THE CYTOLOGIC RESPONSES OF NORMAL BEAGLE DOGS UTILIZING THE SKIN WINDOW TECHNIC

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THE CYTOLOGIC RESPONSES OF NORMAL BEAGLE DOGS UTILIZING THE SKIN WINDOW TECHNIC

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TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

For over a century numerous investigators have studied the structure and functions of the leukocytes and the other inflammatory cells with a variety of technics. Today the study continues with many questions still remaining unanswered.

The major problem in the study of leukocytes and inflammatory cells in inflammation has been the deficiencies in the available technics. Laboratory animals were used in the early studies and often it was necessary to sacrifice them making continuous studies impossible. In addition, it was impossible to serially study the cells from a single lesion.

In 1940 Rebuck introduced a new method, the skin window technic, which allowed the serial study of the leukocytes in acute inflammation in a single lesion. Since this simple technic produced only superficial abrasion of the skin, the experimental animals can be used repeatedly.

Rebuck first used the technic in the rabbit and later applied it to the human. Since that time it has been used many times to study the various functions and structures of the leukocytes and the other inflammatory cells in normal and diseased humans.

Riddle and Barnhart (1965) used the skin window technic on dogs to study the eosinophil as a source for profibrinolysin in acute inflammation. According to their findings they reported

that the cellular exudate in the canine species resembled that reported in the human skin window.

It is the purpose of this investigation to describe the cellular exudate obtained using the skin window technic in the normal beagle dog.

REVIEW OF LITERATURE

In 1829, Durtrochet first reported the migration of the white cells from the blood vessels into an inflamed area (Rebuck and Crowley, 1955). Since then many investigators have studied leukocytes and their functions in inflammatory reactions. In summary, their reviews stressed the importance of lymphocytes and monocytes as sources of macrophages in acute inflammation.

Rebuck (1940) introduced a new *in vivo* technic using the rabbit to study the role of the monocyte in acute inflammation, which later became known as the skin window technic (Sieracki and Rebuck, 1960). The skin window technic allows the serial study of the cellular exudate in a single lesion over any given period of time (Rebuck and Crowley, 1955; Rebuck, 1958; Edwards, 1959). Heretofore, this was not possible because the methods used resulted in considerable trauma or injury and the experimental animals had to be sacrificed.

Using the skin window technic, Rebuck (1949) described the functional and structural changes which occurred in the neutrophils and lymphocytes in acute inflammation in man when the patient was not systemically immunized to the stimulating antigen. The neutrophils were the first cells to migrate into a lesion. Initially they became swollen, and before they shrank and died, the neutrophils completed their enzymatic and phagocytic activities. Fragments of neutrophilic cytoplasm were shed into the fluid exudate and these small particles were then phagocytized by the lymphocytes.

The lymphocytes probably used these pieces of neutrophilic cytoplasm for nutrients.

Lymphocytes appeared in the exudate at two hours and gradually increased in number until they became the dominant cell at 14-18 hours. At the 9-14 hour stage the majority of the lymphocytes began to hypertropy and during the 14-18 hour period they were indistinguishable from tissue macrophages or histiocytes. As the lymphocytes transformed into macrophages, the cytoplasm increased and became more phagocytic (Rebuck, et al., 1958; 1960; 1961b). The nuclei also increased in size, the chromatin separated into finer pieces, the nuclear membrane lost the uniform smooth outline, and the nuclear indentation became larger.

The cellular exudate varied with use of different antigens and with the immunological status of the patient to a specific antigen. When using tuberculin as the antigen, Rebuck and Yates (1954) reported a significant decrease or absence of lymphocytes at the important 12 hour stage in patients who were tuberculin-positive. In contrast, the controls or tuberculin-negative patients responded with a high level of lymphocytes at the 12 hour stage of acute inflammation.

Poliomyelitis vaccines produced the highest level of lymphocytic migration at the 12 hour stage in individuals vaccinated with Salk polio vaccine, while the lymphocyte response in non-vaccinated subjects at the 12 hour stage was very poor (Rebuck and LoGrippo, 1960; 1961). In individuals immune to the typhus rickettsiae, the neutrophils remained the predominant cells for

24 hours. In nonimmune patients, the neutrophils were much less numerous (Wisseman and Tabor, 1965).

Hu, et al. (1961) studied Rus positive individuals and described an eosinophilia along with an increase in lymphocytes and giant cells at 33 hours of inflammation. Eitzman and Smith (1957) observed eosinophilias of 10-93% in infants of 36 hours to 21 days of age. Riddle and Barnhart (1965), in the only report using dogs, induced eosinophilic levels up to 40% on the coverslips by using stimulants of fibrin and fibrinogen.

