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# A study of post-infectious eosinopenia or Simon's "septic factor"

Elizabeth Morgan
*Yale University*

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# A STUDY OF POST-INFECTIOUS EOSINOPENIA OR SIMON's "SEPTIC FACTOR"

Elizabeth Morgan

1971

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# A STUDY

# OF POST-INFECTIOUS EOSINOPENIA

# OR

# SIMON's "SEPTIC FACTOR"

Report of research into eosinophil behavior, using trichinosis and pyelonephritis in mice and rats as an animal model.

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A thesis submitted to

Yale University School of Medicine

in partial fulfillment of the requirements

for the M. D. degree, 1974

by Elizabeth Morgan

The research was done

under the direction of

Professor Paul Beeson
Nuffield Professor of Clinical Medicine
Oxford University

and

Professor Stuart Finch
Professor of Medicine
Yale University

I should like to express my thanks to Professor Stuart Finch of Yale University and Professor Paul Beeson of the University of Oxford for their continuous advice, guidance and encouragement in the preparation of this thesis.

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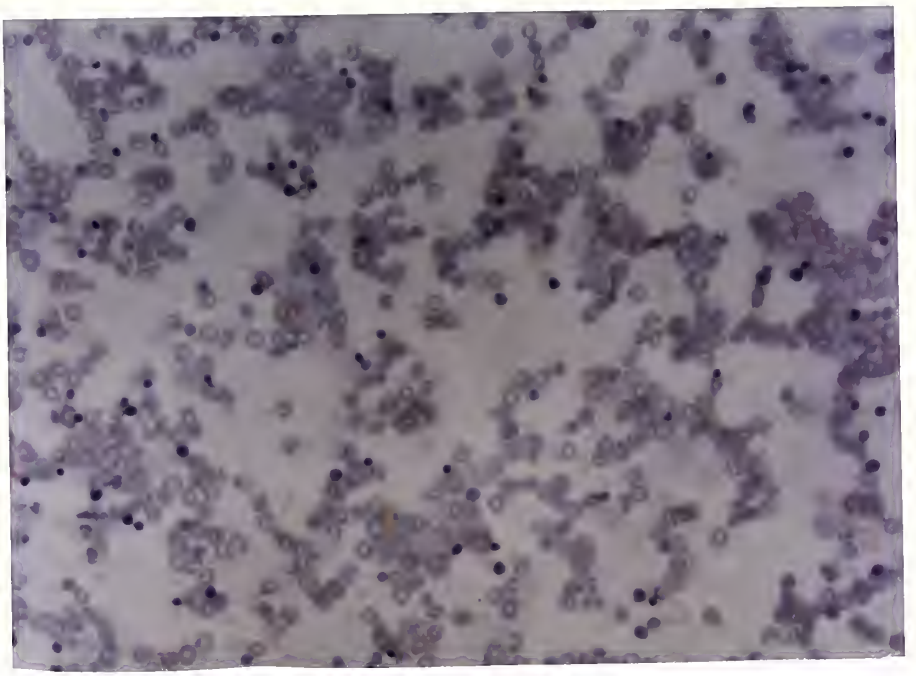

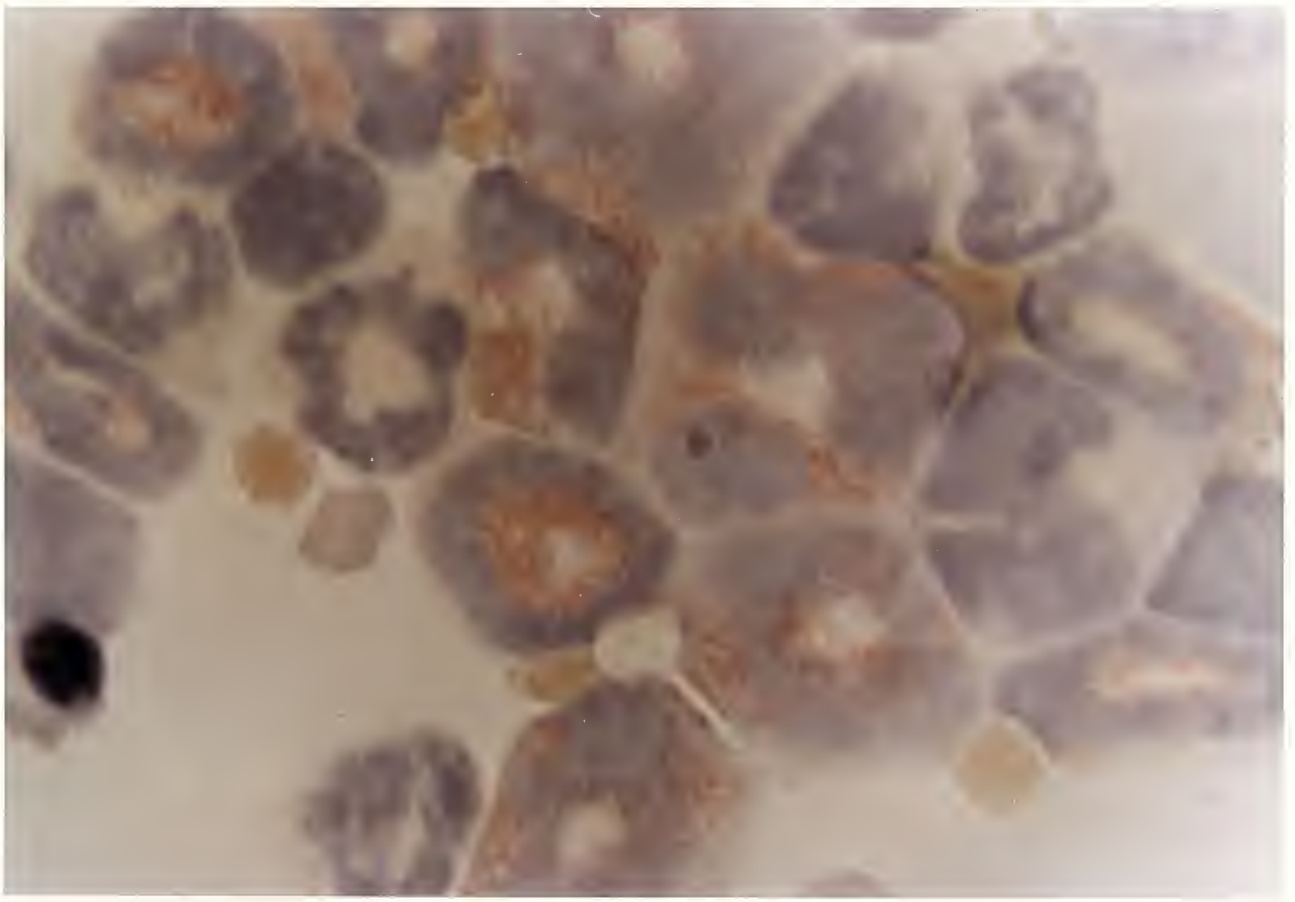

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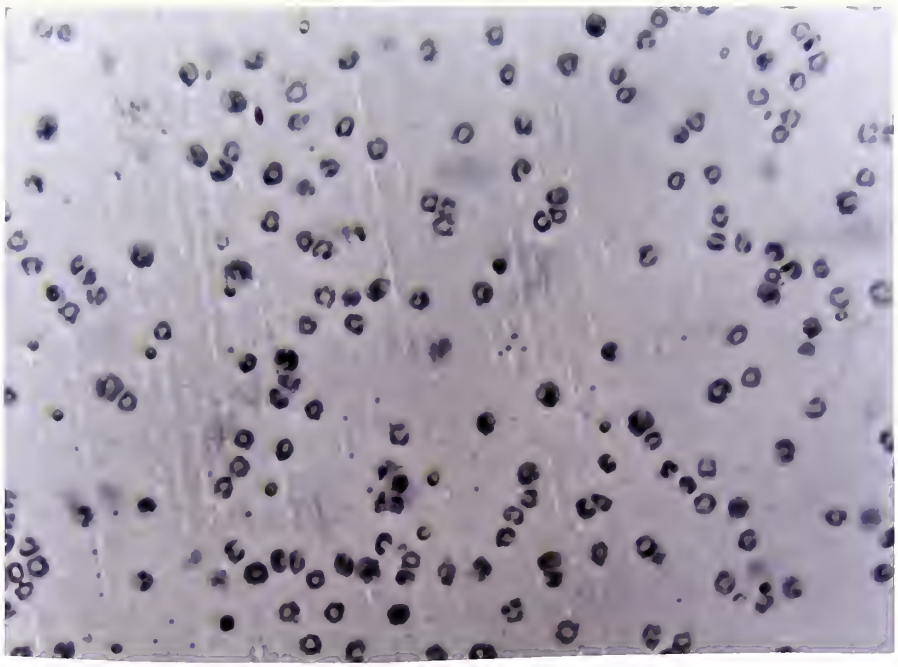
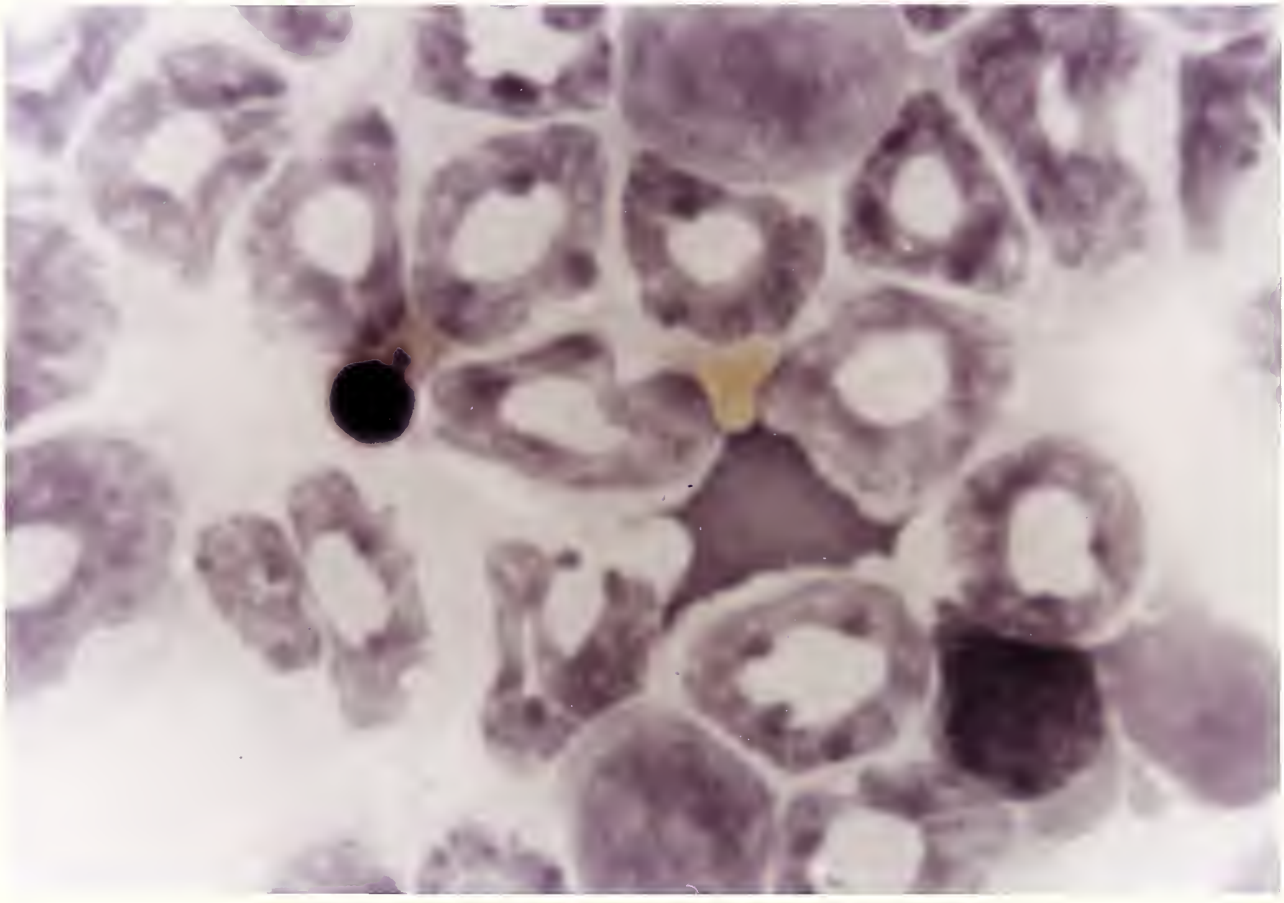




II. Photographs of bone marrow of mouse given Trichinella, followed, on the 18th day, by ureter-ligation and, on the 19th day, by intravenous E. coli. (Bone marrow was taken 48 hours after E. coli injection.) The mouse develops trichinosis, and after the E. coli, acute pyelonephritis. The photograph illustrates "Simon's septic factor" -- the marked eosinopenia which develops after acute bacterial infection despite the eosinophilic stimulus of parasitic infection.

# CHAPTER I

## INTRODUCTION

This thesis is a study of the changes in the bone marrow and peripheral blood eosinophils when an acute pyogenic bacterial infection is superimposed on a chronic parasitic infection. It is also a study of the causes of these changes, and of the relation of eosinophils to neutrophils in the course of this dual infection.

