutrophil production. In this situation the apparent disappearance of eosinophils may be the result of degranulation.

Eosinophils in marrow

The bone marrow has been considered to be the main site of origin of blood cells in adult animals for over 100 years (Neumann, 1868). In 1879, Ehrlich had shown that eosinophils also originated

in bone marrow:

> "The bone marrow is a breeding place in which polymorphonuclear cells are produced in large numbers from mononuclear pre-existing forms".
>
> "... the mononuclear eosinophil cells grow into polynuclear in the bone marrow ...."
>
> (Ehrlich & Lazarus, 1900).

The way in which eosinophils are produced in bone marrow has not been studied until recently because of technical difficulties. Although eosinophils are easy to recognise, normal animals have so few that it is difficult to find enough to analyse. Animals with higher levels are likely to harbour parasites so that eosinophil counts fluctuate unpredictably. The introduction of radioactive labelling techniques (Doniach & Pelc, 1950) and their application to the study of cell kinetics (Quastler & Sherman, 1959; Cleaver, 1967) made the present studies possible.

Cohen, N.S., LoBue & Gordon (1967) studied eosinophil labelling in rat bone marrow after single injections of 3H-thymidine. Labelling was also studied in rats depleted of granulocytes. They suggested that rat eosinophils probably divide more rapidly when stimulated. Later Alexander, Monette, LoBue, Gordon & Chan (1969), using a double labelling technique, found that unstimulated rat eosinophil precursors had a cell cycle time of about 24 h. Eosinophil production in bone marrow has not been measured directly.

Eosinophils in blood

Repeated injections of foreign proteins are known to result in a rapid increase in the numbers of eosinophils in blood (Schlecht, 1910) and tissues (Schlecht & Schwenker, 1912). The increase in eosinophils was maximal about 4 - 6 h after reinjection (Bennett, 1951). Homma (1921) was the first to study carefully the release of mature eosinophils from rat marrow when 'eosinophilic' substances were injected in the rat. Since then the phenomenon has been studied in many ways, and a wide variety of agents have been thought to induce an eosinophilia.

The first kinetic studies of eosinophil release from marrow were made by Foot in 1963 and 1965. She measured the emergence time (T_E) of mature eosinophils in inbred rats of the PVG/C strain given continuous intraperitoneal infusions of 3H-thymidine. Labelled eosinophils were first seen in the blood 67 h later. Later she and her husband found that T_E of eosinophils in Sprague-Dawley rats was 48 h (Blenkinsopp, 1966; Blenkinsopp & Blenkinsopp, 1967). Bro-Rasmussen, Anderson & Henriksen (1967) improved this method by giving only single injections of 3H-thymidine. In Leo rats they found T_E was 36 h. A similar T_E was found by Cohen, N.S., LoBue & Gordon (1967) using Long-Evans rats.

Factors involved in the 'release' of eosinophils have been studied by Cohen, N.S., LoBue & Gordon (1967) who found T_E was reduced when

leucocytes were removed from the peritoneal cavity by lavage. Anderson, Bro-Rasmussen & Haugaard (1969) showed that eosinophil T_E was lengthened by injections of steroids.

Eosinophil half-life ($T\frac{1}{2}$) in normal rat blood was estimated to be 10 $\pm$ 2 h (Foot, 1965). No adequate studies of blood eosinophil $T\frac{1}{2}$ in rats, under conditions associated with an eosinophilia, have been reported.

Eosinophil phagocytosis

In 1895 Mesnil reported that eosinophils could phagocytose particles. They have been shown to phagocytose antigen-antibody complexes *in vivo* (Sabesin, 1963) and antigens in lymph nodes soon after injection of the antigen (Litt, 1964 ; Roberts, 1966). Human eosinophils can phagocytose polystyrene particles, *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*, but this process is not as well developed as in neutrophils (Cline, Hanifin & Lehrer, 1968). There is no evidence that some particles are phagocytosed preferentially by eosinophils rather than by neutrophils or macrophages. Despite this, great effort has been expended in trying to involve eosinophils in antigen 'processing' (Speirs, 1958) and in 'detoxification' of harmful antigen-antibody complexes (Litt, 1963, 1964).

Eosinophils and diseases

Eosinophilia has been associated with a number of diseases since 1889, when Gollasch found an increased number of eosinophils in the blood of patients with asthma. Canon (1892) described their occurrence in chronic skin diseases.

Eosinophilia has come to be recognised as a very common feature of parasitic infections in man. Brown (1898), while he was a medical student at the John Hopkins Medical School, was the first to notice that increased numbers of blood eosinophils occur in patients with trichinosis. Trichinella spiralis has been used in many experimental studies of parasitic infection, and the participation of eosinophils in trichinosis is well described (Gould, 1945, 1970; Larsh, 1963, 1968).

Eosinophils accumulations and functions

The observation of large accumulations of eosinophils in tissues under various circumstances has prompted most of the theories about the nature of eosinophils. However the mechanisms of accumulation are still unknown. Eosinophils can be found in large numbers in tissues into which Trichinella spiralis larvae have migrated. Schwarz (1914) noted that eosinophils do not accumulate in tissues very close

to encysted Trichinella spiralis larvae, but are found mainly in inflamed tissues nearby. Thus the production of 'eosinotactic' agents by the larvae themselves seemed unlikely.

It has been reported that specific substances are 'eosinotactic' in vivo. These include xylose (Chapman & Clark, 1968 b), components of fungi (Chapman & Clark, 1968 a), altered globulins (Cohen, S.G. & Sapp, 1963), histamine (Archer, R.K., 1963, 1968) and antigen-antibody complexes (Litt, 1964), but the subject is still beset by many contradictory findings. For example in studies on antigen-antibody complexes, Speirs & Turner (1969) showed that intraperitoneal injections of tetanus toxoid in mice induced local eosinophil accumulation when the antigen was not complexed with antibody. But Litt (1964) showed that haemocyanin or bovine serum albumin coupled to antibody caused more eosinophil accumulation in guinea pig lymph nodes than that characterising either antigen alone. At present it is not possible to discern any common mechanism by which antigens or antigen-antibody complexes are associated with eosinophil accumulation in tissues.

In vitro studies, using the Boyden chamber technique (Boyden, 1962), have failed to distinguish qualitatively between the responses of eosinophils and neutrophils to chemotactic agents. Substances which attract neutrophils usually also attract eosinophils, but to a lesser extent, and there are also species differences (Keller & Sorkin, 1968, 1969; Ward, 1969).

Anaphylactic reactions induce an eosinophilia and additional numbers of eosinophils localise in tissues (Campbell, 1943; Parish & Coombs, 1968). Kay (1970) has shown that γ_1 antibody, which can transfer anaphylactic reactions, induces eosinophil accumulation in guinea pig skin when the antigen is added. Orange & Austen (1970) showed that eosinophils suppress the release of slow reacting substance of anaphylaxis (SRS-A). Further work on eosinophil accumulation in anaphylaxis should prove particularly rewarding, and this may clarify the mechanisms by which large numbers of eosinophils localise in certain sites.

Mechanisms of eosinophil production

As early as 1900 Ehrlich & Lazarus had clearly outlined a possible mechanism for increased eosinophil production:

> "Under these circumstances [when local eosinophilic suppuration has affected large areas] large amounts of the specific active agents reach the bloodstream by absorption and diffusion. Here it exercises a strong chemiotactic influence on the physiological storage depot of the eosinophils, the bone marrow: leading to an increase of the eosinophils of the blood to a greater or less degree. The bone marrow, according to general biological laws, is by the increased emigration now further stimulated to a fresh production, and during a protracted illness can hence keep up the eosinophilia".

Opie (1904) was also among the first to study the mechanisms of eosinophilia experimentally. He produced trichinosis in guinea pigs

and showed that, whereas oral infection with living larvae induced an eosinophilia, rather surprisingly ground-up larvae given subcutaneously would not induce an eosinophilia. In orally infected guinea pigs the blood neutrophil count fell at the same time as the eosinophil count was rising, which suggested that there were distinct regulatory mechanisms for these two types of cell. Careful histological work enabled him to make other important observations:

> [After oral infection] "..... increase of eosinophil leucocytes, ... is associated with local accumulation in the mesenteric lymph glands and in the lungs. No noteworthy increase of these cells in the intestinal mucosa was noted."

He found that eosinophil accumulations in lymphatic glands and the base of the mesentery could be so great that they resembled small abscesses in which the eosinophil replaced the neutrophil. He also described very large accumulations of eosinophils in parts of the lung 3 - 4 weeks after oral infection, and by studying marrow eosinophils he deduced that marrow was the seat of eosinophil production in guinea pigs. In heavily infested guinea pigs he found that mature eosinophils accumulated in the marrow while eosinophils in blood disappeared. These careful studies laid the basis for much of the future work on eosinophils in vivo.

In 1964, Zaiman & Villaverde showed that some component of blood initiates eosinophilia in trichinosis. Eosinophilia was induced in one of a pair of parabiotic rats when the other was infested with Trichinella spiralis. By separating the rats at intervals from the second day onwards, they showed that the stimulus to eosinophil production passed from the infested rat to the normal rat before the new generation of larvae had begun to migrate in the blood.

The introduction by Basten, Boyer & Beeson (1970) of a reproducible model for studying eosinophil production in rats has proved a great advantage for in vivo investigation of this phenomenon. Rats were given oral or intravenous inoculations of Trichinella spiralis, and daily blood eosinophil counts were compared with normal rats. Basten & Beeson (1970) showed that rats produced an eosinophilia only if they contained a normal complement of lymphocytes. The production of an eosinophilia was inhibited by methods known to damage or prevent the action of lymphocytes. These methods included treatment with antilymphocyte serum and neonatal thymectomy combined with thoracic duct drainage. The stimulus to eosinophil production was associated with viable large pyroninophilic lymphocytes which appeared in the thoracic duct lymph in rats given oral trichinosis. In lymphocyte

transfer studies, these large lymphocytes induced a small but significant eosinophilia in normal rats. An enhanced eosinophilia in response to intravenous larvae was induced in rats given thoracic duct lymphocytes several weeks after the donor was stimulated with oral larvae. Recently, Walls, Basten, Leuchars & Davies (1970) have shown that lymphocytes involved in eosinophil production in mice are thymus dependent.

Aims of this study

The work described in this thesis was done in parallel with the studies reported above. Autoradiographic techniques were used to measure the kinetics of the eosinophilia in rats given larvae intravenously, and to follow the fate of the 'sensitised' lymphocytes which would initiate an eosinophilia in normal recipient rats. The questions which were posed included: What types of eosinophils are present in rat marrow? What is the kinetic pattern of eosinophil production in bone marrow in normal rats and rats given an 'eosinophilic' stimulus? How can the rate of eosinophil production be measured? What factors regulate the release of eosinophils into the circulating blood? Is eosinophil half-life in the blood altered when there is an eosinophilic stimulus? What is the mechanism by which sensitised lymphocytes initiate eosinophil production?

CHAPTER 2

METHODS

Rats

The rats used in the majority of experiments were specific pathogen free (S.P.F.) Wistar rats from the Medical Research Council, Radiobiological Research Establishment, Harwell, Berkshire. These rats were shown to have very low resting eosinophil counts of not more than 150/mm^3. When injected with Trichinella spiralis larvae these rats produced a more reliable and consistent eosinophilia than 8 other strains of rats which were tried. In experiments where cell transfer studies were done, syngeneic rats were used: PVG/C non-S.P.F. rats from the Animal Research Council, Compton, Berkshire and B/H rats derived by caesarian section and barrier maintained from Animal Supplies Ltd., North Finchley, London. Rats weighed 160 - 200 g. Male rats were preferred but in some experiments females were also used.

Rats from outside sources were taken into an animal holding room in which they were kept for several days before being used. Here they were fed on modified Oxoid diet 41B (Berks, Bucks & Oxon Farmers Ltd.,

Reading) with water always available. Twelve hours of artificial light were provided from 9 a.m. to 9 p.m. The temperature varied from 62 to 88°F but was usually about 74°F.

Some rats were bred in the Nuffield Department of Clinical Medicine animal house. Each pair was kept together throughout its breeding period, providing up to 7 litters. They were only handled when necessary by an animal technician who wore plastic gloves, and careful breeding records were kept. Breeding rats were kept in a separate room with light and temperature control. Rats were fed on a sterile autoclaved small animal diet (Spillers Ltd., Gainsborough, Lincolnshire). Rooms were sterilized with formalin vapour twice a year, and cages and bottles were sterilized each fortnight. Sawdust was autoclaved.

Anaesthesia

Rats were anaesthetised in a chamber with a continuous flow of compressed air bubbling through ether. The rate of induction of anaesthesia was regulated by varying the air flow from the cylinder. Anaesthesia was maintained by dripping ether onto small cotton wool swabs over the muzzle.

Trichinella spiralis larvae

The method of Kagan (1960) was used to obtain Trichinella spiralis larvae. Adult rats were given 15-25 larvae/g via a plastic tube passed through the mouth into the stomach, while the animals were under anaesthesia. At least three weeks later the animals were killed, skinned, eviscerated and passed through a mincer. The pieces were put on 4 layers of muslin and supported over the neck of a large glass funnel by a flat circular grid. A centrifuge tube was attached to the tip of the funnel by a rubber sleeve. A digesting mixture was freshly prepared, containing crystalline hog pepsin (Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire) 5 g in one litre of distilled water to which 7 ml of concentrated HCl had been added. About 1 litre of this artificial gastric juice per 100 g of carcass was added to the funnel and left for 6 to 12 hours at 37°C. Motile larvae were digested out of their capsules in the carcasses and settled in the centrifuge tube. Larvae were washed twice in tap water, distilled water, sterile pyrogen-free saline and Parker 199 tissue culture medium, containing 200 units pencillin/ml + 100 µg streptomycin/ml (Wellcome Ltd., London). 0.1 ml of sedimented larvae contained about 50,000 larvae and as many as one million could be obtained from a single rat. When prepared by this method, larvae contained no endotoxin.

Intravenous injections of Trichinella spiralis larvae. The stimulus used to increase eosinophil production throughout this work was intravenous injections of 10,000 Trichinella spiralis larvae (Basten, Boyer & Beeson, 1970). Injections were made into the lateral tail vein using 23 gauge needles while the rats were under light anaesthesia. For injections, Trichinella spiralis larvae in Parker 199 medium were drawn up into a 1 ml syringe, and suspended by agitation of the syringe. The larvae were then injected gradually, with the syringe held vertically. Complete injection of larvae was ensured by detaching the syringe from the needle and drawing up 0.5 ml of sterile saline with the same syringe, while the needle was still in position in the vein, and flushing the saline through the needle with the syringe again held vertically.

Intraportal injections of Trichinella spiralis larvae. After opening the abdomen, the upper intestines were reflected to the rat's left, exposing the portal vein. A loop of tape was passed around the vein and after occlusion of the vessel, injections were made in the same way as intravenous injection of larvae. The hole in the vein was closed with methyl 2-cyanoacrylate adhesive (Ethicon Ltd., Edinburgh). The abdominal wound was closed with sutures.

Autoradiographic methods

The radioactively labelled compounds used were: 3H-Thymidine (methyl-T TRK 120, The Radiochemical Centre, Amersham), specific activity 23 Ci/mM, and 3H-Adenosine (TRA 3, The Radiochemical Centre) specific activity 100 - 1,000 mCi/mM. Glass slides were acid washed and coated with gelatin solution (Rogers, 1967) a few days before they were used. Nuclear Emulsion K2 (Ilford Ltd., Essex) was diluted with half its volume of distilled water, and a dipping technique was used to coat the experimental slides which were dried in the dark and put in plastic microscope slide boxes (A.R. Lamb, Alperton, Middlesex) which were sealed with black tape. In some experiments boxes contained dry silica gel and a small amount of solid CO_2. All slides were incubated at + 4°C for three weeks. Then they were developed for 5 min at 21°C with a solution of KodakD19 developing powder, and fixed with Kodafix (Kodak Ltd., Hemel Hempstead, Herts). Plain and exposed slides were included with each box and processed at the same time. Slides were stained with haematoxylin and Biebrich scarlet or Leishman's stain or methyl-green pyronin by the methods described for marrow preparations, and were examined under the microscope within 12 h of staining.

Marrow preparations

Initially marrow preparations were made by smearing marrow over glass slides. Many of the cells were distorted, damaged or obscured by other cells so that another method was devised using a Cytocentrifuge (Shandon Ltd., London).

Rats were killed by anaesthetising with ether and injecting air intravenously. A complete femur was removed through an incision in the groin. The bone was wiped clear of attached muscles. Bone ends were cut off with bone forceps. In some studies 5 ml of Parker 199 containing 1 unit of heparin/ml was forced through the marrow cavity using a 21 gauge needle on a 5 ml syringe.

In other studies where measurements of the number of eosinophils/mg marrow were made, compressed air at 10 lbs/in^2 was suddenly blown through the needle and a plug of marrow was collected in a plastic Universal container (Sterilin Ltd., Richmond, Surrey) and weighed immediately. 5 ml of Parker 199 containing heparin 1 unit/ml was then added.

The marrow cell suspension was drawn up into a 5 ml syringe using a 23 gauge needle, and gently expressed again. This was repeated twice producing a single cell suspension. Two drops of the cell suspension per slide were centrifuged for 5 min at 22 g on a Cytocentrifuge.

The glass slides used were acid washed and coated with gelatin as for autoradiography. The cell buttons on the slides were dried rapidly in air as soon as the Cytocentrifuge had stopped and fixed for 5 min in absolute methanol. The whole procedure took about 15 min for each rat, and as many as 24 slides were made for each rat. The slides were stored and stained later. With this technique it was found that the eosinophil granules did not overlie the nucleus. This enabled accurate studies to be done using the shape of the nucleus as an index of cell development. It also enabled accurate autoradiography of eosinophils to be done, as geometrical variations in the relationship of nucleus to emulsion were reduced to a minimum.

Marrow eosinophil counting. Counts of the number of eosinophils/mg bone marrow were done on weighed samples made up as single cell suspensions. All marrow cells were counted directly in an improved Neubauer counting chamber and the results were expressed as cells/mg bone marrow. Cytocentrifuged preparations were made at the same time from these single cell suspensions and were stained separately either with Leishman's stain or haematoxylin and Biebrich scarlet. The proportion of nucleated cells to non-nucleated cells was calculated by counting 1,000 cells on the stained marrow preparation. The

percentage marrow eosinophils was counted from among 1,000 nucleated cells. The number of eosinophils/mg of bone marrow could then be calculated. The accuracy of the method was assessed by counting several slides made from one bone marrow suspension that contained many eosinophils. The mean % eosinophils ± S.D. on five slides was 22.1% ± 1.7.

Staining of marrow preparations. Some marrow smears were stained with Leishman's stain as described for blood smears. Others were stained with haematoxylin followed by Biebrich scarlet at pH 9.5. Harris' haematoxylin (Ortho Pharmaceuticals, High Wycombe, Buckinghamshire) was used for 5 minutes followed by rapid differentiation in 1% acid-alcohol. Then the slides were washed in water and put in a blueing agent consisting of $NaHCO_3$. 2g, $MgSO_4$. 20 g, dissolved in 1 litre of distilled water. The slides were stained with 0.04% Biebrich scarlet, water soluble (British Drug Houses) in a Sørensen glycine/NaOH buffer pH 9.5. Staining took place in a few seconds at 30°C. When eosinophils in autoradiographs were being stained, slides were kept in the Biebrich scarlet solution at 25 - 30°C for 15 minutes. Eosinophil granules stained strongly and there was no difficulty in recognising all types of eosinophils. The specificity of Biebrich scarlet stain for eosinophils was carefully checked in several experiments. After

staining marrow smears with Leishman's stain, and noting the position of the eosinophils, the stain was removed with ethanol, and the slides restained with Biebrich scarlet. In another experiment, rat marrow which contained 46.3% eosinophils when stained with Leishman's stain, was found to have 45.7% eosinophils when stained with haemotoxylin and Biebrich scarlet. This showed the Biebrich scarlet staining of eosinophils was consistent and specific for eosinophil granules.

Cell morphology was very clear in these preparations as the cells were flattened on the glass and no cells overlapped. About 5% of the marrow cells on the slides were damaged.

Blood counts

Rats were lightly anaesthetised between 9 a.m. and 4 p.m. and a 23 gauge needle was put into the lateral tail vein. The emerging blood was dripped directly onto a piece of Parafilm (Gallenkamp, London) and when needed onto glass slides. Blood smears were made at once.

Eosinophil counts. Each blood sample was drawn up in a white cell pipette to the 1 volume mark and diluted with a solution consisting of 5 volumes of 1% water soluble eosin yellow shade C.I. 45380 (British Drug Houses Ltd., Poole, Dorset) dissolved in a fluid consisting of 5 volumes of acetone and 90 volumes of distilled water (Discombe, 1946).

The blood and eosin solutions were mixed by rotating the pipette. After removing about half the contents of the pipette, the mixture was run under the cover slip on both sides of an improved Neubauer counting chamber. The cells were allowed to settle and the chambers were kept moist in plastic petri dishes containing damp filter paper. Eosinophils were counted on both sides of the chamber. In Harwell rat blood, eosinophil counts were considered to be raised when there were more than 27 eosinophils on both sides of the counting chamber ($150/mm^3$).

Total blood white cell count. 50 mm^3 of blood was drawn up in a red cell counting pipette and added to 25 ml of Isoton (Coulter Electronics Ltd.) and mixed. 0.2 ml of a solution of 1% saponin in Isoton was added to lyse the red cells. Ten minutes later the cells were counted in a Coulter counter Model D using settings of A.C.3 and T.20. Duplicate counts were often done.

Differential blood white cell counts. Smears of blood cells were fixed and stained with Leishman's stain made by stirring 1.5 g Leishman's stain (British Drug Houses, Ltd.) in 1 litre methanol at 50°C for 20 min. The slides were placed horizontally and covered with a freshly filtered solution of the concentrated stain. Five minutes later they were washed with tap water and covered with a freshly

made solution of diluted buffered stain at pH 6.8. The buffer was made from buffer tablets (Gurr Ltd., London) each day and an equal quantity was added to filtered concentrated Leishman's stain and mixed immediately before staining. The second staining step took ten minutes. Then the slides were washed with tap water and left to dry vertically. Two hundred white blood cells were counted for each slide. Calculations of the number of each type of cell/mm^3 were done rapidly with an Olivetti computer.

The overall accuracy of the counting methods was assessed by repeatedly counting one sample of blood from a normal Harwell rat, (Table 2.1).

	mean ± S.D.	Number counted
Total white cells/mm^3	7,979 ± 720	[27]
Neutrophils/mm^3	1,578 ± 389	[27]
Mononuclear cells/mm^3	6,502 ± 392	[27]

Table 2.1. Accuracy of white blood cell counts

One sample of blood was counted 27 times.

Concentration of blood leucocytes for autoradiography. It was tedious to find eosinophils in smears of blood from normal S.P.F. rats as there were so few present. For this reason a method for concentrating blood leucocytes for blood autoradiograph was devised. Tail vein samples were drawn up into heparinised capillary micro-haematocrit tubes (Hawksley & Sons Ltd., Lancing, Surrey). One end of each tube was blocked with Plasticine. The tubes were then centrifuged for 5 minutes on a Hawksley microcentrifuge. The tubes were broken at the junction of buffy-coat and red cells, and the white cells were removed by pushing a plug of Plasticine down from the open end of the tube with a thin wire. The white cells were pooled in a few drops of Parker 199 containing 1 unit of heparin/ml, and then put on slides using a Cytocentrifuge. White cells were present in high concentrations and because they were flat, morphology was excellent.

Measurement of emergence time (T_E)

Measurements were made of the time intervals between intravenous injection of 1 μCi 3H-thymidine/g rat, and the first appearance of labelled blood eosinophils or neutrophils as seen on autoradiographs of blood. This value for T_E was assumed to be equal to the duration

of cell transfer through the maturation non-proliferating eosinophil compartment added to $T_{G_2} + T_M$ in the last cell division.

Measurements of eosinophil half-life in blood ($T\frac{1}{2}$)

Measurements were made of the fraction of blood eosinophils in rats which were labelled ~~in the blood~~ at intervals following an intravenous injection of 3H-thymidine, 1 μCi/g body weight. Half-life of labelled eosinophils was calculated both from the rate of increase of all labelled cells in the blood by the method of Foot (1965), and also by the rate of disappearance of the most heavily labelled cohort of eosinophils from the blood by the method of van Furth & Cohn (1969).

Splenectomy

Splenectomy was done through a small incision under the left costal margin, with the rat under ether anaesthesia. The vessels to the spleen were tied with 3 or 4 silk sutures and the spleen was removed intact. The abdominal wound was closed with silk sutures.

Adrenalectomy

Rats were anaesthetised with ether, and after shaving the skin on either side of the back, incisions were made just below the costal

margin, about 1 cm lateral to the spinal processes on each side. The abdominal muscles were cut at the lateral side of the spinal muscles. After blunt dissection with forceps in the perinephric fat, the adrenal glands were seen at the cranial end of the kidney. The glands were plucked out and no sutures to the pedicles were needed. The muscles of the back and skin were closed with silk sutures. The rats were kept warm during the procedure on a small heated operating table. After the operations 5 - 10 rats were kept together to enable them to maintain normal body temperature, and they were given 0.9% sodium chloride as drinking water with normal diets.

Histological methods

Tissues were fixed in 10% buffered formol-saline, or Zenker's formol-saline, mounted in wax and sectioned at about 5 μ thickness. Sections were dewaxed and stained with either haematoxylin and Biebrich scarlet or eosin, or methyl-green pyronin or Giemsa's stain by Wolbach's method.

Lungs. At selected intervals after injections of larvae into Harwell rats, they were anaesthetised with ether and the trachea tied in the neck. The thoracic contents were removed whole, avoiding

puncture of the lungs. They were fixed by totally immersing in 10% buffered formol-saline. With this technique the spatial relationships in the lung were well preserved.

Liver, muscle, lymph nodes, spleen. These were fixed and processed as described above.

Thoracic duct lymphocyte collection

Normal rats or rats given 15 Trichinella spiralis larvae/g orally 3-5 days before, had thoracic ducts cannulated by the method of Bollman, Cain & Grindlay (1948), adapted by Gowans & Knight (1964).

Thoracic duct lymphocytes were collected in 5 ml buffered phosphate saline containing 20 units heparin/ml and streptomycin 1mg/ml. Twelve hour collections of cells were sedimented at 100 g for 10 minutes, washed once in Parker 199 and resuspended in a favourable culture medium consisting of 8 parts of Parker 199, 2 parts of isotonic phosphate buffer, pH 7.4, 0.1 part of 50% glucose and 0.1 part of inactivated syngeneic normal rat serum.

Staining of thoracic duct lymphocytes. Lymphocytes were spread on acid washed, gelatine coated slides using a Cytocentrifuge at 22 g for 5 min. The cells were probably flattened equally and morphology

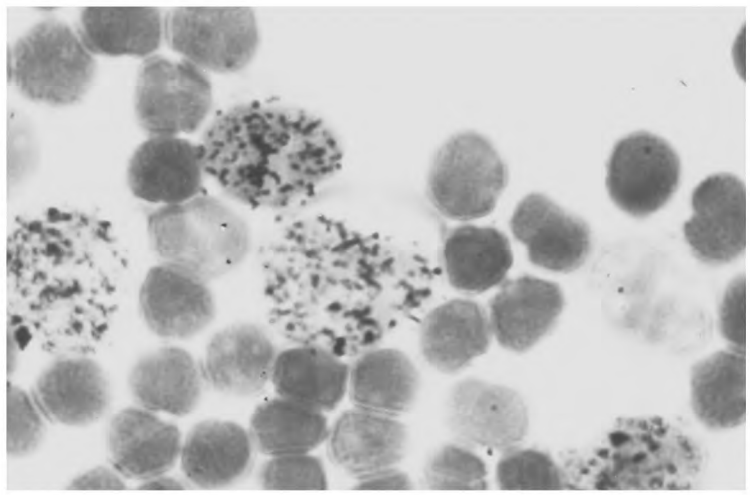


Figure 2.1. Thoracic duct lymphocytes from a rat with oral trichinosis. 80% of the large lymphocytes were labelled with 3H-thymidine *in vitro*. Autoradiograph. Lieshman's stain. x 600.

was excellent. Lymphocytes were stained with methyl-green pyronin (G.T. Gurr) for 5 minutes or Leishman's stain as described for blood smears.

Labelling of lymphocytes. Thoracic duct lymphocytes were incubated with either 3H-thymidine which labelled only large sensitised lymphocytes or 3H-adenosine which labelled all the lymphocytes, by the methods of Gowans (1962).

Large lymphocytes were labelled by adding 0.1 µCi 3H-thymidine/ml to the resuspended lymphocyte suspensions containing about 1 x 10^8 lymphocytes/ml and incubated for 1 h at 37°C in a shaking water bath. Autoradiographs showed that 55-85% of the large pyroninophilic lymphocytes incorporated the radioactive label. The lymphocytes were washed twice in 10 ml Parker 199 and then suspended in 2 ml Parker 199 for injections. Lymphocytes were injected into the lateral tail vein of recipient syngeneic rats within 3 h of collection. Cell viability was assessed by adding 1% trypan blue in saline at room temperature to the cell suspension. Fewer than 5% of the cells took up the dye. Cytocentrifuge preparations of inocula were taken for autoradiography and for staining with methyl-green pyronin (G.T. Gurr Ltd.). An autoradiograph of labelled large lymphocytes from thoracic duct lymph prepared by this method is shown in Figure 2...

Inocula containing labelled small lymphocytes were made by first incubating 12 h collections of thoracic duct lymphocytes in an unfavourable medium consisting of 8 parts of Parker 199, 2 parts of buffered phosphate saline and 0.5 parts of heat inactivated syngeneic normal rat serum (Gowans, 1962). Five to ten x 10^7 lymphocytes/ml were incubated in a shaking water bath with a 5% CO_2 atmosphere for 12 h at 37°C. Methyl-green pyronin stained smears showed that almost all the large lymphocytes were killed by this procedure. The remaining small lymphocytes were then washed once in Parker 199 and incubated at 37°C for 1 h in the favourable culture medium described under 'lymphocyte collections', with 40 µCi of 3H-adenosine/ml. The population of labelled small lymphocytes was washed once in Parker 199 and injected without delay into recipient rats. Ninety to 100% of the small lymphocytes were labelled by this technique.

Labelled lymphocytes in recipient rats

Rats which received the labelled lymphocytes were killed at intervals after the transfers and tissues were taken for autoradiography. The sizes of labelled cells in bone marrow preparations were measured with a calibrated eyepiece measuring graticule (Graticules Ltd., London).

The number of labelled lymphocytes in the tissues could not be assessed by scintillation counting, as the amount of radioactivity in each tissue was small. Some labelled macrophages, which had presumably ingested labelled lymphocytes were seen in tissues; the number of lymphocytes destroyed in this way could not be measured.