Investigation in diseased humans has resulted in a variety of cellular responses. The normal leukocytic response is greatly diminished in patients with a peripheral blood neutropenia, and the subsequent hypertrophied forms of lymphocytes are also decreased in number. It is believed the time and the sequence of the cellular components of the exudate is governed, at least in part, by the neutrophil and that each step in the inflammatory cycle is subject to the preceding event (Page and Good, 1958).

In patients, with acute leukemia in which the circulating mature neutrophilic leukocytes were greatly decreased in number, Perillie and Finch (1960) observed markedly decreased and even acellular exudates on the coverslips. They concluded that this poor response was probably responsible for the susceptibility of these individuals to infection. Boggs (1960) also reported that immature cells were never located in the exudate. However, in patients with chronic leukemia in which the circulating mature neutrophils were near normal in number, a normal cellular exudate

was observed. Immature nucleated red blood cells in the peripheral blood also do not appear in the skin window exudate (Torre and Leikin, 1959).

A patient with polyarteritis nodosa exhibited increased eosinophils at seven hours (Rebuck, et al., 1951). Riis (1959) reported an increase in eosinophils in Felty's syndrome. Eosinophils comprised over half of the cellular exudate in patients allergic to fish extract, grasses or ragweed (Eidinger, et al., 1962). The topical application of a steroid failed to decrease the eosinophilia while oral administration greatly reduced the eosinophilic response (Eidinger, et al., 1964). The systemically acting steroid may possibly have reduced the total number of circulating eosinophils and thereby cause a decrease in eosinophils on the skin window coverslips.

The basophilic leukocyte is a rare participant in exudate of normal individuals. However, in patients with ulcerative colitis it was present at three hours of inflammation and increased steadily to peak between 14-27 hours. The peripheral blood basophilic level in these patients was normal or only slightly increased (Priest, et al., 1960). In contrast, a patient with terminal basophilic granulocytic leukemia, whose blood contained 52% basophils, had very few basophils in the skin window exudate (Rebuck, et al., 1961b). Patients with interstitial cystitis also were characterized by an increased migration of basophils which peaked at 26 hours (Rebuck, et al., 1961a).

The cellular exudate is altered by the actions of cortisone

and ACTH. Rebuck and Mellinger (1953) reported a diminished cellular response after topical application of cortisone at the site of the skin window lesion. The phagocytic capabilities of the exudate were correspondingly decreased due to the diminished number of cells. The same cellular response resulted following the systemic administration of ACTH (Rebuck, et al., 1951).

Perillie, et al. (1960) reported using the skin window technic in the study of the lupus erythematosus phenomenon and evaluated its possible use as a diagnostic aid. They concluded that the value of the technic as a diagnostic aid was as good as other methods available.

Several authors have questioned the ability of lymphocytes to transform into macrophages. Florey (1961) and Medawar (1961) stated that lymphocytes do not stick to glass surfaces. This evidence seriously challenges Rebuck and Crowley's (1955) interpretation of their skin window preparations in which they reported the transformation of lymphocytes into macrophages. Volkman and Gowans (1964) also presented evidence that small lymphocytes are not capable of transforming into macrophages. When tritiated thymidine labeled lymphocytes were transfused into rats at the time inflammation was created, no labeled lymphocytes appeared at the site of the lesion and no labeled macrophages were found on the coverslips. However, when labeled bone marrow cells were injected, many labeled macrophages appeared on the coverslips. This suggests precusors of macrophages other than lymphocytes. In order to avoid this controversy, these cells will be referred

to as small monuclear cells in this paper.

MATERIALS AND METHODS

A concurrent project in the College of Veterinary Medicine under contract with the National Institute of Health was the study of the transmissability of leukemic materials to dogs. Seven purebred beagle dogs from the normal colony of the leukemia project were used in this study. The dogs, three males and four females, ranged in age from 4-6 months and weighed from 15-20 pounds. The animals were well cared for from birth and were in excellent health as determined by physical examination. Total red and white blood cell counts as well as a differential white cell count were made before each animal was used. The blood counts were all within the normal range (Schalm, 1965).

The skin window technic of Rebuck and Crowley (1955) and modified by Riddle and Barnhart (1965) for dogs was used in this investigation. The author adopted a few further modifications to facilitate the work.

Number two, 15 mm square glass coverslips were alcohol cleaned. Two centimeter cardboard squares were cut from unlined file cards. A small, approximately 2 mm by 10 mm, strip of masking tape was folded on itself and stuck onto the cardboard squares. The coverslips were then attached to the cardboard by the use of the remaining adhesive ends of the tape. Attaching the coverslips to the cardboard facilitated their handling and reduced the chance of contamination of the lesion. This also allowed an easy method of identifying the cardboard squares with their accompanying coverslips.