Persistent peripheral eosinophilia is a well-known result of parasitic infections. It is less well-known that eosinopenia may develop during bacterial infections. Attention was drawn to this by Simon's description of a "septic factor" in his Manual of Clinical Diagnosis (1922). Simon reported that eosinopenia was associated with all pyogenic bacterial infections. He described the "septic factor" as an association of neutrophilia and eosinopenia in a bacterial infection, and regarded this as "one of the most valuable symptoms of pyogenic infection." To Simon, a rise in peripheral eosinophils, despite a persistent neutrophilia, was the sign that recovery from a pyogenic infection had begun. Simon's observations were extended by Spink (1934) who reported that the eosinophilia of trichinosis was altered to eosinopenia by super-infection with Staph. aureus, tuberculosis or trypanosomiasis.

Most of the work on eosinophils during combined parasitic and bacterial infections was done many years ago, and was hindered by inaccurate laboratory techniques. With more accurate techniques available, it was decided to investigate the matter once again to determine the effect, if any, of bacterial infection on eosinophils, and what caused it.

## CHAPTER II

## HISTORY

Clinically, eosinopenia has been ignored, largely because the normal eosinophil count is very low. Eosinophilia, on the other hand, has been described in a tremendous variety of diseases. In many of these, for instance malaria and tuberculosis, the eosinophilia has never been shown to be due to the disease itself rather than to an intercurrent parasitic infection. In other diseases, such as most parasitic infections and many allergic, pulmonary and skin disorders, eosinophilia is definitely a part of the clinical picture.

The function of the eosinophil remains unknown, despite its discovery more than a century ago (Jones, 1846), although various roles have been suggested. These include the eosinophil as a secretor of erythrocytes (Duran-Jorda, 1948); as a carrier of toxic histamine to tissues for inactivation (Vaughn, 1953); as the site of profibrinolysin synthesis (Barnhardt et al., 1963); as a histamine antagonist in tissue (Archer, 1963); as a mast cell precursor (Van den Hoof, 1962); as one of the cells involved in the immune response (Basten, Boyer and Beeson, 1970).

Research into the function of the eosinophil has generally fallen into one of the following five categories:

1. Eosinophils as histamine antagonists.
2. The structure and function of eosinophil granules.

3. Eosinophils as part of the immune response.

4. The relation of eosinophils to neutrophils.

5. Eosinophils and their relation to steroids and stress.

The research in each of these areas will be briefly reviewed.

It is often difficult to interpret results of experiments on eosinophils. For instance, Speirs (1953) used mice with "normal" eosinophil counts of 700 per cu.mm., with the lowest count being 155. In the experiments reported here, the normal count never exceeded 77. A count of 155 would be regarded as a definite indication of parasitic disease. Laboratory animals often have various parasitic diseases which raise the marrow and blood eosinophil count. Therefore, in investigating eosinophil reactions the possibility of a hidden parasitic infection must always be borne in mind.

## 1. Eosinophils as Histamine Antagonists

In the 1950's, attention focussed on eosinophils as histamine and mast cell antagonists in inflammatory lesions. Code (1952) reported slightly increased blood histamine levels in patients with eosinophilia, and remarked, " ... the only adult myelocyte for which there is some evidence that the carriage of histamine may be one of its functions is the eosinophil."

Vaughn (1953), from studies of eosinophilia produced by Ascaris extract and histamine in guinea pigs, proposed that the eosinophil acts as a carrier of histamine or a "histamine-like toxic material" from the bone marrow to the tissues for inactivation.

Welsh and Greer (1959) reported that eosinophils phagocytosed mast cell granules released by 48/80, a histamine liberator, and suggested the eosinophil's major role was to respond to histamine release and allergic inflammation.

Also in 1959, Archer reported that eosinophils may contain an anti-histamine, and claimed that intradermal injections of histamine or 5-HT into skin rich in eosinophils were not followed by edema. He confirmed his earlier studies on horses, which showed that histamine may be chemotactic for eosinophils. Archer reported further work (1963) in horses showing that eosinophils were the "physiological opposite" of the mast cell, and operated in the local control of histamine and inflammatory lesions. He claimed that eosinophils contained a substance capable of inhibiting the increased permeability produced by bradykinin.

Although Archer has done many studies on the anti-histamine role of eosinophils, his theory is not widely accepted. Few other workers have been able to reproduce his results. Felarca and Lowell (1968) published studies which showed that histamine -- exogenous, or endogenous released by 48/80 -- was not eosinophilotactic. They suggested that "eosinophils" reported to respond to histamine had in fact been dead or dying cells which stained pink with Wrights' stain and were mistaken for eosinophils.

## 2. The Structure and Function of Eosinophil Granules

The eosinophil is distinguished from other granulocytes by its cytoplasmic granules which stain an intense red

with eosin (hence the name, eosinophil) and other stains, including Biebrick-Scarlet, benzidine and peroxidase stains. In the past ten years, many studies have been done on the chemical composition of these granules, and their role in phagocytosis.

Charcot-Leyden granules have been reported in eosinophils, and Ayres and Starkey (1950) believed they were formed from the nucleus of the eosinophil, not from the granules. Welsh (1959), however, concluded from electron microscopic preparations that the eosinophil granules coalesced to form the nidus and protein-building material for Charcot-Leyden crystals.

Rytömaa (1960) reported increased peroxidase activity in the eosinophil granules in kwashiorkhor, inversely related to the lipid content of the cells. Harris, et al. (1961) studied the radio-opacity of all the blood cells, and showed that the eosinophil and erythrocyte were far more radio-opaque than neutrophils, basophils and lymphocytes. They thought the protein (presumably enzymatic protein) of the granule was responsible for the eosinophil's marked radio-opacity.

Bosworth and Archer (1962) isolated a material from eosinophil granules which they claimed had an opsonin-like activity. Monocytes, and neutrophils to a lesser extent, rapidly phagocytosed bacteria, zymosan particles and sensitized red cells coated with this material. The authors postulated that the eosinophil releases its granules after

phagocytosis in order to increase the phagocytic activity of monocytes and thus enhance the antibody production.

Archer (1963) showed by phase contrast cinematography that horse eosinophils phagocytosed yeast cell walls, foreign erythrocytes and antigen-antibody precipitates, releasing the cytoplasmic granules into or alongside the phagocytic vacuole. His analysis of disrupted granules revealed soluble cathepsin, ribonuclease, arylsulfatase, betaglucuronidase and phosphatases. Insoluble phosphatases and peroxidases were found in the granule residue. Lysozyme and phagocytin were not present. Archer concluded that the high enzymatic protein content of the eosinophil granules makes them behave in a manner similar to that of lysosomes.

Barnhardt et al. (1963), by labelling antibodies with fluorescent material, demonstrated profibrinolysin in eosinophilic granules. Increased profibrinolysin accompanied eosinophil maturation in the marrow, while mature peripheral eosinophils contained less profibrinolysin. They suggested that eosinophils act in "clot lysis and in maintaining the fluidity of the blood".

Miller et al. (1966) used the electron microscope to study eosinophil granules, and found a crystalline core, the "internum" (of 40 Å in man; 30 Å in rodents), rich in arginine, and a cortex rich in phospholipids. They thought the core was probably composed, in whole or in part, of the specific eosinophil peroxidase.

Seeman and Palade (1967) reported acid phosphatase activity in disrupted, but not in intact, eosinophil granules,

and postulated that the disruption of a protective membrane, a protective matrix or an enzyme-inhibitor complex might cause eosinophil enzyme activation.

The work on eosinophil granules has concentrated on their enzymatic lysosome-like role in eosinophil phagocytosis. The cells have often been reported to ingest antigen-antibody complexes, and Litt (1962, 1964, 1966) believes that the primary action of eosinophils is the phagocytosis of antigen-antibody complexes. He suggests that eosinophils are "exquisitely sensitive detectors of antibody, as complex ... It has become increasingly apparent that soluble immune complexes play an important role in the initiation of a number of pathologic states. It is now evident that eosinophilic leucocytes are endowed with the capacity to wall off these noxious agents by phagocytizing them..."

Despite Litt, it is by no means universally accepted that eosinophils are more phagocytic, or more selective in phagocytosis, than neutrophils. In fact, there is considerable evidence that they are much less phagocytic. It remains doubtful whether a major activity of eosinophils and their granules is phagocytosis.

### 3. Eosinophils as Part of the Immune Response

Most of the work on eosinophils as part of the immune response has been on local tissue eosinophilia, or on transitory peripheral eosinophilia. These forms of eosinophilia, which occur within hours of exposure to the foreign antigen, are derived from a mobilization of pre-formed eosinophils.

There is another kind of eosinophilia, a delayed eosinophilia, which takes several days to develop, and results from an increased production and release of eosinophils from the bone marrow. The immediate and delayed forms must be distinguished from one another. They are two distinct kinds of response, presumably arising from different mechanisms.

A. Immediate Eosinophilia.

One of the earliest reports of a role for eosinophils in the immune response was from Bennett (1951) who showed that anaphylaxis in the guinea pig was followed by a peripheral eosinophilia.

Samter (1953) showed a marked transitory eosinophilia in guinea pigs with intraperitoneal implants of lung tissue from animals dying in anaphylactic shock.

In 1958 Speirs showed that mice had a slight local eosinophilia when first injected with Oxyuris extracts, and a progressively greater and more prolonged eosinophilia with each subsequent injection. Speirs suggested that the eosinophil response was essential for antibody production, and that the decreased antibody production associated with cortisone, x-rays, malnutrition and stress was the result of the eosinopenia these agents cause.

In further experiments, Speirs (1963) used labelled tetanus toxin to show an increase of eosinophils and mononuclear cells in the inflammatory exudate of previously sensitized mice. The eosinophils picked up the radioactive label

by contacting degenerating labelled mononuclear cells. Later, the labelled eosinophils were found within macrophages. Speirs concluded that the eosinophil acts as a "transfer mechanism carrying specific information from disintegrating cells, injured by the injection of antigen, to viable macrophages."

Arnason and Waksman (1963) showed local eosinophilia at retest sites of purified protein antigens, but not at virgin sites. They could not show a circulating eosinophilia. The retest reaction was characterized by massive local eosinophilia and depended neither on circulating antibody nor on preformed local antibody, but was inhibited by anti-lymphocyte serum.

Sabesin (1963) showed eosinophil phagocytosis of ferritin-antibody complexes in sensitized animals, and agreed with Speirs that antibody synthesis might depend on the eosinophil response to antigenic stimulation.

Litt (1964) confirmed the eosinophil phagocytosis after local injection of antigen-antibody complexes, and suggested that the post-infectious eosinophilia during convalescence was caused by the appearance at that time of antibody complexing with foreign antigens. In another paper he reported studies of fluorescent antigen-antibody complexes suggesting that eosinophil phagocytosis of antigen-antibody complexes is a defense against the pathogenic effects of the complexes.

These studies on eosinophilia after the injection of antigen, either at primary or retest sites, show that there

is a rapid accumulation of eosinophils at the site of injection. This seems to be associated in some way with the formation of antigen-antibody complexes, although the work of Arnason and Waksman suggests that the increased eosinophilia at retest sites may be mediated by a lymphocyte, rather than caused by an antigen-antibody complex alone.

B. Delayed Eosinophilia.

Rogers et al. (1953) reported that a local and peripheral eosinophilia developed in patients rejecting skin homografts. The eosinophilia subsided as the homograft dermal pad sloughed. The time course of the eosinophilia was not noted in detail, but it reached a peak after several days, as the epithelium sloughed leaving only the dermis intact.

Huntley and Costas (1965) studied a patient with agammaglobulinemia who had a concurrent eosinophilia due to the visceral larva migrans syndrome. They suggested that, since eosinophilia developed in the absence of gamma-globulin, the mechanism of eosinophilia in antigen-antibody reactions was different from the mechanism in parasitic diseases.

Basten, Boyer and Beeson (1970) showed that the delayed eosinophilia which follows oral infection of rats with Trichinella is dependent on intact circulating parasites. It could not be produced by oral infection with homogenized parasites or by intramuscular, subcutaneous or intraperitoneal injections of intact parasites. Anti-larval antibody was not found to correlate closely with the eosinophil response.

A second injection of larvae, twenty days after the first, enhanced the circulating eosinophil response and suggested that the enhanced response was analogous to the anamnestic antibody response.

In a second paper, Basten and Beeson (1970) showed a lymphocyte role in eosinopoiesis. A depletion or inactivation of recirculating lymphocytes significantly reduced the eosinophil response to trichinosis. Normal lymphocytes plus bone marrow made irradiated rats capable of a delayed eosinophilic response to trichinosis. When "memory" cells were substituted for normal lymphocytes, they produced a secondary type of eosinophil response. Primary delayed eosinophilia could be produced adoptively only by live large lymphocytes, and not by cell-free lymph or plasma. The action of the lymphocyte was the result of a diffusible factor, as was shown by enclosing the lymphocytes in cell-tight diffusion chambers.

These experiments demonstrating the lymphocyte's role in inducing the delayed eosinophil response to parasites not only support the theory that eosinophilia is one of the immunologic phenomena, but also suggest a lymphocytic mechanism for the induction of eosinophilia. They show that the eosinophilia of parasitic diseases is not a response distinct from other immune responses, as Huntley and Costas proposed. Rather, they indicate that the increased production of eosinophils in various unrelated disorders can be attributed to a mechanism and function common to all eosinophils.

## 4. Eosinophils, Steroids and Stress

It has been known for many years that stress can decrease the peripheral eosinophil count, an effect that is ascribed to an increase in circulating adrenal steroids.

Dalton and Selye (1939) described the blood picture of the alarm reaction, produced by such stimuli as exposure to cold, muscular exercise, toxic doses of drugs, hemorrhage and surgical intervention. A constant feature of these alarm reactions was leucocytosis, preceded by an eosinopenia, and followed by an eosinophilia.