^{51}Chromium labelled lymphocytes

Normal and stimulated thoracic duct lymphocytes were collected and washed as described above. Two x 10^8 lymphocytes were incubated in 0.5 ml 0.9% saline for 3/4 h at 37°C with 100 µCi sodium ^{51}chromate, specific activity between 3 - 5 µg/Ci (The Radiochemical Centre). The lymphocytes were washed four times in Parker 199 at room temperature. Radioactivity in the washings from the last wash contained $<$ 2% of the radioactivity in the cell button. Two x 10^7 labelled lymphocytes in 1 ml Parker 199 were injected into the lateral tail veins of recipient syngeneic rats. Twenty-two to 23 h later the recipient rats were anaesthetised with ether and bled out by the aorta. Tissues were counted in a Wallac gamma scintillation counter. Radioactivity in each tissue was expressed, after subtraction of background counts, as the percentage of the injected radioactivity. It was assumed that the distribution of radioactivity was an accurate measure of the distribution of lymphocytes. Dresser, Taub & Krantz

(1970) have discussed this assumption and consider it valid if symmetrically designed experiments were done, and if the time between injections and sacrifice is 24 h or less.

Calculation of results

Means, standard deviations, standard errors, regression equations and student T tests were calculated with an Olivetti Programma 101 computer.

CHAPTER 3

MARROW EOSINOPHILS

Introduction

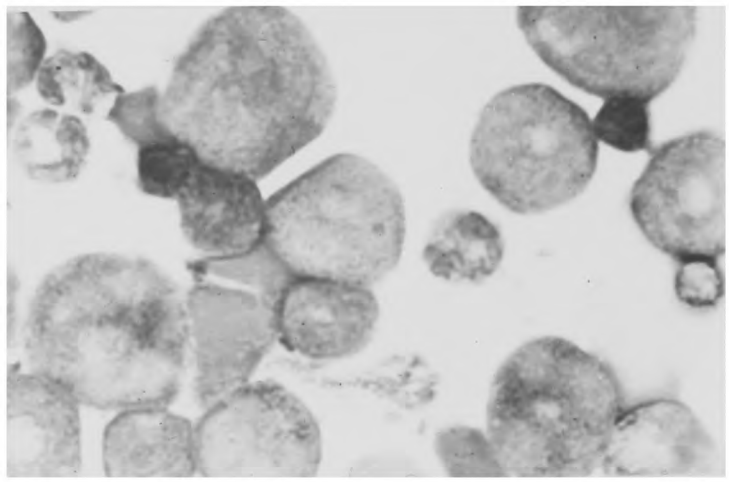
There have been several technical advances in the last decade, which have been applied to the study of bone marrow eosinophils. They include the emphasis on the use of single cell suspensions for counting marrow eosinophils (Hudson, 1963) and the introduction of staining techniques for eosinophils using acidophilic stains at an alkaline pH (Foot, 1965). In 1958 studies of the kinetics of bone marrow granulocytes were begun using autoradiographic techniques (see Cronkite & Vincent, 1969), although the method had been developed some years earlier (Doniach & Pelc, 1950).

This Chapter describes work done on eosinophil kinetics in rat bone marrow using these techniques, in order to discover how normal and increased eosinophil production takes place. The first published work on eosinophil kinetics in normal rat bone marrow has been reported recently from the Graduate School of Arts and Science, New York (Cohen, N.S., LoBue & Gordon, 1967; Alexander, Monette, LoBue, Gordon & Chan, 1969).

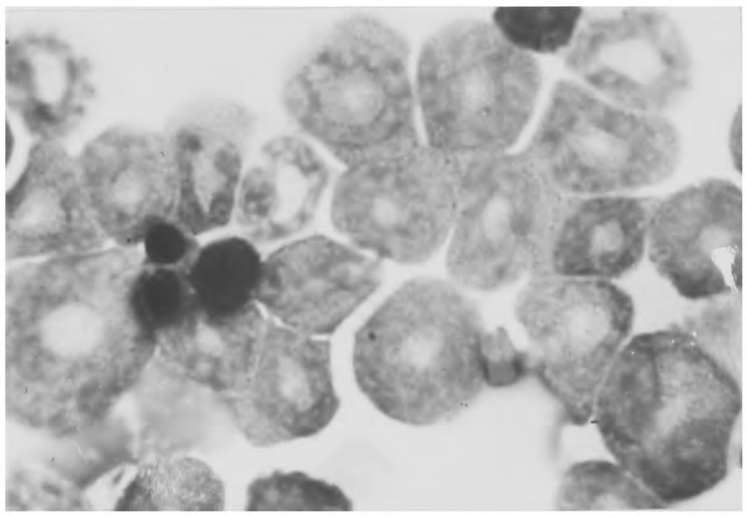
Eosinophils in normal marrow

Marrow preparations with the Cytocentrifuge. Using conventional methods of making bone marrow preparations it was difficult to delineate morphologically different types of eosinophils and to study the relationships between them. In order to see if marrow eosinophil morphology was improved when marrow was prepared with a Cytocentrifuge, (Dore & Balfour, 1965) bone marrow from a normal rat was both spread out on a glass slide by conventional methods, and also centrifuged onto a slide with the Cytocentrifuge as described in Chapter 2. The Cytocentrifuged sample showed eosinophil morphology more clearly. The cells did not overlap, they were pressed flat against the slide, eosinophil granules did not overlie the nuclei and only occasional eosinophil nuclei appeared to be distorted. Twenty-five percent of the marrow eosinophils on slides prepared by the smearing method had nuclei with a central hole, but on the Cytocentrifuged slides made from the same bone marrow sample, there were 51%. This suggested that eosinophil nuclei with central holes were being smeared on glass slides in such a way that the ring character of the nuclei was obscured.

Types of marrow eosinophils. To classify the different types of marrow eosinophils the obvious variables included: the presence or absence of a nuclear hole; the number of eosinophil granules in the



(a)



(b)

Figure 3.1. Two types of rat eosinophils which divide in bone marrow. (a) Eosinophils 'type A' have a solid nucleus. (b) Eosinophils 'type B' have a central nuclear hole. Leishman's stain, x 600.

cytoplasm; the size of the nuclear hole; the size of the eosinophils; and the presence of basophilic cytoplasm. As the property of possessing a nuclear hole was so distinctive, this was chosen as the basis for classification.

Three types of marrow eosinophils could be defined: type A (solid nucleus), type B (ring nucleus but excluding type C) and type C (ring nucleus identical to blood eosinophils). Later type C eosinophils were shown to be incapable of incorporating 3H-thymidine, and this was helpful in distinguishing them from eosinophils type B on marrow autoradiographs (Figure 3.1).

In 24 normal 200 g female Harwell rats, marrow eosinophils were classified as types A, B and C. Four hundred eosinophils were counted for each rat. An equal number of eosinophils type A and B were present, with relatively few type C, (Table 3.1).

	Type A (solid nuclei)	Type B (ring nuclei)	Type C (blood type)
Mean	45.8%	44.5%	9.3%
± S.D.	11.8	9.0	6.0

Table 3.1. % Marrow eosinophils of each type in 24 normal rats

Marrow eosinophil size. Most marrow eosinophils were less than 15 μ in diameter when measured with an eyepiece calibrated graticule. Eight percent of types A and B were 15 - 20 μ in diameter. All eosinophils type C were 11 - 15 μ in diameter. There was a relationship between eosinophil size and eosinophil mitosis. All eosinophils seen in prophase were greater than 15 μ in diameter. Eosinophils in mitosis had a mean diameter ± S.D. of 16.8 ± 14 μ. An occasional eosinophil with visible chromosomes was seen with a diameter of 9 - 12 μ but these had probably just divided, as the two separating parts of eosinophils in telophase were of this size. It was concluded that eosinophil size increased to a maximum just before cell division, and became smallest immediately after cell division.

Marrow eosinophils type C. Only about 10% of the marrow eosinophils were type C. If these were the progeny of cell division from the compartment of eosinophils type B, it was important to account for this small number. In many species, including the guinea pig, 75% of bone marrow eosinophils are band and segmented forms (Hudson, 1968). Eosinophils type C might have remained in the bone marrow only a short time, but on the other hand eosinophil T_E was found to be between 17 and 40 h (p. 83). The possibility remained that eosinophils type C left the bone marrow a few hours after they had been formed, and that

they matured in some other organ such as the spleen. This focused attention on the spleen as a site for eosinophil production. Two experiments were designed to test the hypothesis that rat spleens are sites in which eosinophils type C mature before they are released into the general circulation.

Single cell suspensions of spleens from 10 normal B/H Animal Supply rats were made. The cells were centrifuged onto glass slides and stained with Leishman's stain, or haematoxylin and Biebrich scarlet as described for bone marrow cell preparations. Among 1,000 splenic eosinophils studied, 95% were types B and C, and none were seen in mitosis. Then 4 normal rats were given injections of 400 µCi of 3H-thymidine intravenously, and spleen eosinophils were studied 1 h and 40 h later. At 1 h, one among 500 splenic eosinophils was labelled. At 40 h 15% of 250 splenic eosinophils were labelled, before labelled eosinophils appeared in the blood.

These results supported the concept that eosinophil cell division did not occur in the spleen, but that some of the eosinophils in the spleen were maturing there prior to release into the blood.

Development sequence of marrow eosinophils. In several experiments dividing marrow eosinophils were shown to be different morphologically from non-dividing eosinophils. Thirty female Harwell rats weighing

about 200 g were given 3H-thymidine, 1 μCi/g intravenously. Marrow was taken for autoradiography one hour later. No label was found in eosinophils type C, but label was found in eosinophils type A and B (see also p. 66). This showed that in normal rat bone marrow, about 90% of the eosinophils were in the 'dividing compartment'.

Label was only found occasionally in the smallest diameter eosinophils of types A and B in the normal rats which confirmed the finding of Alexander, Monette, LoBue, Gordon & Chan (1969). In marrow samples taken from rats which were producing many eosinophils on the other hand, labelling in the smallest eosinophils of types A and B occurred as frequently as in the larger cells. This suggested that T_{G_1} was very short in stimulated dividing eosinophils (see p. 67).

Morphological differentiation of eosinophils type B (dividing) from type C (non-dividing) was difficult, as the size of the hole in the nucleus was uniformly variable. Nevertheless, in practice, it was possible to distinguish marrow eosinophils which were identical to circulating eosinophils and these were not labelled 1 h after an intravenous injection of 3H-thymidine. Evidence is presented on p. 66 that eosinophils type A were precursors of eosinophils type B.

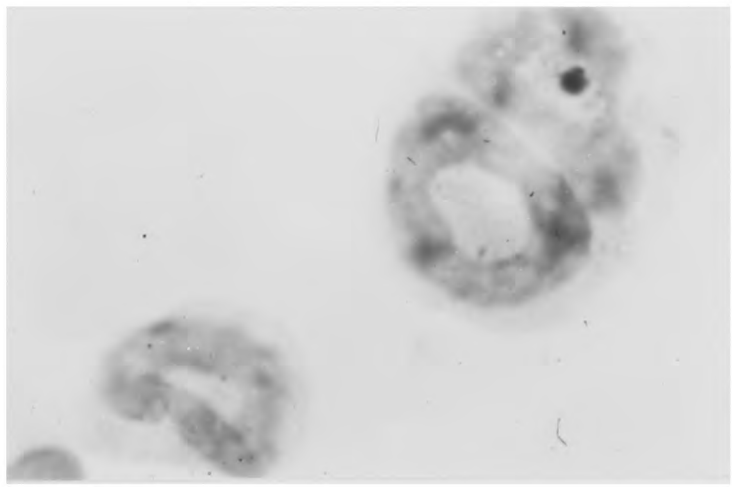


Figure 3.2. An abnormal eosinophil found in stimulated rat bone marrow. The eosinophil is binucleate with a nuclear satellite. A normal eosinophil is present in the lower part of the figure for comparison. Haematoxylin and Biebrich scarlet, x 800.

Abnormal eosinophils in bone marrow. Abnormal eosinophils were very rare in bone marrow. Cells with circular, darkly staining nuclei, which could be classified as 'pyknotic', frequently took up 3H-thymidine, so that they were not considered abnormal. However, abnormal eosinophils in marrow were occasionally found in rats given Trichinella spiralis intravenously. Some eosinophils type A with two separate nuclei were seen. Three mature marrow eosinophils with double nuclei were seen and one is shown in Figure 3.2.

Small 'packets' of eosinophil granules with variable amounts of nuclear remnant were also seen in some bone marrow and blood samples. Some may have been preparative artifacts, but they were seen on smears and in wet preparations supravitally stained by the method of Discombe (1946). It was thought that these 'packets' might be eosinophil fragments produced in tissues, but none were seen in thoracic duct lymph draining the intestine in which many eosinophils are found. It seemed more likely that 'packets' of eosinophil granules were derived from bone marrow eosinophils which had failed to develop normally in vivo, and which had not been removed completely by the reticulo-endothelial system. The small quantities found, made it unlikely that they had any significant physiological role.

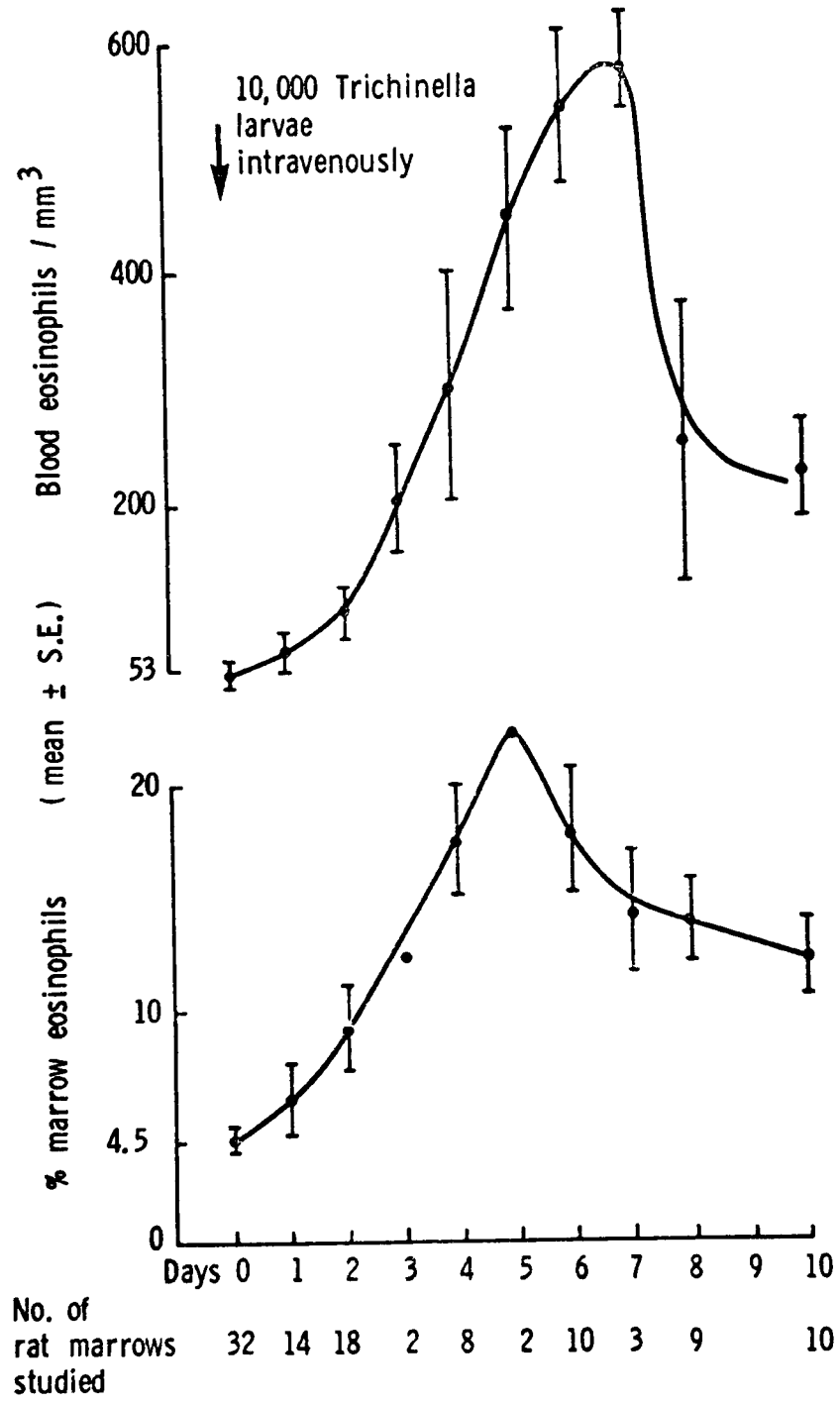


Figure 3.3. The relationship between blood and bone marrow levels of eosinophils in rats given Trichinella spiralis larvae intravenously. Bone marrow levels altered 1-2 days before blood levels.

Eosinophils in stimulated marrow

Bone marrow: blood eosinophil relationships. In earlier work in this laboratory on eosinophil production (Basten, Boyer & Beeson, 1970; Basten & Beeson, 1970; Boyer, Basten & Beeson, 1970), it was assumed that blood levels of eosinophils would mirror bone marrow levels. In order to test this important assumption 108 female Harwell rats, weighing about 200 g were studied. Thirty-two were normal and 76 were given a single injection of Trichinella spiralis larvae intravenously, and were killed for marrow eosinophil counts at intervals thereafter. Injections were given on various days, but always between 9.45 - 10.15 a.m. Blood eosinophil counts were done at the time of injection and at death, but not at other times. The results are shown in Figure 3.3.

The marrow eosinophil level was maximal about 2 days before the greatest blood eosinophil count was reached. The shapes of the two curves were similar. Bone marrow levels increased from 4.5% to 22% (x 4.9), and blood levels increased from 53 to 600/mm^3 (x 11.3). This was evidence that, in this system, changes in marrow eosinophil levels were mirrored by changes in peripheral blood eosinophil levels.

In the same experiment, it was possible to compare blood eosinophil counts with marrow eosinophil levels, at the time each rat was killed. Results in the 93 rats are shown in Figure 3.4.

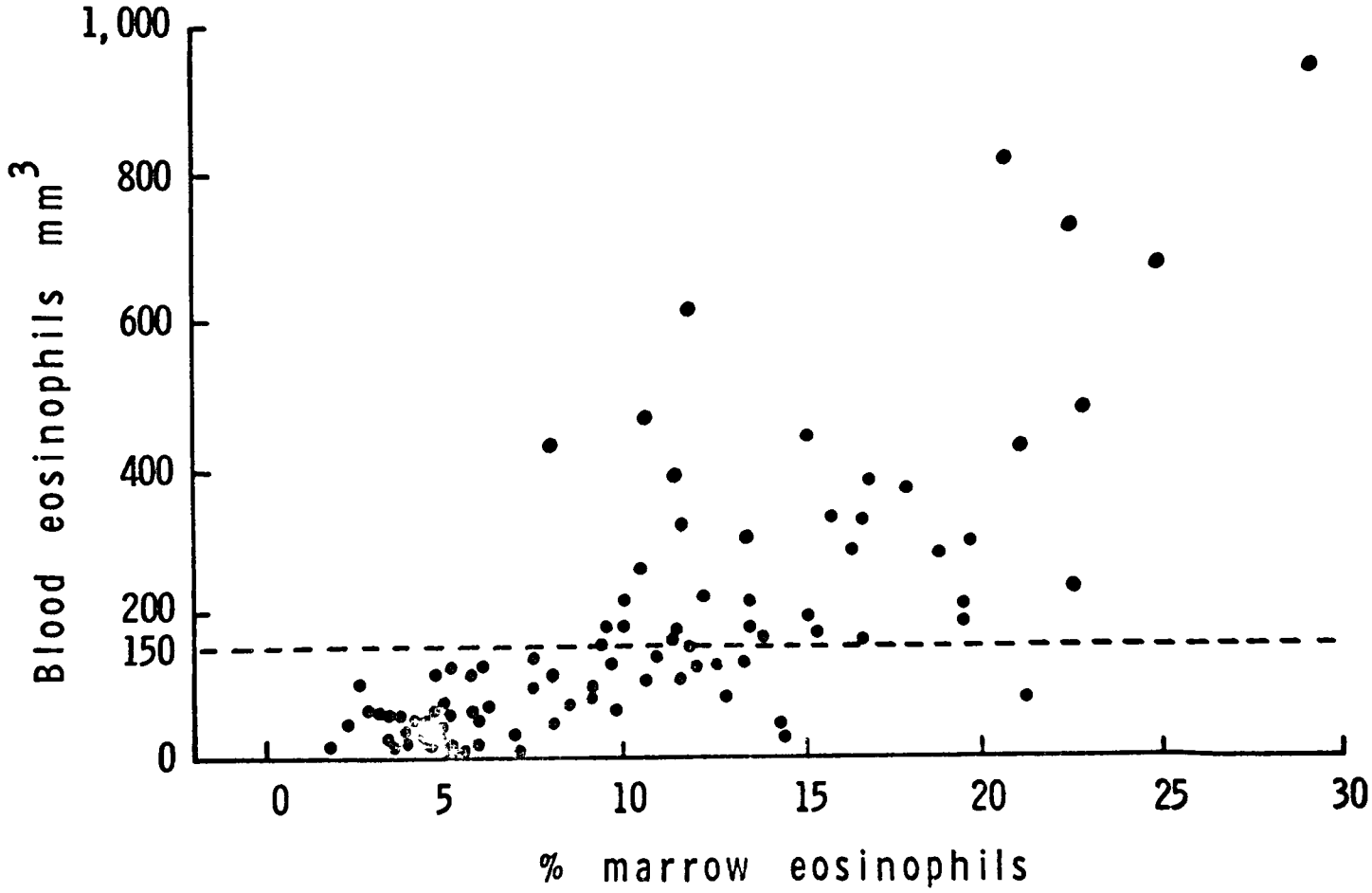


Figure 3.4. The relationship between bone marrow eosinophil percentage and blood eosinophils/mm^3 in normal rats and rats given Trichinella spiralis larvae intravenously. Raised blood eosinophil levels were only found when the percentage of marrow eosinophils was twice normal.

A best fit regression straight line had the relationship $y = 6.96 + 0.02\ x$, where x is the blood eosinophil count/mm^3 and y the marrow eosinophil %. Where x = y, the correlation coefficient was 0.80.

In stimulated bone marrow, one third of blood eosinophil counts which were within the normal range were associated with eosinophil levels in the marrow above the normal range. This was because marrow eosinophil counts rose before peripheral blood levels were increased in the stimulated rats.

Blood eosinophil counts of over 150/mm^3 were always associated with marrow eosinophil levels of over 9%, i.e., twice the normal level. Moreover, amongst the 93 rats studied, none had an eosinophil count of over 150/mm^3 associated with a normal bone marrow content of eosinophil. This provided direct confirmation for the assumption that with this method of inducing eosinophilia blood levels of 150/mm^3 or more were associated with increased numbers of bone marrow eosinophils.

Very high eosinophilia. About 1% of rats showed a greater than than normal eosinophil response. One rat developed an eosinophilia of 6,250/mm^3 11 days after Trichinella spiralis larvae were given intravenously. The blood mononuclear cells and neutrophils also increased by a factor of three. The eosinophil count had returned to

normal by day 17. Most of the eosinophils in the blood were mature but a small proportion had non-fenestrated nuclei. In another rat the blood eosinophil count rose to 2,000/mm^3 and the bone marrow was found to contain 47% eosinophils at that time.

The mechanism of these abnormally high responses is not known, but it may depend on continuing excess bone marrow eosinophil proliferation possibly due to additional stem cells entering the eosinophil production sequence.

In one rat which had an unusually great eosinophil response to the stimulus, the carcass was found to contain 101 living larvae 3 weeks after injection, showing that some larvae had passed through the lungs. However this response did not occur in 4 other rats in which larvae were found to have migrated through the lungs after intravenous injection. The magnitude of these abnormally high eosinophil responses demonstrates the capacity for eosinophil production that may be available in rats.

Marrow eosinophil kinetics. An experiment was designed to give as much information as possible about the kinetics of eosinophil production in rats which had received a single stimulus to eosinophil production, compared with normal rats. Labelling of marrow eosinophils after single injections of 3H-thymidine was followed.

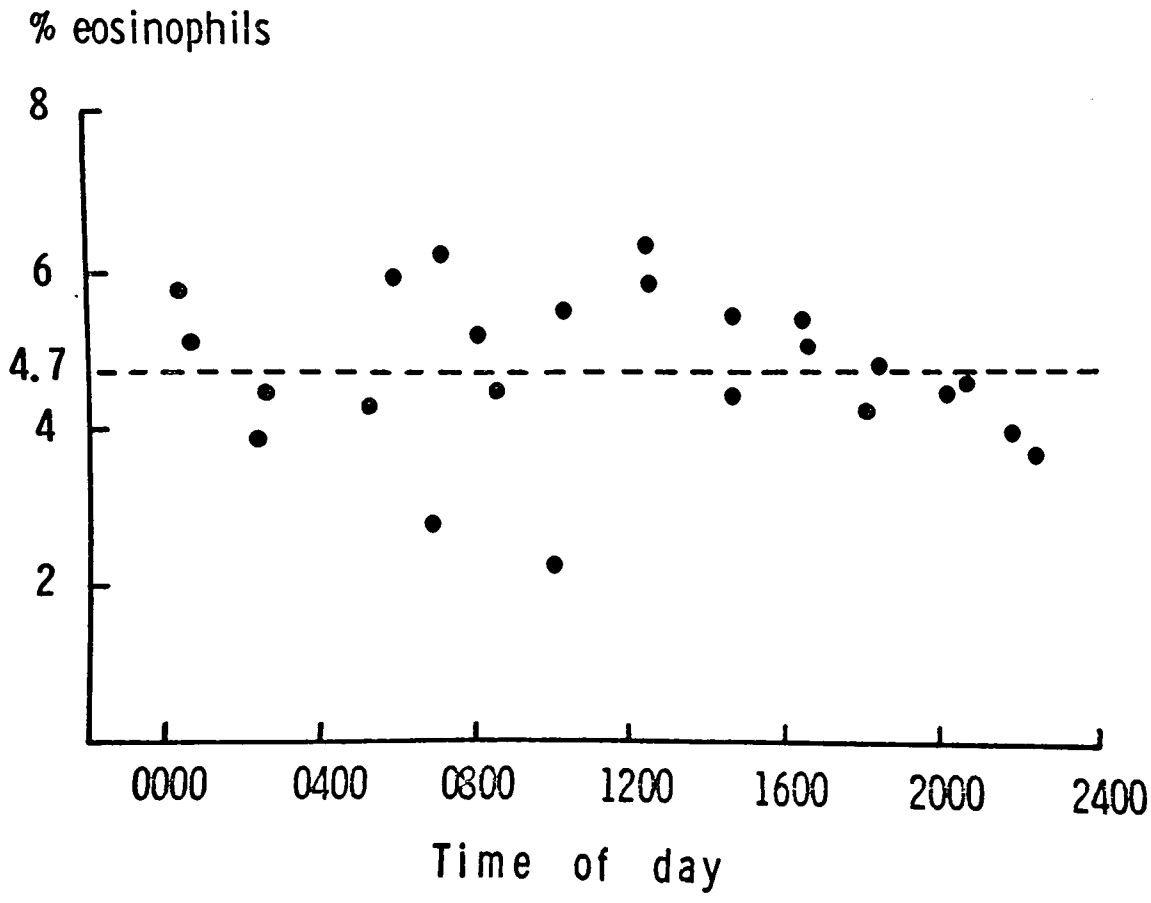


Figure 3.5. The number of eosinophils/100 nucleated cells in normal marrow during 24 h. There was no apparent diurnal variation.

Methods. Two groups of female 200 g Harwell rats were used. The first group of 23 rats was injected with Trichinella spiralis larvae intravenously at 10.0 a.m. and the second consisted of 24 normal rats. Rats were from the same litters and were handled in the same way, so that each stimulated rat had a comparable control. Twenty-four hours after injection of larvae into the first group of rats, both groups were given 3H-thymidine 1 μCi/g intravenously. At 2 hourly intervals thereafter pairs of rats were sacrificed from the control and stimulated groups. Blood and marrow eosinophils were studied.

Percent marrow eosinophils. Normal rat marrow contained 4.7 ± 1.0% (24) eosinophils. Hulse (1964) who used Wistar rats of the same strain as those used here, also found 4.7% eosinophils in normal rat marrow. Rytömaa (1960) has reviewed some of the marrow eosinophil percentages which have been reported for rats previously. There did not appear to be a diurnal variation in the % eosinophils in bone marrow (Figure 3.5).

In our stimulated rats the eosinophil content of the marrow did not begin to rise until several hours after the injection of larvae (Figure 3.6)

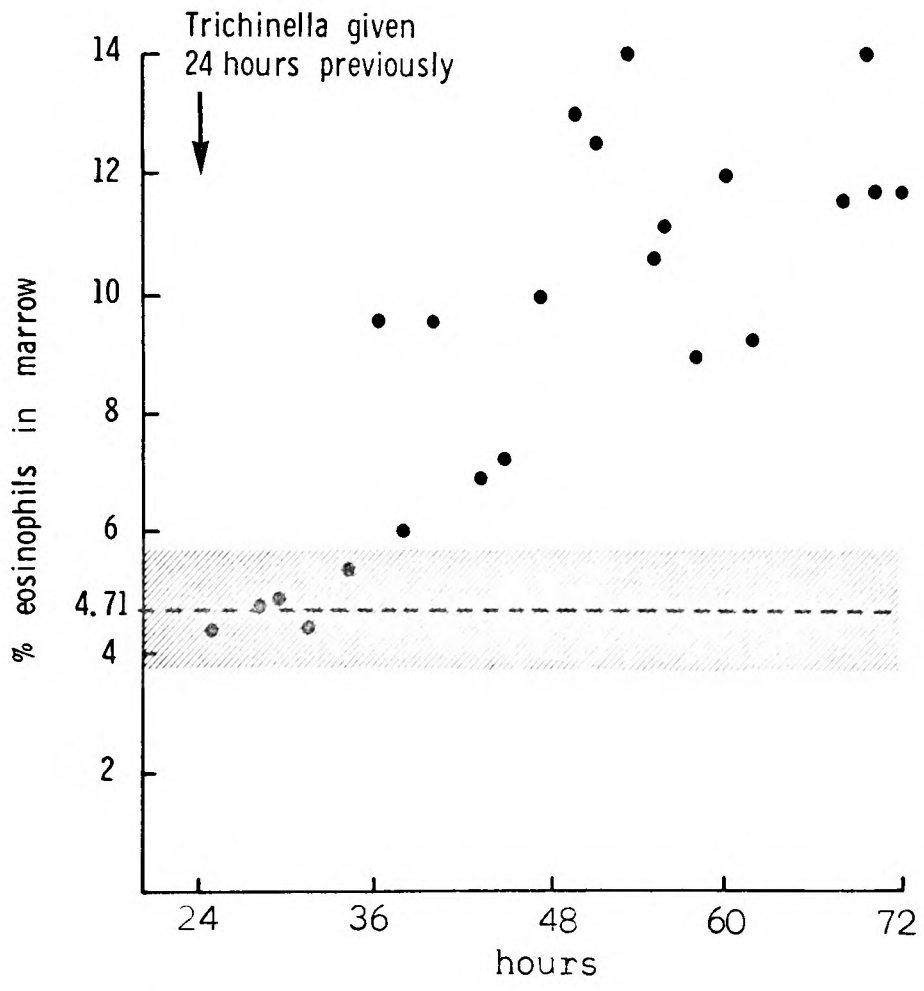


Figure 3.6. % Eosinophils in the marrow of rats given larvae intravenously. The range of normal values is shown in the hatched area.