The coverslips and the cardboard squares were wrapped in aluminum foil, placed in petri dishes and sterilized. Each individual kit could be removed from the petri dishes without contaminating the unused kits (Fig. 1).

To minimize chemical and mechanical trauma, the dog's skin was prepared for experimentation at least 15 hours prior to the creation of the lesion. The hair was clipped from the dog's back, shoulders and thorax. A commercial cream depliatory* was spread over this area and allowed to react for 15-25 minutes. The hair and cream were scraped away and washed with warm tap water. The area was then washed with Germicidal Soap** and allowed to dry.

A strip of two inch adhesive tape, twelve inches in length, was applied over the thoracic vertebral column. Another twelve inch strip of tape was applied about 15-20 mm below and parallel to the first piece of tape. Five short strips of tape about 15-20 mm apart were arranged perpendicular to the first two strips. This resulted in four small 15-20 mm square areas of exposed skin suitable for the experimental lesions. The application of the tape could be repeated on the opposite side of the dog. This would allow the study of a total of eight lesions simultaneously.

A small pledget of cotton was soaked in 70% alcohol and placed over the exposed skin. The cotton was held loosely in place with another piece of adhesive tape until the experiment

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*Nair, Carter Products, Inc., New York, New York.

**Park-Davis & Company, Detroit, Michigan.

was to begin.

At this time a Elizabethian collar was cut from a cardboard box and placed around the dog's neck. The hind legs were hobbled by tying them together with a piece of gauze. This prevented the dog from either biting or scratching at the taped areas.

A small lesion, 3-5 mm in diameter, was created by repeatedly scraping a sterile No. 20 Bard-Parker blade over the exposed skin. When fine bleeding points were observed and blood began to collect on the blade, the correct depth of the lesion was reached. The experiments were timed from this point.

A sterile platinum loop was used to apply one to two loops of an inflammatory stimulant, Diptheria Toxoid* or Old Tuberculin**. To study the phagocytic properties of the cellular exudate, trypan blue or Pelikan*** ink was also applied to eight of the experimental lesions. The lesions were immediately covered with the sterile coverslip and cardboard square which was held in place with a piece of adhesive tape (Fig. 2).

The cells migrated and adhered to the underside of the coverslips. The first coverslip was removed at two hours and replaced immediately with a second coverslip. The subsequent coverslips were removed every two hours until the experiment was terminated at the 24 hour stage.

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*Parke, Davis & Company, Detroit, Michigan.
**Parke, Davis & Company, Detroit, Michigan.
***John Henschel and Co., Inc., 425 Park Avenue, South New York 16, New York.

The coverslips were rapidly air dried and mounted on microscope slides with Permount*. The cells were then stained like blood smears with either Wright's-Leishman or May-Grünwald-Giemsa stains. The technic followed for the Wright's-Leishman stain was essentially that described by Schalm (1961), however, it was necessary to increase the staining time to 12 minutes. The procedure for the May-Grünwald-Giemsa stain was described by Rebuck and Crowley (1955).

A total of 384 coverslip preparations from 30 lesions were studied.

*Fisher Scientific Co., Fair Lawn, New Jersey.

RESULTS AND DISCUSSIONS

The diptheria and tuberculin antigens chosen for this investigation were agents to which the dogs were not systemically immunized. The cellular responses to the two antigens was essentially the same probably because the animals were nonimmune to them. To seven lesions no antigen was employed and the cellular response was qualitatively the same but quantitively decreased as compared to lesions stimulated with the nonimmune antigens. This was especially true in the latter stages of each experiment. Because the responses were very similar, the results of all these experimental lesions will be described together.

TWO HOUR STAGE The cellular exudate was sparse and the primary cell present was the neutrophilic leukocyte. The majority of the neutrophils were 12-14 microns in diameter with the cytoplasmic portion increased. The profiles of the cells were nearly round and the cell membranes appeared stretched. The neutrophilic granules were more widely dispersed throughout the cytoplasm than in normal cells. A few fragments of neutrophilic cytoplasm were scattered throughout the preparation (Fig. 3). A paucity of small mononuclear cells, measuring 10-12 microns in diameter, was present. A few macrophages and eosinophils were also scattered throughout the exudate.

FOUR HOUR STAGE The neutrophils were present in great numbers (Fig. 4). They appeared much as described in the two hour

stage, but an increase in cytoplasmic fragments was observed. The small mononuclear cells also increased in number but still measured from 10-12 microns in diameter with a few as large as 20 microns. The small mononuclear cell appeared exactly like a peripheral blood lymphocyte with a large round, dark staining nucleus that contained a coarse, clumped chromatin pattern. The nuclear membrane was smooth and thick. The eccentric, slightly indented nucleus was surrounded by a thin ring of basophilic staining cytoplasm (Fig. 5). Some cells also contained a few azurophilic granules.