Thorn et al. (1948) found that eosinopenia developed with the injection of pituitary adrenocorticotropic hormone. When the level of circulating eosinophils failed to fall in response to that stimulus, deficient capacity of the adrenal cortex was presumed to exist.

In 1953, Speirs, mentioned above, reported the use of the eosinopenic response as an assay of the activity of adrenocorticotropic preparations.

Bröchner-Mortensen (1952) reported that two preparations of ACTH, tested in human subjects, did not produce eosinopenia, but rather a massive eosinophilia within one to three weeks. However, this eosinophilia has been regarded not as an action of ACTH, but as sensitivity to contaminants in the early ACTH preparations.

Visscher and Halber (1955) reported daily cycle variations in circulating eosinophils, with a peak from 8 to 10 a.m. This cycle was affected by various environmental factors, but

predominantly controlled by the adrenal cortical steroids. Removal of the adrenals or a cortical insufficiency abolished the daily cycle.

The effects of steroids on circulating eosinophils can be produced by injections of ACTH (Thorn et al. 1948), synthetic corticotrophins, for instance Synacthen (Greig et al. 1967) or exogenous steroids (Quittner 1951). The circulating eosinophils, as mentioned above, immediately disappear from the blood. This is thought to be the result of increased eosinophil destruction in the spleen. This eosinopenia is usually seen within six hours of an injection.

There is considerable confusion over the steroid effect on marrow eosinophils. Both cortisone and ACTH have been reported to have no specific effect on marrow eosinophils (Rosenthal et al. 1950).

Quittner et al. (1951) reported that, although the eosinophil ratio in the marrow did not change, the peripheral granulocytosis and marrow myelocytosis seen twelve to twenty-four hours after cortisone, was "compatible with the concept of blocking of the marrow."

On the other hand, Cardinali et al. (1964) reported that mitotic activity decreased for all marrow elements during the first twelve to eighteen hours after high doses of cortisone.

Andersen and Bro-Rasmussen (1968) reported that dexamethasone given to rats for sixteen days reduced by two-thirds the number of proliferating eosinophils in the marrow. This effect is thought to result from only very long-term injections of steroids.

The most widely held belief at the present time, based on the work of Hudson (1963, 1964) is that steroids block eosinophil release from the marrow and thus raise the number of eosinophils in the marrow.

However, the results reported here do not agree with Hudson's findings. In the experiments reported here, high doses of cortisone acetate, given once a day reduced the marrow eosinophils by more than three-fourths within three days.

Clearly, there is still considerable uncertainty about the effect of steroids on the marrow eosinophils.

5. The Relation of Eosinophils to Neutrophils

As already mentioned, the relation of eosinophils to neutrophils is a major concern of this thesis. The first report suggesting that eosinophils and neutrophils might be inversely related was that of Brown (1898). He reported large numbers of eosinophils and neutrophils in the peripheral blood and muscle of patients with trichinosis. He noted that with the largely eosinophilic leucocytosis in three patients with acute trichinosis there was a "coincident decrease in the quantity of neutrophilic elements." He saw, in the peripheral blood, cells which "might be regarded as forms in transition between neutrophiles and eosinophiles" and suggested that the eosinophils in trichinous muscle were transformed neutrophils.

The work of Simon (1922) has been mentioned above. His "septic factor" was a neutrophilia associated with eosinopenia,

but he found that the relation between the two blood cells was not necessarily inverse, since the eosinophils could begin to rise before the neutrophils declined.

The work of Spink (1934) involved the superinfection of trichinous guinea pigs with trypanosomiasis, tuberculosis and Staphylococcus aureus. He found that trichinous animals, super-infected with tuberculosis or trypanosomes, developed, besides the decrease in peripheral eosinophils, less muscular destruction, less edema, and less infiltration around the encysted Trichinella larvae. He examined the bone marrow but found no corresponding eosinopenia when peripheral eosinophils declined. Spink's results do not agree with the findings reported here. It is felt that, owing to the limited techniques available at that time, his bone marrow studies might be misleading.

McNaught (1939) confirmed earlier reports of an eosinophil decline in the presence of neutrophilis, and reported that "eosinophilia may be absent in trichinosis complicated by such concomitant bacterial infections as furunculosis, bronchopneumonia, etc. and also in fulminating cases of trichinosis terminating fatally."

Rogers (1953) reported a rise in eosinophils during rejection of skin homografts in human patients, and noted, "... the number of eosinophiles in a differential count increases at the expense of the polymorphonuclear cells, thus establishing an inverse relationship between these two types of cells ..."

Observations on the relation of eosinophils to neutrophils have in the past been largely confined to peripheral eosinophilia, owing to the difficulty of studying bone marrow cells with the techniques then available. Estimates of eosinophils are unreliable when done by differential counts of peripheral smears. It was not until the introduction of the counting chamber that accurate counts of eosinophils per cu.mm. of blood could be done (Discombe 1946).

Moreover, the data from clinical experience were unreliable because the time between the trichinosis and bacterial infections was not always known. Work on experimental animals was limited because there were only crude techniques available to estimate the inoculated dose of Trichinella.

The cytocentrifuge now makes it possible to count bone marrow cells more accurately. Methods of accurately assessing the inoculated number of Trichinella are also available (Basten 1969). For these reasons, it seemed worthwhile to investigate Simon's "septic factor" once again.

The primary interest of these experiments was the relation between eosinophils and neutrophils in the presence of two infections stimulating the production of the two kinds of cells. To produce this dual infection, Trichinella was used to produce an eosinophilia, and ureteral ligation followed by Escherischia coli was used to produce a neutrophilia.

It is not, however, possible to investigate one aspect of the eosinophil apart from the others. Hence, the initial study reported here led to investigation of the other aspects

of eosinophil activity mentioned above. Thus, the development of trichinosis in the mice showed that undoubtedly some form of immune response was being elicited. The ureteral ligation, as a severe stress, certainly altered steroid secretion, and thus the peripheral eosinophil pattern. The disappearance of the eosinophils after bacterial infection led to consideration of possible mechanisms of eosinopenia, including degranulation and a change in the chemical composition and staining properties of the cytoplasmic granules.

# CHAPTER III

## MATERIALS AND METHODS

### 1. Experimental Animals

#### A. Mice

Mice were the animals used in most of the experiments. The mice were four-month-old, male and female, C3H inbred mice, weighing 20 to 25 g. The mice came originally from Animal Suppliers (London) Ltd., and were subsequently bred in the laboratory where the experiments were carried out.

#### B. Rats

In a few experiments, female rats were used. These came from the Harwell Laboratory, and were about four months old, and weighed roughly 200 g.

### 2. Collection of Trichinella Larvae for Infection

*Trichinella* larvae were the Culbertson strain, obtained from Professor G. S. Nelson of the London School of Hygiene and Tropical Medicine. The larval strain was maintained in the laboratory through infecting rats which then served as reservoirs of larvae. To get larvae for infecting rats or mice, an infected rat was skinned and eviscerated. Then one half of the rat carcass was digested at 37°C. for eight hours in 1 liter of distilled water with 7 ml. of concentrated hydrochloric acid and 5 g. of hog pepsin. The yield of worms,

usually 0.6 - 1.0 ml. of settled worms, was washed with tap water six times, with sterile saline twice and with Parkers' 199 Medium[1] twice. One-tenth of a millilitre of settled worms contained approximately $5 \times 10^4$ larvae (Basten 1969). Then the larvae were resuspended in Parkers' 199 at the desired concentration for infection.

3. Oral Infection with Trichinella

A. Mice

Mice were infected by depositing fresh muscle-stage larvae in their stomachs. First, the mice were anesthetized with ether. Then, a short, straight steel cannula was pushed down the back of their throats, into the stomach. If the cannula entered the trachea, as signaled by violent coughing, the tube was withdrawn and re-inserted. With the tube in the stomach, 0.2 ml. of Parkers' 199, containing 200 larvae, was injected through the cannula. Immediately after this, 0.1 ml. of sterile saline, and then 0.1 ml. of air were injected into the stomach to make sure that the worms reached the stomach, and were neither regurgitated, nor aspirated into the lungs.

---

[1] This medium, Morgan, Morton and Parker's Medium Number 199 (1950) is a balanced salt solution containing a mixture of amino acids and vitamins. The formula of this medium is to be found at the end of this chapter on page 27.

B. Rats

Rats were infected in the same way, except that the dose was 2,000 larvae/200 g. rat; a long plastic tube replaced the steel cannula; the procedure often made the rat stop breathing and resuscitation was necessary.

4. Peripheral Eosinophil and Total White Blood Cell Counts

Mice were anesthetized with ether, and three capillary tubes filled with blood from the retro-orbital sinus. The counts were done between 8:30 and 11:00 a.m. to avoid, as far as possible, the diurnal variations in peripheral counts.

A. The Eosinophil Count

One-tenth of a millilitre of blood was diluted 1:10 in eosinophil staining fluid: 4 ml. of acetone; 5 ml. of concentrated eosin; 91 ml. of distilled water. Both sides of a Neubauer counting chamber were filled with this diluted blood, and the eosinophils on both sides of the chamber counted. The number of eosinophils x 5.5 = eosinophils per cu.mm.

B. The Total White Blood Cell Count

Five-hundredths of a millilitre of blood was diluted 1:20 in white blood cell diluting fluid, i.e. a crystal of methylene blue in 2% glacial acetic acid. One side of a Neubauer chamber was filled with the diluted blood, and the nucleated cells in the sixty-four large squares were counted. The number of nucleated cells x 50 = nucleated cells per cu.mm.

5. Ureter Ligation and Sham Operation

A. Ureter Ligation

Using ether anesthesia, mice were first shaved in the right lower quadrant of the abdomen, then spread-eagled on a cork board, and all four limbs taped down with Lasso tape. The abdomen was washed with Methcol, and a diagonal incision was made into the right lower quadrant. The peritoneum was incised, care being taken not to perforate the intestines. The kidney was located, and the ureter traced down, freed and ligated with Ethicon surgical silk. The peritoneum was closed with a continuous suture, and the skin closed either with clips or with interrupted silk sutures.

B. Sham Operation

The same procedure as above was followed, except that the ureter, once identified and dissected free, was not ligated.

6. Infection with Escherischia Coli

The E. coli parent strain was originally obtained from a patient with a urinary tract infection. The strain was cultured and stored at -20°C. Twenty-four hours before the mice were infected, the parent strain was defrosted and used to inoculate three blood agar plates, which were incubated overnight at 37°C. (The parent strain was refrozen at -20°C.)

The next morning a loopful of E. coli from the blood agar plates was incubated in 5-10 ml. of beef heart broth (the volume of broth depending on the number of mice to be infected) for two hours at $37^{o}$C.

After incubation, the bacteria per cu.mm. were counted, using the Neubauer counting chamber. Because of the great number of bacteria, only sixteen of the smallest squares needed to be counted for a fairly accurate estimate of bacterial concentration. The number of bacteria x 25 x $10^{4}$ = bacteria per cu.mm.

The mice were infected intravenously with the E. coli. Ether anesthesia was used, and the inoculum injected, using a 25-gauge needle and tuberculin syringe, in the right lateral tail vein. To make the vein more prominent and accessible, the tail was first dipped in hot tap water. An inoculating dose of 3.5 x $10^{7}$ bacteria per mouse was used.

## 7. Collection and Staining of Bone Marrow

### A. Mice

The mouse was killed by cervical dislocation. The femur was exposed by cutting away skin and scraping the bone as clean as possible with a fresh scalpel blade. The proximal end of the femur was cut with a pair of sharp, heavy scissors. The middle of the femur was held with fine forceps as the distal end of the femur was cut with the bone scissors.

A 21-gauge needle with a tuberculin syringe attached was put into the proximal open end of the femur, and

1 ml. of Parkers' 199 Medium forced through the bone shaft to expel the marrow. The marrow was collected in a small plastic jar containing 2 ml. of Parkers' 199. Using first a 21-, and then a 23-gauge needle, a single-cell suspension of marrow cells was made by gently pipetting the marrow and medium up and down in a 1 ml. tuberculin syringe. Two drops by pipette (four by 23-gauge needle) of the single-cell suspension was pipetted into each cytocentrifuge cylinder. The cells were spun at 500 r.p.m. for 5 minutes. The slides were airdried, and immediately fixed in 100% methanol.

At the end of an experiment, all the marrow slides from that experiment were stained simultaneously in Haematoxylin and Biebrick-Scarlet. This was to avoid variations in staining, as the strength of the stain varied from one batch to another.

The marrow slides were coded and counted double-blind. The cells were counted as one of three groups:

1. Neutrophils, identified by their doughnut-shaped nucleus.

2. Eosinophils, identified by their red cytoplasmic granules.

3. All other nucleated cells. These were usually immature and mononuclear cells.

At least one thousand marrow cells were counted for each slide. Marrow eosinophils were expressed as eosinophils per 1,000 nucleated cells.

B. Rats

The same procedure for marrow preparation was used for rats as for mice, except that rats were killed with ether, and 5-10 ml. of Parkers' 199 Medium was used to dilute the marrow before cytocentrifugation.

8. Steroid Injections

The mice were lightly anesthetized with ether to prevent wild struggling, and hence a great increase in steroid release. Cortisone acetate, in doses of 0.5, 2.5 or 12.5 mg. was dissolved in sterile saline, and injected intraperitoneally with a 25-gauge needle. The volume of the injection never exceeded 0.2 ml.