Where the time after injection of larvae is x and the % marrow eosinophils is y, a best fit regression line had the form $y = 0.67 \pm 0.18x$ with a correlation coefficient where x = y of 0.81. From this it was calculated that the percent eosinophil in marrow had begun to rise at 22.7 h and had doubled at 49.2 h after injection of larvae.

Percent marrow eosinophils types A, B and C. When the proportions of the eosinophils types A, B and C were counted, it was found that they did not alter in any rat marrows during the period 1 - 3 days after the stimulus was given. The mean results for this period ± S.D. are shown in Table 3.2.

Eosinophils	% eosinophils	
	Normal rats	Stimulated rats
Type A	45.8 ± 11.8	43.4 ± 7.9
Type B	44.5 ± 9.0	49.1 ± 5.5
Type C	9.4 ± 6.0	5.5 ± 2.7

Table 3.2. The proportion of eosinophils types A, B and C in normal marrow and marrow 1 - 3 days after intravenous larvae

As there was no alteration in the relative proportions of the three types of eosinophils, it was concluded that the stimulus to eosinophil production initially acted on all eosinophil types in proportion to their concentrations in marrow tissue.

Percent marrow eosinophils in mitosis (mitotic index). The eosinophil mitotic index of normal rats was 0.74 ± 0.33 (24). There was no clear diurnal variation, but so few were found that this conclusion could not be made with confidence. Dustin & de Harven (1954) found an eosinophil mitotic index of 0.44% in normal rat marrow. This is similar to the myeloid mitotic index of rats of 0.5% (Kindred, 1942). In the stimulated rats the mean mitotic index was raised to 1.94 ± 0.67 (23) an increase of x 2.6. Here too there was no clear diurnal pattern, but there may have been a peak in mitotic activity 40 h after injection of larvae (Figure 3.7).

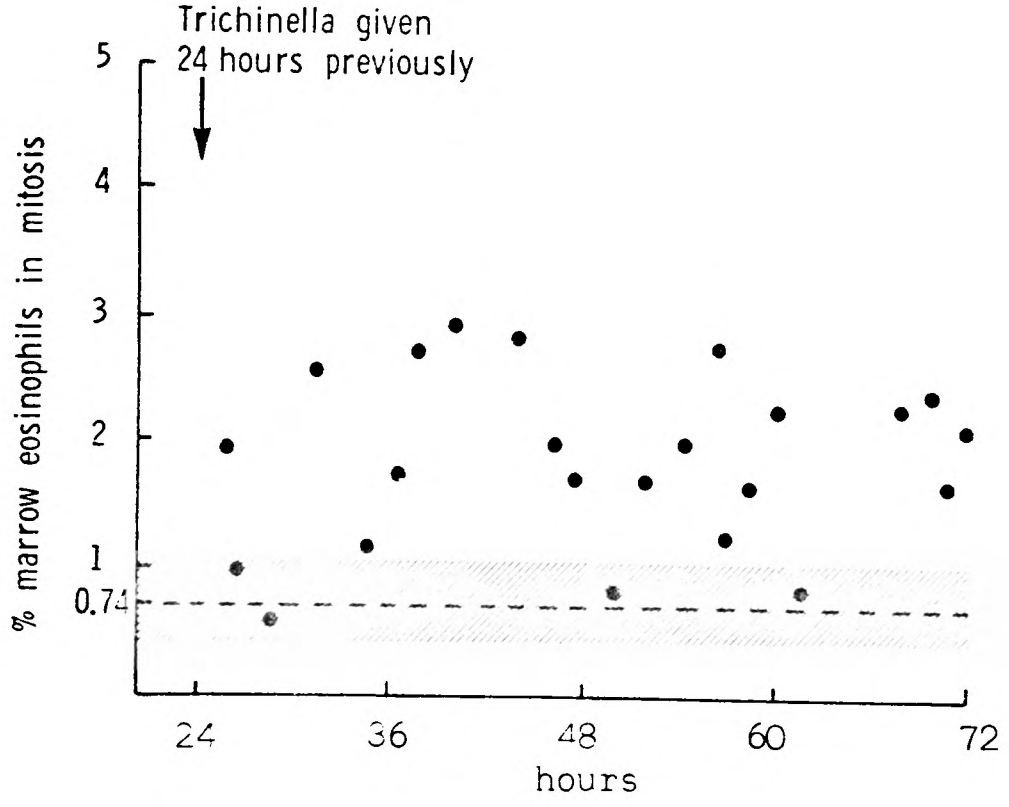


Figure 3.7. The mitotic index of marrow eosinophils 1-3 days after intravenous injections of larvae. The range of normal values is shown in the hatched area.

Eosinophil mitosis in each phase of cell division. As so few eosinophil mitoses were found in marrow (1 in 2,800 nucleated cells in normal marrow and 1 in 500 in stimulated marrow) and as cells in prophase may be difficult to recognise, care was taken not to overlook eosinophils in mitosis. The proportions of eosinophil mitoses in prophase metaphase, anaphase, and telophase in both normal and stimulated rats are shown in Table 3.3. The proportions of the stages in eosinophil mitoses are similar to those reported for normal rat marrow by Dustin & de Harven (1956).

	Normal rats		Stimulated rats	
	No.	%	No.	%
Prophase	68	38.4	171	40.4
Metaphase	72	40.6	150	35.5
Anaphase	11	6.2	43	10.1
Telophase	26	14.7	59	13.9
Total:	177		423	

Table 3.3. The % eosinophil mitoses in each phase of cell division in normal and stimulated rats.

Labelling of marrow eosinophils types A and B. Marrow eosinophils types A and B were studied at 2 h intervals after injection of 3H-thymidine. In the normal rats the proportion of labelled eosinophils type A was falling when maximal labelling of eosinophils type B was taking place. This suggested that eosinophils type A were developing into eosinophils type B. Results are shown in Figure 3.8.

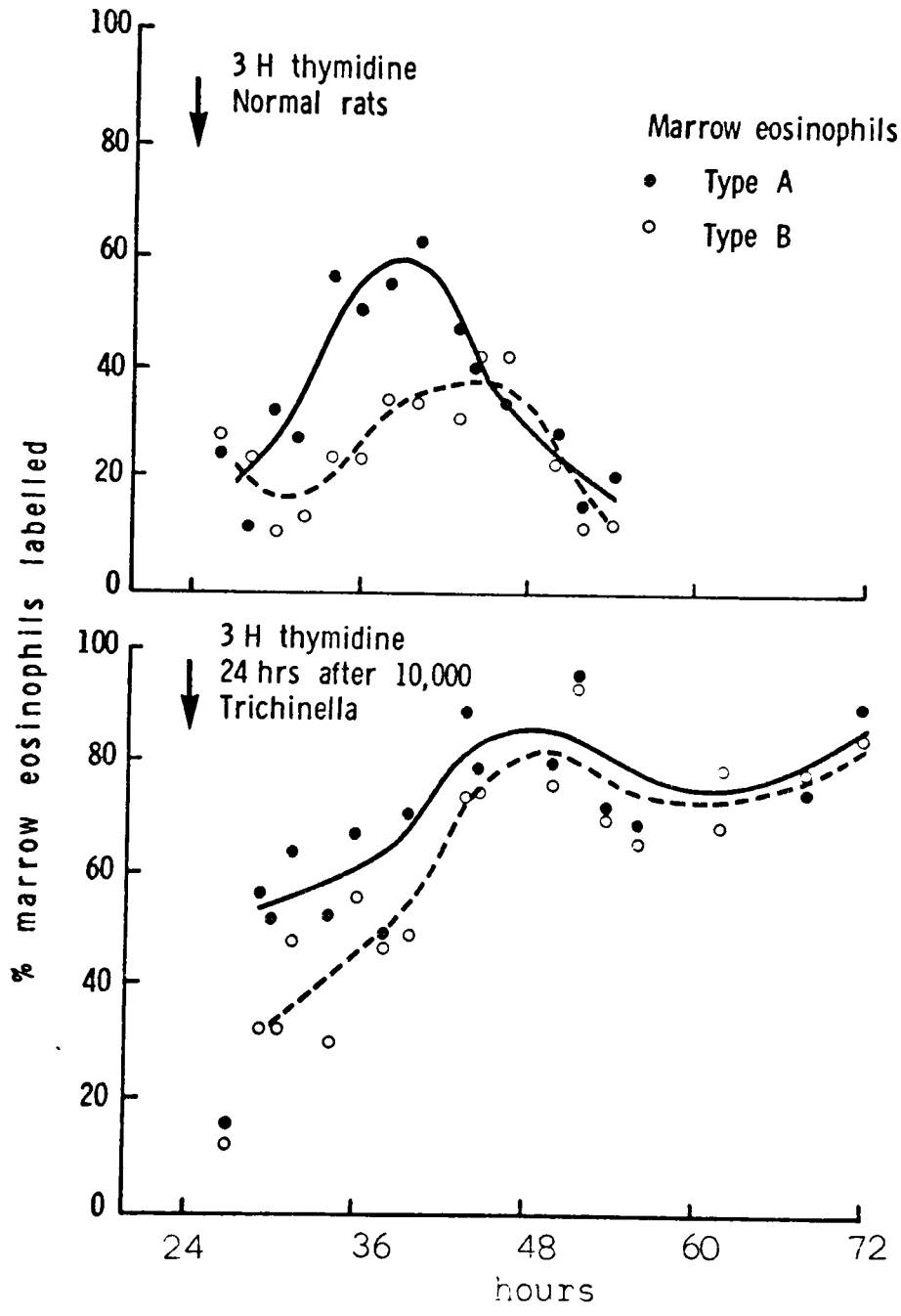


Figure 3.8. The % eosinophils types A and B labelled by a single injection of 3H-thymidine in normal rats (upper figure) and stimulated rats (lower figure).

	Eosinophil cell cycle times (h)			
	Normal rats		Stimulated rats	
	Median	Mean ± S.D.	Median	Mean ± S.D.
T_C median	30.0	(see Figure 3.11)	9.0	(see Figure 3.11)
$T_{G_1} + \frac{1}{2}T_M$	9.0	9.1 ± 1.3	0.8	0.9 ± 0.5
T_S	18.5	21.6 ± 12.8	7.3	7.4 ± 0.7
$T_{G_2} + \frac{1}{2}T_M$	2.8	3.9 ± 3.9	1.9	2.1 ± 1.1

Table 3.4. Eosinophil cell cycle times in the marrow of normal rats and rats given Trichinella spiralis larvae, calculated from eosinophil mitosis labelling curves.

The stimulus decreased eosinophil cell cycle times by a factor of 3, and the main effect was on T_{G_1} and T_S.

Marrow eosinophil cell cycle parameters. The bone marrow slides from normal and stimulated rats were assessed for the presence of label over eosinophil mitoses at 2 h intervals after the injection of 3H-thymidine. Up to 10 slides from each rat were scanned to locate 100 eosinophil mitoses. The results are shown in Figure 3.9.

The number of grains over the eosinophil mitoses were counted in both groups of rats. The amount of 3H-thymidine incorporated in human granulocytes varies during S and most label is incorporated during the middle of S. Grain counts over labelled rat eosinophil mitoses also showed this effect (Figure 3.10).

The results of the % labelled eosinophil mitoses were processed by Dr Gordon Steel, Biophysics Department, Royal Marsden Hospital, Sutton, Surrey, using a computer programme in which a mathematical model was set up where G_1, S and G_2 were lognormally distributed, and the best fit form of this model was produced (Barrett, 1966). The median cell cycle time for normal eosinophils was 30 h, and for stimulated eosinophils 9 h as shown in Table 3.4.

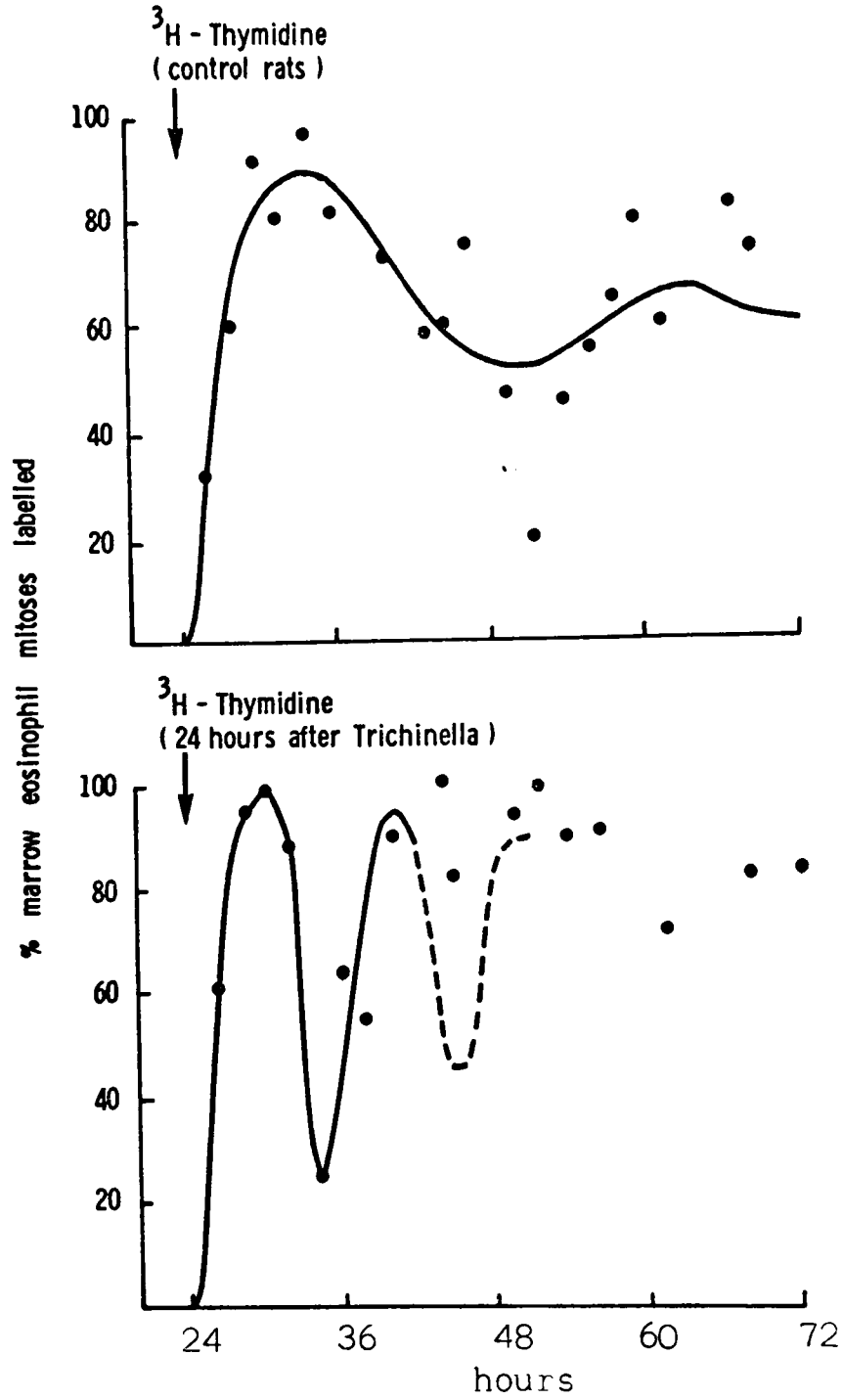


Figure 3.9. % marrow eosinophil mitoses labelled in normal rats (upper figure) and rats given Trichinella spiralis larvae (lower figure). Dividing eosinophils in the stimulated marrow had cell cycle times about 1/3rd as long as the normal rats. The curves fitted to the experimental points were computed, using Barrett's method and have a good fit for the first cell cycle in each group of rats.

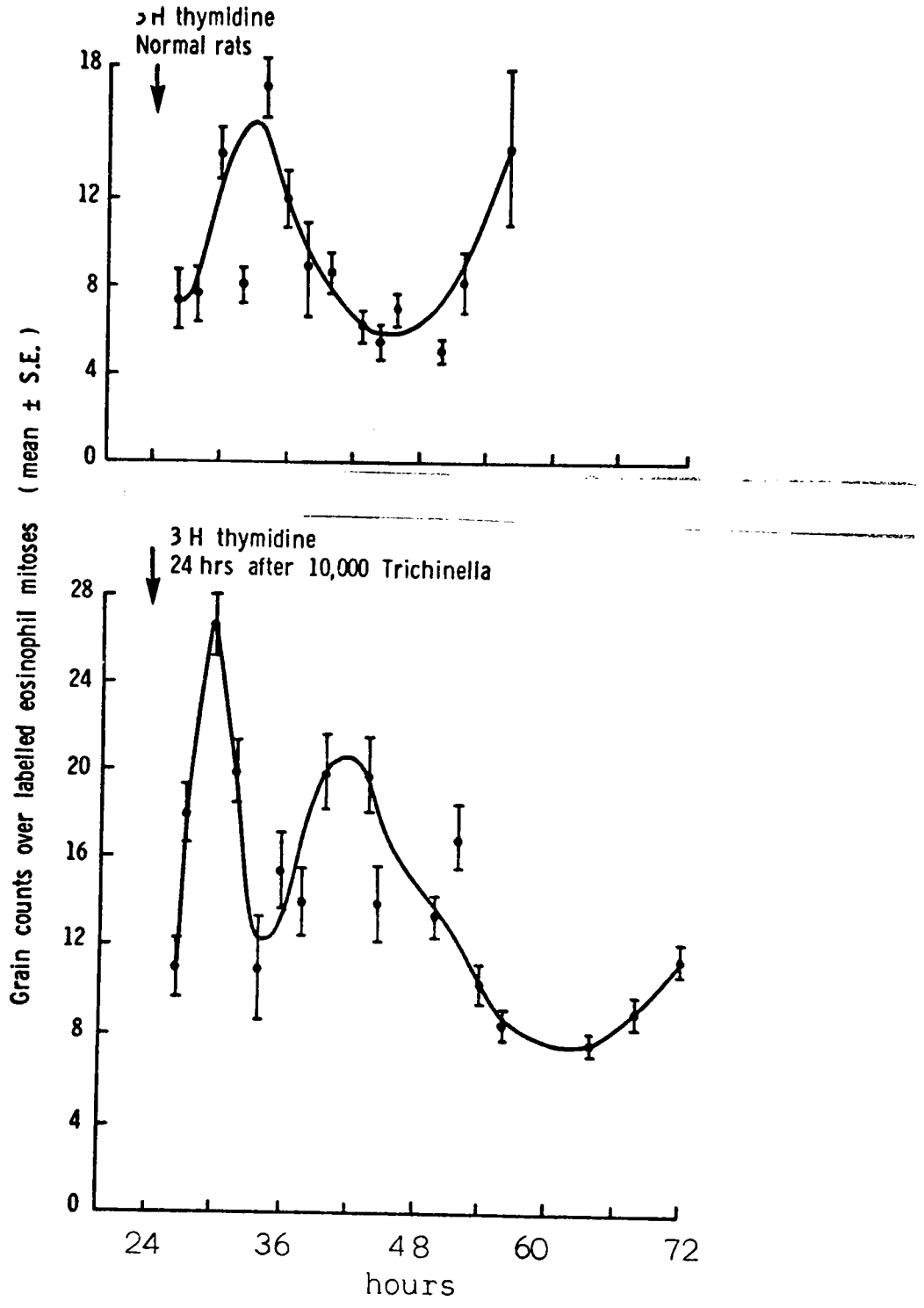


Figure 3.10. The number of grains over labelled eosinophil mitoses after single injections of 3H-thymidine in normal rats (upper figure) and rats given Trichinella spiralis larvae intravenously, (lower figure). A striking periodicity in grain counts was found, probably due to variations in the amount of thymidine taken up at different times in the S phase. The curves were drawn by eye.

A further analysis of the cell cycle times of the first group of labelled mitoses was done by Dr Gordon Steel. The frequency distribution of these times in normal and stimulated rats is shown in Figure 3.11.

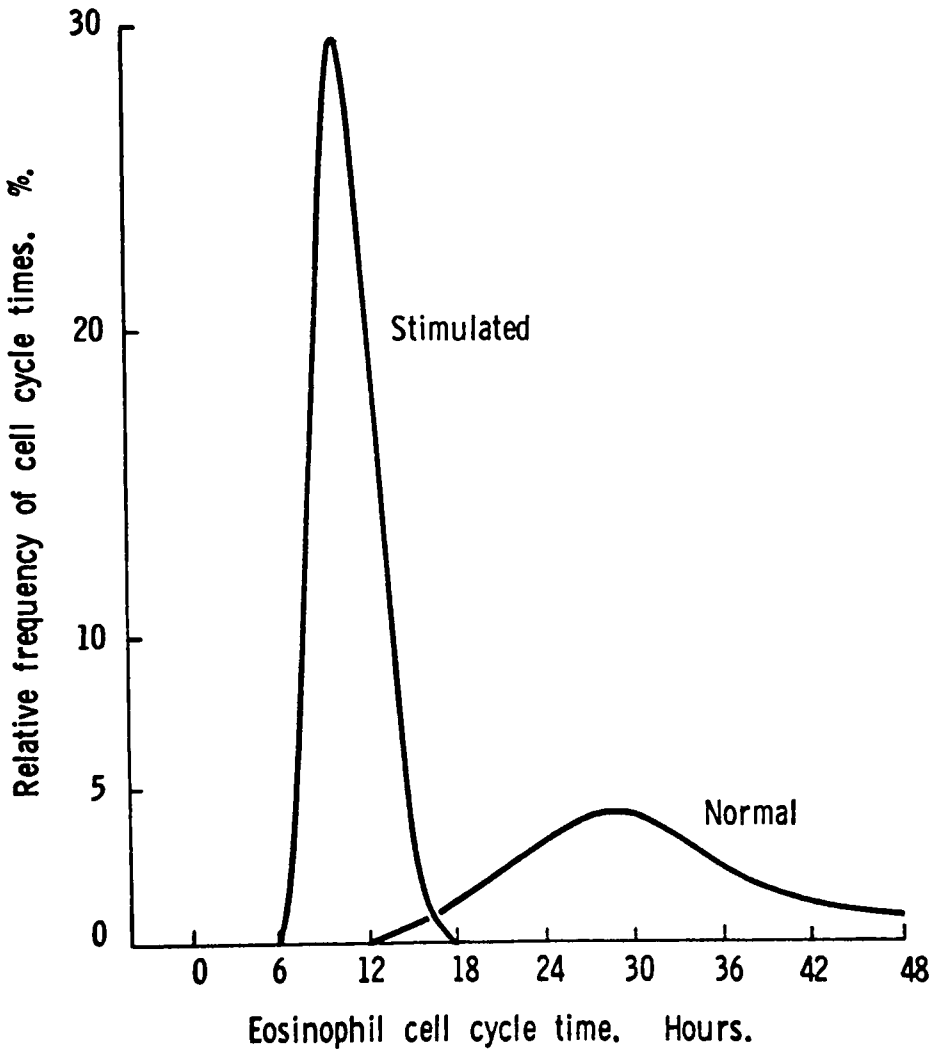


Figure 3.11. The frequency distribution of eosinophil cell cycle times in the marrows of normal and stimulated rats. In the stimulated rats the cell cycle times were shorter and the spread was also reduced.

	Normal rats		Stimulated rats	
	Compartment A	Compartment B	Compartment A	Compartment B
Birth rate/100 cell/h	2.3	2.3	7.7	7.7
Number of divisions in the compartment	2.3	2.0	7.7	2.0
Compartment transit times (h)	69.0	60.0	69.0	18.0

Table 3.5. Compartment parameters for eosinophils type A and B in normal and stimulated rat marrow

(calculated from the cell cycle times shown in Table 3.4)

Eosinophil production rates.

Indirect measurements. From the cell cycle times for normal and stimulated rats it was possible to calculate for eosinophils type A (compartment A) and B (compartment B) several compartment parameters, where

$$\text{Birth rate} = \frac{\log_e 2}{T_C} \quad \text{(Tarbutt \& Blackett, 1968)}$$

$$\text{Number of division (m)} \quad 2^m = \frac{\text{flow out}}{\text{flow in}}$$

$$\text{Compartment transit time} = T_C \times m$$

At least two assumptions are necessary: that cell cycle times for marrow eosinophils as a whole can be applied to eosinophils types A and B separately, and that flow rate into compartment A is about 1%/h (similar to the flow into the proerythrocyte compartment of rats of similar age to those studied here, Tarbutt, 1967). Using these equations and the cell cycle times shown in Table 3.4, the following results were calculated (Table 3.5).

These calculated results can be combined with figures obtained for T_E (p. 85) to give a total marrow transit time: 7 days for normal eosinophils, and 4.5 days for stimulated eosinophils. This may be

compared with the total transit time for erythrocytes in young rats of 6.5 days (Roylance, 1968). In stimulated rats there is an initial delay of 23 h before the marrow eosinophil counts begin to rise, so that the total time for stimulation of the earliest eosinophils until they emerge into the blood is 5.5 days. This time is equivalent to the point at which a maximal blood eosinophilia was found in the blood of stimulated rats. Thus the maximal effect of the stimulus was only seen in the blood, when the earliest eosinophil precursors had finally left the marrow.

The calculations showed that in stimulated marrow, an additional 5 - 6 divisions occurred among eosinophils type A. This would result in about 32 - 64 times the normal number of eosinophils being made, during the 6 days after the marrow has been stimulated.

Direct measurements. It is possible to estimate marrow eosinophil production rates (Kp)directly, using the formula:

$$Kp = \frac{N \times E \times LI}{T_S}$$ (Lala, Maloney & Patt, 1964)

Kp = marrow production rate (new eosinophils produced/mg bone marrow/h).

N = number of nucleated cells/mg bone marrow

E = proportion of marrow nucleated cells which were dividing eosinophils, types A and B.

LI = proportion of eosinophils labelled 1 h after injection of 3H-thymidine

T_S = duration of eosinophil S phase (h)

Kp would provide a direct marrow assay for substances influencing eosinophil production in vivo.

Nucleated cells/mg marrow. Measurements of the total numbers of nucleated cells/mg bone marrow are difficult to make for technical reaons. This is shown by the variety of results that have been reported for rats: 2.0×10^6 (Kindrid, 1942); 8.65×10^5 (Plum, 1943); and $2\text{-}3 \times 10^6$ (Burke & Harris, 1959). Here particular care was taken to weigh the marrow free from bone fragments. The mean number of marrow nucleated cells/mg $\pm$ S.D. was found to be $1.81 \pm 0.54 \times 10^6$ (24). It did not vary significantly between normal or stimulated rats.

	Normal rat	Stimulated rats		
Time after Trichinella injections (days):	0	+ 5	+ 6	+ 7
Marrow eosinophils %	2.9	22.5	29.0	8.0
Marrow eosinophils types types A and B %	92.0	84.0	75.0	83.0
Labelling index eosinophils types A and B %	20.5	30.0	19.0	13.5
Eosinophil production rate new cells/mg/h	0.5×10^3	14.1×10^3	10.2×10^3	2.2×10^3

Table 3.6. Eosinophil production rate in rats

The rate of production/mg bone marrow/h increased by a factor of x 28, 5 days after injection of larvae intravenously.

In an experiment to measure eosinophil production rates directly, 4 female Harwell rats, weighing about 200 g were used. One was normal. Three others were given larvae intravenously 5, 6 and 7 days previously. The 4 rats were killed 1 h after injections of 3H-thymidine, 1 μCi/g intravenously. Marrow was weighed, counted and processed for eosinophil counts and autoradiography. The LI of cell types A and B were measured together. It was assumed that T_S in the normal rats was 18.5 h and 7.3 h in the stimulated rats, and that N was 1.81×10^6. Results are shown in Table 3.6.

The production rate of eosinophils in normal rats was 0.5×10^3 eosinophils/mg/h. If it is assumed that the total volume of bone marrow in a rat is $1.18 \pm 0.53 \times 10^3$ mm^3/100 g rat (Kindrid, 1942) the total number of eosinophils being produced/h in the normal 200 g rat was 9.4×10^5 eosinophils/h. In the rat given larvae intravenously 5 days before, it was calculated that 276×10^5 eosinophils/rat/h were produced. This is an increase of 28 times over the normal rate. The magnitude of this response can be compared with the peripheral blood changes of eleven times over the same period, and the bone marrow % eosinophil change of five times.

Conculusions

This Chapter has described experiments which were done to study eosinophil production in bone marrow. After delineating several types of eosinophil, well known autoradiographic techniques were used to measure eosinophil cell cycle times and other kinetic parameters, in normal and stimulated rats. The results showed that after a delay of 23 h, all the marrow eosinophils in the dividing compartment were affected and there was a considerable reduction in cell cycle times, and 5 to 6 additional divisions were induced. Indirect and direct measurements showed that eosinophil production rate increased 30 times above normal. The stimuli to increased production could have acted in several ways to produce these effects, and this is discussed in Chapter 7.

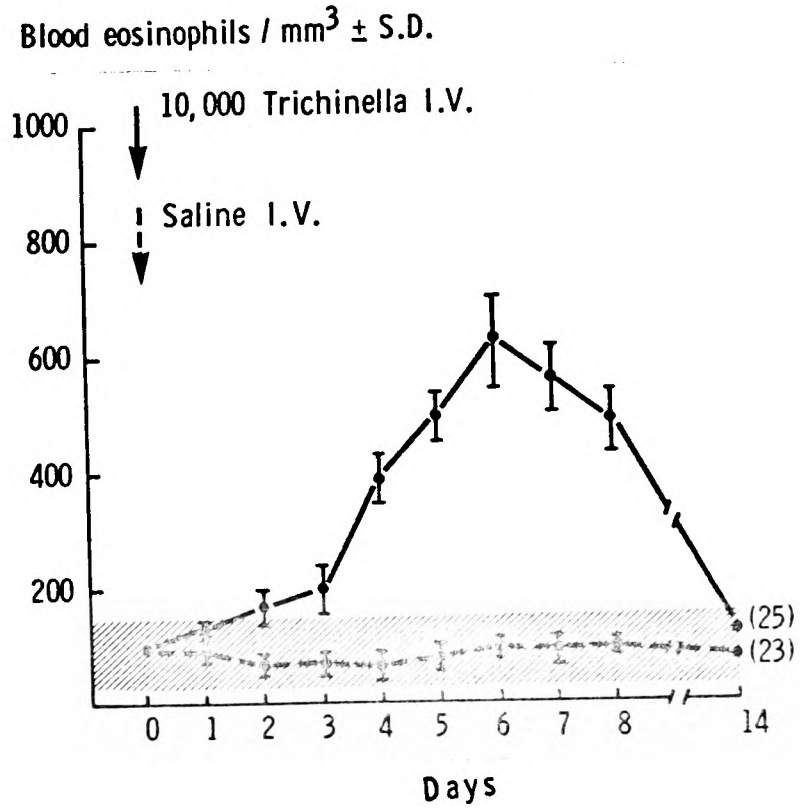


Figure 4.1. Blood eosinophil/mm^3 in Harwell rats given *Trichinella spiralis* larvae or saline intravenously. The normal level of blood eosinophils $\pm$ 2 S.D. is shown in the hatched area. The eosinophil response to injected larvae was uniform and predictable (Basten, 1970).