Eosinophils likewise had increased in number. These cells appeared swollen and were most numerous in areas associated with fibrin. The eosinophilic leukocyte comprised approximately 2% of the cellular exudate.

SIX HOUR STAGE The ratio of neutrophilic leukocytes to mononuclear cells was about two to one. Many of the neutrophils were less distinct and the exudate contained an increased amount of cytoplasmic fragments. A considerable number of the neutrophils had lost all their cytoplasm to the fluid exudate and their nuclei remained naked on the coverslips. The intact neutrophils often contained vacuoles within their cytoplasm.

Many of the mononuclear cells increased in size up to 20 microns and were fixed in a variety of elongated shapes suggesting they were actively motile at time of fixation. The nuclear membrane, which in the earlier stages was thick and round, became thin and irregular conforming to the over-all shape of the cells.

The chromatin pattern was finer and the distribution between chromatin and parachromatin was more obvious (Fig. 6).

The eosinophilic leukocyte was present at about the same percentage as in the previous stage, however, many were fragmented and free eosinophilic granules were present in the fluid exudate. They were most numerous in areas associated with fibrin (Fig. 7). This supports Riddle and Barnhart's (1965) conclusion that eosinophils are selectively attracted to fibrin in areas of inflammation. A considerable number of the eosinophilic granules, either intracellular or extracellular, were fused resulting in an occasional eosinophilic mass up to 5 microns in diameter (Fig. 8).

EIGHT HOUR STAGE The percent of neutrophils were decreased to 50% of the cellular exudate (Fig. 9). More of the neutrophils were degenerated with clumped chromatin and pyknotic nuclei their salient features. Many of the neutrophils were also dehydrated and shrunken (Fig. 10).

The mononuclear cells were increased to 50% of the cellular response and like the previous stage many were fixed in irregular shapes. The chromatin was broken into finer pieces and the nuclear membrane remained irregular in outline. The cytoplasm, at this stage, contained a few small vacuoles and stained basophilic. The amount of cytoplasm in relation to nuclear size had increased but still remained relatively small when compared to the other cells in the exudate (Fig. 11).

The eosinophilic leukocyte decreased in number and could only

occasionally be located in the exudate.

TEN HOUR STAGE The neutrophils for the first time in the experiment comprised less than 50% of the cellular exudate. A few neutrophils appeared normal but the great majority of them were shrunken with degenerate and pyknotic nuclei. Most of the cytoplasmic fragments were absent, possibly because they were phagocytized by the mononuclear cells.

The mononuclear cells made up over 50% of the cells on the coverslip. The majority of these cells were 12-14 microns in diameter because of an increase in cytoplasm. The basophilic staining cytoplasm contained vacuoles and an increased number of large azurophilic granules. Some of the mononuclears also had phagocytized nuclear lobes of dead neutrophils (Fig. 12).

The ten hour stage was also characterized as the point in which the majority of the lesions reached their peak cellular response. The peak response in dogs occurs approximately two hours earlier than the important peak 12 hour stage in humans (Rebuck and LoGrippo, 1960).

TWELVE TO TWENTY-FOUR HOUR STAGES The previously described changes continued and became more pronounced. The intact or relatively normal neutrophils markedly decreased in number, while dying and shrunken forms persisted throughout the experiment.

The mononuclear cells increased until they reached approximately 75% of the cellular exudate (Fig. 13). The average size of the mononuclear cells continued to increase. The majority of

these cells reached a diameter of 18-22 microns, although small mononuclear cells such as seen at the two and four hour stages were still present on the coverslips until the experiment was terminated at 24 hours. The nucleus increased in size and the chromatin masses continued to break into smaller pieces. The cytoplasm also increased in volume and often contained many large vacuoles. Azurophilic granules were prominent throughout the later hours. After the 14 hour stage, the mononuclear cells were very similar to tissue macrophages and morphologically were indistinguishable from macrophages (Fig. 14). The total mononuclear cell response began to decrease in number at about the 16 hour stage.

EFFECT OF VITAL DYES Vital dyes, trypan blue or Pelikan ink, in addition to the diptheria toxoid or tuberculin were applied to eight lesions at the beginning of the experiment to study the phagocytic ability of the exudate. The cytological response was essentially the same as previously described. The first evidence of phagocytosis occurred at four hours. A few neutrophils contained small accumulations of either trypan blue or Pelikan ink in their cytoplasm. At six hours more neutrophils had ingested the vital dyes and a few of the larger mononuclear cells also had phagocytized the dyes. After six hours, the neutrophils became less important in phagocytosis of the dyes, but the enlarging mononuclear cells became more phagocytic. They retained this ability until the experiment ended at the 24 hour stage (Fig. 15).