9. Synacthen Injections

Synacthen, a synthetic adrenocorticotropic hormone, was dissolved in sterile saline and injected intraperitoneally, as for the steroid injections. The dose of hormone was either 0.5 or 1.0 units.

10. Cortisol Levels

The mouse blood cortisol levels were assayed by Mr. Higgins of the Biochemistry Department of the Radcliffe Infirmary, Oxford, using the fluorimetric method. One millilitre of blood was needed for the cortisol determination. To get the blood as rapidly as possible, and thus avoid stress-induced steroid release, a mouse was decapitated with a sharp

scalpel blade, and the blood allowed to drip into a small heparinized tube. As one mouse yielded about 0.5 ml. of blood, two mice were bled into each tube. The blood was stored at 4°C. until it was used.

11. Bilateral Adrenalectomy

Bilateral adrenalectomy was done in Harwell rats, using ether anesthesia. The rat was spread-eagled on his stomach on a cork board, and secured with clips. The central part of the back was shaved. A vertical incision about an inch long was made in the middle of the back, beginning just above the last thoracic vertebra. Skin to the left and right of the incision was freed from the underlying connective tissue. A stab-wound was made to the left of the spine in the costovertebral angle of the twelfth rib. This stab-wound was lateral to the bulk of the spinal muscles. Through the stab-wound, the peritoneum was incised. Using a bright light, the adrenal gland could be seen slightly above and medial to the stab-wound. The gland was grasped at the hilum with forceps and gently pulled out through the stab-wound. The vessels and connective tissue were cut distal to the forceps. This procedure was then repeated on the right side. Each stab-wound was closed with one suture of 000 - silk, and the skin incision was closed with three or four skin clips. Ureter ligation was always done after the adrenalectomy. The rat was turned onto his back, and a right ureter ligation was done, as described above.

After the operation, the rats were given normal saline to drink.

## Morgan, Morton and Parker's Medium No. 199 (1950)

Quantities in milligrams per 1000 ml.

| | | | |
|---|---|---|---|
| L-Arginine | 70.0 | Riboflavin | 0.010 |
| L-Histidine | 20.0 | Pyridoxine | 0.025 |
| L-Lysine | 70.0 | Pyridoxal | 0.025 |
| L-Tyrosine | 40.0 | Niacin | 0.025 |
| DL-Tryptophan | 20.0 | Niacinamide | 0.025 |
| DL-Phenylalanine | 50.0 | Pantothenate | 0.01 |
| L-Cystine | 20.0 | Biotin | 0.01 |
| DL-Methionine | 30.0 | Folic acid | 0.01 |
| DL-Serine | 50.0 | Choline | 0.50 |
| DL-Threonine | 60.0 | Inositol | 0.05 |
| DL-Leucine | 120.0 | p-aminobenzoic acid | 0.05 |
| DL-Isoleucine | 40.0 | Vitamin A | 0.10 |
| DL-Valine | 50.0 | Calciferol | 0.10 |
| DL-Glutamic acid | 150.0 | Menadione | 0.01 |
| DL-Aspartic acid | 60.0 | Vitamin E | 0.01 |
| DL-Alanine | 50.0 | Ascorbic acid | 0.05 |
| L-Proline | 40.0 | Glutathione | 0.05 |
| L-Hydroxyproline | 10.0 | Cholesterol | 0.2 |
| Glycine | 50.0 | Oleic acid | 20.0 |
| Cysteine | 0.1 | Sodium acetate | 50.0 |
| Adenine | 10.0 | L-Glutamine | 100.0 |
| Guanine | 0.3 | Adenosine triphosphate | 10.0 |
| Xanthine | 0.3 | Adenylic acid | 0.2 |
| Hypoxanthine | 0.3 | Ferric nitrate | 0.1 |
| Thymine | 0.3 | Ribose | 0.5 |
| Uracil | 0.3 | Deoxyribose | 0.5 |
| Thiamin | 0.010 | | |

## CHAPTER IV

## NORMAL MICE AND THE RESPONSE TO TRICHINOSIS

The aim of these experiments was to study the "septic factor" -- the effects on eosinophils of trichinosis and pyelonephritis. Before beginning, it was necessary to know the eosinophil counts of the peripheral blood and the bone marrow in normal mice. Also, it was necessary to know the pattern of eosinophil response to trichinosis alone, without a superimposed pyelonephritis.

### 1. Eosinophils in the Normal Mouse

Peripheral eosinophil counts were done in thirty-six normal C3H mice, male and female. The counts were not done on one group of mice alone. Twenty were from the original shipment. The other sixteen came from later shipments, or from mice bred from the original shipment.

The normal peripheral eosinophil count never exceeded 77 eosinophils per cu.mm. The average count was 18 eosinophils per cu.mm. (Graph I, Day 0). All the counts were done between 8 and 10 a.m., the time of the maximum peripheral eosinophilia. The daily variation between maximum and minimum counts in normal mice was insignificant, since the maximum counts were already close to zero. However, in mice with a raised eosinophil count from trichinosis, the difference between the daily maximum and minimum counts was often very marked.

The bone marrows of normal mice had an eosinophil count corresponding to the low peripheral count. Twelve thousand cells were counted; two thousand for each of six mice. The average eosinophil count in the marrow was 11 eosinophils per 1000 nucleated cells. (Graph II, Day 0.) All six normal marrow counts were very low. There was little variation from one marrow to another. It was felt that these low counts, coupled with the low peripheral counts, indicated that blood and marrow eosinophils in normal C3H mice were consistently low.

2. The Accuracy of the Marrow Counts

To be certain that one or two marrow counts were an accurate reflection of a total marrow cell population, comparative marrow counts were done between:

a) 1000 cells in the upper and lower part of each slide.

b) 1000 cells from different slides, prepared from the same marrow.

c) 1000 cells from the right and left femurs of the same mouse.

Comparative counts were done throughout the experiments on a total of fifty-three mice. The average variation, for 159 marrow slides, was 11.2%. A variation of a few cells could be expected in a normal count of 10 eosinophils per 1000 nucleated cells. Table I gives examples of some of the comparative counts.

TABLE I

SAMPLE VARIATIONS IN MARROW COUNTS

Comparative counts were done on 53 mice.

5000 cells were counted for each mouse:

1. two counts, 1000 each, same slide, left femur.
2. two counts, 1000 each, same slide, right femur.
3. one count of 1000 from a different slide, same marrow, i.e. left femur.

Total variation: 11.2%, mean.

$p < 0.20$, $> 0.15$ for right vs. left marrow

$p < 0.49$, $> 0.48$ for first vs. second count

$p < 0.20$, $> 0.15$ for different slide, same marrow

Therefore, the difference in counts is not significant.

* * * * * *

Sample Counts

| | 1. Same Slide | 2. R vs. L | 3. Different slide, L marrow |
|---|---|---|---|
| High Counts | 227-263 | 132-135 | 205-196 |
| | 232-217 | 212-220 | 245-265 |
| | 162-170 | 232-253 | 285-316 |
| | 172-162 | 155-162 | 231-240 |
| | 155-166 | 158-148 | 301-328 |
| | 124-133 | 223-217 | 321-338 |
| Low Counts | 93-87 | 71-66 | 66-59 |
| | 19-17 | 81-83 | 89-101 |
| | 56-59 | 11-13 | 25-25 |
| | 66-71 | 19-18 | 17-15 |
| | 11-13 | 31-27 | 30-33 |
| | 23-28 | 16-14 | 12-8 |

3. The Normal Response to Trichinella

The eosinophil response was followed in thirty-six mice from several litters. All the mice had peripheral counts every two or three days after infection. Each day that a peripheral count was done, at least two mice were used for marrow counts. During the peak peripheral eosinophilia following trichinosis, four to six mice were used for marrow. The number of marrow counts was increased during the peak response, since this was the time that the mice would be given pyelonephritis. It was important to know the variation of the response to trichinosis alone, in order to avoid ascribing the eosinophil fluctuations of trichinosis to operations or infection.

Graph I shows the peripheral counts following infection with trichinosis. A slight eosinophilia developed during the first ten days after trichinosis. The mean peripheral count on day ten was 63 eosinophils per cu.mm. By the twelfth day of infection, the peripheral eosinophils had risen to a mean count of 390 eosinophils per cu.mm. This peak response lasted from day twelve to day fifteen. On the seventeenth day, the mean count declined to 296 eosinophils per cu.mm., a level which was maintained for the next ten days as a continuous response to chronic infection with Trichinella.[1]

[1] Two mice kept for six weeks, had final counts on the forty-second day of infection of 341.0 and 429.0 eosinophils per cu.mm.

Graph II shows the marrow counts following infection with trichinosis. The rest of the marrow eosinophils resembled that of the peripheral eosinophils. On the tenth day there was a slight rise in marrow eosinophils: 69 eosinophils per 1000 nucleated cells. On the twelfth day, the count was 243 per 1000 nucleated cells. This declined to 177 per 1000 nucleated cells on the seventeenth day, and stayed in that range for the next ten days.

The variation in marrow counts for the same mouse, as noted earlier, was 11.2%. With a count of 20 from the left femur, any recount should be approximately between 190 and 210 eosinophils per cu.mm. There was no greater variation in counts from different femurs than in two counts from the same slide. It seemed fairly certain that a marrow count from one femur would be representative of the eosinophil population of the total marrow.

4. Unusual Responses in Normal Mice to Trichinosis

In every group of mice infected with trichinosis, one or two mice developed an unusual response. There were two kinds of unusual response. One response was a slight peripheral eosinophilia, up to 100 per cu.mm., with no further rise. This was probably the result of a chance inoculation with a low dose of Trichinella larvae.

The other response was a slight eosinophilia, below 100 per cu.mm., until the eighteenth day of infection, or later, followed by a sudden rise in eosinophils, sometimes

to over 1000 eosinophils per cu.mm. This response might have been due to a delayed maturation of the larvae, or delayed migration into the muscles. Another possibility, in the light of later experiments, is that these mice had an intercurrent bacterial infection.

Any mouse with a peripheral eosinophil level below 120 per cu.mm. on the twelfth day was excluded from the results and from further experiments. Otherwise, an eosinophil rise or fall might have been attributed to pyelonephritis, when in fact it was an unusual response to the initial Trichinella infection.

## CHAPTER V

## EXPERIMENTS ON TRICHINOSIS AND PYELONEPHRITIS

The aims of the first experiments on the "septic factor" were:

1. To determine the time that eosinopenia appeared after a pyogenic infection.

2. To compare this eosinopenia with the normal peripheral eosinophil pattern.

3. To find if changes in the bone marrow accompanied changes in the eosinophils of the peripheral blood.

4. To look for a relation between the changes in the eosinophils and those of the neutrophils.

The inbred mice, C3H, had a normal peripheral eosinophil count of 12.3 per cu.mm. (St. Error = 2.5). The highest eosinophil count in a normal animal was 77. With such low normal counts, an eosinopenia from a pyogenic infection would not be noticed. So a peripheral eosinophilia was produced with Trichinella. The first task was to determine the dose of larvae.

### 1. The Appropriate Larval Dose

The mice were infected using the method of Basten (1969). The larval inoculations were calculated using his figure of $5 \times 10^4$ worms per 0.1 ml. The estimated figure

roughly approximated the number of injected larvae. A moderate variation in the number of larvae was not particularly significant. The object of infection was to produce a sustained eosinophilia, not an eosinophilia at a specific level.

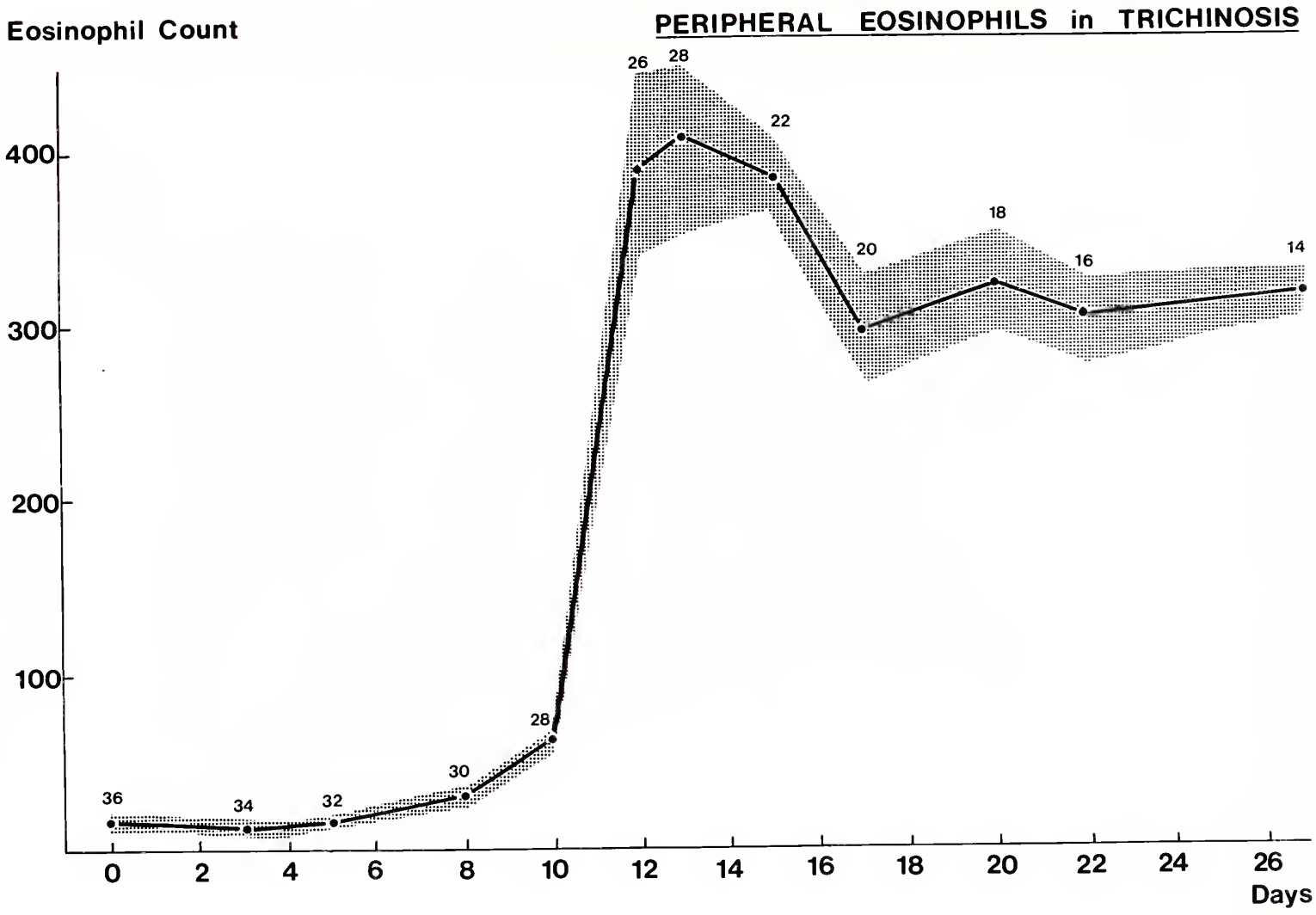
Two groups of three mice were given doses of 300 and 400 larvae. Four mice died in 24 hours; the remaining two in four days. Three mice given 100 larvae had a peak eosinophilia of only 100 eosinophils per cu.mm. Two hundred larvae (about 10 larvae per gram) was taken as the inoculating dose. This dose was used in all subsequent experiments.