CHAPTER 4

BLOOD EOSINOPHILS

Introduction

As variations in the blood concentration of eosinophils are easy to follow, eosinophil counts are commonly used to study alterations in eosinophil production and utilisation. This is shown in the study of Basten, Boyer & Beeson (1970), who found that intravenous injection of Trichinella spiralis larvae induced an eosinophilia in rats, (Figure 4.1).

Blood eosinophil levels can be altered in many ways: by changes in marrow production rate and emergence time; changes in distribution in the vascular compartment including sequestration and margination: changes in the size of intravascular eosinophil compartments; and changes in the rate of eosinophil removal from the blood. Hence measurements of blood eosinophil levels do not by themselves indicate how a stimulus may alter the eosinophil concentrations in blood.

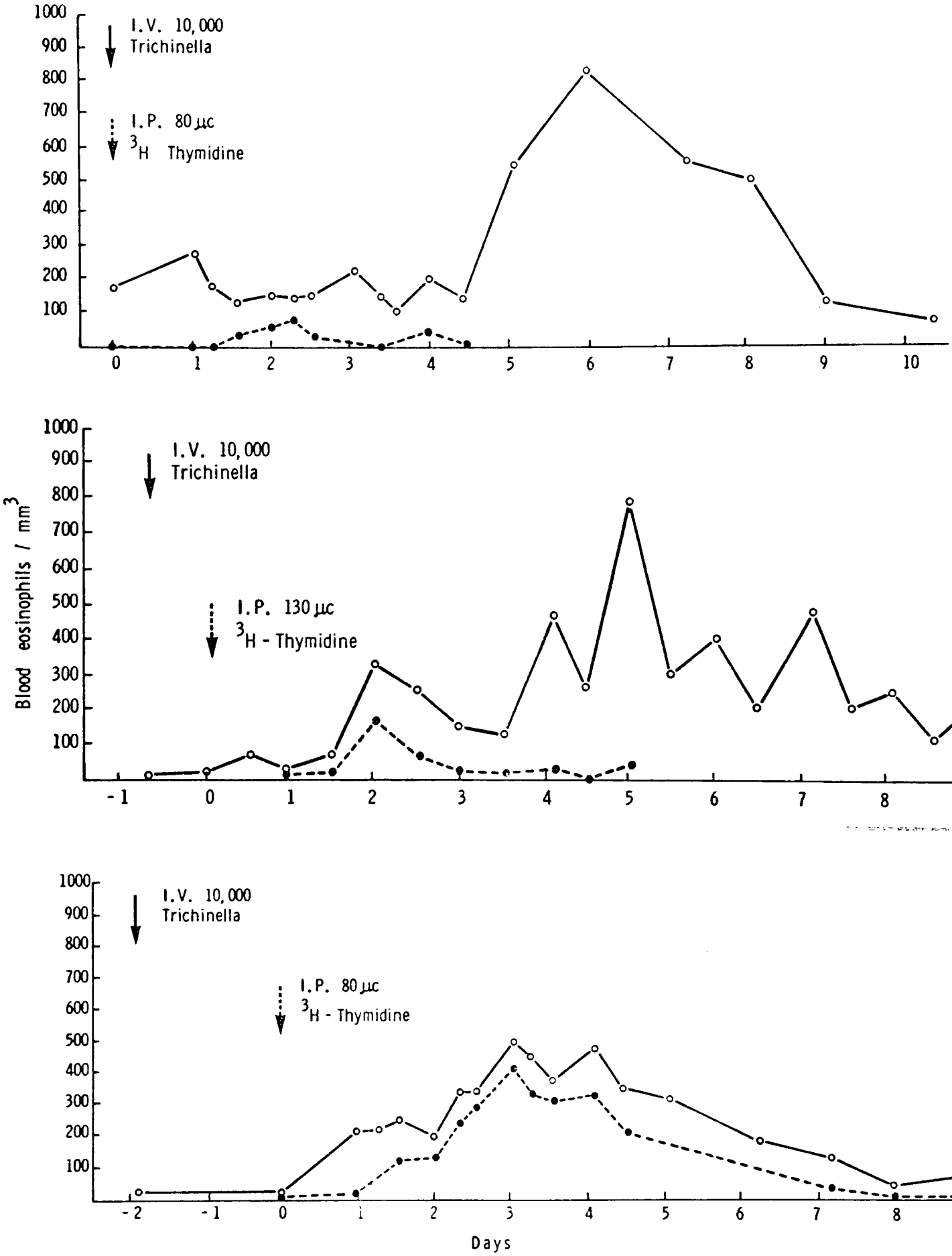


Figure 4.2. Blood eosinophils/mm^3 (solid lines) and blood labelled eosinophils/mm^3 (dotted lines) after injection of larvae and 3H-thymidine. The largest number of eosinophils was labelled when the 3H-thymidine was injected 2 days after the larvae.

This Chapter describes experiments which were done to study the relative importance of these mechanisms in the regulation of blood eosinophil levels in normal rats, and rats in which an eosinophilia had been induced by injection of larvae. The aim of the work was to determine how an eosinophilia occurs, both in terms of the kinetics of eosinophils in the blood, and the processes responsible for the changes that may take place. The kinetics of blood eosinophils have been studied in normal states, and this is reviewed by Andersen & Bro-Rasmussen (1969).

Percent blood eosinophils labelled by single injections of 3H-thymidine.

First, experiments were done to estimate the flow of newly formed eosinophils into the blood, as an extension of the work on bone marrow production described in the last Chapter.

3H-Thymidine was either administered immediately before injecting larvae, or 24 h or 48 h later. When the 3H-thymidine was injected with the larvae a maximum of 42% of the blood eosinophils became labelled $2\frac{1}{2}$ days later. When the injection of label was delayed for 24 h, 60% were labelled and when the delay was 48 h, 83% were labelled, (Figure 4.2). This showed that the eosinophilia was caused by increased number of newly formed eosinophils over 80% of which were synthesising D.N.A., two days after injection of larvae. This agreed

with the previous finding(p.67) that T_S/T_C is greater in stimulated than normal rats. In addition there may have been some reutilisation of label in the stimulated rats to account for the high proportion labelled by a single injection of 3H-thymidine.

Eosinophil emergence time (T_E)

A number of experimental studies on marrow eosinophils have suggested that there is a pool of mature marrow eosinophils available for release. Hudson (1968) classified guinea pig marrow eosinophils which had finished dividing and which were morphologically band and segmented forms as "the marrow reserve pool". He found that release of these mature eosinophils occurred within 29 hours of reinjection of antigens.

It was of importance to see whether a similar phenomenon was taking place in rats injected with larvae by the intravenous route, as eosinophils were found in large numbers in lung lesions within 24 h (Chapter 5).

Blood eosinophils of 17 rats were counted immediately before larvae were injected intravenously. Four to 24 h later a further blood count was done. There was no signficant difference between the initial blood eosinophil counts/mm^3 of 41.4 $\pm$ 15, and the final counts of 37.6 $\pm$ 24.6. Because the blood eosinophil counts did not fall during this period, newly formed eosinophils were probably entering the circulating blood as rapidly as they were being removed into the lungs.

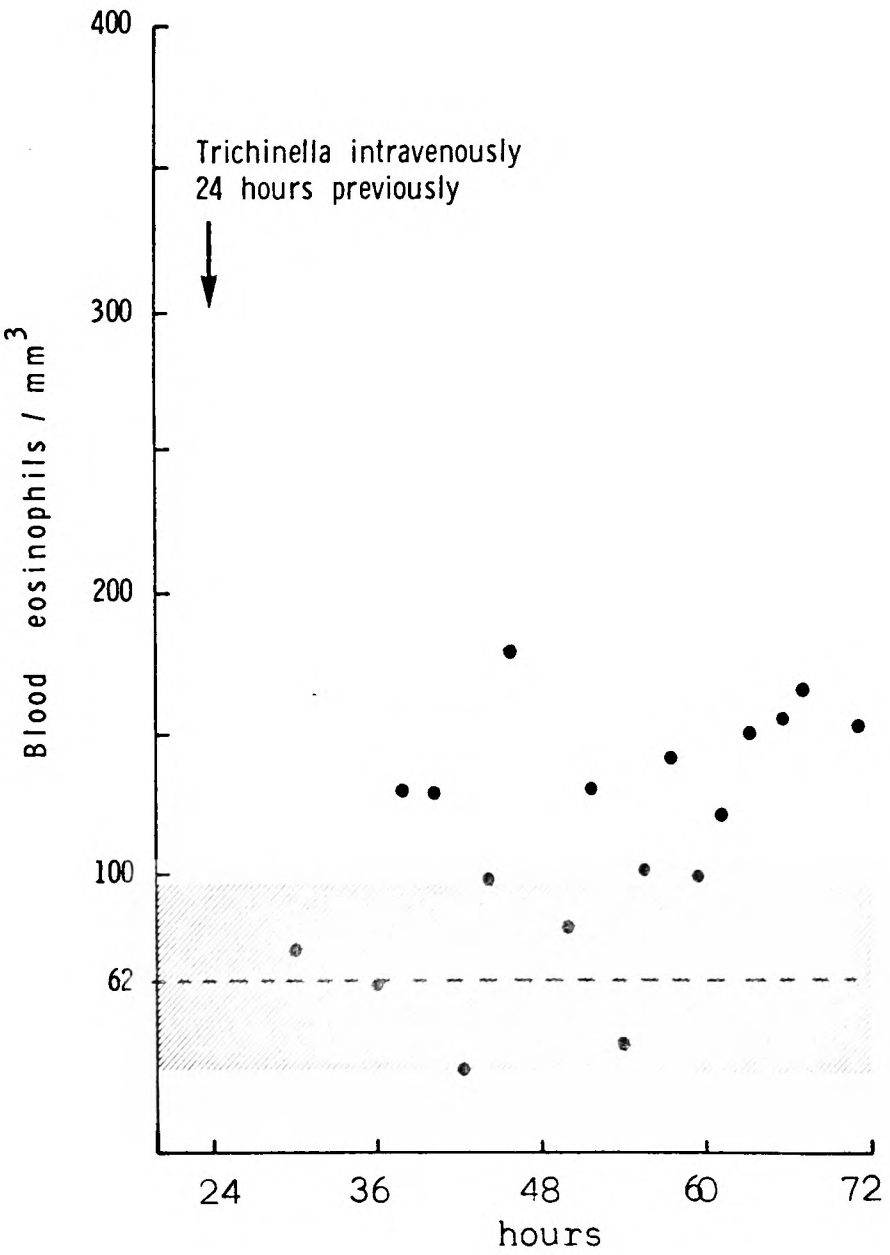


Figure 4.3. Eosinophil counts in rats given Trichinella spiralis larvae intravenously. The counts began to rise 36 - 48 h later. The shaded area outlines the range of blood eosinophil counts in 24 normal rats studied at the same time.

If newly formed eosinophils were being released sooner than normal, in response to the stimulus, then a shortening of the normal T_E should be expected. This possibility was studied in 23 rats given single intravenous injections 1 µCi 3H-thymidine/g, 24 h after larvae had been injected intravenously. There were no alterations in blood mononuclear cell or neutrophil counts during this period but the blood eosinophil counts rose above the normal levels as shown in Figure 4.3.

The % blood eosinophils labelled in these stimulated rats after the injection of 3H-thymidine was also studied. Labelled eosinophils began to appear in the blood of normal rats 40.9 h after the injection of 3H-thymidine, but in the stimulated rats labelled eosinophils emerged after only 17.7 h, (Figure 4.4.)

It was concluded that eosinophil emergence time was reduced in stimulated rats. This mechanism, which results in more eosinophils leaving the marrow, is clearly different from that proposed in the concept of a "marrow reserve pool" (Hudson, 1968).

Grain counts over labelled blood eosinophils in the stimulated rats showed that the most heavily labelled eosinophils were present in blood 28 h after injection of 3H-thymidine. The time interval between the first appearance of labelled eosinophils in the blood and the time when the second wave of labelled cells emerged was about 25 h, (Figure 4.4

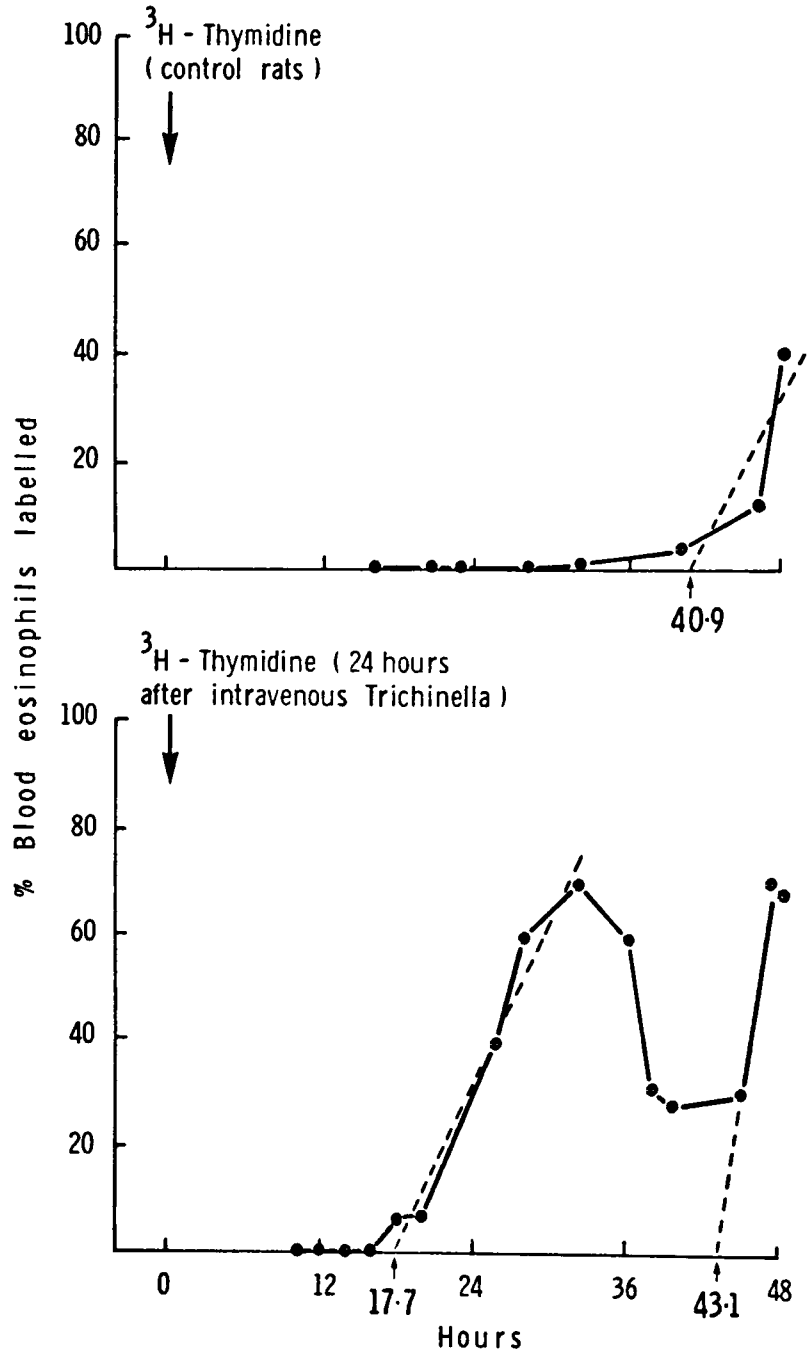


Figure 4.4. The % labelled blood eosinophils after a single injection of 3H-thymidine in normal rats (upper figure) and rats given larvae intravenously (lower figure). The emergence time was 40.9 h in normal rats, but was reduced to 17.7 h in stimulated rats. These figures were calculated from regression equations plotted as dotted lines.

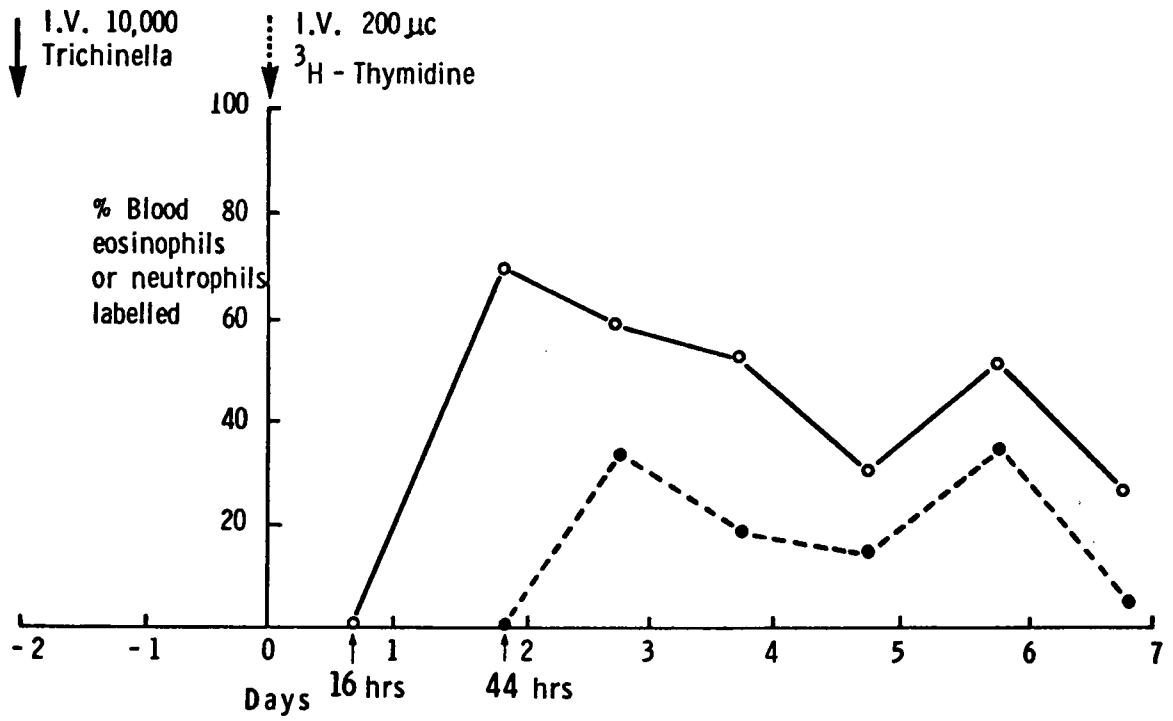


Figure 4.5. The % labelled blood eosinophils (continuous line) and % labelled neutrophils (dotted line) in a rat given larvae and 3H-thymidine intravenously. In daily samples of blood labelled eosinophils appeared 28 h before labelled neutrophils, suggesting that their emergence times were regulated separately.

Comparison of eosinophil and neutrophil T_E

It was interesting to find that in the previous experiment, no change in neutrophil T_E occurred when eosinophil T_E was halved. This result was confirmed by a similar study on one rat which was injected with larvae and given 1 μCi/g 3H-thymidine 24 h later. Daily blood samples were taken and the % eosinophils and neutrophils labelled was measured. Results are shown in Figure 4.5.

Eosinophil 'release'

It has not proved possible to study separately the pools of marginated and circulating eosinophils which comprise the total blood intravascular pool of eosinophils. Athens (1969) used a radioactive di-isopropyl fluorophosphate label to study the marginated pool of neutrophils in man, but eosinophils are not labelled by this technique. So experiments were done to assess indirectly the importance of the eosinophil mobilising processes from marginated sites, by measuring the blood eosinophil count itself. It was assumed that if increased blood eosinophil levels occurred then these additional eosinophils were probably sequestered or marginated cells. This short term increase in blood eosinophil counts which was presumed not to come about by additional eosinophil production, was termed eosinophil 'release'.

Plasma. Experiments were designed to test for eosinophil 'releasing' activity in the plasma of Harwell rats 1.5, 4, 6 and 23 h after they were injected with Trichinella spiralis larvae intravenously. Pairs of Harwell rats were given 0.5 to 1.8 ml of the plasma intravenously, and eosinophil counts were done at the time of injection and four hours later. Five of 6 recipients had an eosinophilia when injected with plasma, taken 6 and 23 h after injection of the larvae. Eosinophil counts rose from 39 - 55/mm^3 to 111 - 133/mm^3 in these recipients. In a control study normal plasma injected into 5 normal rats did not produce this effect.

In another experiment, 28 S.P.F. Harwell rats were used as recipients of plasma. Donor Harwell rats were either normal animals or had larvae injected intravenously 12 - 24 h previously. Donor rats were given an intravenous injection of 100 units heparin, and were bled out from the aorta under ether anaesthesia. Plasma was separated and 2 ml volumes were injected intravenously within an hour into normal recipients. Blood eosinophils were counted at the time of injection of plasma and 3, 6 and 24 h later. These counts showed a two-fold increase at 3 and 6 h in 19 recipients given plasma from the rats which had intravenous larvae. In 9 rats given normal plasma, there was no alteration in blood eosinophil counts. Statistical differences at 3 h were $P<0.005$; at 6 h $P<0.001$; but at 24 h $P>0.25$ (Figure 4.6).

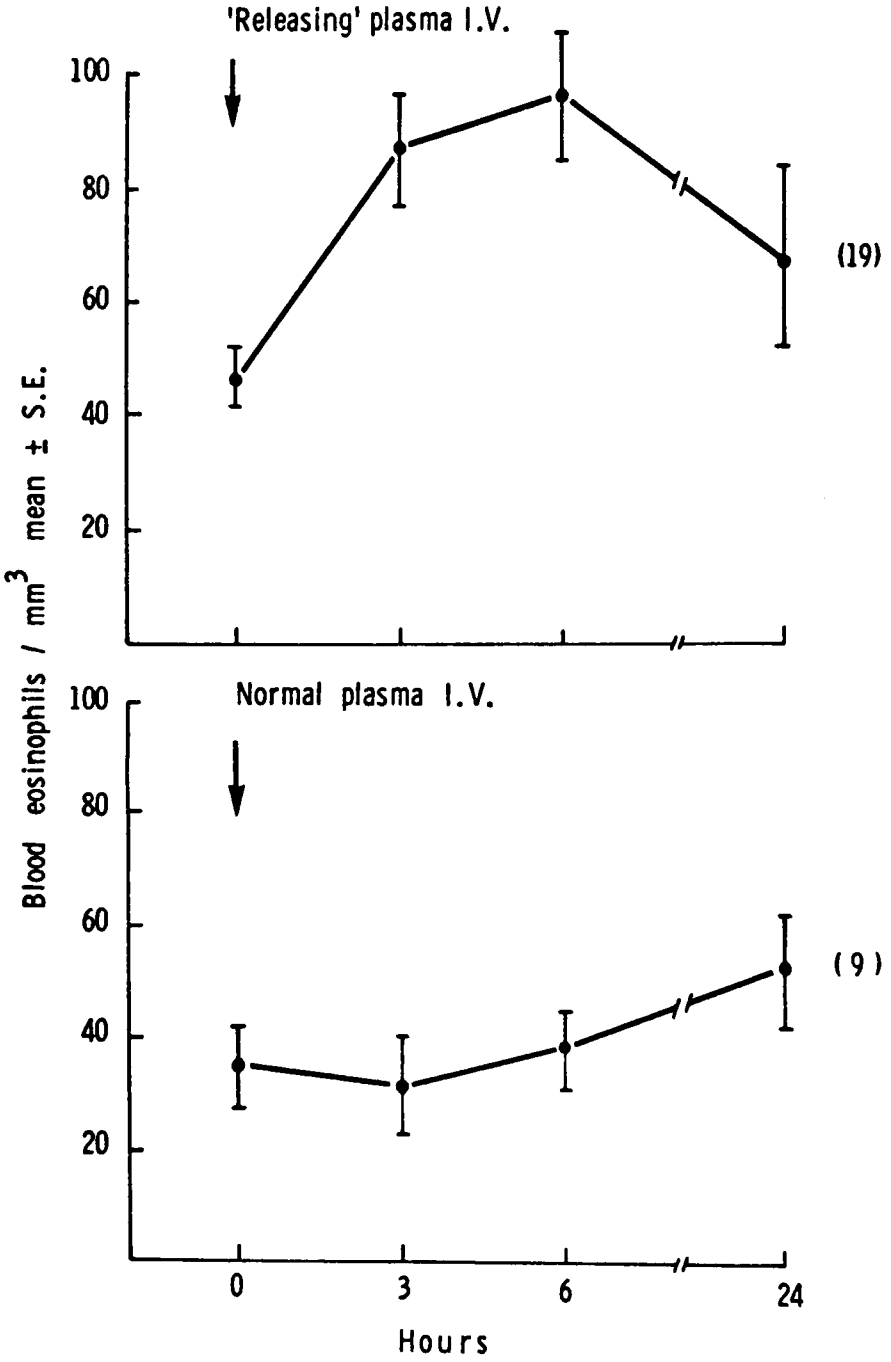


Figure 4.6. Blood eosinophil counts in rats given injections of normal plasma (lower figure) and plasma from rats given Trichinella spiralis larvae intravenously 12-24 h before (upper figure). The plasma from the stimulated rats induced a small but significant eosinophilia at 3 and 6 h.

The mean height of the 6 h blood eosinophil response was 87/mm^3. This small number was probably partly related to the small 'reserve' of non-circulating eosinophils in these S.P.F. rats. In non-S.P.F. Animal Supply rats, 'releasing' plasma in a group of 3 rats induced a rise of blood eosinophils from a mean of 100/mm^3 to over 300/mm^3.

Further tests were made to determine whether the 'stress' of injection had lowered the eosinophil count to some extent in the face of a rise brought about by eosinophil 'releasing' plasma. Harwell rats were adrenalectomized and at various time intervals afterwards were given 'releasing' plasma intravenously. Rats injected within 2 days of adrenalectomy showed wide variations in blood eosinophil response to the plasma, but this effect lessened as the time interval increased to 12 days after adrenalectomy. No increase in the mean eosinophil response to plasma occurred, so it was concluded that adrenalectomy had not provided a more 'sensitive' in vivo assay system.

Ten ml active plasma derived from rats 24 h after injection of Trichinella spiralis larvae was dialysed against 2 changes of 2 L buffered normal saline, pH 7.4 for 24 h at + 4°C, but showed no reduction in 'releasing' activity when tested in 4 normal recipient Harwell rats, 3 of which showed a doubling of blood eosinophil counts 4 - $5\frac{1}{2}$ h after injection. In other experiments the plasma activity did not appear to be lost by storage at -20°C for several days.

Splenectomised rats which had higher than normal resting levels of eosinophils responded to 'releasing' plasma in the same way, with maximal eosinophil counts 6 h later, but the % rise was not increased. Similarly adrenalectomised and splenectomised rats did not prove to be any more useful for assaying 'releasing' plasma than normal rats.

Lung tissue. It was possible that eosinophil 'releasing' activity was produced by damaged lung tissue. To investigate this, lung tissue was taken from 3 Harwell rats injected 16 h previously with larvae intravenously. The lungs were cut up with scissors, homogenised with a shredding homogeniser in Parker 199 and treated with ultrasound for 1 minute while cooled with ice, and centrifuged. The supernatant (0.5 ml, equivalent to about 1/8th of the soluble part of one lung) was given to each of 4 Harwell rats intraperitoneally. No eosinophilia occurred in the recipients 2, 4 and 6 h later. This provided no evidence of 'releasing' activity in the lungs themselves, although it might be admitted that such activity could have been destroyed by the extraction procedures.

Damaged eosinophils. The possibility was considered that damaged eosinophils themselves could generate eosinophil 'releasing' activity. This was tested by using peritoneal eosinophils removed from 4 rats by peritoneal lavage. These animals were given trichinosis, followed

by splenectomy combined with intravenous larvae 3 weeks later. Peritoneal lavage was carried out to remove mast cells. Five days later an additional lavage yielded an exudate rich in eosinophils. Of 4.1×10^8 cells, forty-eight percent were eosinophils, 50% mononuclear cells, 2% mast cells and less than 1% neutrophils.

Half this cell suspension was frozen to -20°C and thawed rapidly and injected intravenously in 3 ml Parker 199 into a normal syngeneic rat. Eosinophil counts at 3, 6 and 24 h later did not rise above $30/mm^3$. The blood neutrophil count also did not alter. The remainder of the cell suspension was incubated in Parker 199 containing 1 unit of heparin/ml with 5,000 Trichinella spiralis larvae in a volume of 2 ml. The suspension was left shaking in a water bath for $\frac{1}{2}$ h at 37°C. Supernatant from this culture did not induce an eosinophil or neutrophil leucocytosis in a normal syngeneic recipient rat at 3, 6 or 24 h after injection. These two experiments did not support the hypothesis that damaged eosinophils could initiate the release of eosinophils from marginated sites.

Eosinophil half life ($T\frac{1}{2}$) in the blood. Rat eosinophil $T\frac{1}{2}$ was first measured accurately by Foot (1963) using continuous infusions of 3H-thymidine into restrained PVG/C rats. She showed that eosinophil $T\frac{1}{2}$ was 10 ± 2 h.

Eosinophil $T\frac{1}{2}$ has not been measured in rats which are unrestrained and specific pathogen-free, or in conditions where blood eosinophil $T\frac{1}{2}$ might be altered. There are considerable technical difficulties in studying labelled blood eosinophils when the eosinophil count is less than $100/mm^3$. Therefore the method for concentrating blood leucocytes for autoradiography was devised, as described on p. 37. The effect of 'stress' on the kinetics of eosinophils was minimised either by bleeding each rat atgreater than 12 h intervals, or by sampling only once after injections were made. Calculations of eosinophil $T\frac{1}{2}$ were done by analysing the rate of increase in the percentage of labelled eosinophils in the blood by the method of Foot (1963).

In 6 normal Harwell rats, sampled only once, 40 to 45 h after an intravenous injection of 2 μCi 3H-thymidine/g, eosinophil $T\frac{1}{2}$ was 6.6 h. Another group of 6 Harwell rats were given larvae intravenously followed after 24 h by 1 μCi 3H-thymidine/g intravenously. Blood eosinophils were sampled once, 14-32 h after injection of label. Eosinophil $T\frac{1}{2}$ in the stimulated rats was calculated to be 6.1 h. As eosinophil $T\frac{1}{2}$ had not decreased appreciably in the stimulated rats, it seemed likely that the marginated pool of eosinophils had increased. This was confirmed in the histological study in which many marginated eosinophils were seen in blood vessels near the lesions induced by larvae (p. 101).

Blood eosinophil $T\frac{1}{2}$ was measured in 2 PVG/C rats, 2 and 5 days after giving larvae intravenously. One μCi 3H-thymidine was injected intravenously and the rats were sampled at 12 h intervals. Eosinophil $T\frac{1}{2}$ was 9.7 and 19.2 h respectively. As blood eosinophil $T\frac{1}{2}$ was found to be increasing in stimulated rats, as the blood eosinophil counts rose, it seemed likely that the total blood granulocyte pool had increased to such an extent that it masked any effect that increased eosinophil migration into the tissues might have in reducing blood eosinophil $T\frac{1}{2}$. The eosinophil $T\frac{1}{2}$ was probably longest at the height of the eosinophilia when the rate of removal was returning to normal. In another PVG/C rat which had been splenectomised, blood eosinophil $T\frac{1}{2}$ was 12.1 h, 2 days after injection of larvae. This was suggestive that splenectomy prolonged eosinophil $T\frac{1}{2}$ in blood, and here too, the mechanism may involve an increase in the size of the total intravascular pool of eosinophils.