SUMMARY AND CONCLUSIONS

Using Rebuck's skin window technic, a cytological study was undertaken to determine the migration of leukocytes and inflammatory cells in acute inflammation in normal beagle dogs. A few modifications of Rebuck's technic to facilitate its use on dogs were described by the author.

Diptheria toxoid and tuberculin, antigens to which the dogs were not systemically immunized, were used in this study. Vital dyes were also employed to study phagocytosis.

The quantity of cells adhering to the coverslips was very numerous, and immature or mitotic cells were never observed. The great majority of these cells were probably carried to the area of inflammation by the blood vascular system and are of hematogenous origin.

The first cells to migrate in any appreciable number were the neutrophilic leukocytes. They increased in number and size for the first six hours. They persisted throughout the 24 hour experiment, but in relatively decreasing numbers after six hours. The neutrophils first appeared as normal peripheral blood cells, then became swollen and later lost small pieces of their cytoplasm to the fluid exudate along with further clumping of their nuclear chromatin. They were capable of phagocytizing vital dyes.

An occasional small mononuclear cell, morphologically the same as a peripheral blood lymphocyte, was present at two hours. These cells increased in size and in number and reached their peak

migratory point at approximately ten hours. The comparable stage in humans was not reached until 12 hours of inflammation. The small mononuclear cells changed in structure until at 16-18 hours the majority could not be distinguished from macrophages. This transformation was accomplished first by an increase in cytoplasm accompanied by an increase in azurophilic granules and intracytoplasmic vacuoles. As the cytoplasm increased, phagocytosis correspondingly increased. The nuclear size likewise increased and the nuclear membrane became thinner and irregular. The coarse clumped chromatin, characteristic of the early migrating small mononuclear cell, separated into finer pieces.

The eosinophilic leukocyte composed about 2% of the cellular exudate from four to six hours of inflammation. After six hours their numbers decreased rapidly. The eosinophils, like the neutrophils, first appeared as peripheral blood eosinophils, then became swollen and later lost pieces of cytoplasm and eosinophilic granules to the fluid exudate. The eosinophils were often associated with fibrin suggesting an attraction for the fibrin strands present in the exudate.

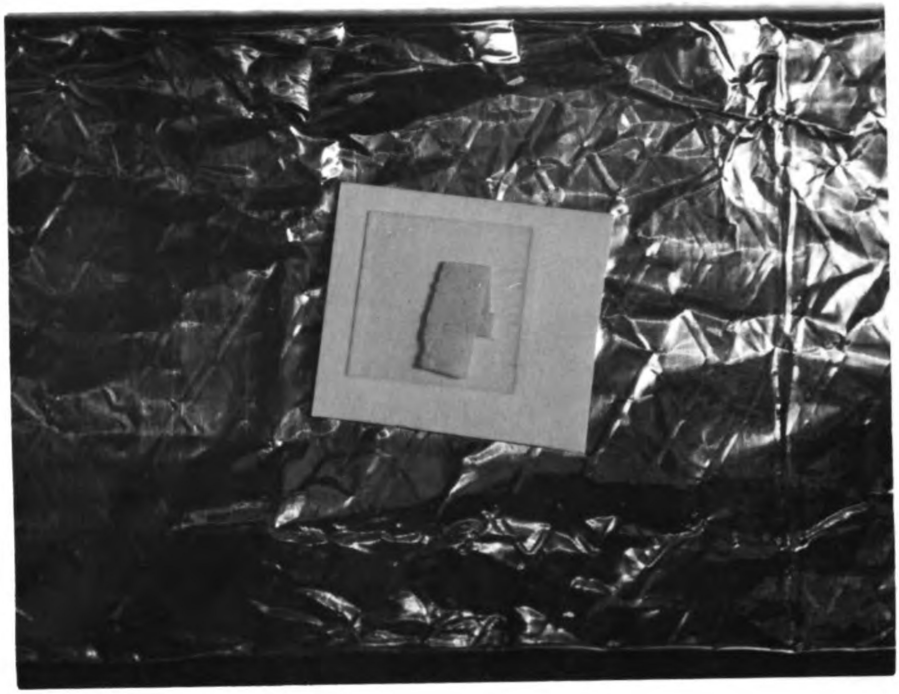
The cellular response in the dog was very similar to cellular response in humans (Rebuck and Crowley, 1955). The most important difference was the time needed to reach the stage of peak cellular migration. This stage occurred at ten hours in dogs as opposed to 12 hours in humans. This indicated that the dog was capable of mobilizing its greatest cellular defense mechanism to an irritant more rapidly than man.

FIGURE 1

Coverslip kit. Note coverslip attached to cardboard square.