Mice given 200 larvae developed a slight eosinophilia by day eight and a peak eosinophilia between days twelve and fifteen. After this, the eosinophil count fluctuated from day to day, but did not fall significantly. (Graph I.)

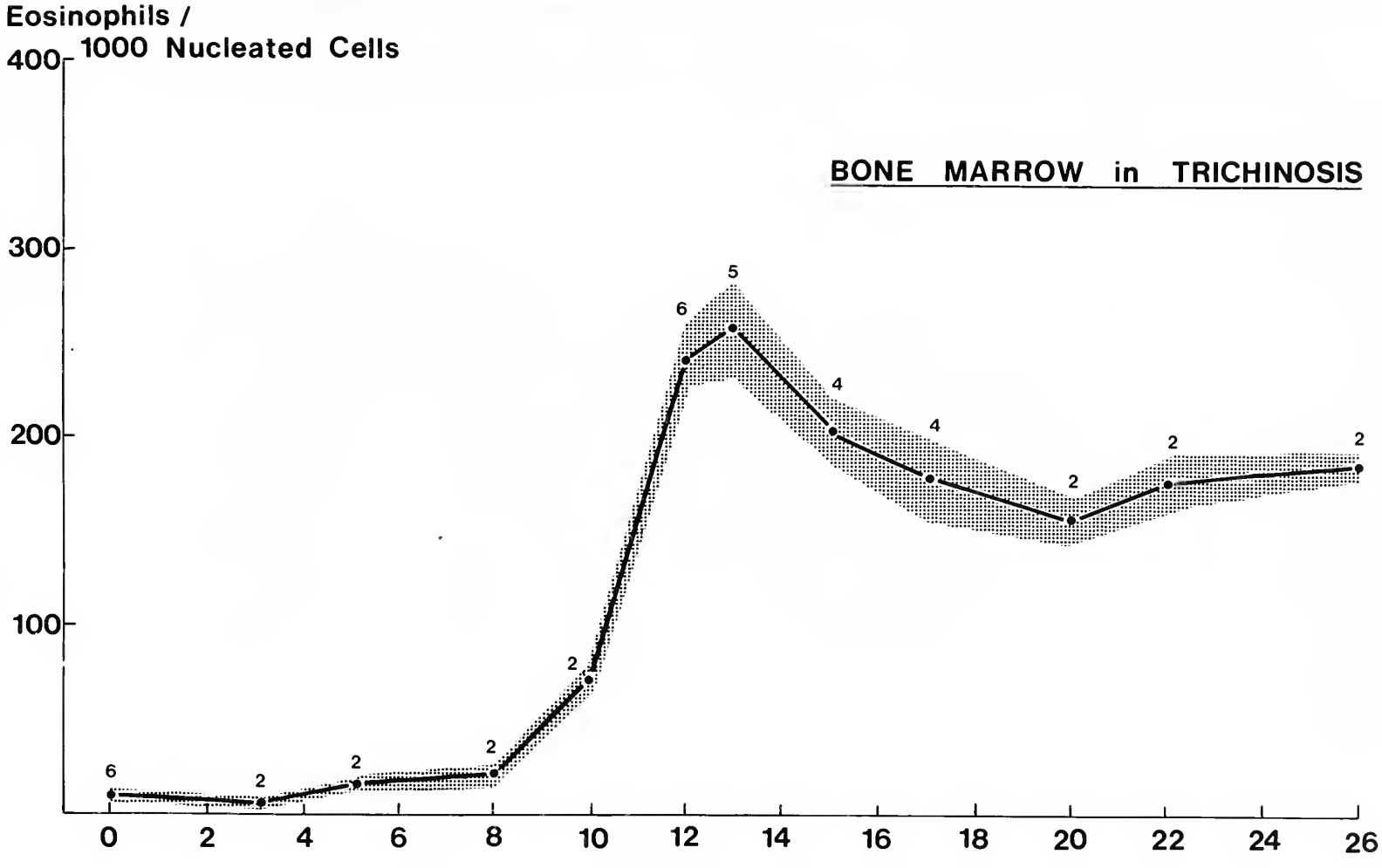
There were variations in the eosinophilia from one mouse to another. Some had maximum eosinophil counts of 900 per cu.mm.; others of 200 per cu.mm. This variation was assumed to be due to variations in the dose of larvae.

In every group of thirty mice, there were one or two which showed no eosinophilia, or only a very slight rise, with a maximum below 100 eosinophils per cu.mm. It was assumed that these mice had received a low larval dose. They were excluded from the results, and from further experiments.

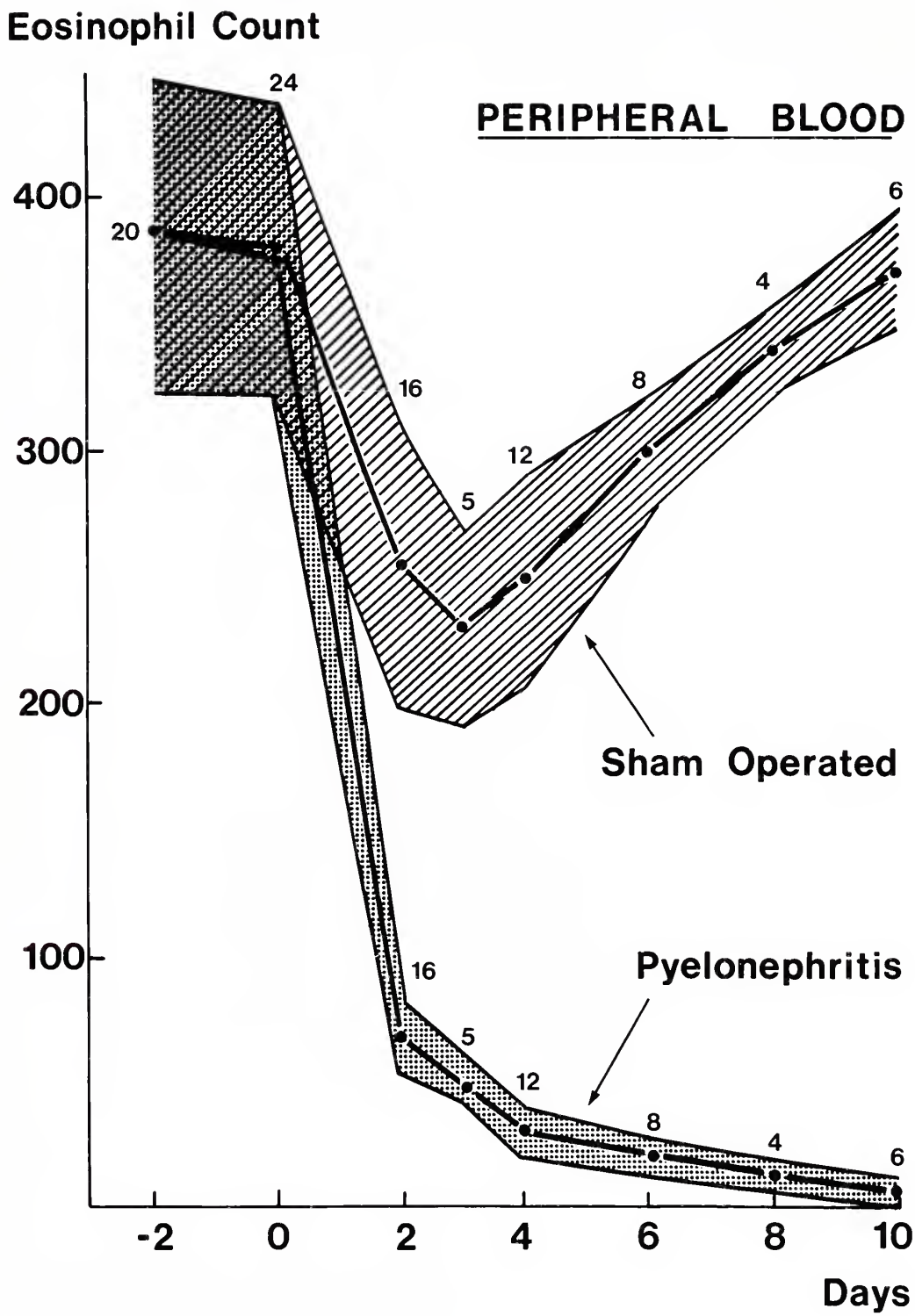
A series of experiments was done, the combined results of which are shown in Graphs III and IV.



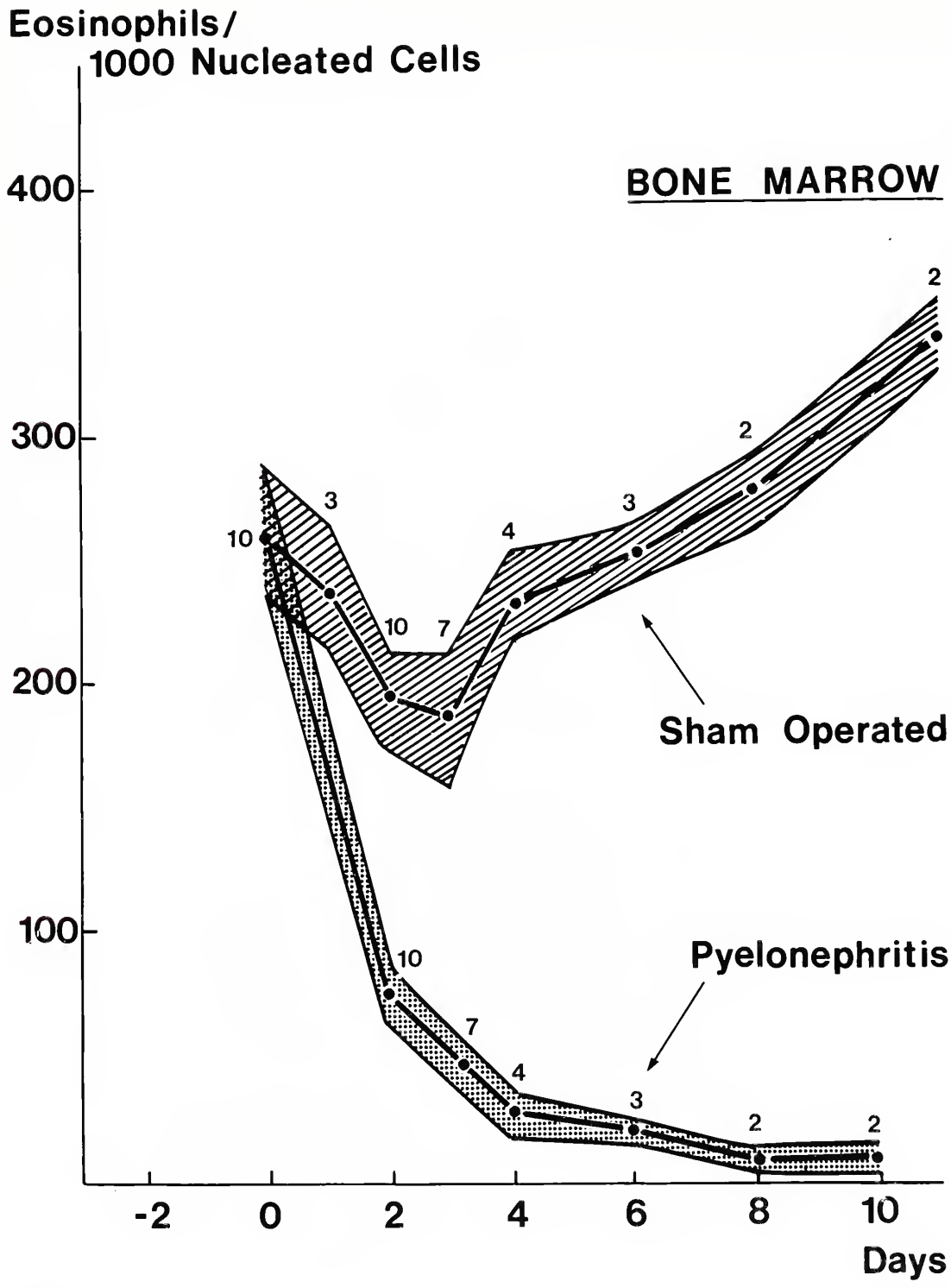

I. Changes in peripheral eosinophils in mice with trichinosis. Graph illustrates the sudden peripheral eosinophilia which develops on the 12th day after Trichinella infestation, which is maintained from the 12th day onwards. The number above each graph point is the number of mice used. Shaded area represents the standard error.