Conclusions

Eosinophil concentrations in the circulating blood depend on several interrelated processes. Input of newly formed eosinophils is regulated by alterations in the emergence time of eosinophils, which is controlled independently of neutrophil emergence time. Once in the circulation, eosinophils are partitioned between

circulating and marginated compartments. Although the sizes of these compartments could not be measured, it was shown by measuring eosinophil half-life in the blood that the sizes of both compartments probably increases when there is an eosinophilia. A serum factor is present in stimulated rats which releases eosinophils into the circulating compartment, so that more cells become available at distant sites.

The general conclusions are reached that eosinophils become available within 24 h of a stimulus by mechanisms which involve a redistribution of eosinophils. The effects of increased production influence blood eosinophil levels after the acute response has been initiated. These findings are discussed further in Chapter 7.

CHAPTER 5

TISSUE EOSINOPHILS

Introduction

Increased bone marrow eosinophil production was initiated by events which occurred during the first 24 h after intravenous injection of Trichinella spiralis larvae. Circulating white blood cells taken during this period would transfer the eosinophil stimulus to normal rats (Basten & Beeson, 1970). In addition single injections of methotrexate only inhibited the development of the eosinophilia when given within 24 h of the injection of larvae (Boyer, Basten & Beeson, 1970). The initial reaction between larvae and tissues was therefore of importance in the induction of increased eosinophil production. This chapter describes experiments which were done to determine the nature of this phase of the response.

The inflammatory reaction induced by Trichinella spiralis larvae

The histological events which occur when larvae come in contact with tissues, were studied in several sites.

Lung. Dr Walter Sheldon of the Johns Hopkins University, cut and stained many of the lung specimens, and part of this report is based on his findings (Boyer, Spry, Beeson & Sheldon, 1970).

In uninjected rats there was some lymphoid bronchiectasis. A few eosinophils were seen in perivascular sites. Occasional eosinophils were found in alveolar septae. In rats injected intravenously with Trichinella spiralis larvae, the following changes were found in the lungs (Figure 5.1).

One hour after injection, many larvae were found in precapillary vessels, and some neutrophils were present on the surface of the larvae. Tissue mast cell numbers were decreased, (Figure 5.1 (a)).

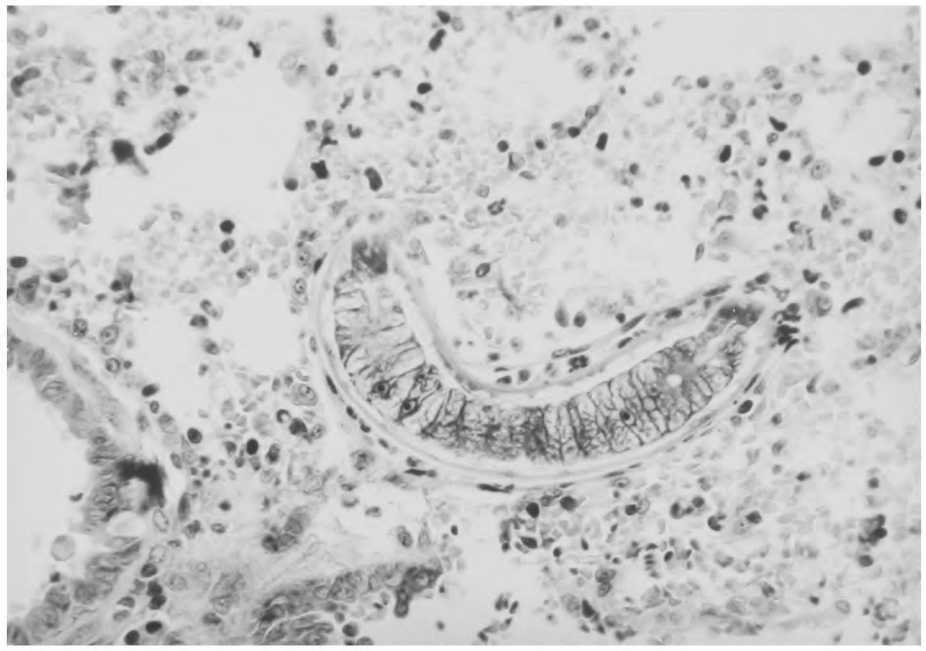
At 2 h, the neutrophil response was increased and predominated, with more eosinophils present than at one hour. Some mast cells had discharged granules.

At 4 h, a further increase in the inflammatory response was present. Mast cells were greatly reduced.

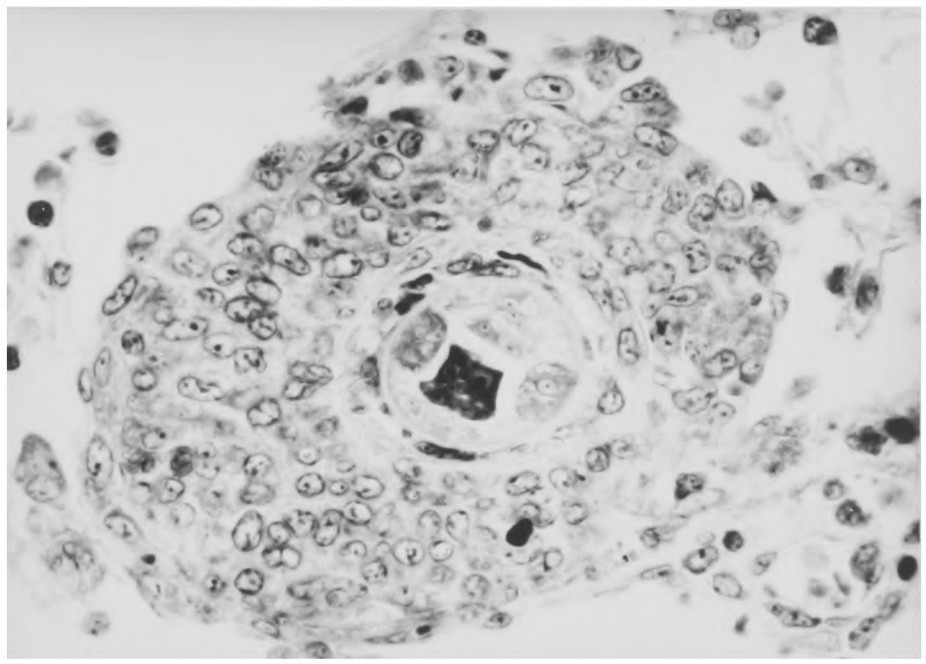
At 6 h, mononuclear cells were present in increasing numbers around the larvae. There were more eosinophils but mononuclear cells were more numerous than granulocytes. Granulated mast cells were present in normal amounts.

Figure 5.1

(a)



(b)



(c)

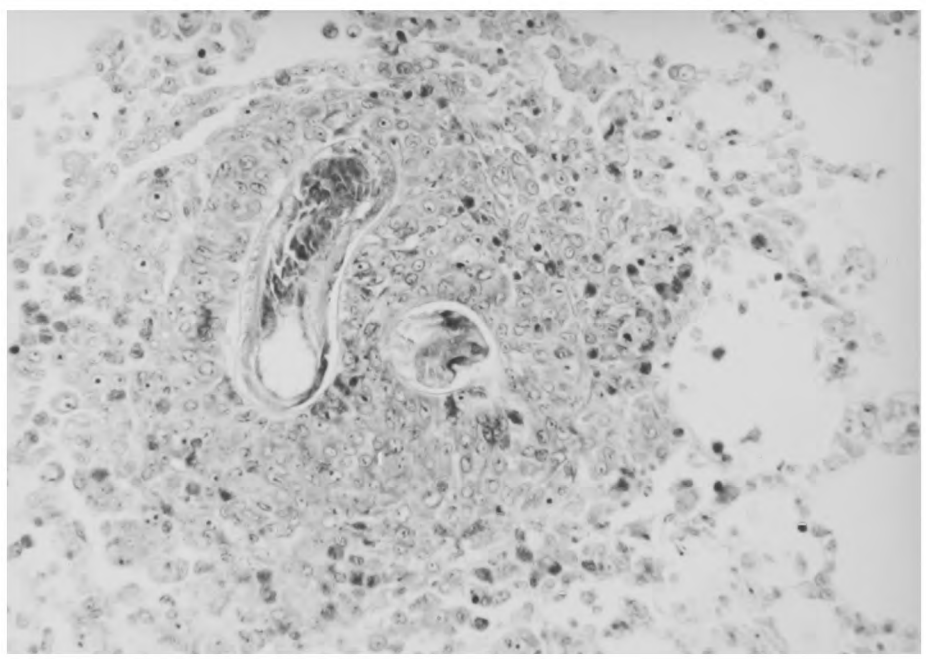


Figure 5.1. Rat lung after intravenous injections of *Trichinella spiralis* larvae. Giemsa stain.

(a) 45 min after injection. The inflammatory reaction around the larva had begun. There was haemorrhage into the lung around the larva, and most of the cells in the neighbourhood were blood cells, mononuclear cells and neutrophils. About x 200.

(b) 12 h after injection. A pallisade of mononuclear cells enclosed the larva. Eosinophils were present in increased numbers in the lesion, but there were few neutrophils. There were some signs of degeneration in the larval cuticle. About x 200

(c) 24 h after injection. Therewere a considerable number of eosinophils around the periphery of the large granulomas. The larvae showed some degeneration. There was some giant cell formation. Some plasma cells were present, but there were few neutrophils. About x 100.

These photographs, and the slides from which they were taken, were made by Dr Sheldon.

At 12 h, compact granulomatous areas were beginning to form. They consisted mainly of large mononuclear cells with eosinophils. Some of the eosinophils showed nuclear degenerations. Some free eosinophil granules were present (Figure 5.1 (b)).

At 18 h, the integuments of the larvae were becoming acidophilic. Definite granuloma formation was present with palisades of mononuclear cells around the larvae, and there was a thinner cuff of the eosinophils outside the mononuclear cells. Again some free eosinophil granules were seen. Occasionally, large aggregates of eosinophils were seen in perivascular regions, and eosinophils appeared to be migrating into the inflammatory sites from the blood. A striking feature of the 12 h and 18 h sections was the finding that there were more eosinophils in the lesion at these stages than neutrophils. This occurred despite a great preponderance of neutrophils in the circulating blood at this time.

At 24 h, the lesions showed destruction of the larvae, and giant cell formation was evident. Many eosinophils were present with occasional immunoblasts and plasma cells, but few neutrophils, (Figure 5.1 (c)).

At 48 h, the size of granulomas was maximal.

At 3 and 7 days, the size of granulomas was reduced. Mononuclear cells and eosinophils were present with some plasma cells, and there were many multinucleate giant cells. The lesions were almost completely resolved at 15 days.

Liver. Larvae injected intraportally induced lesions in which mononuclear cells and eosinophils also predominated, as in the lung lesions. The consistency of the liver tissue made it an easier site in which to assess the cellular reactions than lungs. The reactions were similar in both sites, but eosinophils were less numerous in liver than lung at comparable times after injection. Mononuclear cells, some neutrophils and plasma cells were found, followed by giant cells and granuloma formation as in the lung. The liver showed evidence of damage around each larva. As in the lungs, the paucity of neutrophils compared with their concentration in the blood was particularly noticeable. In the liver, larvae were destroyed within 3 days, but some small granulomas persisted for two weeks.

Muscle. The histology of larvae in muscle was not studied in detail as this has been reported on many occasions by others (Gould, 1945) and electron micrographs of the capsule surrounding the encysted larvae have been reported recently by Bruce (1970). But it was important to compare the degree of eosinophil accumulation and the nature of the cells found around Trichinella spiralis larvae in muscle with liver and lung. Eight days after oral infection some small migratory larvae were found in thigh muscles. There was very little cellular infiltrate around the larvae, but destruction of adjacent

muscle tissue was observed. At 18 days the larvae were larger and easier to find. There were well formed 'capsules' consisting of homogenous tissue containing nuclei with large nucleoli.

The inflammatory reaction was slight compared with that in lung or liver. Most of the mononuclear cells and eosinophils were scattered irregularly in the connective tissue of the muscle. Neutrophils were uncommon as had also been noted in lung and liver lesions.

Response to inert plastic spheres. The specificity of the reactions to larvae was studied by injecting intravenously 5 x 10^5 inert plastic microspheres. The spheres had a mean diameter of 11.3 μ $\pm$ 2.3, and when they were injected into Harwell rats they were found lodged in the lungs. Histologically only a very mild reaction was produced, in which neutrophils were present with mononuclear cells. The lungs did not contain additional eosinophils and no granuloma formation occurred. There was no alteration in the peripheral blood eosinophil counts for eleven days after injections of plastic beads. Thus mechanical blockage of pulmonary capillaries did not effect eosinophil production or localisation. Similar results showing little response to injection of plastic beads have been reported by Kellermeyer & Warren (1970).

Survival of larvae after intravenous injection. When larvae were injected intravenously, only a very few survived. In the carcasses of 10 rats digested one month after giving 10,000 larvae intravenously, no larvae were found in 7. The 3 other carcasses contained 1, 9 and 12 living larvae.

Conclusions from histological findings. The early events which could be important in the initiation of eosinophil production, as shown in the histological studies, include mast cell degranulation at 1 - 4 hours, accumulation of mononuclear cells at 6 - 12 hours close to the larvae, and eosinophil arrival from 6 - 24 hours in areas around the mononuclear palissades. With these possibilities in mind, experiments were carried out to try to determine if any of these events were essential in the induction of eosinophil production.

Mast cell degranulation

Mast cell degranulation was a feature of the early response to larvae. Because of this and frequent reports that eosinophils are in some way associated with mast cells and histamine, (see Archer, R.K., 1963; Fernex, 1968) it was important to consider whether mast cell degranulation itself could initiate eosinophil production.

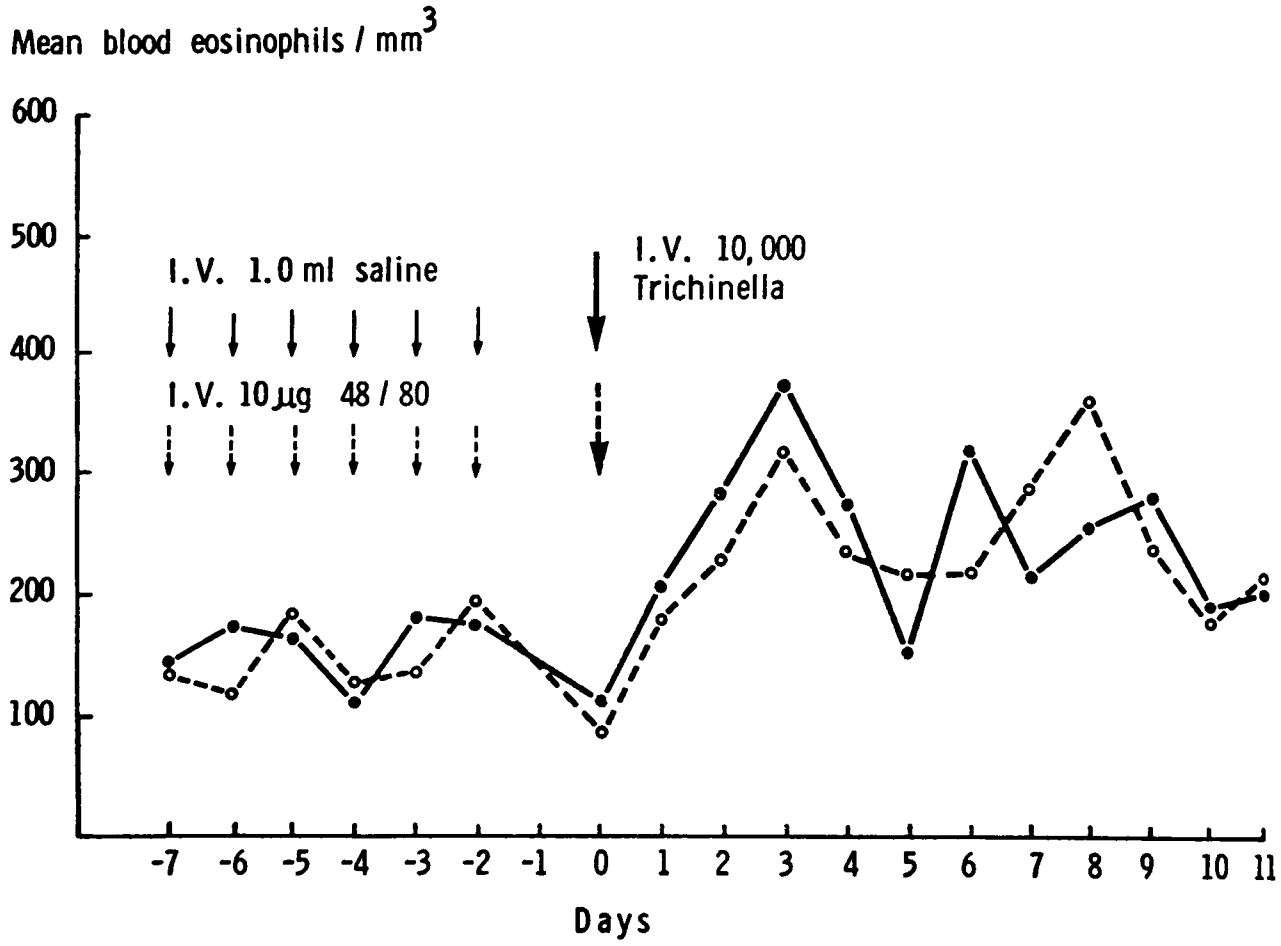


Figure 5.2. Mean blood eosinophil counts in 6 PVG/C rats given daily injections of 10 µg Compound 48/80 and then injected intravenously with larvae (dotted line). The mean response of 6 PVG/C rats given daily injections of 1 ml saline, followed by an intravenous injection of larvae was compared (solid lines). There was no eosinophilia in response to injections of Compound 48/80, and the blood eosinophil response to larvae was unaffected.

Ten µg of compound 48/80 (Wellcome) which is known to be a powerful tissue histamine liberator from mast cells *in vitro* (Paton, 1958; Horsfield, 1965) and from lung *in vivo* (Feldberg & Talesnik, 1953) was injected daily into PVG/C rats. The lethal dose for a rat was 100 µg. Repeated injections of compound 48/80 did not induce an eosinophilia. Moreover there was no difference in the peripheral blood eosinophil response to larvae, whether animals had been pre-treated with 48/80 or saline. This did not support the hypothesis that mast cell degranulation is an essential step in eosinophil production (Figure 5.2).

Mononuclear cell accumulation around larvae

Several experiments suggested that the interaction of larvae with macrophages, which was such a prominent feature of the histology of larvae in the lung and liver, inhibited the induction of eosinophil production. When larvae were injected intraperitoneally, many mononuclear cells were found to be adherent to the larvae when they were removed from the peritoneal cavity at 24 h. No eosinophilia occurred unless 3 times the usual quantities of larvae were given intraperitoneally. In other experiments it was found that when larvae coated with peritoneal cells were injected intravenously, a smaller eosinophil occurred than when normal larvae were used.

Twenty-four hours after injecting larvae into the lung, some cells which looked like lymphocytes or immunoblasts were seen. For this reason, and because of the known affect of larvae on lymphocytes in lymph nodes (p. 108), experiments were done to study lymphocyte responses to larvae. In vitro, thoracic duct lymphocytes, unlike peritoneal macrophages, did not accumulate around larvae. Nor did thoracic duct lymphocytes adhere to larvae in culture media containing antilarval antibodies under conditions in which peritoneal cells and blood leucocytes attached firmly within a few minutes. In vivo, injected thoracic duct lymphocytes labelled with tritiated nucleic acid precursors did not migrate into the mononuclear accumulations around larvae (p.123).

Eosinophil accumulation around larvae.

It was considered possible that eosinophil accumulation in inflammed tissues may initiate the stimulus to eosinophil production. In support of this suggestion was the finding that there was a great reduction in eosinophil accumulation around larvae in the lungs when eosinophil production had been inhibited with a single injection of prednisolone. However, when stimulated rats were given Corynae-bacterium anaerobium intravenously, although the blood eosinophilia

was inhibited, there was only slight diminution in eosinophil accumulation in lung lesions (Boyer, 1970). For this reason it was felt that eosinophil accumulation around larvae would not by itself initiate eosinophil production. Moreover homogenised lung tissue which had contained larvae for 24 h when given intraperitoneally to normal rats, did not induce an eosinophilia 2-10 days later.

Lymph nodes draining sites containing larvae

As there was no evidence that the stimulus for eosinophil production was derived from the cells which were involved in the inflammatory reaction in immediate contact with larvae, and as eosinophil production was initiated by large pyroninophilic lymphocytes found in the thoracic duct lymph and blood (Basten & Beeson, 1970), attention was directed to the lymph nodes draining sites in which larvae lodged.

The lymphoid tissue in the wall of the upper small intestine and draining lymph nodes in the mesentery were studied in 2 rats given oral trichinosis three days before, and 4 normal rats. The rats were given ^{3}H-thymidine 1 μCi/g intravenously 1 h before sacrifice. The mesenteric lymph nodes of the infected rats contained large numbers

of labelled large pyroninophilic lymphocytes in the cortical, paracortical and medullary regions. Some of the follicles also contained labelled large pyroninophilic cells.

In the infected rats few labelled pyroninophilic cells were found in the duodenal and jejunal villi, and there were only occasional lymphoid aggregates in the bowel wall unlike normal rats. This showed that large pyroninophilic lymphocytes, some of which are involved in eosinophil production, probably originate in the draining lymph nodes of the intestine.

A similar stimulation of lymphocytes in draining lymph nodes may occur when larvae lodge in the lungs. Unfortunately normal rats had a mild lymphoid bronchiectasis and the neck nodes were full of actively dividing large pyroninophilic lymphocytes. This meant that the effect of larvae on draining lymph nodes of the lung could not be studied.

Conclusions

This Chapter has described invetigations concerned with the tissue reactions responsible for the induction of eosinophils production. Eosinophil accumulation occurred rapidly in the sites of acute inflammation, but the mechanism for this response is not known. The

results of several experiments did not favour the possibility that local events in the sites of contact between parasites and tissues initiated eosinophils production. The important process appeared to be stimulation of draining lymph nodes.

CHAPTER 6

LYMPHOCYTES IN TRICHINOSIS

Introduction.

Basten & Beeson (1970) found that when living large pyroninophilic lymphocytes from the thoracic duct of rats with oral trichinosis were injected into normal syngeneic rats, an increase in eosinophil production occurred, as shown by an eosinophilia 2 - 6 days after transfer. There were several possible mechanisms for the induction of increased eosinophil production in the recipients. The lymphocytes could have localised in sites at a distance from marrow or they could have lodged in bone marrow, and initiated eosinopoiesis by short range processes. In the second case it should be possible to find these lymphocytes in bone marrow at an early stage after transfer.

With these possibilities in mind, experiments were done to determine the fate of transferred labelled lymphocytes from the thoracic ducts of rats with oral trichinosis. All transfers were done between syngeneic rats. The fate of the lymphocytes was assessed

initially using tritium labelled large lymphocytes and autoradiography, and later by using ^{51}Chromium labelled lymphocytes and whole tissue γ irradiation counting.

Distribution of labelled large lymphocytes in normal rats.

Eight normal rats were killed 3, 12, 21, 22, 40, 46, 48 and 72 h after transfer of labelled large lymphocytes from the thoracic duct of rats given oral trichinosis. Table 6.1a lists the characteristics of the lymphocytes given to the 8 normal rats and Table 6.1b shows the tissues in which they were found.

Labelled lymphocytes in bone marrow. Some labelled lymphocytes were always found in bone marrow. The largest number were noted in tissue taken 21 h after transferring 6 x 10^7 labelled large lymphocytes. In this recipient rat the labelled cells appeared to be randomly distributed, but the technique of preparing bone marrow slides had disaggregated cell groups. Forty-two labelled lymphocytes had a hundred close neighbours: 64 were mononuclear cells, 31 neutrophils and 5 eosinophils. These cells were found in similar proportions over other parts of the same slides. Thus the labelled lymphocytes appeared to have no spatial relationship to eosinophils or other cell types.

Cannulation after infection (days)	Thoracic Duct Lymphocytes				Time recipient killed after transfer (h)
	Total No. x 10^8	% large	% large labelled	Total large labelled x 10^7	
4	6.4	6	79	4.9	3
3.5	3.0	10	84	4.1	12
5	3.1	23	56	6.0	21
3.5	1.8	7	72	1.5	22
3	1.3	5	76	0.4	40
3.5	1.3	13	77	1.4	46
4.5	6.2	25	55	8.6	48
4.5	15.0	12	58	10.0	72

Table 6.1a. Characteristics of labelled large lymphocytes from the thoracic ducts of rats with with trichinosis.

The number of labelled large lymphocytes transferred to 8 normal recipients and the time after transfer when recipients were killed is shown.

Femoral marrow	Small intestine		Colon	Spleen	Mesenteric node	No of labelled cells in	
	Upper ½	lower ½				300 blood mononuclear cells	3 sections of thymus
+	+++	+++	+	+	++	0	0
+					+++		10
++	+++		+	+++	+++	0	12
		+++	+++			2	
	+++	++		+	++	0	0
		++			+	0	
+		++	+	+	++	0	1
	++	+	++	++	++	0	1

0 = no labelled cells seen; + = labelled cells found with difficulty
++ = labelled cells found easily; +++ = several labelled cells in many high power fields.

Table 6.1b. The distribution of labelled large lymphocytes in syngeneic rats, 3 to 72 h after transfer

Most of the lymphocytes were found in the small intestine and lymph nodes, but some were found in bone marrow.

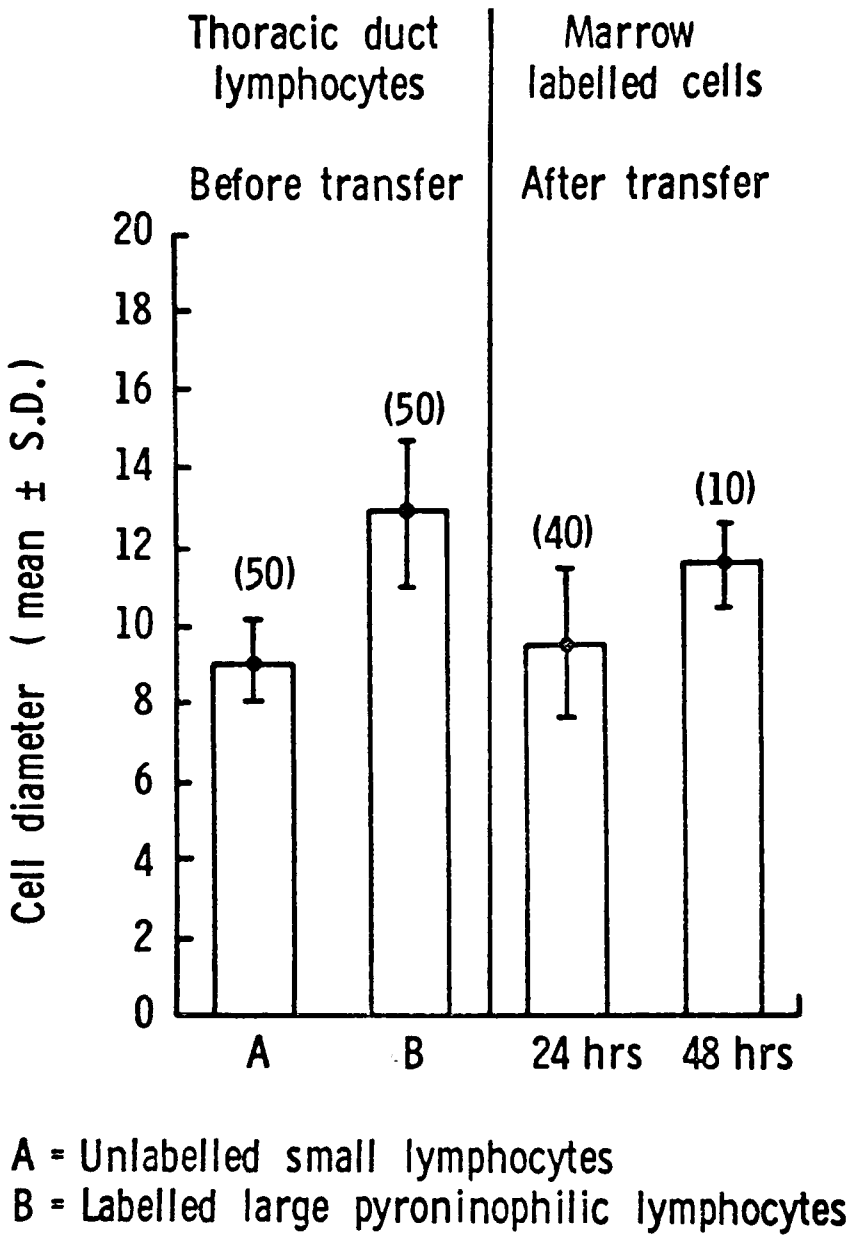


Figure 6.1. The diameter of large labelled thoracic duct lymphocytes and their progeny in bone marrow. Twenty-one and 22 h after transfer the labelled lymphocytes became the same size as small lymphocytes and at 48 h those still remaining in the bone marrow were plasma cells. The number of cells studied are in brackets.

The labelled lymphocytes were found to change in size and morphology after transfer. Most had become small lymphocytes by 24 h. By 48 h the labelled lymphocytes in bone marrow had become larger than at 24 h, and had more cytoplasm. They had the appearances of plasma cells. The sizes of the labelled cells at these times are shown in Figure 6.1.

Labelled lymphocytes in peripheral blood. Only 2 labelled cells were found among 2,100 blood mononuclear cells studied at intervals after transfer of labelled large lymphocytes. Both were small mononuclear cells. Thus there appeared to be little circulation of labelled lymphocytes or their progeny through the blood stream, after intravenous injection.

Labelled lymphocytes in lung. Following intravenous injection, no clumps of labelled lymphocytes were found lodged in the lung. Labelled lymphocytes were, however, found outside the vascular system of the lung within three hours of injection, in lymphoid aggregates around medium sized bronchi. Only a few labelled lymphocytes were found in lung parenchyma, so this was clearly not a major site for the cells to settle.

Labelled lymphocytes in intestine. Large numbers of the injected labelled large lymphocytes settled in the small intestine, as described by Gowans (1962), Gowans & Knight (1964) and Hall & Smith (1970), in the intestinal villi, in the tissue normally rich in mononuclear cells, plasma cells and eosinophils. Later they were distributed along the whole length of the villi in the lamina propria. They were not clumped together, and morphologically they were indistinguishable from the other mononuclear cells found there. The jejunum and ileum contained equal numbers of labelled cells, but the colon had distinctly less. In the colon the labelled cells were rarely found in the villi, most being in the basal regions.

Using the haematoxylin and Biebrich scarlet technique, it was not uncommon to find labelled cells in apparent contact with eosinophils. Twenty-one hours after injecting labelled large lymphocytes, 40 of 342 labelled large lymphocytes (12%) in the jejunum were adjacent to eosinophils. It was difficult to tell whether this relationship was specific as both cell types were relatively common in the villi. If the spatial relationship between eosinophils and labelled lymphocytes was not due to chance this might reflect some shared role for these two cells.

Occasionally, labelled cells appeared to be in the mucosal epithelial cells. The presence of lymphocytes in mucosal cells has been reported in biopsies of human intestine (Shields, Touchon & Dickson, 1969). It is not known if lymphocytes migrate into the lumen of the intestine. None were found in the lumen of the gut in the sections studied here.