FIGURE 2

Dorsal thoracic area of dog with four skin window preparations. Window I and II in place. Window III coverslip and cardboard square in place. Window IV is exposed ready for creation of lesion.




I
II

FIGURE 3

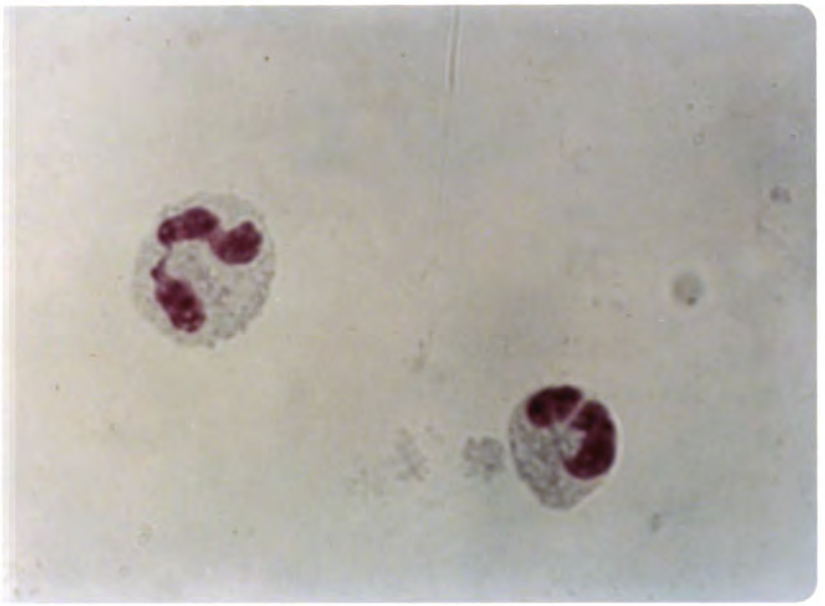
Two neutrophilic leukocytes. Two hour stage. One neutrophil is swollen. Note cytoplasmic fragments.

May-Grünwald-Giemsa; x 1320.

FIGURE 4

Low power view. Four hour stage. Note the predominance of neutrophils.

May-Grünwald-Giemsa; x 330.