II. Changes in bone marrow eosinophils in mice given trichinosis. Graph illustrates the eosinophil changes in the bone marrow which parallel those in the peripheral blood: the eosinophilia on day 12 which persists thereafter. The number above each graph point is the number of mice used. Shaded area represents the standard error.




III. Changes in peripheral eosinophils in mice with trichinosis after (1) sham-operation and E. coli or (2) ureter ligation and E. coli (pyelonephritis). Day 0 is the day of operation. Day 1 is the day of E. coli injection. The high eosinophil count of Day 0 shows the effects of trichinosis. By Day 2, mice with pyelonephritis show the marked fall in peripheral eosinophils which is characteristic of post-infectious eosinopenia. The number above each graph point is the number of mice used. Shaded area represents the standard error.




IV. Changes in bone marrow eosinophils in mice with trichinosis after (1) sham-operation and E. coli or (2) ureter ligation and E. coli (pyelonephritis). Day 0 is the day of operation. Day 1 is the day of E. coli injection. The high eosinophil count of Day 0 shows the effects of trichinosis. By Day 2, mice with pyelonephritis show the marked fall in bone marrow eosinophils which parallels the fall in peripheral eosinophils, and which is characteristic of post-infectious eosinopenia. The number above each graph point is the number of mice used. Shaded area represents the standard error.

2. The First Experiment

Knowing the normal eosinophil count, and that following trichinosis, the eosinopoietic stimulus was challenged by pyogenic infection. The infection was produced by ureter ligation followed by intravenous E. coli. It has been shown that about 10 percent of some strains of mice will develop pyelonephritis with the injection of E. coli alone, without ureter ligation. However, the C3H strain of mice was very resistant, and in no case developed pyelonephritis without ureter ligation. The controls were mice with trichinosis which were sham-operated, and then given E. coli. These were to show the effect, if any, of stress and endotoxin on peripheral and marrow eosinophils.

Twenty-one mice[1] were infected with Trichinella. Eosinophil and total white blood cell counts were done every second day. Bone marrow slides, done on days 0, 8 and 12 were used to chart the changes in marrow eosinophils and neutrophils. On day sixteen, one-half of the mice were sham-operated. The others had the right ureter ligated. The next day, all the mice received E. coli. The mice were killed 2, 4, 6 and 10 days after operation. A peripheral count was done before each mouse was killed for the bone marrow count.

This experiment showed a significant drop in peripheral eosinophils 24 hours after E. coli infection. The marrow showed a corresponding drop to normal levels within 24 hours.

[1] The experiment began with 21 mice. There was a total of five deaths from anesthesia, operation and E. coli.

The neutrophils of the peripheral blood and marrow are inversely related to the eosinophils. The neutrophils fell as the eosinophils rose during the development of trichinosis. Then, the neutrophils rose as the eosinophils fell after E. coli infection. The post-operative marrow counts should be viewed with caution as they are from only eight animals: four after sham-operation and four after ureter ligation.

Despite the small numbers, the results suggested that a specific factor from the infection might be responsible for the changes in the peripheral and marrow eosinophils. Naturally, there would be a transient eosinopenia from the stress-induced steroids of the operation, but the peripheral eosinopenia which was observed was not transient. The changes in the marrow could not be attributed to a steroid effect. There is nothing to suggest that eosinopenia in the bone marrow can result from the transient release of steroids from a short-term stress. The fall within 24 hours of the marrow eosinophils was puzzling. This was too sudden to be accounted for by a decreased eosinopoiesis.[1] It was decided to do more experiments to confirm or refute the preliminary results.

### 3. The Second Experiment

In this experiment, and the next two, the day of operation was changed to the twelfth day after Trichinella infection.

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[1] The work of Spry (D. Phil. thesis, Oxford, 1970) shows the emergence time of eosinophils in rats with trichinosis is 17 hours.

(In the first experiment it was on the sixteenth day.) By making the operation earlier, at the time of the maximum rise in eosinophils, it was hoped that the greatest possible change in eosinophils could be seen. It was not certain whether the stress of the numerous blood counts of the first experiment had affected the peripheral blood picture. To avoid this difficulty, three mice were set aside for peripheral blood counts only, and the rest were used for bone marrow studies.

Peripheral counts were done on day 0 to be sure of a low normal eosinophil count; on day twelve to be sure of an eosinophil response to the Trichinella; on the second post-operative day to show persistent eosinophilia in the sham-operated animals. The counts on days 0 and 12 were as expected. However, the pre-operative counts in the three sham-operated mice were 385, 962.5 and 1188. These counts were much higher than in the first experiment, where the peak eosinophil count on day twelve was 330 per cu.mm.

In this experiment the eosinophils of ligated and sham-operated mice declined together. This did not agree with the results of the first experiment. It was thought that the low marrow counts might be the result of the extremely high peripheral eosinophilia. A great release of eosinophils into the peripheral blood might conceivably deplete the marrow. It was decided to repeat the experiment with more detailed peripheral counts.

## 4. The Third Experiment

In repeating the previous experiment, the peripheral eosinophils were not followed before the operation, but a peripheral count was done on each mouse before it was killed for bone marrow. Mice were killed before operation, and at intervals after *E. coli* injection. In this experiment the peripheral and bone marrow eosinophils of the sham-operated and ligated mice decreased after *E. coli* injection, as they had done in the second experiment. However, the eosinophils of the sham-operated mice were consistently higher than the ligated mice.

It was possible that the peripheral counts might be affected by variations in the dose of larvae, and obscure a post-infectious eosinopenia. For example, a sham-operated mouse with a low larval dose, sacrificed on the same day as a ligated mouse with a high larval dose might obscure the differences of the eosinophil response in the two groups of mice. The variations in the larval dose would presumably affect the bone marrow picture, as well as the peripheral count. To check this possibility, the fourth experiment was carried out.

## 5. The Fourth Experiment

In this experiment, peripheral counts were done on each mouse at the time of operation. Sham-operated and ligated mice were matched in pairs, according to their

pre-operative eosinophil count. Pairs of animals were killed for bone marrow 4, 12, 24, 48 and 96 hours after E. coli injection.

The results of this experiment resemble those of the first experiment in showing a sharp fall in the peripheral and marrow eosinophils following ureter ligation and E. coli. This fall is more marked than that of the second and third experiments. On the other hand, they resemble the results of the second and third experiments in showing a moderate decline in the eosinophils in the sham-operated mice.

## 6. Conclusions

After a review of the results of trichinosis alone (Graphs I and II), and the results of these four experiments (Graphs III and IV), the following decisions were reached:

### A. Day of Operation

The day of ureter ligation should be postponed until the eighteenth day after Trichinella infection, when a stable eosinophil level was reached. Otherwise, it would not be clear whether the eosinophil decline in the control and experimental mice was due to the natural course of trichinosis, the stress of the operation, the endotoxin of the E. coli or the pyogenic infection.

Operation on day twelve, the peak day of eosinophilia, was far more stressful than on a later day. In the original experiment, when ligation was on day sixteen, two of ten mice died during the operation. The second, third

and fourth experiments, with operation on day twelve, had mortality rates of fifty percent or more. The days of peak eosinophilia are felt to be the days of greatest reaction to Trichinella larvae.

B. The Effect of Steroids

To differentiate the eosinophil effect of stress-induced steroids from that of infection, it was necessary to study the effects of steroids alone on mice with trichinosis.

C. Timing of Marrow Counts After E. Coli

A review of the marrow slides showed that too many mice had been killed too early after E. coli infection. In each experiment, several mice had been killed less than 24 hours after the E. coli. Although these early changes in eosinophils were of interest, the nature of the delayed eosinophil response to infection had not been clearly defined. Since time and techniques limited the number of mice which could be used in any one experiment, they would be better used later in the experiments.

D. Control for Ureter Ligation

It was decided to add a second set of controls in the light of a recent article (Foster et al. 1970) which reported that ureter ligation alone could affect marrow leucopoiesis. The second control group would be mice with trichinosis and ligated ureters, without E. coli infection. The subsequent experiments would have three groups of animals:

i. The experimental group, with trichinosis, ligated ureters and *E. coli* infection. These would show the effect of infection on eosinopoiesis.

ii. The first control group, with trichinosis, a sham-operation and *E. coli*. These would show the effects of bacterial endotoxin on eosinopoiesis.

iii. The second control group with trichinosis and ligated ureters, but no infection. These would show the effect of ureter ligation on eosinopoiesis.

E. Accuracy of Bone Marrow Counts

The bone marrow preparations were reviewed, and the inaccuracy of any one count of 1000 cells was striking. It was decided to increase the count to 2000 for each marrow preparation. In addition, it was decided to count marrow slides of both femurs, instead of only the right femur. This would act as a further check on the accuracy of the cell counts, and would increase the marrow count to 4000 for each mouse.

## CHAPTER VI

## URETER LIGATION vs LIGATION AND E. COLI IN MICE WITH TRICHINOSIS

The first four experiments indicated that the eosinophilia of trichinosis declined to normal levels in the face of a superimposed pyelonephritis. Since sham-operated mice showed an initial, transient fall with rapid recovery in eosinophil levels, the stress of operation and anesthesia had been excluded as a major cause of prolonged "post-infectious" eosinopenia.

It was possible that ureter ligation, rather than the infection itself was responsible for "post-infectious" eosinopenia. To find out if this were so, ureter ligation alone was compared with pyelonephritis. There were three groups of animals:

1. Trichinosis, sham-operation, intravenous *E. coli*.
2. Trichinosis, ureter ligation and intravenous *E. coli*.
3. Trichinosis and ureter ligation.

The operation was on the eighteenth day of trichinosis. *E. coli* was given 24 hours later. Marrow counts were done on each of the first four post-operative days. Peripheral

counts were done on the day a mouse was killed for bone marrow.[1]

Table II shows the results of the experiment. The mean peripheral eosinophil count on the day of operation was 300 eosinophils per cu.mm. At twenty-four hours, before E. coli was given, the peripheral count had fallen to 73.5 eosinophils per cu.mm. in ligated mice; 71.0 eosinophils per cu.mm. in the sham-operated mouse. During the three days after E. coli, the peripheral counts fell still further in mice with pyelonephritis (i.e. ureter ligation and E. coli). Peripheral counts rose to pre-operation levels both in sham-operated mice and in mice with ureter ligation.

The marrow eosinophil counts declined in mice with pyelonephritis. Sham-operated mice had high marrow eosinophil counts. Counts in mice with ureter ligation alternately rose and fell, the final count being 86 eosinophils per 1000 nucleated cells on the fourth post-operative day (i.e. 72 hours after E. coli).

The peripheral eosinophil response to ureter ligation excluded ureter ligation as the cause of post-infectious eosinopenia. The marrow counts after ureter ligation were ambiguous, but did not show the steady decline seen in mice with pyelonephritis. It was evident that the inevitable

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[1] Deaths during operation and within the first 24 hours after operation were very high. Ten mice died out of twenty-eight. To avoid more deaths, the number of peripheral counts was restricted, since these are stressful in themselves and because of the anesthesia they require.

TABLE II

EFFECTS ON EOSINOPHILS OF URETER LIGATION

ALONE vs LIGATION AND E. COLI

19 mice, 200 larvae given to each.

On day 18 of trichinosis:

Group I. Control. no operation. Eosinophil counts done day 18.

Group II. Sham-operation; *E. coli*, 24 h. later.

Group III. Ureter Ligation

A. No *E. coli*.

B. *E. coli*, at 24 h., i.e. pyelonephritis.

GROUP I

Trichinosis only. Day 18

| Peripheral | Marrow |
|---|---|
| 264.5 | 156 |
| 357.0 | 161 |
| 286.0 | 180 |

| Hours Post-Op | GROUP II | | GROUP III-A | | GROUP III-B | |
|---|---|---|---|---|---|---|
| | Periph. | Marrow | Periph. | Marrow | Periph. | Marrow |
| 24 | 71.5 | 82 | 60.5 | 213 | | |
| | | | 44.0 | 173 | | |
| | | | 104.5 | 128 | | |
| 48 | 99.0 | 117 | 115.5 | 127 | 60.5 | 85 |
| | | | | | 55.0 | 93 |
| 72 | 214.5 | 190 | 462.0 | 168 | 11.0 | 53 |
| | | | | | 33.0 | 46 |
| 96 | 313.5 | 225 | 368.5 | 86 | 27.5 | 25 |
| | | | | | 16.5 | 13 |

eosinopenia of both bone marrow and blood after pyelonephritis could not be the result of ureter ligation alone. However, it was conceivable that ureter ligation had a slight depressive effect on marrow eosinophils, distinct from that caused by infection.

Operation, anesthesia and ureter ligation had been eliminated as causes of "post-infectious" eosinopenia. It seemed therefore that the eosinopenia was probably a direct result of pyelonephritis.