Labelled cells were common around the edges of lymphoid aggregates in the bowel wall, especially at 24 hours, but more labelled cells were found in the villi than in the lymphoid aggregates.

At no stage was label found in eosinophils. Thus there was no evidence for the view that eosinophils are formed from lymphocytes in the intestine.

Labelled cells in spleen. Labelled cells were found in splenic tissue at all times after injection, they were commonest in the loose connective tissue of the spleen, under the splenic capsule. They did not exhibit any spatial relationship to groups of eosinophils which were found in all the spleens studied. At 21 hours, 22 of 100 labelled cells had become small in size and were in pairs. This was evidence that division of large labelled lymphocytes occurred in the spleen.

Labelled cells in the mesenteric lymph nodes. In the first 24 h after injection, labelled cells were found in large numbers in the mesenteric lymph nodes, initially clustered around the post-capillary venules and in the looser connective tissue in the marginal sinuses, later spread throughout the lymphoid tissue. They were never found in lymphoid follicles. At 21 h, 14 of 100 labelled cells were small lymphocytes in pairs, as was found in the spleen. It was not possible to interpret grain counts over these cells, but the impression was reached that these cells in pairs had fewer grains than cells examined at earlier times which would support the suggestion that they had divided in the nodes. No labelled cells were seen in mitosis in these sites however. At 3 days all the labelled cells in the mesenteric lymph nodes were small, 5 - 8 μ in diameter.

Labelled cells in thymus. Some labelled lymphocytes were found in thymic tissue 12 and 21 h after injection. In sections of pancreas, which were used as a control, only one labelled cell was seen at any time. It thus seemed that slightly more labelled large lymphocytes migrated into the thymus than could be accounted for by non-specific effects.

The later fate of transferred labelled large lymphocytes.

An experiment was done to study the long term fate of labelled large lymphocytes. 4.0×10^7 labelled large thoracic duct lymphoctyes from each of two rats with oral trichinosis were transferred to 2 normal rats. The second rats were left for 9 days and then given oral trichinosis and labelled cells were looked for in thoracic duct lymph and other tissues four days later.

Autoradiographs of the second rats' thoracic duct lymph showed that all of 50 labelled cells found were small lymphocytes. In 2 spleen sections only 6 labelled cells were found, and five of these were large and pyroninophilic. In 3 sections of jejunum, only 9 labelled cells were seen and 3 of these were large pyroninophilic lymphocytes.

This experiment suggested that the majority of large pyroninophilic lymphocytes in the thoracic duct lymph of rats with oral trichinosis are short lived or continue to divide repeatedly until their label has disappeared, as very few labelled cells were found in recipient rats 13 days after transfer. Fifty-eight of the 65 labelled cells found were small lymphocytes, and the majority of these cells were in the recirculating pool of small lymphocytes. These recirculating small lymphocytes may contain 'memory' to the antigen challenge with larvae (Gowans & Uhr, 1966).

Lymphocytes in inoculum				Recipient rats*	
Cannulation after infection (days)	Total No. x 10^8	% viable large	Label used	Time Lymphocytes injected	Time Rats Killed
4	6.5	25	Tritiated Thymidine	+ 1.5 hours	+ 4 hours
3.5	4.2	7		+ 45 min	+ 3 hours
3.5	5.0	10		- 3 min	+ 3 hours
3.5	6.2	Not done		- 3 min	+ $3\frac{1}{2}$ hours
Normal	4.2	0**	Tritiated	+ 1.5 hours	+ 6 hours
Normal	6.2	0**	Adenosine	- 3 min	+ 3 hours

* Trichinella spiralis larvae were injected into recipient rats at zero time.

** The large lymphocytes were killed by incubation for 12 hours in an unfavourable medium, (p. 43).

Table 6.2. The characteristics of labelled large or small lymphocytes injected into recipient rats which were also given larvae intravenously.

Distribution of labelled large and small thoracic duct lymphocytes in rats given Trichinella spiralis larvae intravenously.

Many mononuclear cells appeared in the inflammatory reaction in lung produced by injecting larvae intravenously (p. 98). To test the possibility that some of these mononuclear cells were lymphocytes, rats were injected intravenously with labelled large or small thoracic duct lymphocytes at various times before or after the injection of larvae. Autoradiographs of tissue were studied to see if labelled lymphocytes appeared in the inflammatory sites.

Four rats received 3H-thymidine labelled large lymphocytes, and 2 other rats were injected with 3H-adenosine labelled small lymphocytes. There were very few labelled large lymphocytes in the inocula of labelled small lymphocytes as virtually all the large lymphocytes had been killed previously by incubation for 12 h in an unfavourable environment (see p. 43). The sizes of the inocula and the times injected are shown in Table 6.2.

Sites of inflammation in lung. Sections of lung showed no labelled lymphocytes at any time in the inflammatory reactions. A few labelled cells were seen in other parts of the lung unrelated to the larvae. This experiment provided no evidence that signficant numbers of mononuclear cells in the inflammatory reactions in lung caused by larvae are lymphocytes.

	Percentage transferred radioactivity in recipient rats (mean ± S.D.)		
Donor:	Trichinosis	Trichinosis	Normal
Recipient:	Normal	Trichinosis	Normal
Tissue in recipient			
Liver	19.5 ± 2.0	19.3 ± 1.3	17.0 ± 1.2
Spleen	14.1 ± 0.4	14.5 ± 1.1	17.7 ± 0.7
Mesenteric node	3.9 ± 0.2	5.2 ± 0.7	7.0 ± 0.7
Neck nodes	2.4 ± 0.5	2.0 ± 0.2	5.1 ± 0.1
Ileum	3.0 ± 0.7	3.4 ± 0.4	3.7 ± 0.8
Jejunum	2.3 ± 0.9	3.3 ± 1.1	3.5 ± 0.4
Blood	0.9 ± 0.04	0.9 ± 0.04	3.3 ± 0.2
Colon, caecum	3.2 ± 0.3	2.4 ± 0.2	2.2 ± 0.4
Heart, lungs	2.9 ± 0.2	2.7 ± 0.2	2.2 ± 0.2
I Femur	0.4 ± 0.1	0.4 ± 0.03	0.2 ± 0.1
Thymus	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.2
Diaphragm	0.3 ± 0.3	0.2 ± 0.2	0.2 ± 0.1

Table 6.3. The percentage injected ^{51}Chromium labelled thoracic duct lymphocytes found in several tissues and organs of rats 22 h after the lymphocytes were injected intravenously.

Less than 0.5% of the radioactivity was found in each femur.

Other tissues. 3H-Thymidine labelled large lymphocytes were found in the jejunum, ilium, abdominal lymph nodes and bone marrow as described in the last section. Labelled small lymphocytes were also distributed in lymphoid areas and intestine in the same areas as labelled large lymphocytes had been found.

Distribution of ^{51}Chromium labelled lymphocytes.

As tissue autoradiography allowed only approximate estimates of the distribution of lymphocytes in recipient rats to be made, ^{51}Cr labelled lymphocytes were used to provide quantitative measurements.

Two x 10^7 ^{51}Cr labelled normal thoracic duct lymphocytes were injected into 3 normal syngeneic recipients which were killed 22 h later and the % of the injected ^{51}Cr in each tissue was counted. Two x 10^7 lymphocytes, 29% of which were large pyroninophilic cells were obtained from the thoracic ducts of rats orally infected with larvae 4 days before. The lymphocytes were labelled with ^{51}Cr and injected intravenously into 3 normal syngeneic rats, and 3 syngeneic rats with oral trichinosis induced 4 days previously. The distribution of radioactivity 22 h later is shown in Table 6.3.

These experiments showed that the femur was not a preferred site for emigration of thoracic duct lymphocytes from the blood, but

slightly more radioactivity was found in the marrow when inocula containing 29% large pyroninophilic lymphocytes were injected. The distribution of labelled thoracic duct lymphocytes was not affected by the presence of the inflammatory response to Trichinella spiralis larvae in the intestine. These findings in relation to eosinopoiesis and eosinophil accumulation, are discussed in the next Chapter.

CHAPTER 7

GENERAL DISCUSSION

Introduction

In the previous chapters experiments have been described which set out to study eosinophils from several points of view, using techniques which have proved valuable in investigations of mechanisms of cell production, kinetics of cells *in vivo*, and responses to immunological stimuli.

The experiments showed that in rats given intravenous *Trichinella spiralis* larvae, there was a marked proliferation of eosinophils in bone marrow, followed by a peripheral blood eosinophilia. It was possible to study the response in the marrow by making autoradiographs of Cytocentrifuge preparations which were stained with haematoxylin and Biebrich scarlet. The kinetics of blood eosinophils during the eosinophilia were also studied, and attempts were made to find out how large pyroninophilic lymphocytes initiated eosinopoiesis. This Chapter discusses these findings and attempts to relate them together.

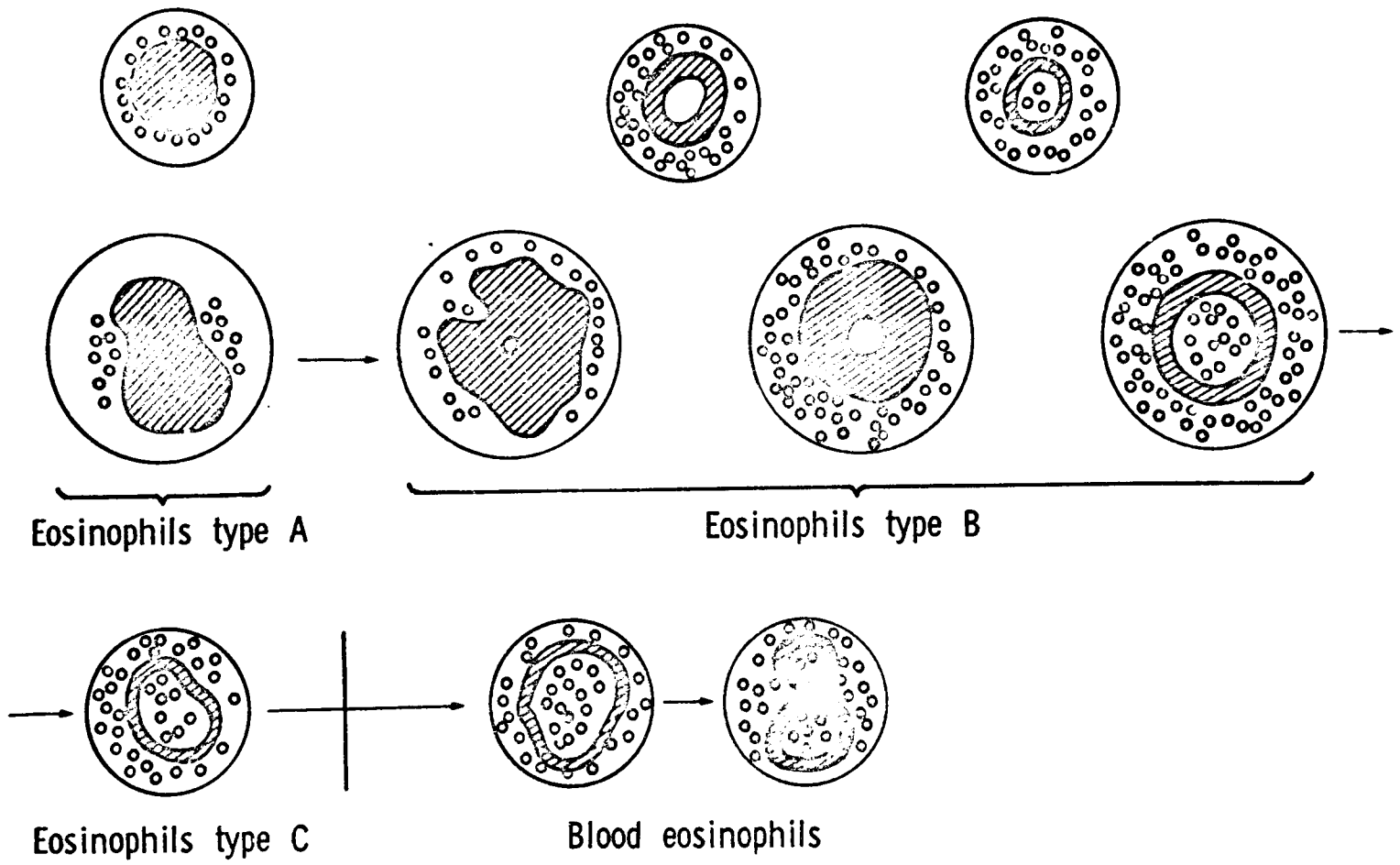


Figure 7.1. Types of eosinophils in rat bone marrow. The most primitive eosinophils (type A) have no central nuclear hole, which is acquired when the cell is more mature. Eosinophils type A also have a basophilic cytoplasm and less granules than mature cells. Eosinophil size did not correlate with maturity. Eosinophils type C did not divide and appeared identical to blood eosinophils.

The kinetics of eosinophil production

Eosinophils in bone marrow. There have been several suggestions about the types of eosinophils in rat marrow (Endicott & Ott, 1945; Hulse, 1964; Alexander, Monet, LoBue & Gordon, 1969). Here 3 types of eosinophils were defined, which were classified by morphological criteria as eosinophils type A, B and C (Figure 7.1).

Eosinophils types A and B were in the proliferating compartment as they were pulse-labelled by 3H-thymidine. Eosinophils type B were morphologically closely related to type C and contained more eosinophil granules than type A. Sequential studies of the labelling of eosinophils types A and B in normal rats showed that labelled eosinophils type B were still being produced when the number of labelled eosinophils type A was declining. When considered together, these findings made it probable that the development sequence was eosinophil type A to B to C. Similar conclusions were reached by Sin & Sainte-Marie (1965) who studied eosinophil development in granulocyte 'islands' in rat thymus, using morphological criteria only.

Alexander, Monette, LoBue & Gordon (1969) were unable to classify the smallest marrow eosinophils, as they were not labelled 1 h after injections of 3H-thymidine. The problem was clarified by making

measurements of the diameter of dividing eosinophils, when it was found that the smallest eosinophils had just divided, and so were in G_1 when DNA synthesis is not taking place. Cronkite (1969) has found that in man also, neutrophil size is smallest immediately after cell division, and mature neutrophils are not smaller than immature neutrophils.

Eosinophils in marrow and spleen. Differential counts of eosinophils in bone marrow of normal rats showed that the compartment of eosinophils type C was small relative to types A and B. This may be a peculiarity of the rat, as in other animals, such as the guinea pig, as many as 75% of marrow eosinophils are band and segmented forms (Hudson, 1968). Many reports on eosinophils in rat spleen have suggested that eosinophils are produced there (e.g. Rytömaa, 1960), and Foot (1965) found that 14% of splenic eosinophils were labelled 12 h after starting a continuous infusion of 3H-thymidine, 2 days before eosinophils were found to be labelled in other sites outside the marrow.

But in the present study no dividing eosinophils were found in the spleen and no splenic eosinophils were found to be labelled 1 h after injection of 3H-thymidine. But 15% of the splenic eosinophils

were labelled 40 h after a single injection of 3H-thymidine, before labelled eosinophils appeared in the blood. This meant that eosinophils type C must have left the marrow after they were formed, and matured in the spleen before they became circulating eosinophils. A similar phenomenon has been described in man, in whom platelets also mature in the spleen (Shulmann, Watkins, Itscoitz & Students,1968).

Diurnal variations in eosinophil count. There was a diurnal variation in eosinophil counts which were usually highest in the morning and lowest at night (see Figure 4.2 and Basten, 1970).. This may be due to a steroid effect on circulating eosinophil half-life or there may be a diurnal variation in the number of newly formed eosinophils released into the blood. Support for the second possibility was derived from experiments in stimulated rats given single injections of 3H-thymidine. There was found to be an interval of 24 hours between the first appearance of labelled eosinophils in the blood and the appearance of a second cohort of labelled cells (Figure 4.4.). A 24 hour cyclical emergence of labelled eosinophils into the blood of normal rats after single injections of 3H-thymidine has been reported also by Anderson & Bro-Rasmussen (1968).

Mechanisms of increased eosinophil production.

Theoretically, increased production of bone marrow eosinophils could come about by a number of mechanisms.

1. Increased numbers of eosinophils produced.
   (a) Increased stem cell entry into eosinophil production
   (b) Increased number of cell divisions in the dividing compartment.
   (c) Stimulation of eosinophils in G_o to enter the dividing compartment.
2. Decreased 'ineffective' production of eosinophils.
3. 'Transformation' of other cells into eosinophils.

The evidence produced here showed that increased eosinophil production took place by increased numbers of divisions taking place in the compartment of dividing eosinophils in the marrow. Some of the other possible mechanisms were excluded on a number of grounds. Abnormal eosinophils were studied in the bone marrow in view of the suggestion that increased granulocyte production might occur by a decrease in ineffective production as has been invoked for increased human neutrophil production by Patt & Maloney (1963-4). Abnormal eosinophils were more frequent in stimulated rats, but the number was very small. There was no sign that eosinophils were being destroyed in any marrow preparations, except when the cells were morphologically abnormal.

Despite the fact that bone marrow stem cells are morphologically similar to lymphoid cells (Wu, Till, Siminovitch & McCullock, 1968; Miller, 1969; Haskill & Moore, 1970), there was no evidence that lymphocytes transformed into eosinophils in experiments in which labelled thoracic duct lymphocytes of various kinds were transferred into recipient rats.

Comparisons with erythrocyte and neutrophil production. As erythrocyte and neutrophil production have been extensively studied, it is important to compare the mechanisms proposed here for the regulation of eosinophil production, with those known to be involved in erythropoiesis, and neutrophil production.

Increased eosinophil production might come about by a mechanism which is known to be involved in erythrocyte production: by alteration in the flow of stem cells into the proliferative compartment, (Lajtha, 1963; 1967; Quastler, 1963; Morse, Rencricca & Stohlman, 1970). For each additional cell introduced at this early stage, 2^n additional cells would be produced where n is the number of divisions the cell undergoes. An additional regulatory process involves a committed cell, the 'erythropoietin sensitive cell' (Bruce & McCulloch, 1964). In chronic anaemia in rats induced by phenylhydrazine, flow from the unrecognisable to the recognisable erythroid dividing compartment is

increased by a factor of about 4 (Tarbutt, 1969). However, the rate and extent of erythrocyte production is also altered during acute and chronic anaemia by a second mechanism involving an increase in the number of divisions in the proliferative compartment, combined with a shortening of cell cycle times (Hanna, 1968; Hanna, Tarbutt & Lamerton, 1969; Tarbutt, 1969).

Less is known about the kinetics of neutrophil production, which has been studied principally in man and dogs (Cronkite & Vincent, 1969). Kinetic studies of neutrophil production in infections have not been done until recently. In four patients with acute infections the myelocyte to blood transit times, and the cell cycle times were not altered, (Bishop & Athens, 1970) but this work has yet to be confirmed, and studies need to be done on stimulated bone marrow itself, before a complete picture can emerge of the mechanisms for increased neutrophil production.

Slow cyclical variations in blood levels of neutrophils in man have been described, and considered to be due to feed-back control of neutrophil production by levels of neutrophils outside the marrow (Morley & Stohlman, 1970). This slow control may regulate the flow of stem cells into the proliferative pool. It would be difficult to demonstrate this phenomenon in the case of the eosinophil in S.P.F. rats, in view of their small number, but if it occurs, the periodicity would be about 7 days.

The results of the kinetic study of eosinophil production in rats described in this thesis showed that the mechanisms by which rapid increases in eosinophil production occur, have many similarities with the response of erythrocyte precursors to acute demand. Increased eosinophil production was associated with an increased number of divisions in the dividing compartment with shorter cell cycle times. The rate of stem cell entry was not measured, but it may not have changed in the rats in which increased production was of only short duration. However it is possible that the very high eosinophilia found in occasional rats ten days after stimulation was due to an increase in the rate of stem cell entry.

Regulation of eosinophil cell cycle times. The cell cycle times of rat eosinophils can be compared with other cell types (Baserga, 1968). In rapidly dividing tissues, such as rat erythroid precursors and intestinal mucosa, T_C is about 8 - 11 h and T_S is about 6 - 8 h, and on stimulation T_{G_1} and T_S are shortened. When eosinophil precursors were stimulated, T_{G_1} was reduced from a median of 9 h to 0.7 h and T_S from 18.5 h to 7.3 h. T_{G_2} altered from 2.8 h to 1.9 h. The change in eosinophil T_{G_1} was intermediate in extent between the changes found in continual renewal systems, such as the rat erythroid series, and conditional renewal systems, such as the liver or kidney,

where proliferation is normally very slow but may become rapid when stimulated (Lamerton, 1969).

The mechanism of control of the rate of cell division is not known, but may involve stimulation of cells in G_1. On stimulation these cells enter an activated state of G_1 in which there is considerable biochemical activity (Baserga, 1968). One site of action of the eosinophil stimulus may be on eosinophil precursors in G_1. The concept that precursor cells may be in a resting state (G_o) has been applied to stem cells (Lajtha, Gilbert, Porte Alexanian, 1963-4). As eosinophil stem cells have not been studied, it is not known whether eosinophil precursors also have a G_o state.

The delay between stimulus and response. There was a delay of 23 h after injection of larvae before the percentage of bone marrow eosinophil began to rise. This delay may be because several events need to take place in sequence: lodgement of larvae in the lungs, contact with tissues, and production and distribution of one or more stimuli. If the stimulus to increased production was produced when lymphocytes became sensitized, the autoradiographic study would suggest that several hours delay could be explained by the time necessary for lymphocytes to travel to tissues and divide.

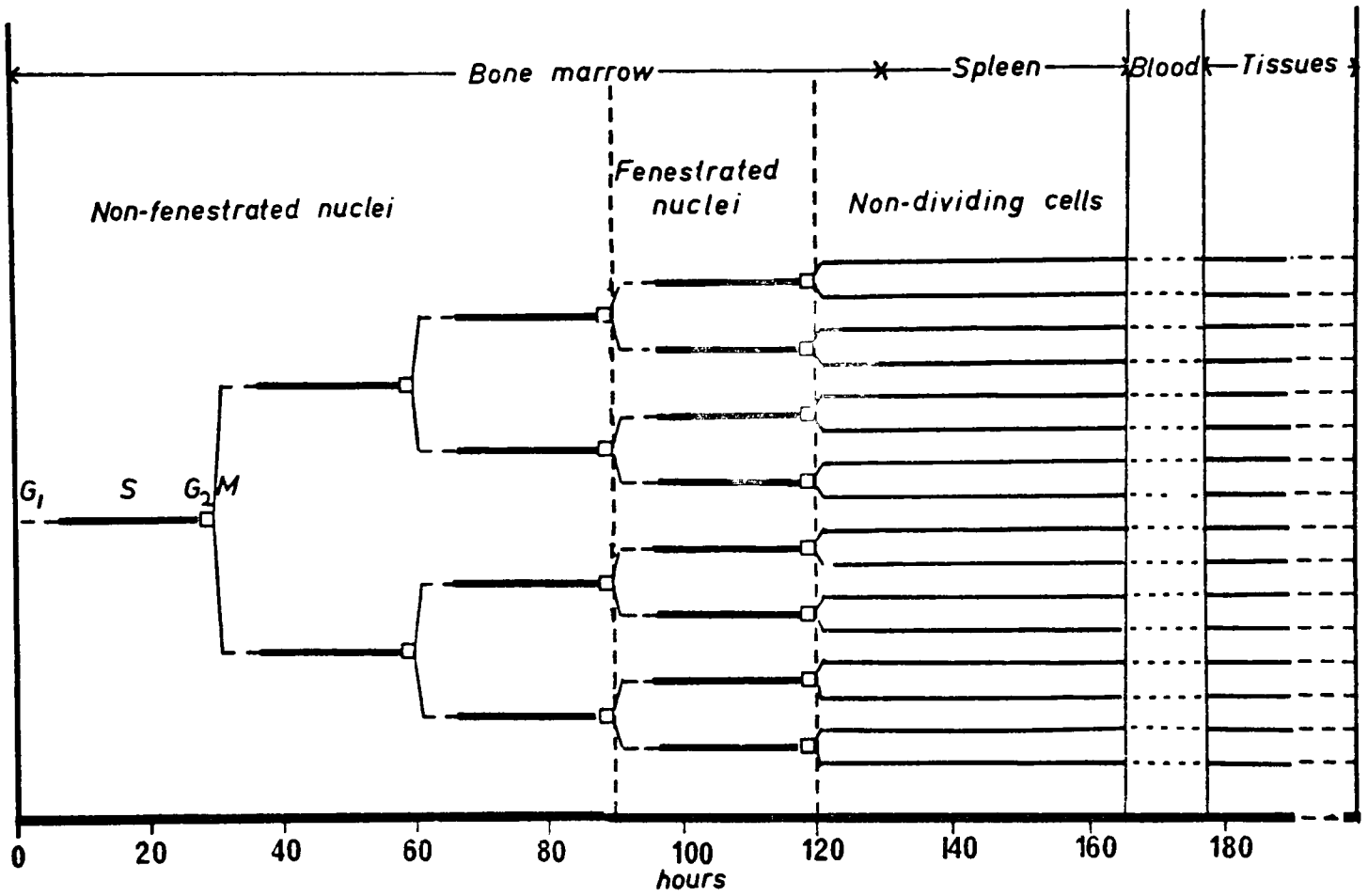


Figure 7.2. Prposed pathway for eosinophil production in normal rats. Doubling divisions of eosinophil precursors are shown to take place in the marrow, from non-fenestrated to fenestrated forms, which then mature in the spleen before entering the blood and emerging into the tissues.

In many other tissues, including the rat kidney (Saetren, 1970), there is a delay between stimulation and response of about 18-36 h (Baserga, 1968; Lamerton, 1969). This may be due to the time interval needed for synthesis of enzymes necessary for S. In mouse skin where an inhibitor of epidermal cell proliferation has been described ('chalone') (Iversen, 1969), the delay between stimulus and response is 3-4 h only. 'Chalones' have been postulated to be involved in the control of mouse granulocyte production (Rytömaa & Kiviniemi, 1968), but recent experiments have shown that mouse granulocyte 'chalone' does not inhibit the development of mouse colony forming units in the spleen, or suppress the growth of granulocyte colonies in vitro (Lajtha, 1970).

The model of eosinophil production in bone marrow. The results of the kinetic study of eosinophil production in bone marrow were used to calculate several parameters involved in the generation of mature circulating eosinophils. When these calculations and the times for eosinophil emergence and transit in the blood are put together, a model of eosinophil production in normal rats can be produced, (Figure 7.2). A similar model of neutrophil production in man has been produced by Cronkite (1969). In the model presented here, 16 mature progeny are derived from each earliest recognisable

eosinophil precursor. In stimulated rats, when eosinophils divide an additional 5-6 times, with shortening of cell cycle times to 1/3rd normal, over a thousand progeny would be produced during the same period.

Lymphocytes and eosinopoiesis.

There has been a great deal of interest in the last few years on the cooperative role of lymphocytes in the induction of immunological processes (Miller & Mitchell, 1969; Davies, 1969). Some of the experiments done here can be seen in relation to this work. Experiments have been described which set out to study the way in which lymphocytes stimulate eosinophil production, as a continuation of the work of Basten & Beeson (1970), who showed that large pyroninophilic lymphocytes from the thoracic ducts of rats with trichinosis induce a delayed eosinophilia in normal syngeneic rats. These lymphocytes were found to localise in tissues and divide into small lymphocytes, or become plasma cells. The distribution was not different from that found for large pyroninophilic lymphocytes from uninfected rats as previously described by Gowans & Knight (1964). However a slightly higher proportion of lymphocytes from rats with trichinosis was found in bone marrow. This raised the possibility that lymphocyte:eosinophil contact might occur in this site.

It is not known where cooperation between thymic processed and bone marrow derived lymphocytes takes place in the induction of antibody formation, but it has been suggested that this may occur in the spleen (Claman & Chaperon, 1969). However, as there is a traffic of lymphocytes through bone marrow of rats (Bond, Feinendegan, Heinze & Cottier, 1963-4; Everett, Caffrey & Reike, 1963-4; Scott & Howard, 1970), it is possible that bone marrow may provide an important site for lymphocyte cooperation. The finding that lymphocytes which are involved in the initiation of eosinophil production can settle in bone marrow provides some evidence that cooperation between large pyroninophilic lymphocytes and eosinophil precursors may occur there, resulting in increased eosinophil production. This would mean that eosinophil production would provide some similarities with bone marrow derived lymphocyte activation. As most of the transferred lymphocytes settled in sites outside the bone marrow it would be necessary to postulate that there was a small sub-population of lymphocytes in the thoracic duct lymph which would localise in bone marrow and initiate eosinophil production. The experiments reported here have not settled this important question, or excluded the possibility that there are long range processes which initiate eosinophil production, but they do show that short range processes could occur. If this is the mechanism

responsible for initiating increased eosinophil production, it would account for the consistent failure to find a stimulus in plasma (Basten, 1970).

There is evidence that soluble serum factors are involved in the control of granulocyte production (McCulloch, 1968). These factors influence the growth of colonies of granulocytes derived from mouse marrow cells cultured in vitro (Bradley & Metcalf, 1966). Colony stimulating factors, which may act as regulators of granulopoesis in vivo and inhibitors have been described (Chan & Metcalfe, 1970; Paran, Ichikawa & Sachs, 1969). However there is no evidence at present that there are any similar factors which regulate eosinophil production.

Lymph nodes showed a marked lymphocyte proliferation in response to stimulation with Trichinella spiralis larvae. The thymus dependant areas were involved (Waksman, Arnason & Janković, 1962; Parrott, de Sousa & East, 1966) and the medullary areas showed a marked lymphocytic proliferation. The importance of the thymic processed lymphocytes has been demonstrated by Walls, Basten, Leuchars & Davies (1970) who found that in mice, initiation of eosinophil production requires the presence of an intact thymus.

This adds weight to the suggestion that eosinophils in the bone marrow respond to stimulation by thymic processed lymphocytes, in much the same way as bone marrow derived lymphocytes cooperate with thymus processed lymphocytes in antibody production. If this proves to be the case, then this kinetic study of eosinophil production may provide an analagous process to the events which follow the interaction of two types of cooperating lymphocytes.

Regulation of circulating eosinophil numbers.

A number of experiments were done to study the factors regulating eosinophil concentration in the blood. 3H-thymidine labelling proved very suitable for following the flow of bone marrow cells from the time when they have finished dividing until they enter the tissue. It was found that single injection of 3H-thymidine in stimulated rats resulted in the labelling of 80% of blood eosinophils. There are several possible mechanisms for the high percentage labelling, including re-utilization of label but it seems most likely that where the eosinophil half-life in the blood is less than the generation-time in the marrow, maximal percentage blood eosinophil labelling represents the ratio T_S/T_C of the earliest bone marrow eosinophil precursors. This high proportion of labelled eosinophils has also been reported in normal rats (Anderson & Bro-Rasmussen, 1968) but these

animals may not have been S.P.F. In the normal S.P.F. rats studied here, only 40% of the blood eosinophils were labelled by single injections of 3H-thymidine.