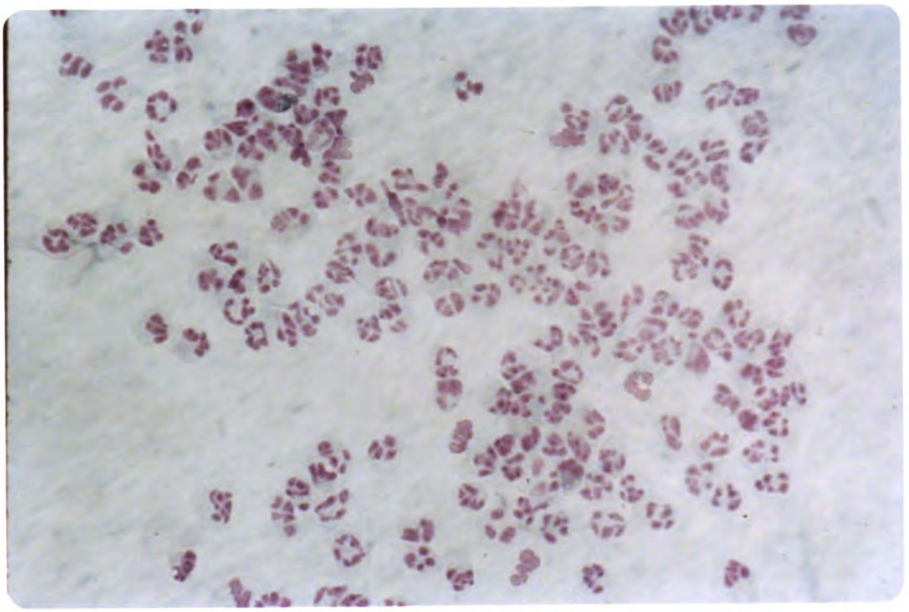


FIGURE 5

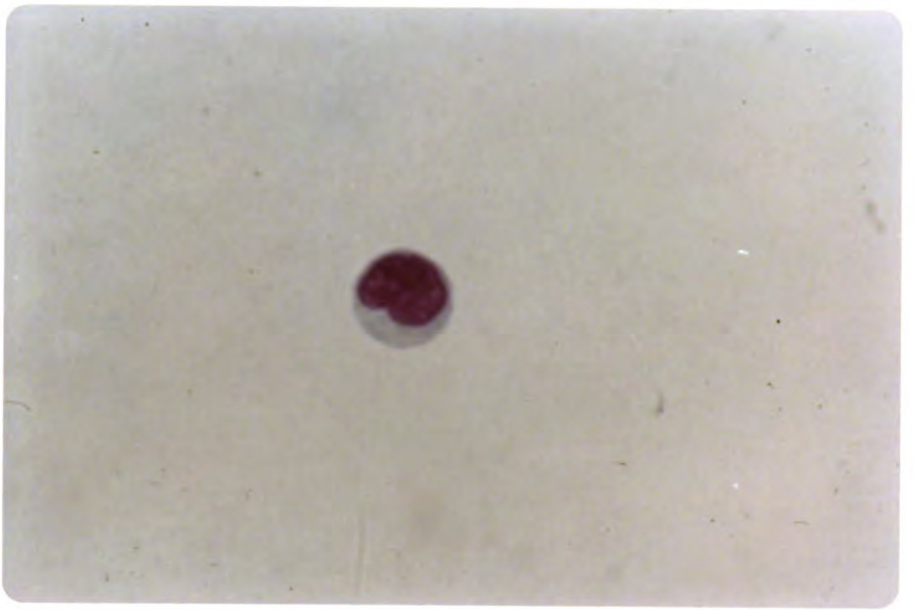
One small mononuclear cell. Four hour stage.

May-Grünwald-Giemsa; x 1320.

FIGURE 6

Two neutrophilic leukocytes and five enlarging mononuclear cells with irregular outlines and finer chromatin pattern. Six hour stage.

Wright's-Leishman; x 1320.



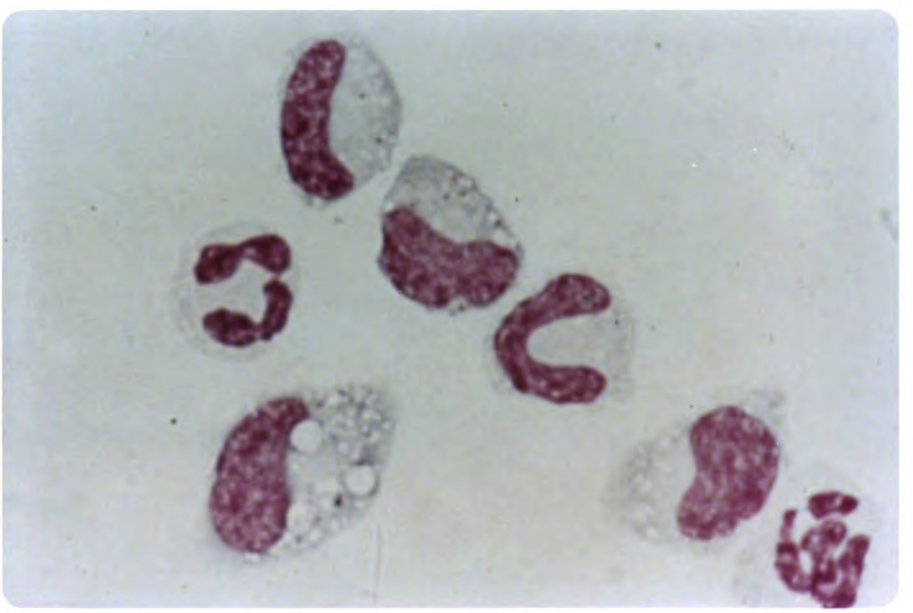


FIGURE 7

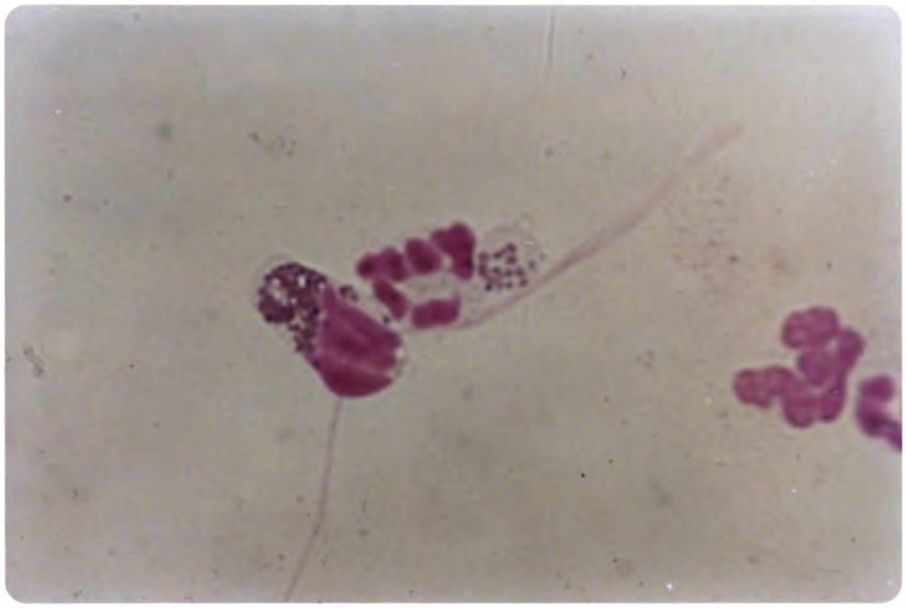
Eosinophilic leukocyte and two degenerating neutrophilic leukocytes. Six hour stage. Note fibrin strands and free cytoplasm from eosinophil.