## CHAPTER VII

## THE ROLE OF STEROIDS IN POST-INFECTIOUS EOSINOPENIA

The sham-operated mice seemed to have eliminated stress-induced steroids as the cause of post-infectious eosinopenia. Adrenal steroids are known to decrease peripheral eosinophils. It is generally thought that steroids increase marrow eosinophils by inhibiting their release from the marrow (Hudson 1963). It therefore seemed unlikely that steroids were responsible for the marrow and circulating eosinopenia that was observed after infection. However, it was not impossible that they were involved, and several experiments were done comparing the effects of steroids with that of pyelonephritis in trichinous mice.

### 1. The Results of Injections of Cortisone Acetate

#### A. Large Doses of Cortisone Acetate

A large dose of cortisone acetate, 12.5 mg., was given by intra-peritoneal injection to 14 mice on the eighteenth day of trichinosis. The injection was repeated every day for three days. Each day four mice were killed for marrow and peripheral counts.

The results are shown in Table III. The rapid fall in peripheral eosinophils which was evident 24 hours after the first injection of cortisone had been expected. The fall in marrow eosinophils had not been expected.

TABLE III

EFFECT ON EOSINOPHILS OF CORTISONE ACETATE INJECTIONS

HIGH DOSE

14 mice, 200 larvae given to each mouse.

On days 18, 19, 20 of trichinosis:

12.5 mg. cortisone acetate injected intraperitoneally, each day. All counts done 24 h. after previous injection.

| Day | Dose | Mouse | Eosinophils, Peripheral Blood (per cu.mm.) | Eosinophils, Bone Marrow (in 1000 marrow cells) |
|---|---|---|---|---|
| Day 18 | Before Cortisone | ( 1) | 275.0 | 208 |
| | | ( 2) | 165.0 | 165 |
| Day 19 | 24 h. after 12.5 mg. | ( 3) | 5.5 | 232 |
| | | ( 4) | 11.0 | 66 |
| | | ( 5) | 5.5 | 155 |
| | | ( 6) | 16.5 | 184 |
| Day 20 | 48 h. after 25.0 mg. | ( 7) | 0 | 98 |
| | | ( 8) | 5.5 | 31 |
| | | ( 9) | 11.0 | 63 |
| | | (10) | 5.5 | 87 |
| Day 21 | 72 h. after 37.5 mg. | (11) | 0 | 32 |
| | | (12) | 0 | 23 |
| | | (13) | 5.5 | 6 |
| | | (14) | 11.0 | 12 |

Marrow eosinophils began to decline within 24 hours, were definitely decreased at 48 hours and were in the normal range by 72 hours. A rise in marrow eosinophils had been expected. Not only was the decline unexpected, but it seemed to follow the same time pattern as "post-infectious" eosinopenia of marrow and blood seen after pyelonephritis.

The dose, 12.5 mg., was extremely high, far beyond the physiological range. It was possible that it had produced an effect unrelated either to "post infectious" eosinopenia, or indeed to any physiological mechanism.

B. Lower Doses of Cortisone Acetate

The experiment was repeated using 0.5 mg. of cortisone, instead of 12.5 mg. The results, Table IV, show that on days two and three, half of the mice had low counts, and half had high counts. The mice with high counts weighed roughly five grams more than those with low counts. It appeared that 0.5 mg. was a borderline dose of cortisone acetate.

There is an increase in marrow and peripheral eosinophils on the third day, and a fall on the fourth. The initial rise in the bone marrow, on the third day, agrees with Hudson's findings. Yet it appears that prolonged steroids, possibly by a cumulative effect, lead to eosinophil depression in the marrow as well as in the peripheral blood.

TABLE IV

EFFECT ON EOSINOPHILS OF CORTISONE ACETATE INJECTIONS

LOW DOSE

14 mice, 200 larvae given to each mouse.

On days 18, 19, 20 of trichinosis:

0.5 mg. cortisone acetate injected intraperitoneally, each day. All counts done 24 h. after previous injection.

| Day | Dose | Mouse | Eosinophils, Peripheral Blood (per cu.mm.) | Eosinophils, Bone Marrow (in 1000 marrow cells) |
|---|---|---|---|---|
| Day 18 | Before Cortisone | ( 1) | 176.0 | 138 |
| | | ( 2) | 203.5 | 312 |
| Day 19 | 24 h. after 0.5 mg. | ( 3) | 374.0 | 323 |
| | | ( 4) | 16.5 | 205 |
| | | ( 5) | 11.0 | 285 |
| | | ( 6) | 220.0 | 89 |
| Day 20 | 48 h. after 1.0 mg. | ( 7) | 11.0 | 231 |
| | | ( 8) | 665.5 | 301 |
| | | ( 9) | 258.5 | 347 |
| | | (10) | 0 | 312 |
| Day 21 | 72 h. after 1.5 mg. | (11) | 0 | 80 |
| | | (12) | 0 | 150 |
| | | (13) | 27.5 | 66 |
| | | (14) | 181.5 | 106 |

2. Synacthen Compared to Cortisone

An attempt was made to reproduce, with synthetic ACTH, the effects of injected steroids. ACTH increases steroid release from the adrenal cortex, and should reproduce the effects of steroids.

Synthetic ACTH (Synacthen), 0.5 units, was given to seven mice, and had no effect on circulating eosinophils. Counts were done six hours after the injection, and the results are shown in Table V. It was assumed that the dose was too low.

Two days later, the seven mice were divided into two groups. Group I, four mice, received 1 unit of Synacthen. Group II, three mice, received 2 mg. of cortisone acetate.

Synacthen, 1.0 units, showed no more effect than 0.5 units on the peripheral eosinophils at six hours. The cortisone acetate, as expected, reduced the eosinophil level to normal.

Follow-up counts at forty-eight hours showed low counts in the cortisone-injected mice.[1] However, in the mice given the Synacthen, the peripheral eosinophils, instead of falling or even staying the same, had tripled. All the counts were over 1000 eosinophils per cu.mm. A count over 1000 per cu.mm. in these mice is rare, and this uniform rise could only

[1] Autopsy after injections of cortisone show white deposits in the peritoneum. Cortisone is not very water-soluble, and seems to precipitate in the peritoneum. This may account for its prolonged effect.

TABLE V

EOSINOPHIL EFFECTS OF SYNACTHEN AND CORTISONE

7 mice, 200 larvae given to each mouse.

On day 18: 0.5 units Synacthen injected intraperitoneally. Peripheral count at 6 hours.

On day 20: Group A: 1 unit Synacthen injected intraperitoneally, count at 6 hours.

Group B: 2 mg. cortisone acetate injected intraperitoneally. Peripheral count at 6 hours.

On day 22: Recount of Group A and Group B.

On day 28: Recount of Group A and Group B.

| Day | Day 18 | Day 20 | | Day 22 | | Day 28 | |
|---|---|---|---|---|---|---|---|
| | 0.5 units Synacthen to all mice | | (per cu.mm.) | | (per cu.mm.) | | (per cu.mm.) |
| Peripheral eosinophil counts | 176.0 | Group A: | 247.5 | No | 1243.0 | No | 339 |
| | 176.0 | Synacthen | 467.5 | drug | 1257.0 | drug | 334 |
| | 429.0 | 1.0 units | 489.5 | given | 1650.0 | given | 272 |
| | 192.5 | given to 4 mice | 610.5 | | 1045.0 | | 383 |
| | 352.0 | Group B: | 16.5 | No | 0 | No | 89 |
| | 126.5 | Cortisone | 11.0 | drug | 16.5 | drug | 67 |
| | 258.5 | Acetate 2 mg. given to 3 mice | 11.0 | given | 0 | given | 167 |

be attributed to the Synacthen. There was no explanation for the massive eosinophilia forty-eight hours (day 22) after the second dose of Synacthen. It seemed that the Synacthen had reduced, instead of increased, the output of the adrenal cortex.

3. Changes of the Adrenal Gland with Synacthen

It is possible that trichinosis makes the adrenal cortex incapable of responding to Synacthen. If this is so, the size of the adrenal gland might be smaller than in a normal mouse. The normal adrenal is a very small, pink sphere, 1 mm. in diameter, a few millimetres medial and superior to the upper pole of the kidney. Autopsies of four mice with trichinosis showed no difference in the size or position of the gland.

Four normal mice given Synacthen had adrenal glands which on microscopic examination seemed to be larger and paler than normal glands. The glands were nearer to the upper pole of the kidney, possibly the result of enlargement of the gland.

Trichinous mice given Synacthen did not show this change, and had adrenal glands similar to normal mice. This suggests that mice with trichinosis may not respond in a normal fashion to increased ACTH production.

4. Blood Cortisol Levels

Blood cortisol levels were done on nine mice, as a preliminary experiment. Four were normal; four were normal mice given 1 unit of Synacthen; and one was a mouse with trichinosis and pyelonephritis, 72 hours after operation and 48 hours after E. coli.

Since blood cortisol levels require 1 millilitre of blood, and since it is difficult to get blood from mice, levels were done on a mixture of two blood samples from different mice. As there was only one mouse with trichinosis and pyelonephritis, the blood cortisol level for this mouse is only a suggestive value. The results are shown in Table VI. The cortisol levels are higher in mice given Synacthen,[1] which shows that the Synacthen was active in these mice. The earlier finding of eosinophilia after giving Synacthen, section 2 above, could not be the result of Synacthen inactivity. Although it is a doubtful value, the cortisol level of 125 in the mouse with trichinosis and pyelonephritis is much higher than normal. It might be an indication that the eosinopenia following pyelonephritis is the result of increased steroid release.

5. Blood Cortisol Levels in Mice with Trichinosis

Before the above values could be interpreted, more values for normal mice were needed, as well as values for mice with trichinosis alone. Accordingly, of a group of 44 mice, 30 mice were killed to determine normal blood cortisol levels. The remaining 14 were given trichinosis and then had blood cortisol determinations.

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[1] It was later found that 60 minutes was the correct time to sample for peak cortisol levels following Synacthen injections.

TABLE VI

CORTISOL LEVELS IN NORMAL MICE

BEFORE AND AFTER SYNACTHEN

| Mice | Procedure | Cortisol, gamma/100 ml. |
|---|---|---|
| 1 & 2 | Normal | 35 |
| 3 & 4 | Normal | 22 |
| 5 & 6 | Normal, 6 h. after 1 unit Synacthen | 45 |
| 7 & 8 | Normal, 6 h. after 1 unit Synacthen | 79 |
| 9 | Day 20 Trichinosis 72 h. after ureter ligation 48 h. after *E. coli*. | 125 |

Our results are shown in Table VII. The results were not at all what had been expected. The mice with trichinosis alone had cortisol levels far higher than normal. This cast doubt on the earlier hypothesis that the eosinophilia of trichinosis was due to an inhibition of steroid release, caused by the trichinosis, and that the "post-infectious" eosinopenia was the result of renewed release of steroids by bacterial infection. The cortisol level in normal mice is 44 gamma and rises to 121 g. one hour after an injection of Synacthen. The cortisol level in trichinous mice is 105 g. and rises to 257 g. one hour after Synacthen, and falls to 64 g. six hours later. In trichinous mice, both before and after Synacthen, the cortisol levels are more than twice the corresponding normal levels. The cortisol level of the mouse with trichinosis and pyelonephritis was the same as the cortisol level of the mice with trichinosis alone.

These experiments with steroids are inconclusive. However, these cortisol levels suggest that "post-infectious" eosinopenia is not the result of steroid changes alone.