Eosinophil emergence time. In normal rats eosinophils first appeared in the blood about 40 h after injection of 3H-thymidine. Foot (1965) found T_E was 67 h in restrained PVG/C rats. But in unrestrained rats given single injections of 3H-thymidine, Bro-Rasmussen, Anderson & Henricksen (1967) showed that T_E was 36 h in Leo rats, which agreed with the findings here. It was found that eosinophil T_E was shortened to 17 h, 2 days after injection of larvae, but neutrophil emergence was not affected, which showed that the emergence times of the two types of granulocytes were regulated independently. The emergence times of neutrophils in rats with pyogenic infections have not been studied, but in man, Cronkite (1969) has shown that neutrophil T_E was reduced from 6 to 2 days in a patient with pneumonia.

Blenkinsopp (1966) reported that T_E was not altered in rats given a second injection of tetanus toxoid, but it was not known whether the second injection altered eosinophil counts or turnover. Cohen, N.S., LoBue & Gordon (1967) found no change in T_E when rats had repeated peritoneal lavage which removed many eosinophils, but as labelled eosinophils were found in tissues 12-16 h after the injections of 3H-thymidine, eosinophil T_E must have been shortened.

Hudson (1968) has described a "marrow reserve pool" of eosinophils in guinea pig bone marrow. By counting the content of eosinophils in single cell suspensions of bone marrow, he showed that a population of mature eosinophils disappeared from the marrow following the last of several injections of foreign proteins. In the present study rapid mobilisation of newly formed eosinophils was achieved by speeding up their passage through the maturation compartment, rather than by releasing a 'pool' of marrow eosinophils.

Eosinophil release. The studies on lung histology showed that eosinophils collect in the lung beginning six hours after injection of larvae. The blood eosinophil count was only slightly altered between 6 and 18 h, so that it was necessary to postulate that eosinophils were being mobilised into the cirulating blood from other sites. New eosinophil production did not take place fast enough to provide these additional cells. This phenomenon of recruitment of eosinophils between the time of injection of larvae and 24 h was termed eosinophil "release", on the assumption that it was due to the release of marginated or maturing eosinophils into the circulation.

A component of plasma, taken from rats 24 h after stimulation induced a doubling of blood eosinophil counts six hours after injection. No similar activity was found in lung or damaged peritoneal eosinophils.

There was no effect of this plasma on blood neutrophil counts.

Gordon, Handler, Siegel, Dornfest & LoBue (1963-4), list many factors which have been found to increase blood eosinophil levels. Bierman (1963-4) also describes substances in tissues ('leucopoeitin G') which have this effect. It is possible that they mobilise eosinophils through a common mechanism by activating a constituent of plasma. Clearly further experiments need to be done to clarify the mechanism of rapid 'release' of eosinophils.

Many papers have described studies on eosinophil accumulation in tissues 12-48 h after stimuli have been applied, and have contained deductions about the mechanisms of the response (e.g. Archer, R.K., 1963; Litt, 1963; Parish & Coombs, 1968; Speirs & Turner, 1969). However these authors have not differentiated between eosinophil production and eosinophil release mechanisms. Although both eosinophil production and eosinophil release may be effected together, the mechanisms for each process are quite different, as has been stressed here. These two processes have not been confused in studies on neutrophils, as knowledge of neutrophil kinetics has been available for some time (see Cronkite, 1969; Cronkite & Vincent, 1969; Athens, 1969).

Eosinophil half-life in blood. The half-life of eosinophils in normal S.P.F. rats was calculated from blood labelling curves and shown

to be about 6 - 7 h. As stress reduces blood eosinophil levels (Blenkinsop & Blenkinsop, 1967) repeated blood sampling was not done at less than 12 h intervals, and in many of the experiments, each rat was only sampled once.

In rats injected with larvae 2 days previously, blood eosinophil half-life remained unchanged, even though many more eosinophils than normal were collected in the lungs. However a decreased half-life of intravascular cells does not necessarily occur while more cells than normal are being removed from the blood into the tissues, when the total number of cells available in the blood is greater than normal. In order to assess the significance of changes in eosinophil half-life in the blood one must also know the sizes of the intravascular and marginated pools at the time the half-life measurement is done. This problem has been discussed in relation to blood neutrophil kinetics in man by Cartwright, Athens, Haab, Raab, Boggs, & Wintrobe (1963-4). As yet no experiments have been done to measure the sizes of the pools of intravascular eosinophils in man or animals. But as eosinophil half-life in rat blood increased when the peripheral blood eosinophilia occurred, the sizes of both marginated and circulating pools of eosinophils must have increased.

The rate of removal from the blood of the most heavily labelled cohort of eosinophils was exponential in both normal and stimulated rats. This provided direct evidence that eosinophil removal from the blood was a random event, not dependent on the length of time that eosinophils had been present in the blood which confirms the earlier work of Foot, (1963).

Similarly, neutrophil removal from the blood of rats takes place by a random process. This has been shown recently by Gerecke, Schultze & Maurer (1970) who gave continuous infusions of 3H-thymidine to rats and found some labelled neutrophils appeared in sputum when the first neutrophils were beginning to appear in the blood. By measuring the rate of increase of labelled blood neutrophils, they calculated that rat neutrophil T_E was 42 - 48 h and their half-life was 6.0 - 7.9 h when calculated from the rate of disappearance of labelled cells. These figures are very similar for blood eosinophils in rats described here.

Eosinophil accumulation in tissues

The reactions of rat lung to Trichinella spiralis larvae are somewhat similar to those produced when ova of Schistosomes or Ascaris are injected intravenously (von Lichtenberg, 1962). However, larvae

induce a maximal inflammatory response at 24 h, and lesions resolve completely. Ova produce a slower, protracted inflammatory reaction. Eosinophils accumulate in the lesions around larvae at six hours but do not appear before 5 days when ova are present. Domingo & Warren (1967, 1968 a & b) have shown that the reaction to Schistosome ova is a delayed hypersensitivity response. These differences suggest that eosinophil accumulation around ova and larvae are probably notsimilar processes.

Mechanisms of eosinophil accumulation. The role of interactions between lymphocytes and macrophages in the local accumulation of eosinophils is being studied at the present time in our laboratory. However, transferred labelled lymphocytes were not found in the lesions in lung, and larvae coated with macrophages induced a smaller eosinophilia than normal. Lymphocytes are found only rarely in acute inflammatory lesions (Koster & McGregor, 1970), but David, Al-askari, Lawrence & Thomas (1964), Dumonde, Wolstencroft, Panay, Matthew, Morley & Howson, (1969) and MacLennan & Harding (1970) have shown that small numbers of lymphocytes can affect a larger number of other cells in a variety of ways. Therefore, these experiments did not exclude the possibility that a small number of very active lymphocytes accumulated in the lesions and induced eosinophil accumulation.

In intestine, 12% of transferred labelled large lymphocytes were found adjacent to eosinophils. This has not been reported previously, probably because methyl-green pyronin, which does not distinguish eosinophils from other cells, has been used. But with haematoxylin and Biebrich scarlet, a spatial relationship between labelled lymphocytes and eosinophils stood out clearly.

This relationship may have been because both large pyroninophilic lymphocytes and eosinophils are common in the lamina propria of the intestine. Alternatively the lymphocytes may have been secreting a substance which attracts eosinophils, similar to the 'leucotactic' factor described by Ward, Remold & David (1969) but there is no evidence on this possibility. If this relationship is not fortuitous, eosinophils and large lymphocytes might share a common role in the small intestine. This suggestion needs to be studied further in view of the finding of Sundell (1958) that following an injection of corticosteroids, which are lympholytic, many eosinophils accumulate in the small intestine. Most lymphocytes in the intestines secrete IgA (Vaerman & Heremans, 1970) and in rabbits with trichinosis, there are many IgA containing lymphocytes in the intestine (Crandall, Cebra & Crandall, 1966). IgA is the main immunoglobulin of external secretions, and eosinophils are distributed in the tissues in contact with external surfaces so that it is possible

to relate them together on thesegrounds, but experimentally, possible links between IgA and eosinophils remain unexplored.

The temptation to conclude this discussion with other hypotheses about the possible functions for eosinophils is easy to resist, mainly because very little is known about eosinophil functions in tissues where they are normally found. But it is hoped that further studies of the activities of eosinophils will be assisted by the work discussed here on eosinophil kinetics and the mechanisms by which eosinophil production and distribution are regulated.

Summary.

This chapter has discussed the mechanisms of eosinophil production and turnover in the marrow, spleen, and blood. Eosinophil production was shown to be analogous to the processes which are involved in erythrocyte and neutrophil production. The role of stimulated large pyroninophilic lymphocytes was discussed, and it was suggested that the evidence at present available on the mechanisms by which lymphocytes stimulate eosinophil production favour short range processes in the marrow itself. The numbers of eosinophils in blood is considered to be regulated by several distinct processes, which involve a plasma factor and a mechanism by which the emergence time of newly formed eosinophils is reduced.

It is hoped that this quantitative study of eosinophil production will provide a basis for further work on the response and behaviour of eosinophils in normal and abnormal states. This may lead to the solution of the enigma which faces daily those who work on eosinophils - their function.

APPENDIX

NEUTROPHIL PRODUCTION

Introduction

Many stimuli increase neutrophil synthesis in bone marrow. Despite this, there has been a consistant failure to explain how neutrophil production in bone marrow is initiated, (Patt & Maloney, 1963; Cronkite & Fliedner, 1964).

Lymphocytes and neutrophil production

As Basten & Beeson (1970) had shown that lymphocytes would transfer an eosinophilic stimulus from rats with trichinosis to normal rats, it was decided to see whether lymphocytes in lymph draining an inflammatory reaction could transfer a neutrophil stimulus to normal rats.

The inflammatory lesion chosen was acute pyelonephritis induced after ureteric ligation by giving E. coli intravenously. A few days later thoracic duct lymphocytes were collected and transferred to

normal recipient rats. The effect of the transferred lymphocytes on the recipients' blood neutrophils was followed with daily blood counts for three weeks.

Methods. Pyelonephritis was induced in 200 g male PVG/C syngeneic rats by the method of Guze & Beeson (1956). A strain of E. coli was derived from the urinary tract of a patient with acute pyelonephritis. It was passaged twice through rats by inducing pyelonephritis and then stock cultures were made in broth and stored at -20°C. A fresh batch of the original culture was taken for each experiment to obviate variability of the organisms from experiment to experiment. Bacteria were counted in broth both by direct microscopy using a modified Neubauer counting chamber, and by colony counts made by spreading out measured volumes of serial dilutions of the bacterial suspensions on blood agar plates. Between 0.5 to 9 x 10^7 bacteria given intravenously induced pyelonephritis 24 h after a ureter had been ligated.

Rats were anaesthetised with ether and the hair over the abdomen was shaved. The skin was cleaned with ether, and using sterile instruments, but no other special precautions, the abdomen was opened through a right subcostal incision. Sterile 1" tape was used to pack the cavity exposing the lower pole of the right kidney and the ureter

Donor lymphocytes and cell free lymph				Recipient rats	
Type of rat	Period infected (days)	No of lymphocytes in inoculum x 10^8 mean	range	No of rats	No with blood neutrophils $> 6{,}000/mm^3$ 2-21 days later
Normal	-	3.7	1.9 - 7.5	10	1
E. coli Pyelonephritis	1 - 2	3.6	2.9 - 5.4	3	1
	2 - 6	3.9	1.4 - 7.1	6	2
	2 - 6	10 ml cell free lymph		2	0
	2 - 6	3.7	2.3 - 4.6*	3	0
	2 - 6	3.0	1.7 - 4.4**	2	0
Hydronephrosis	-	3.2	1.3 - 3.7	7	0
Parker 199	-	2 ml culture medium		6	2

* The transferred lymph contained endotoxin, as the recipient rats developed high blood neutrophil counts 1 day later.

** The donor rats were treated with streptomycin intravenously during the period when the lymph was collected.

Table A.1. Characteristics of donated rat thoracic duct lymphocytes and cell free lymph with blood neutrophil responses in recipient rats.

This experiment was designed to study whether lymphocytes from rats with pyelonephritis could induce a neutrophil leucoytosis in normal recipient rats.

which ran along the medial side of the renal fat. The ureteric blood vessels acted as useful markers. A silk 4/0 suture was tied firmly around the ureter. Then the pack was removed and the abdomen was closed with two layers of silk sutures. Twenty-four hours later the rats were given about 7×10^7 E. coli in broth intravenously. During the next 4 days the blood neutrophil counts rose to about 20,000/mm^3. The thoracic ducts were cannulated at various time intervals after injection of E. coli. The infected right kidney was not disturbed. Dulbecco A solution containing 20 units heparin/ml and 100 µg streptomycin/ml was used as lymph collecting fluid. 10 µg/ml streptomycin was bacteri c idal in broth to the E. coli strain used. The donor rats were infused with saline, 2 units of heparin in 2 ml/h, but were not given antibiotics.

The lymphocytes were collected for 12 h periods and then washed twice in Parker 199 and after counting and taking smears for staining, the cells were injected into normal recipient rats. The recipient rats had blood neutrophil counts daily, beginning two days before the cells were transferred. Control experiments were done at the same time by injecting similar rats with other lymphocytes or other media. The time at which the thoracic duct lymph was collected from the donor animals was also varied. The number of lymphocytes injected and the types of solutions used for each experiment is shown in Table A.1.

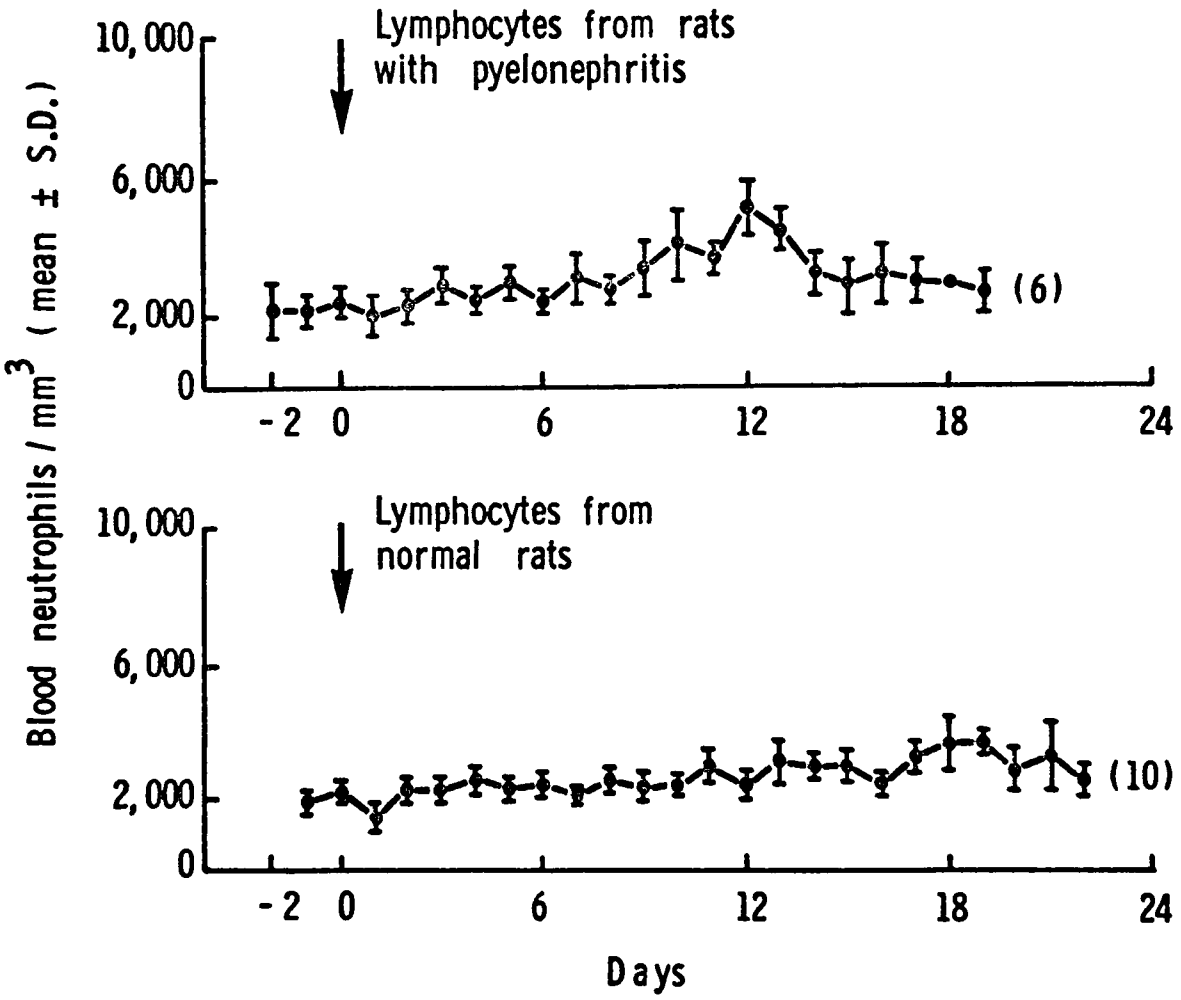


Figure A.1. Blood neutrophils/mm^3 in rats given thoracic duct lymphocytes from either normal syngeneic rats (lower graph) or syngeneic rats with acute pyelonephritis (upper graph). A small, but probably insignificant, increase in blood neutrophil counts occurred on the 12th day in rats given lymphocytes from rats with pyelonephritis.

Results. The initial neutrophil counts ± S.D. in recipient rats for 2 days before transfer of lymphocytes were 2,292 ± 860 (96). Two of 6 rats given lymphocytes from rats with pyeloneophritis had neutrophil counts which reached 6,360 and 7,429/mm^3 on the 12th day after lymphocyte transfer. However 2 rats which received Parker 199 intravenously also developed blood neutrophil counts of 7,052/mm^3, on day 6 and 9,460/mm^3, on day 17, and one rat given normal lymphocytes had 6,631/mm^3, on day 21, and one rat given lymphocytes from rats with one to 2 day old pyelonephritis had 6,210/mm^3, on day 20.

When graphs of the blood neutrophil counts in rats given lymphocytes from either normal rats or rats with 2 - 5 day pyelonephritis were drawn, a slight rise was found in the latter group at day 12, but this was considered to be insignificant, (Figure A.1). It was concluded that lymphocytes in thoracic duct lymph draining a pyelonephritic kidney did not induce neutrophil production on transfer to normal rats.

Granulocytes and neutrophil production.

When a stimulus for neutrophil production was not found in lymphocytes, other experiments were designed to see whether a stimulus could be found in granulocytes or plasma.

Damaged cells are probably released into the blood from tissues when there is an inflammatory reaction, and an attempt was made to study this by making counts of the numbers of abnormal cells in thoracic duct lymph of rats with pyelonephritis.

In rats with pyelonephritis the volume of thoracic duct lymph and the total number of cells in the lymph were not significantly different from those present in normal rats. The lymph also contained normal numbers of large pyroninophilic lymphocytes. However, thoracic duct lymph of rats with pyelonephritis also contained cells not normally found there. These included pyknotic and damaged lymphocytes, neutrophils and some eosinophils (Figure A.2).

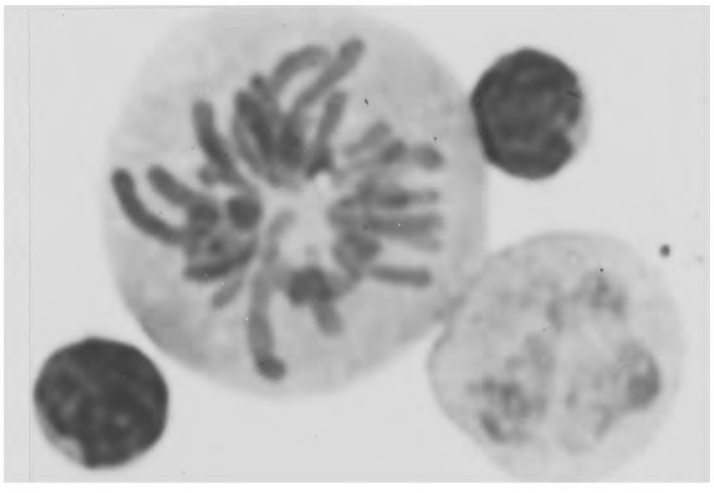


Figure A.2. Some cells found in thoracic duct lymph of a rat with pyelonephritis. The largest is a lymphocyte in mitosis. The smallest cells are small lymphocytes. The cell in the right lower corner is an eosinophil. Leishman's stain; x 900.

In 4 rats with 4 day old pyelonephritis a mean of 5×10^5 neutrophils/h and 6×10^3 eosinophils/h were released into the thoracic duct lymph. In all 4 animals the flow of granulocytes into the lymph was stopped almost completely after a continuous infusion of streptomycin was given intravenously to the rats. The number of neutrophils returned to the blood in this way were very small compared to the total number in the blood. Over a 24 h period the number of neutrophils returning to the blood would only represent 1% of the number of circulating neutrophils if the rat had 10,000 neutrophils/mm^3.

The findings of granulocytes in thoracic duct lymph draining infected kidneys was not surprising. Menkin & Freund (1929) first described the presence of neutrophils in efferent lymph from lymph nodes draining infected skin. Smith & Wood (1949) confirmed also that neutrophils could be found in lymph node afferent lymph.

It was possible that neutrophil accumulation in tissues provided a stimulus for neutrophil synthesis. To test this possibility, large numbers of neutrophils from the blood of donor rats were injected into the skin of normal rats.

Four Harwell rats were given 1 mg endotoxin intravenously and 24 h later, after anticoagulating with 0.5 units of heparin/g, were bled out by the aorta. The blood contained 17,000 - 25,000 neutrophils/mm^3.

White blood cells were separated by incubating for half an hour in a solution of Methocel (Dow Co., Midland, Michigan, U.S.A.) and Triosil 45 (Glaxo Laboratories Ltd., Greenford, Essex) as described by Hulliger & Blazkovec,(1967). After separation of the white cells from red cells, the white cells were washed twice in 10 ml of Eagles' medium. One, 1.25 and 1.9 x 10^8 white blood cells, 50% of which were neutrophils, were given subcutaneously into the back of the neck of three normal rats. Daily neutrophil counts showed that no rise above the normal occurred during the next 7 days.

This experiment suggested that the neutrophil leucocytosis that is produced in response to inflammatory reactions is not initiated by the presence of large numbers of neutrophil leucocytes in tissues.

Plasma and neutrophil production

Many substances induce a neutrophil leucocytosis soon after injection. Endotoxin is particularly effective in inducing this effect of 'neutrophil release' (Boggs, Chervenick, Marsh, Cartwright & Wintrobe, 1968). It was possible that 'neutrophil release' might act as a stimulus for neutrophil production. Two experiments were done to test this.

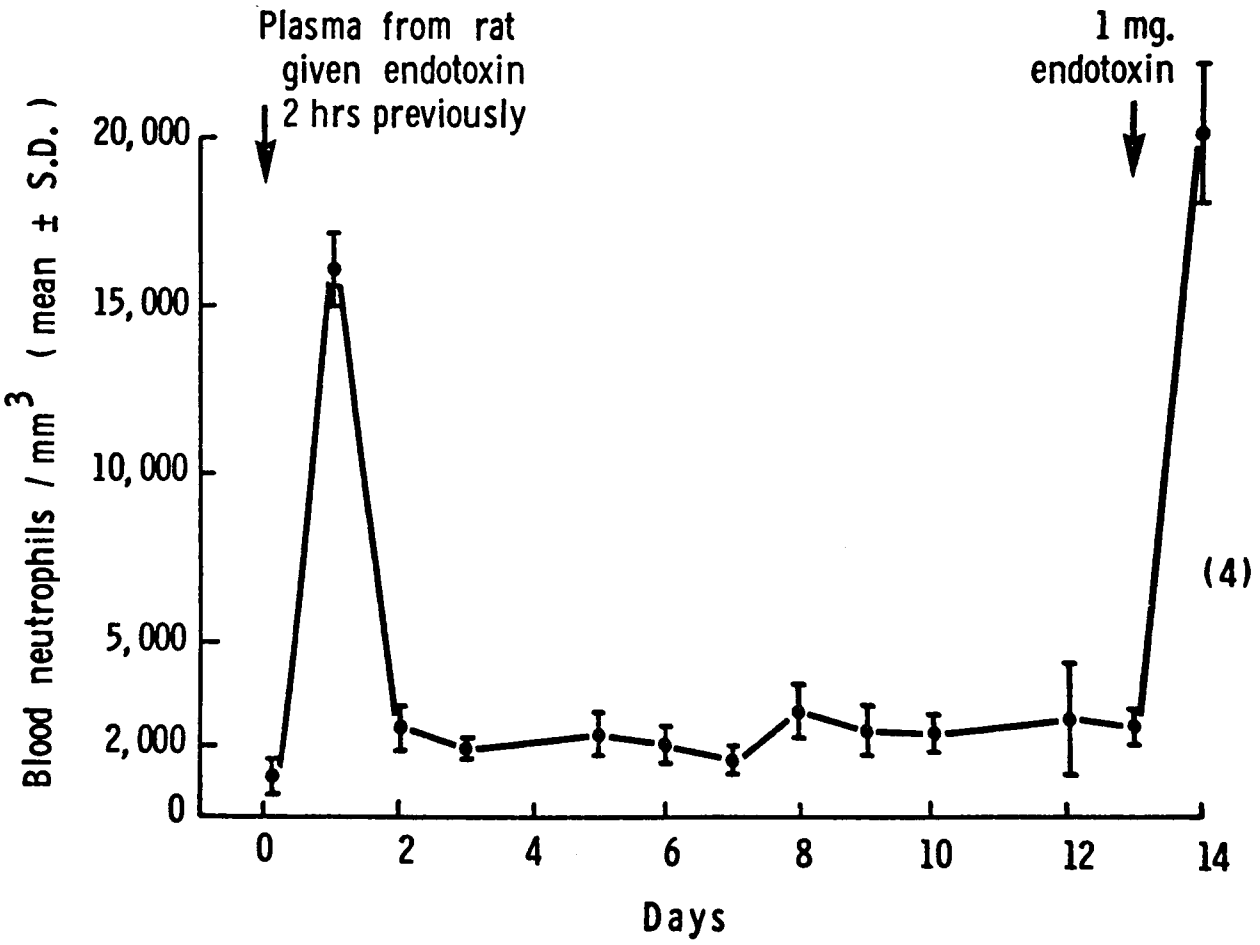


Figure A.3. Blood neutrophil counts in rats given neutrophil releasing plasma and 1 mg endotoxin 2 weeks later.

Endotoxin was injected and after the initial neutrophil leucocytosis blood counts were followed for 2 weeks. One mg endotoxin dissolved in 1 ml saline was given intravenously to 6 200 g rats and a neutrophil leucocytosis occurred 24 h later, returning to normal at 48 h. There was no change in the blood mononuclear cell counts, but eosinophils disappeared from the blood during the 48 h period. Blood neutrophil counts did not rise above the normal level after the first 48 h.

In a second experiment, plasma from rats injected with 1 mg endotoxin 2 h previously, was injected into 4 normal rats. This induced a neutrophil leucocytosis of $16,000/mm^3$ 24 h later. The magnitude of the response to neutrophil 'releasing' plasma was nearly as large as that produced when 1 mg endotoxin itself was injected 13 days later in the same animals. There was no neutrophil leucocytosis in these rats during the 12 days between injections (Figure A.3).

These two experiments suggested that neutrophil release itself had not initiated sufficient additional neutrophil synthesis to be evident in peripheral blood levels.

Discussion

The conclusion was reached that lymphocytes in lymph draining a pyogenic lesion do not transfer a neutrophil stimulus to normal rats. It is unlikely that inadequate numbers of lymphocytes were transferred, as innocula contained as many as 7×10^8 lymphocytes. Strober & Gowans (1965) induced accelerated skin homograft rejection in rats with 7×10^7 sensitized thoracic duct lymphocytes.

Studies of Walls, Basten, Leuchars & Davies (1970) agreed with this general conclusion. They showed that neutrophil production in mice was not inhibited when thymic dependent lymphocytes were not available.

As no stimulation of neutrophil production was found when lymphocytes, plasma or granulocytes were injected into normal rats, it is possible that neutrophil production is regulated in other ways. There have been previous attempts to find a stimulus for neutrophil production in plasma, and positive results were achieved by Nowell (1959) who found that plasma from rats with staphylococcal empyaema increased the mitotic index of bone marrow cells in culture at 20 h, whereas the inflammatory exudate itself was inactive. However it would seem that the mechanisms by which neutrophil production is regulated remain largely unknown.

REFERENCES

ALEXANDER, P., MONETTE, F.C., LoBUE, J., GORDON, A.S. & CHAN, P.-C. (1969). 'Mechanisms of leucocyte production and release. X. Eosinophil proliferation in rats of different ages. Scand. J. Haemat., 6, 319.

ANDERSON, V. & BRO-RASMUSSEN, F. (1968). 'Autoradiographic studies of the kinetics of eosinophils'. Ser. Haemat., 1. 33.

ANDERSON, V., BRO-RASMUSSEN, F. & HOUGAARD, K. (1969). 'Autoradiographic studies of eosinophil kinetics: effect of cortisol'. Cell Tissue Kinet. 2, 139.

ARCHER, G.T., AIR, G., JACKAS, M., MORRELL, B.D. (1965). 'Studies on rat eosinophil peroxidase'. Biochim. biophys. Acta, 99, 96.

ARCHER, G.T. & BLACKWOOD, A. (1965). 'Formation of Charcot-Leyden crystals in human eosinophils and study of the composition of isolated crystals'. J. exp. Med., 122, 173

ARCHER, G.T. & HIRSCH, J.G. (1963a). 'Isolation of granules from eosinophil leucocytes, and study of their enzyme content'. J. exp. Med., 118, 277

ARCHER, G.T. & HIRSCH, J.G. (1963b). 'Motion picture studies on degranulation of horse eosinophils during phagocytosis. J. exp. Med., 118, 287.

ARCHER, R.K. (1963). The Eosinophil Leucocyte. Philadelphia: F.A. Davis Co.

ARCHER, R.K. (1968). 'The eosinophil leucocytes'. Ser. Haemat., 1,3.

ATHENS, J.W. (1969). In 'Human tumor cell kinetics'. ~~In~~ "Granulocyte kinetics in Health and disease" U.S. Dept. of Health, Education and Welfare, Bethesda, Maryland. Nat. Cancer. Inst. Monograph 30, 135

BARRETT, J.C. (1966). 'A mathematical model of the mitotic cycle and its application to the interpretation of percentage labelled mitoses data.' J. nat. Cancer Inst. 37, 44.

BASERGA, R. (1968). 'Biochemistry of the cell cycle: a review'. Cell Tissue Kinet. 1. 167.

BASTEN, A. (1969). 'The mechanisms of eosinophilia in parasitic infestation'. D. Phil. Thesis: University of Oxford.

BASTEN, A. & BEESON, P.B. (1970). 'Mechanism of eosinophilia. II. Role of the lymphocyte'. J. exp. Med. 131, 1288.

BASTEN, A., BOYER, M.H. & BEESON, P.B. (1970). 'Mechanism of eosinophilia. I. Factors affecting the eosinophil response of rats to Trichinella spiralis'. J. exp. Med. 131, 1271.

BENNETT, I.L. (1951). 'Comparison of leukocyte changes produced by pyrogens and by anaphylaxis in the guinea pig'. Proc. Soc. exp. Biol. Med. 77, 772.