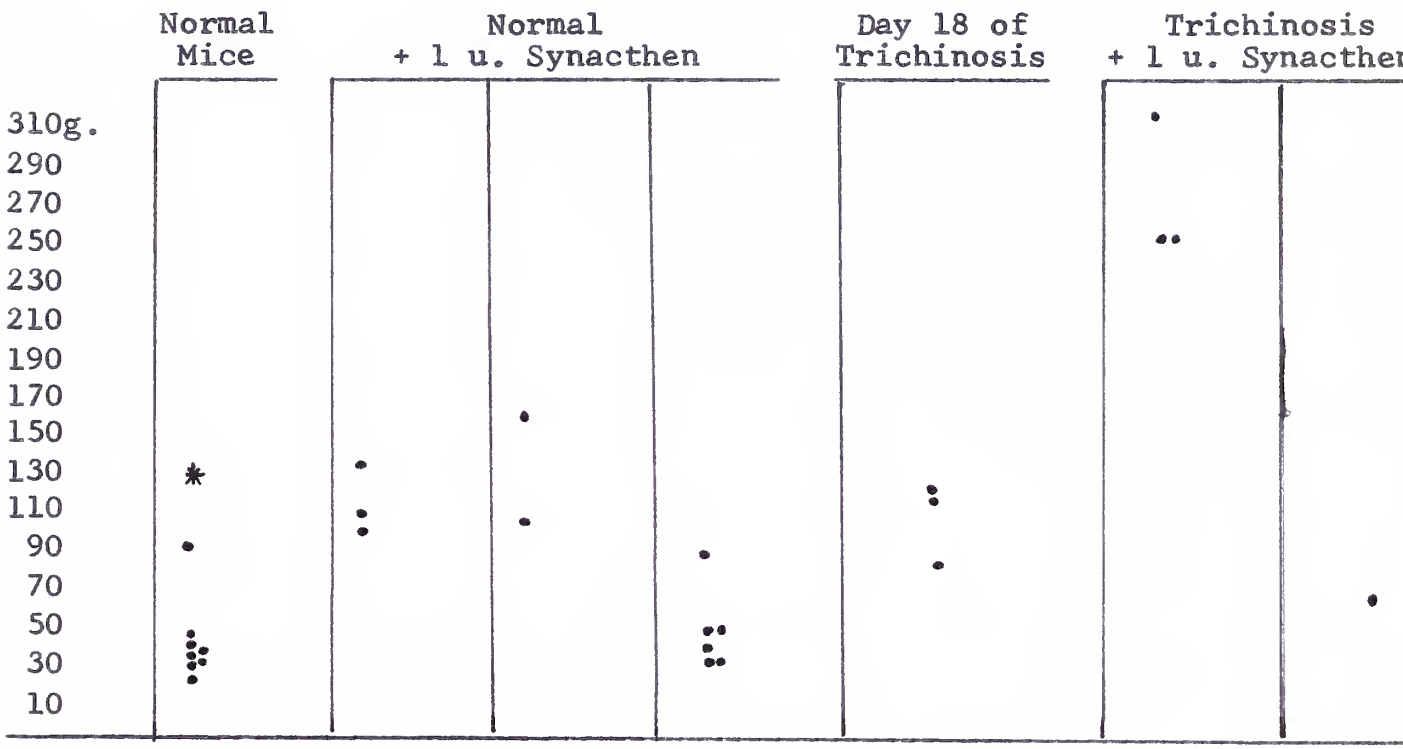
TABLE VII

EFFECT OF TRICHINOSIS AND/OR SYNACTHEN ON CORTISOL LEVELS IN MICE

| Normal Mice | Normal + 1 u. Synacthen | | | Day 18 of Trichinosis | Trichinosis + 1 u. Synacthen | |
|---|---|---|---|---|---|---|
| | 30' | 60' | 6 hours | | 60' | 6 hours |
| 35g. | 134g. | 150g. | 45g. | 120g. | 310g. | 64g. |
| 22 | 100 | 92 | 79 | 115 | 230 | |
| 37 | 96 | | 45 | 75 | 230 | |
| 87 | | | 28 | | | |
| 30 | | | 28 | | | |
| 41 | | | 32 | | | |
| 42 | | | | | | |
| 31 | | | | | | |

* The cortisol level of one mouse with trichinosis and pyelonephritis was 125 gamma.

Graph of Cortisol Levels (shown in table above)




* Pyelonephritis and Trichinosis.

## CHAPTER VIII

## TRICHINOSIS, PYELONEPHRITIS AND BILATERAL ADRENALECTOMY IN RATS

The experiments had raised the question whether corticosteroids were involved in post-infectious eosinopenia. To investigate this further, an attempt was made to combine bilateral adrenalectomy, unilateral ureter ligation and *E. coli* intravenously in trichinous mice. If post-infectious eosinopenia did not develop, it would be an indication that steroids were responsible for post-infectious eosinopenia.

Unfortunately, the mice did not survive the combined operation. Twenty-four hours post-operatively, one mouse of the original ten was still alive. Then the experiment was repeated using Harwell rats, since they are tough animals and their response to trichinosis is known in detail (Basten 1969).

### 1. First Experiment

In a preliminary experiment, five rats were given trichinosis. On the eighteenth day of trichinosis, four rats had a simultaneous unilateral ureter ligation and bilateral adrenalectomy. The fifth was used for control. One of the four was killed for a marrow count forty-eight hours after the operation (i.e. 24 hours after *E. coli*), and the remaining three were killed for marrow counts seventy-two hours after the operation (i.e. 48 hours after *E. coli*).

This preliminary experiment showed no post-infectious eosinopenia, either in the blood or in the bone marrow even forty-eight hours after the injection of E. coli, suggesting that adrenal steroids might be necessary for post-infectious eosinopenia.

2. Second Experiment

The results of this preliminary experiment were encouraging, and the experiment was repeated with twelve rats, divided into three groups:

i. Control, killed before operation, on the 18th day of trichinosis - 2 rats.

ii. Bilateral adrenalectomy and pyelonephritis, the operation being done on the 18th day of trichinosis - 6 rats.

iii. Pyelonephritis, the operation being done on the 18th day of trichinosis - 4 rats.

Four rats in this experiment died during the operation. The combined results from the eight survivors and the five rats of the preliminary experiment are shown in Table VIII.

Although rats with trichinosis do not seem to show the dramatic bone marrow post-infectious eosinopenia seen in mice, the peripheral counts show a marked fall. However, with bilateral adrenalectomy, i.e. decreased steroids, there is no sustained eosinopenia either in the blood or in the bone marrow following pyelonephritis. The results of these experiments, although they are not conclusive, suggest that the removal of the adrenals and hence absence of steroids, inhibits or prevents the destruction of eosinophils seen with combined parasitic and bacterial infection.

TABLE VIII

EFFECT OF ADRENALECTOMY

ON POST-INFECTIOUS EOSINOPENIA

| Experimental Group | Peripheral Blood Eosinophils (per cu.mm.) | Bone Marrow Eosinophils (of 1000 marrow cells) |
|---|---|---|
| i. Trichinosis only | 427 | 404 |
| | 429 | 398 |
| ii. Trichinosis and Pyelonephritis | | |
| 24 h. after *E. coli* | 28 | 321 |
| | 172 | 142 |
| 48 h. after *E. coli* | 49.5 | 255 |
| iii. Trichinosis, Pyelonephritis and Bilateral Adrenalectomy | | |
| 24 h. after *E. coli* | 55 | 312 |
| | 0 | -- (poor preparation. No reliable count.) |
| 48 h. after *E. coli* | 267 | 241 |
| | 150 | 270 |
| | 210 | 419 |
| | 247.5 | 450 |
| | 199.0 | 414 |

## CHAPTER IX

## SUMMARY AND CONCLUSIONS

These experiments have established the effects on eosinophils of Trichinella infection in C3H mice. Trichinosis produces a marked eosinophilia in the circulating blood and in the marrow, beginning about the tenth day of infection and persisting for some weeks. There is a close correlation between the number of eosinophils in the blood and the number in the bone marrow.

Acute Escherischia coli pyelonephritis in trichinous mice produces a marked fall in blood and bone marrow eosinophils, reducing them to normal or near normal levels within twenty-four to forty-eight hours. This fall in eosinophils is accompanied by a simultaneous rise in neutrophils.[1] This reduced level persists for at least a week, the period of observation. This reciprocal relation between eosinophils and neutrophils suggests that the mechanism causing the neutrophilia may be involved in simultaneous eosinopenia.

The role of steroids in this post-infectious eosinopenia in trichinous mice was investigated. The results of the experiments were inconsistent with one another, but the findings were the following:

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[1] Although not reported in this thesis, neutrophil counts done on trichinous mice showed a rise from 6000 W.B.C./cu.mm. to 20,000/cu.mm. or more, 48 hours after super-infection with E. coli. Control mice given E. coli but having a sham operation did not show this rise.

1. Cortisone acetate in trichinous mice causes a rapid decline in peripheral eosinophils in six hours. High doses cause a decline in marrow eosinophils within forty-eight hours in mice with trichinosis.

2. ACTH would be expected to produce a similar effect to that of cortisone. However, Synacthen, a synthetic ACTH, in trichinous mice, causes no fall in peripheral eosinophils, but within forty-eight hours causes a rise in peripheral eosinophils. By ninety-six hours, there is a massive rise in eosinophils, far above the eosinophil levels of trichinosis alone.

3. Normal mice given Synacthen have increased blood cortisol levels. This indicates that Synacthen is active in these mice, although number 2 above raised the possibility that it was not.

4. Autopsy shows that normal mice may have adrenal gland enlargement following Synacthen injection. This enlargement is not seen in mice with trichinosis, suggesting an inhibition of the normal response to ACTH.

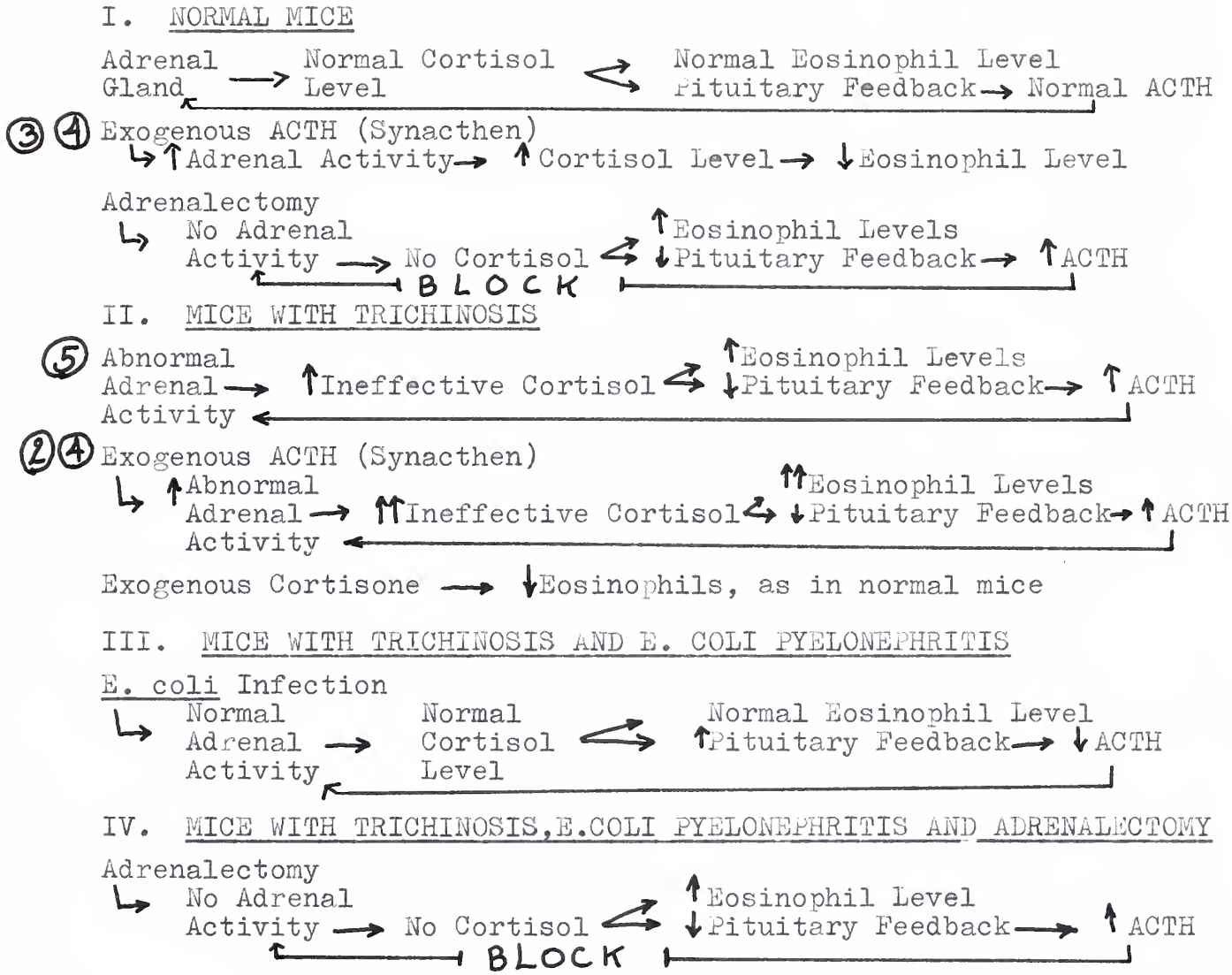
5. However, mice with trichinosis alone were found to have blood cortisol levels significantly higher than those of normal mice, indicating increased secretion of adrenal corticol steroids. Synacthen given to these trichinous mice raises blood cortisol levels higher still.

6. The removal of steroids, by bilateral adrenalectomy, in rats with trichinosis and pyelonephritis seemed to inhibit the development of post-infectious eosinopenia.

These experiments have provided an experimental model of the well-known clinical phenomenon of eosinopenia, following acute bacterial infection, first noted by Simon in 1922, and called by him the "septic factor." Exogenous steroids can produce, in trichinous mice, an eosinopenia which resembles that which follows acute E. coli pyelonephritis. However, it has not been possible to identify the mechanism of post-infectious eosinopenia. The results have established abnormally high cortisol levels in trichinosis. But, it has not been possible to define the relation of the steroid abnormality to the eosinophil changes seen in trichinosis and in super-imposed pyelonephritis.

Although the results appear contradictory, the following mechanism was postulated that might explain the findings: that trichinosis alters the adrenal production of cortisol and leads to the release of an aberrant and ineffective cortisol. Such an ineffective cortisol would lead to eosinophilia, as is seen in trichinosis. It might also cause increased ACTH release, because of a break in the feedback cycle. The stimulus of acute E. coli pyelonephritis might reverse the effects of trichinosis, and renew the production of normal cortisol. The re-establishment of normal cortisol, and feedback would produce normal levels of ACTH, normal levels of cortisol and an eosinopenia.

This hypothesis is diagrammed below. The numbers indicate the experimental evidence, listed at the beginning of this chapter, which the hypothesis explains.




This hypothesis does not explain how E. coli infection re-establishes normal cortisol release. It does explain the other results. Although the hypothesis may not be entirely correct, it is suggested that one approach to research investigating eosinophil behavior might be a study of the behavior of the adrenal gland and the structure of cortisol produced during trichinosis.[1]

[1] Further research on this problem is being carried out under the direction of Professor Paul Beeson, Nuffield Professor of Clinical Medicine, at the Radcliffe Infirmary, University of Oxford.

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