BIERMAN, H.R. (1963-4). 'Characteristics of Leukopoietin G in animals and man'. Ann. N.Y. Acad. Sci., 113, 753.

BISHOP, C.R., & ATHENS, J.W. (1970). 'Studies of granulocytopoiesis in abnormal conditions'. In Symposium on Hemopoetic cellular proliferation', p. 229. Ed. by Stohlman, F. London: Grune and Stratton.

BLENKINSOPP, E.C. (1966). 'Eosinophil turnover in the rat during primary and secondary antibody response to tetanus toxoid'. J. Path. Bact. 91, 617.

BLENKINSOPP, E.C. & BLENKINSOPP, W.K. (1967). 'Effect of a glucocorticoid (dexamethasone) on the eosinophils of the rat'. J. Endocr. 37, 463.

BOGGS, D.R., CHERVENICK, P.A., MARSCH, J.C., CARTWRIGHT, G.E., & WINTROBE, M.M. (1968). 'Neutrophil-releasing activity in plasma of dogs injected with endotoxin'. J. Lab. Clin. Med. 72, 177.

BOLLMAN, J.L., CAIN, J.C. & GRINDLAY, J.H. (1948). 'Techniques for the collection of lymph from the liver, small intestine or thoracic duct of the rat'. J. Lab. Clin. Med. 33, 1349.

BOND, V.P., FEINENDEGEN, L.E., HEINZE, E. & COTTIER, H. (1963-4). 'Distribution of transferred tritiated cytidine-labelled leucocytes and red cells in the bone marrow of normal and irradiated rats'. Ann. N.Y. Acad. Sci., 113, 1009.

BOYDEN, S. (1962). 'The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes'. J. exp. Med. 115. 453.

BOYER, M.H., (1970). D. Phil. thesis. University of Oxford.

BOYER, M.H., BASTEN, A. & BEESON, P.B. (1970). 'Mechanism of eosinophilia. III. Suppression of eosinophilia by agents known to modify immune responses'. Blood, 36, 458.

BOYER, M.H., SPRY, C.J.F., BEESON, P.B., & SHELDON, W.H. (1970). 'Mechanism of eosinophilia. IV. The pulmonary lesions resulting from intravenous injection of Trichinella spiralis larvae'. Unpublished.

BRADLEY, T.R. & METCALF, D. (1966). 'The growth of mouse bone marrow cells in vitro'. Aust. J. exp. Biol. Med. Sci., 44, 287.

BRO-RASMUSSEN, F., ANDERSON, V. & HENRIKSEN, O.(1967). 'The kinetics of eosinophil granulocytes in rats. Autoradiographic studies'. Scand. J. Haemat. 4, 81.

BRO-RASMUSSEN, F. & EGEBERG, J. (1966). 'The ultrastructure of eosinophilic granulocytes in the peritoneal cavity of rats following injection of ferritin'. Scand. J. Haemat. 3, 257.

BROWN, T.R. (1898). 'Studies on trichinosis, with especial reference to the eosinophilic cells in the blood and muscle, the origin of these cells and their diagnostic importance.' J. exp. Med. 3, 315.

BRUCE, R.G. (1970). 'The structure and composition of the capsule of Trichinella spiralis in host muscle'. Parasitology, 60, 223.

BRUCE, W.R. & McCULLOCH, E.A. (1964). 'The effect of erythropoietic stimulation on the hemopoietic colony-forming cells of mice'. Blood, 23, 216.

BUDDECKE, E., ESSELLIER, A.F. & MARTI, H.R. (1956). 'Über die chemische Natur der Charcot-Leydenschen Kristall'. Hoppe-Seylers Z. physiol. Chem. 305, 203.

BURKE, W.T. & HARRIS, C. (1959). 'Total cell counts of the bone marrow of normal albino rats from 1 to 50 weeks of age'. Blood, 14, 409.

CAMPBELL, D.H. (1943). 'Relationship of the eosinophile response to factors involved in anaphylaxis'. J. infect. Dis. 72, 42.

CANON, P. (1892). 'Über eosinophile Zellen und Mastzellen im Blute gesunder und Kranker'. Dtsch. med. Wschr. 10, 206.

CARTWRIGHT, G.E., ATHENS, J.W., HAAB, O.P., RAAB, S.O., BOGGS, D.R., & WINTROBE, M.M. (1963-64). 'Blood granulocyte kinetics in conditions associated with granulocytosis'. Ann. N.Y. Acad. Sci., 113, 963.

CHAN, S.H. & METCALF, D. (1970). 'Inhibition of bone marrow colony formation by normal and leukaemic human serum'. Nature (Lond.) 227, 845.

CHAPMAN, J.S. & CLARK, J. (1968a). 'Components of fungi in relation to eosinophilia'. Amer. Rev. resp. Dis. 97, 399.

CHAPMAN, J.S. & CLARK, J. (1968b). 'Chemical stimulus to eosinophils'. Amer. J. clin. Path. 49, 815.

CLAMAN, N.H. & CHAPERON, E.A. (1969). 'Immunological complementation between thymus and marrow cells - A model for the two-cell theory of immunocompetence'. Transplantation Revs. 1, 92.

CLEAVER, J.E. (1967). 'Thymidine metabolism and cell kinetics'. Amsterdam: North-Holland Publishing Co.

CLINE, M.J., HANIFIN, J., & LEHRER, R.I. (1968). 'Phagocytosis by human eosinophils'. Blood, 32, 922.

COHEN, N.W., LoBUE, J. & GORDON, A.S. (1967). 'Mechanisms of leukocyte production and release. VIII. Eosinophil and neutrophil kinetics in rats'. Scand. J. Haemat. 4, 339.

COHEN, S.G. & SAPP, T.M. (1963). 'Experimental eosinophilia. VIII. Cellular responses to altered globulins within cutaneous tissue'. J. Allerg. 36, 415.

CONNELL, J.T. (1968). 'Morphological changes in eosinophils in allergic disease'. J. Allerg. 41, 1.

COTRAN, R.S. & LITT, M. (1969). 'The entry of granule-associated peroxidase into phagocytic vacuoles of eosinophils'. J. exp. Med. 129, 1291.

CRANDALL, R.B., CEBRA, J.J. & CRANDALL, C.A. (1966). The relative prepations of IgG, IgA and IgM containing cells in rabbit tissues during experimental Trichinosis'. Immunology 12, 147.

CRONKITE, E.P. (1969). In Human tumour cell kinetics. U.S. Dept. of Health, Education and Welfare, Bethesda, Maryland. "Kinetics of granulocytopoiesis'. Nat. Cancer. Inst. Monograph, 30, 135.

CRONKITE, E.P., & FLIEDNER, J.M. (1964). 'Granulocytopoiesis'. New Eng. J. Med. 270, 1347 and 1403.

CRONKITE, E.P. & VINCENT, P.C. (1969). 'Granulocytopoiesis'. Ser. Haemat. 2, 3.

DAVID, J.R., AL-ASKARI, S., LAWRENCE, H.S. & THOMAS, L. (1964). 'Delayed hypersensitivity in vitro. I. Specificity of inhibition of cell migration by antigens. J. Immunol. 93, 264.

DAVIES, A.J.S. (1969). 'The thymus and the cellular basis of immunity'. Transplantation Revs. 1, 43.

DIENER, E., SHORTMAN, K. & RUSSELL, P. (1970). 'Induction of immunity and tolerance in vitro in the absence of phagocytic cells'. Nature (Lond.), 225, 731.

DISCOMBE, G. (1946). 'Criteria of eosinophilia'. Lancet, i, 195.

DOMINGO, E.O. & WARREN, K.S. (1967). 'The inhibition of granuloma formation around Schistosoma Mansoni eggs. II. Thymectomy'. Amer. J. Path. 51, 757.

DOMINGO, E.O. & WARREN, K.S. (1968a). 'The inhibition of granuloma formation around Schistosoma Mansoni eggs. III. Heterologous antilymphocyte serum'. Amer. J. Path. 52, 613.

DOMINGO, E.O. & WARREN, K.S. (1968b). 'Endogenous desensitisation. Changing host granulomatous response to Schistosome eggs at different stages of infection with Schistosoma Mansoni.' Amer. J. Path. 52, 369.

DONIACH, I. & PELC, S.R. (1950). 'Autoradiography technique'. Brit. J. Radiol. 23, 184.

DORE, C.F. & BALFOUR, B.M. (1965). 'Cytocentrifuge and blast cell transformation of lymphocytes in culture.' Immunology 9, 403

DRESSER, D.R., TAUB, R.N. & KRANTZ, A.R. (1970). 'The effect of localised injection of adjuvant material on the draining lymph node'. Immunology, 18, 663.

DUMONDE, D.C., WOLSTENCROFT, R.M., PANAY, G.S., MATTHEW, M., MORLEY, J., & HOWSON, W.J. (1969). ' "Lymphokines": non-antibody mediators of cellular immunity generated by lymphocyte activation'. Nature (Lond). 224, 38.

DUSTIN, P. & de HARVEN, E. (1954). 'La regulation hormonale de l'eosinophilie sanguine et son mechanism'. Rev. Hemat. 9, 307.

EHRLICH, P. (1879). 'Beitrage zur Kenntniss der granulirten Bindegewebs-zellen und der eosinophilen leukocyten.' Arch. Anat. Physiol, Lpz. 3, Physiol. Abt. 166. 'Uerber die specifischen granulationen der Blutes'. Arch. Anat. Physiol. Lpz. 3, Physiol Abt. 571.

EHRLICH, P. & LAZARUS, A. (1900). Histology of the Blood. Normal and Pathological. Cambridge University Press. [An English translation of 'Die Anaemi, Nothnagel's Specielle Pathologie und Therapie' vol. VIII, done under the personal guidance of P. Ehrlich]. Ed. by Myers, W.

ENDICOTT, K.M. & OTT, M. (1945). 'The normal myelogram in albino rat'. Anat. Rec. 92, 61.

EVERETT, N.B., CAFFREY, R.W. & REIKE, W.O. (1963-4). 'Recirculation of lymphocytes'. Ann. N.Y. Acad. Sci, 113, 887.

FEDORKO, M. (1968). 'Formation of cytoplasmic granules in human eosinophilic myelocytes: an electron microscope autoradiographic study'. Blood, 31, 188.

FELDBERG, W. & TALESNIK, J. (1953). 'Reduction of tissue histamine by compound 48/80'. J. Physiol. 120, 550.

FERNEX, M. (1968). 'The mast cell system, its relationship to Atherosclerosis, Fibrosis and Eosinophils'. London: Academic Press Inc.

FLIEDNER, T.M., FACHE, I. & ADOLPHI, C. (1966). 'Über die Umsatzkinetik der Leukocyten bei keimfreien Mäusen'. Schweiz. med. Wschr. 96 1236.

FOOT, E.C. (1963). 'Eosinophil turnover in the rat'. Nature (Lond.) 198, 297.

FOOT, E.C. (1965). 'Eosinophil turnover in the normal rat'. Brit. J. Haemat. 11, 439.

van FURTH, R. & COHN, Z.A. (1969). 'The origin and kinetics of mononuclear phagocytes'. J. exp. Med. 128, 415.

GERECKE, D., SCHULTZE, B. & MAURER, W. (1970). 'Autoradiographische Bestimmung der mittleren Verweilzeit neutrophiler Granulozyten im Blut der Ratte mittels Dauerinfusion van 3H-Thymidin'. Experientia 26, 311

GOLLASCH, - (1889). 'Zur Kenntniss des asthmatischen Sputums'. Fortschritte der Medizin (Berlin), vii, 361

GORDON, A.S., HANDLER, E.S., SIEGEL, C.D., DORNFEST, B.S., & LoBUE, J. (1963-4). 'Plasma factors influencing leucocyte release in rats'. Ann. N.Y. Acad. Sci., 113, 766.

GOULD, S.E. (1945). 'Trichinosis'. Illinois: Thomas Springfield.

GOULD, S.E. (1970), ed. 'Trichinosis in man and animals'. Illinois: Thomas Springfield.

GOWANS, J.L. (1962). 'The fate of parental strain small lymphocytes in F_1 hybrid rats'. Ann. N.Y. Acad. Sci, 99, 432.

GOWANS, J.L. & KNIGHT, J. (1964). 'The route of recirculation of lymphocytes in the rat'. Proc. Roy. Soc. (Lond.) Ser. B. 159, 257.

GOWANS, J.L. & UHR, J.W. (1966). 'The carriage of immunological memory by small lymphocytes in the rat'. J. exp. Med. 184, 1017.

GRICELLI, C., VASSALLI, P. & McCLUSKEY, R.T. (1969). 'The distribution of large dividing lymph node cells in syngeneic recipient rats after intravenous injection'. J. exp. Med, 130, 1427.

GROSS, R. & SCHMIDT, G.H.H. (1954). Über die relatinität der antagonismus zwischen corticoiden und bluteosinophilen'. Klin. Wschr. 32, 245.

GUZE, L.B. & BEESON, P.B. (1956). 'Experimental pyelonephritis. I. Effect of ureteral ligation on the course of bacterial infection in the kidney of the rat'. J. exp. Med. 104, 803.

HALL, J.G., SMITH, M.E. (1970). Homing of lymph-borne immunoblasts to the gut'. Nature (Lond.) 226, 262.

HANNA, I.R.A. (1968). 'An early response of the morphologically recognisable erythroid precursors to the bleeding'. Cell Tissue Kinet. 1, 91.

HANNA, I.R.A., TARBUTT, R.G. & LAMERTON, L.F. (1969). 'Shortening of the cell-cycle time of erythroid precursors in response to anaemia'. Brit. J Haemat. 16, 381.

de HARVEN, E. & DUSTIN, P. (1956). 'La régulation hormonale de l'éosinophilie'. Rev. Hémat. 11, 131.

HASKILL, J.S. & MOORE, M.A.S. (1970). 'Two dimensional cell separation-comparison of embryonic and adult haemopoietic stem cells'. Nature (Lond.) 226, 853.

HOGARTH-SCOTT, R.S. (1968). 'Naturally occurring antibodies to the cuticle of nematodes'. Parasitology, 58, 221.

HOMMA, E. (1921). 'Pathologische und biologische untersuchungen über die Eosinophilzellen unde die Eosinophile'. Virchow. Arch. Path. Anat. Physiol. 233, 11.

HORSFIELD, G.I. (1965). 'The effect of compound 48/80 in the rat mast cell'. J. Path. Bact. 90, 599.

HUDSON, G. (1963). 'Changes in the marrow reserve of eosinophils following re-exposure to foreign protein'. Brit. J. Haemat. 9, 446.

HUDSON, G. (1968). 'Quantitative study of the eosinophil granulocyte.' Seminars Haemat., 5, 166.

HULLIGER, C. & BLAZKOVEC, A.A. (1967). 'A simple and efficient method of separating peripheral-blood leucocytes for in-vitro work'. Lancet i, 1304.

HULSE, E.V. (1964). 'Quantitative cell counts of the bone marrow and blood and their secular variations in the normal adult rat.' Acta haemat. (Basel), 31, 50.

IVERSEN, O.H., BJERKNES, R. & DEVIK, F. (1968). 'Kinetics of cell renewal, cell migration and cell loss in the hairless mouse dorsal epidermis'. Cell Tissue Kinet. 1, 351.

IVERSEN, O.H. (1969). In Homeostatic Regulators, p. 29, 'Chalones of the skin'. Ed. by Wolstenholme, G.E.W. & Knight, J. London: J.A. Churchill Ltd.

KAGAN, I.G. (1960). 'Trichinosis: a review of biological, serological and immunological aspects'. J. infect. Dis, 107, 65.

KAY, A.B. (1970). 'Studies on eosinophil leucocyte migration. 1. Eosinophil and neutrophil accumulation following antigen-antibody reactions in guinea pig skin'. Clin. exp. Immunol. 6, 75.

KELLER, H.V. & SORKIN, E. (1968). 'Chemotaxis of leucocytes'. Experientia (Basel) 24, 641.

KELLER, H.V. & SORKIN, E. (1969). 'Studies on chemotaxis. XIII. Differences in the chemotactic response of neutrophil and eosinophil polymorphonuclear leucocytes'. Int. Arch. Allergy. 35, 279.

KELLERMEYER, R.W. & WARREN, K.S. (1970). 'The role of chemical mediators in the inflammatory response induced by foreign bodies: comparison with the schistosome egg granuloma'. J. exp. Med. 131, 21

KINDRED, J.E. (1942). 'A quantitative study of the hemopoietic organs of young albino rats'. Amer. J. Anat. 71, 207.

KOSTER, F. & McGREGOR, D.D. (1970). 'Rat thoracic duct lymphocytes: types that participate in inflammation'. Science, 167, 1137.

LAJTHA L.G. (1963). 'On the concept of the cell cycle'. J. cell. comp. Physiol. 62, supplement 1, 143.

LAJTHA, L.G. (1967). 'Bone marow stem cell kinetics'. Seminars haemat. 4, 293.

LAJTHA, L.G. (1970). Personal communication.

LAJTHA, L.G., GILBERT, C.W., PORTEOUS, D.D. & ALEXANIAN, R. (1963-64). 'Kinetics of a bone-marrow stem-cell population'. Ann. N.Y. Acad. Sci., 113, 742.

LALA, P.K., MALONEY, M.A. & PATT, H.M. (1964). 'A comparison of two markers of cell proliferation in bone marrow'. Acta haemat. (Basel) 31, 1.

LAMERTON, L.F. (1969). 'Cell population kinetics in relation to homeostasis'. In 'Homeostatic regulators', p. 5. Ed. by Wolstenholme, G.E.W. & Knight, J. London: J.A. Churchill Ltd.

LARSH, J.E., Jr. (1963). 'Experimental trichinosis'. Advanc. Parasitol. 1, 213.

LARSH, J.E., Jr. (1968). 'Experimental trichinosis'. Advanc. Parasitol. 6, 361.

von LICHTENBERG, F. (1962). 'Host response to eggs of S. mansoni. I. Granuloma formation in the unsensitized laboratory mouse'. Amer. J. Path. 41, 711.

LITT, M. (1963). 'Studies in experimental eosinophilia. V. Eosinophils in lymph nodes of guinea pigs following primary antigenic stimulation'. Amer. J. Path. 42, 529.

LITT, M. (1964). 'Eosinophils and antigen-antibody reactions'. Ann. N.Y. Acad. Sci., 116, 964.

McCULLOCH, E.A. (1968). 'Differentiation of hematopoietic stem cells'. XII Congress international Society of hematology, New York, 260.

MACLENNAN, I.C.M. & HARDING, B. (1970). 'The role of immunoglobulins in lymphocyte-mediated cell damage, in vitro. II. The mechanism of target cell damage by lymphoid cells from immunized rats'. Immunology, 18, 405.

MENKIN, V. & FREUND, J. (1929). 'Leucocyte content of regional lymphatics in inflammation'. Arch. Path. 8. 263.

MESNIL, M.A. (1895). 'Sur le mode de résistance des vertébrés inférieurs aux invasions microbiennes artificielles'. Ann. Inst. Pasteur, 9, 301.

MILLER, F., de HARVEN, E. & PALADE, G.E. (1966). 'The structure of eosinophil leuckocyte granules in rodents and in man'. J. cell. Biol. 31, 349.

MILLER, J.F.A.P. (1969). 'The role of the blood cells in immunity'. Brit. J. Haemat., 16, 331.

MILLER, J.F.A.P. & MITCHELL, G.F. (1969). 'Thymus and antigen-reactive cells'. Transplantation Revs. 1, 3.

MORGAN, E. & BEESON, P.B. (1970). Personal communication.

MORLEY, A. & STOHLMAN, F. (1970). 'Cyclophaphamide-induced cyclical neutropenia. An animal model of a human periodic disease'. New. Eng. J. Med. 282, 643.

MORSE, B.S., RENCRICCA, N.J. & STOHLMAN, F., Jr. (1970). 'The mechanisms of action of erythropoietin in relationship to cell cycle kinetics'. In 'Symposium on Hemopoietic Cellular Proliferation', p. 160. Ed. by. Stohlman, F. London: Grune & Stratton.

MURAKAMI, I., OGAWA, M., AMO, H. & OTA, K. (1969). 'Studies on kinetics of human leucocytes in vivo with 3H-thymidine autoradiography (II). Eosinophils and basophils'. Acta haemat jap, 32, 384.

NEUMANN, E. (1868). 'Über die Bedeutung des Knockenmarkes für die Blutbildung'. Zentralblatt für die medizinischen Wissenschaften 6, 689.

NOWELL, P.C. (1959). 'Stimulation of mitoses in rat marrow cultures by serum from infected rats'. Proc. Soc. exp. Biol (N.Y.), 101, 347.

OPIE, E.L. (1904). 'An experimental study of the relation of cells with eosinophile granulation to infection with an animal parasite (Trichinella spirella)'. Amer. J. med. Sci. 127, 477.

ORANGE, R.P. & AUSTEN, K.F. (1970). 'Cellular and humoral mechanisms in anaphylaxis and allergy'. Basel: Karger (in press).

PARAN, M., ICHIKAWA, Y. & SACHS, C. (1969). 'Feedback inhibition of the development of macrophage and granulocyte colonies, II. Inhibition by granulocytes'. Proc. Soc. Nat. Acad. Sci, 62. 81.

PARISH, W.E., & COOMBS, R.R.A. (1968). 'Peripheral blood eosinophilia in guinea pigs following implantation of anaphylactic guinea pig and human lung'. Brit. J. Haemat. 14, 425.

PARROTT, D.M.V., de SOUSA, M.A.B. & EAST, J. (1966). 'Thymus-dependent areas in the lymphoid organs of neonatally thymectomized mice'. J. exp. Med., 123, 191.

PATON, W.D.M. (1958). 'The release of histamine'. Progr. Allergy, V, 79.

PATT, H.M. & MALONEY, M.A. (1963). 'An evaluation of granulocytopoiesis'. In 'Cell Proliferation'. Symposium at University of Dublin, p. 157. Ed. by Lamerton, L.F. & Fry, R.J.M. Oxford: Blackwell Scientific Publications.

PATT, H.M. & MALONEY, M.A. (1963-4). 'A model of granulocyte kinetics'. Ann. N.Y. Acad. Sci., 113, 515

PLUM, C.M. (1943). 'Zur granulocytopoiese bei Ratten'. Fol. haemat. 67, 119.

PRESENTEY, B. (1969). 'Cytochemical characterization of eosinophils with respect to a newly discovered anomoly'. Amer. J. clin. Path. 51, 451.

QUASTLER, H. (1963). 'The analysis of cell population kinetics'. In 'Cell proliferation', p. 18. Symposium at University of Dublin. Ed. by Lamerton, L.F. & Fry, R.J.M. Oxford: Blackwell Scientific Publications.

QUASTLER, H. & SHERMAN, F.G. (1959). 'Cell population kinetics in the intestinal epithelium of the mouse'. Exp. Cell. Res. 17, 420.

ROBERTS, A.N. (1966). 'Rapid uptake of tritiated antigen by mouse eosinophils'. Nature (Lond.) 210, 266.

ROGERS, A.W. (1967). 'The Techniques of autoradiography', p. 210. Amsterdam: Elsevier Publishing Co.

ROSS, R. & KLEBANOFF, S.J. (1966). 'The eosinophilic leucocyte. Five structure studies of changes in the uterus during the estrus cycle'. J. exp. Med. 124, 653.

ROYLANCE, P.J. (1968). 'An evaluation of erythropoiesis in the young rat'. Cell Tissue Kinet. 1, 299.

RYTÖMAA, T. (1960). 'Organ distribution and histochemical properties of eosinophil granulocytes in rat'. Acta Path. Microbiol. Scand. 50, Suppl., 140.

RYTÖMAA, T. & KIVINIEMI, K. (1968). 'Control of cell production in rat chloroleukaemia by means of the granulocyte chalone'. Nature (Lond.) 220, 136.

SABESIN, S.M. (1963). 'A function of the eosinophil: phagocytosis of antigen-antibody complexes'. Proc. Soc. exp. Biol. (N.Y.), 112, 667.

SAETREN, H. (1970). 'The first mitotic wave released among rat kidney tubule cells by partial nephrectomy.' Acta path. microbiol. Scand. 78A, 55.

SCHAEFER, H.E. & FISCHER, R. (1968). 'Eine spezifische Färbung eosinophiler granulocyten mit Biebricher scharlah'. Klin. Wschr. 46, 396.

SCHLECHT, H. (1910). 'Über die Einwirkung von Seruminjektionen auf die Eosinophilen und Mastzellen des Menschlichen und tierischen Blutes'. Deutsch. Arch. Klin. Med. 98, 308.

SCHLECT, H. & SCHWENKER, G. (1912). Über lokale eosinophilie in den bronchien und in der lunge beim anaphylaktischen meerschweinchen'. Arch. Exptl. Pathol. Pharmakol. 68, 163

SCHULTZE, F. (1856). Arch. Mikroskop. Anat. Entwichlungsmech., 1,1.

SCHWARZ, E. (1914). 'Die lehre von der allgemeinen und örtlichen "Eosinophilie".' Ergeb. Allge. Pathol. Pathol. Anat. 17, 137.

SCOTT, D.W. & HOWARD, J.C. (1970). Personal communication.

SHIELDS, J.W., TOUCHON, R.C. & DICKSON, D.R. (1969). 'Quantitative studies on small lymphocyte disposition in epithelial cells'. Amer. J Path. 54, 129.

SHULMAN, N.R., WATKINS, S.P., Jr., ITSCOITZ, S.B. & STUDENTS, A.D. (1968). 'Evidence that the spleen retains the youngest and hemostatically most effective platelets'. Trans. Assn. Amer. Physicians. 302.

SIN, Y.M., & SAINTE-MARIE, G (1965). 'Granulocytopoiesis in the rat thymus. I. Description of the cells of the neutrophilic and eosinophilic series. Brit. J. Haemat, 11, 613.
II. Pattern for granulocyte formation based oncell counts in granulocytopoietic islands'. Brit. J. Haemat, 11, 624.

SMITH, R.O., & WOOD, W.B. (1949). 'Cellular mechanisms of antibacterial defense in lymph nodes. I. Pathogenesis of acute bacterial lymphadenitis. II. The origin and filtration effect of granulocytes in the nodal sinuses during acute bacterial lymphadenitis'. J. exp. Med. 90, 555.

SOULSBY, F.R. (1963). 'The nature and origin of the functional antigens in helminth infections'. Ann. N.Y. Acad Sci., 113, 492.

SPEIRS, R.S. (1958). 'Advances in the knowledge of the eosinophil in relation to antibody formation'. Ann. N.Y. Acad. Sci., 73, 283.

SPEIRS, R.S. & TURNER, M.X. (1969). 'The eosinophil response to toxoids and its inhibition by antitoxin'. Blood, 34, 320.

SPICER, S.S. & LILLIE, R.D. (1961). 'Histochemical identification of basic proteins with Biebrich scarlet at alkaline pH.' Stain. Technol. 36, 365.

STROBER, S & GOWANS, J.L. (1965). 'The role of lymphocytes in the sensitisation of rats to renal homografts'. J. exp. Med. 122, 347.

STRYCHMANS, P.A., CRONKITE, E.P., FACHE, J., FLIEDNER, T.M. & RAMOS, J. (1966). 'Deoxyribonucleic acid synthesis time of erythropoietic and granulopoeitic cells in human beings'. Nature (Lond.) 211, 717.

SUNDELL, B. (1958). 'Variations in the level of eosinophils in the wall of the small intestine of the rat'. Acta endocr. 28, Supp. 39, 9.

TARBUTT, R.G. (1967). 'A study of erythropoiesis in the rat'. Exp. Cell Res. 48, 473.

TARBUTT, R.G. (1969). 'Cell population kinetics of the erythroid system in the rat. The response to protracted anaemia and to continuous γ-irradiation'. Brit. J. Haemat, 16, 9

TARBUTT, R.G. & BLACKETT, N.M. (1968). 'Cell population kinetics of the recognizable erythroid cells in the rat'. Cell Tissue Kinet. 1, 65.

THOMPSON, J. & van FURTH, R. (1970). 'The effect of glucocorticoids on the kinetics of mononuclear phagocytes'. J. exp. Med. 131, 429.

VAERMAN, J.-P. & HEREMANS, J.F. (1970). 'Origin and molecular size of immunoglobulin-A in the mesenteric lymph of the dog'. Immunology, 18, 27.

WAKSMAN, B.H., ARNASON, B.G. & JANKOVIĆ, B.D. (1962). 'Role of the thymus in immune reactions in rats. III Changes in the lymphoid organs of thymectomized rats'. J. exp. Med. 116, 187.

WALLS, R.S. (1970). Personal communication.

WALLS, R.S., BASTEN, A., LEUCHARS, E. & DAVIES, A.J.S. (1970). 'Eosinophil and neutrophil leucocyte responses in thymus-deprived mice'. Unpublished.

WARD, P.A. (1969). 'Chemotaxis of human eosinophils'. Amer. J. Path. 54, 121.

WARD, P.A., REMOLD, H.G. & DAVID, J.R. (1969). 'Leucotatic factor produced by sensitized lymphocytes'. Science, 163, 1079.

WHARTON JONES, T. (1846). 'The blood capsule considered in its different phases of development in the animal series'. Phil. Trans. R. Soc. 1, 63.

WU, A.M., TILL, J.E., SIMINOVITCH, L. & McCULLOCH, E.A. (1968). 'Cytological evidence for a relationship between normal hemato-poietic colony-forming cells and cells of the lymphoid system'. J. exp. Med. 127, 455.

ZAIMAN, H. & VILLAVERDE, H. (1964). 'Studies on the eosinophilic response of parabiotic rats infected with Trichinella spiralis'. Exp. Parasit. 15, 14.

ZUCKER-FRANKLIN, D. (1968). 'Electron microscopic studies of human granulocytes: structural variations related to function'. Series Haemat. 5, 109.

ACKNOWLEDGEMENTS

To Professor Paul Beeson, I extend the greatest appreciation for supervising this work. It has been a privilege to work closely with him throughout this study.

This thesis is a contribution to a team project in which Tony Basten, Mark Boyer, Ronald Walls and Elizabeth Morgan have taken part. For their work as teachers, helpers, advisers and critics, I am extremely grateful. I should also like to thank Dr MacLennan for useful discussions and for reading the manuscript.

I should also like to thank the following: Sarah Davies who gave technical assistance, especially by doing many eosinophil counts, autoradiographs and histological sections; Mike Clark and his staff who looked after the rats, and Carole Lander who carried out rat breeding programmes skillfully; Fleur Walsh who typed the thesis; and Miss P. Dewhurst, Mr Haines and Mr D. Luckett who prepared the illustrations.

Many other people provided help, and advice and discussed the work at various times. These included Dr A. Rogers, Dr R. Berry,

Dr Z. Cohn, Dr P. Murphy, Dr J. Hirsch, Dr N. Blackett, Dr G. Gordon Steel, Professor Lamerton and Professor Lajtha. I thank them all.

It is a particular pleasure to thank the Rector and Fellows of Exeter College for providing the generous hospitality and stimulating environment of the College during the tenure of the Staines Medical Research Fellowship.

Grants were provided by the Medical Research Council, Nuffield Benefaction and Wellcome Trust.

This thesis is dedicated to my wife, Marion.

INDEX