May-Grünwald-Giemsa; x 1320.

FIGURE 8

Two eosinophilic leukocytes with one containing fused eosinophilic granules and two degenerating neutrophils. Six hour stage.

May-Grünwald-Giemsa; x 1320.



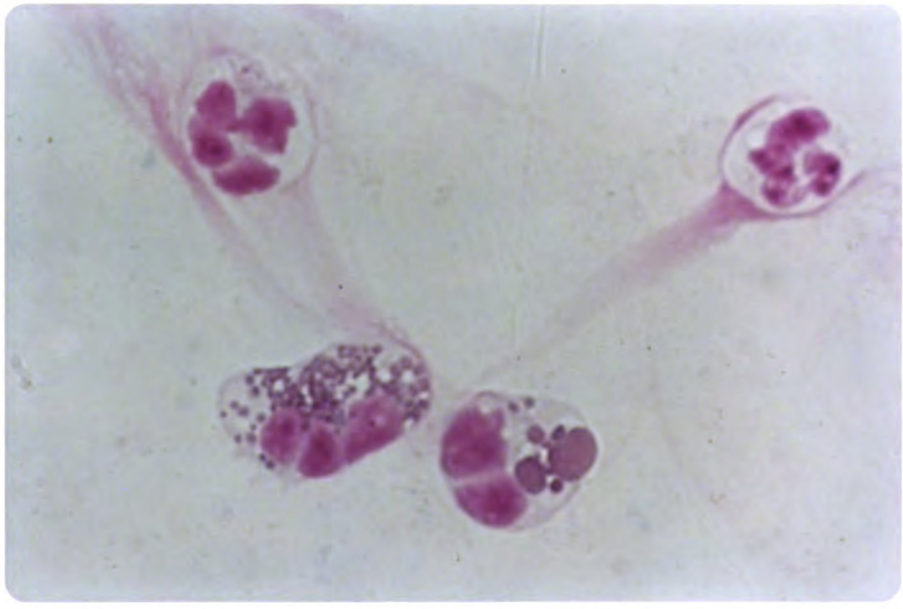


FIGURE 9

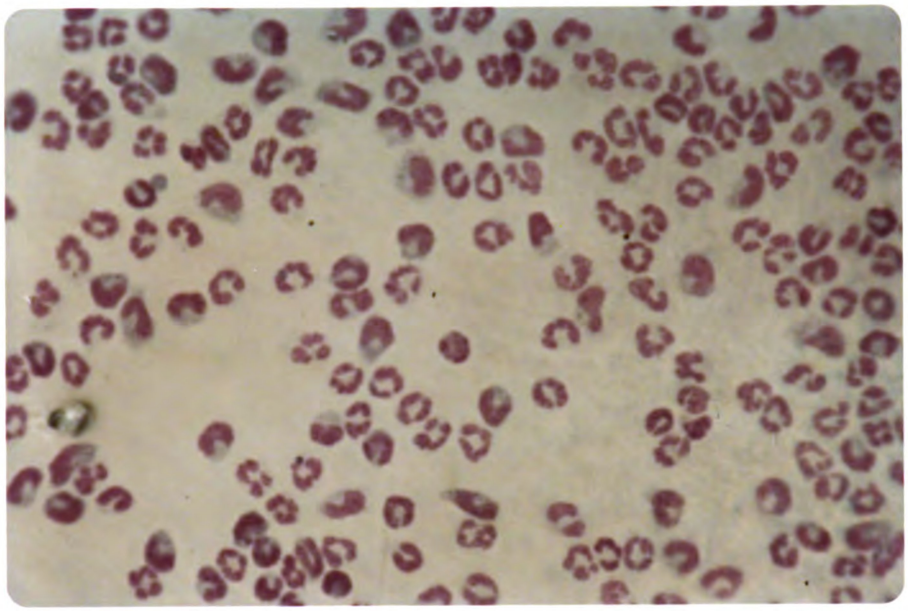
Low power view. Eight hour stage. Note approximate equal numbers of neutrophils and mononuclear cells.

Wright's-Leishman; x 330.

FIGURE 10

Five degenerating neutrophils and two mononuclear cells. Eight hour stage.

Wright's-Leishman; x 1320.



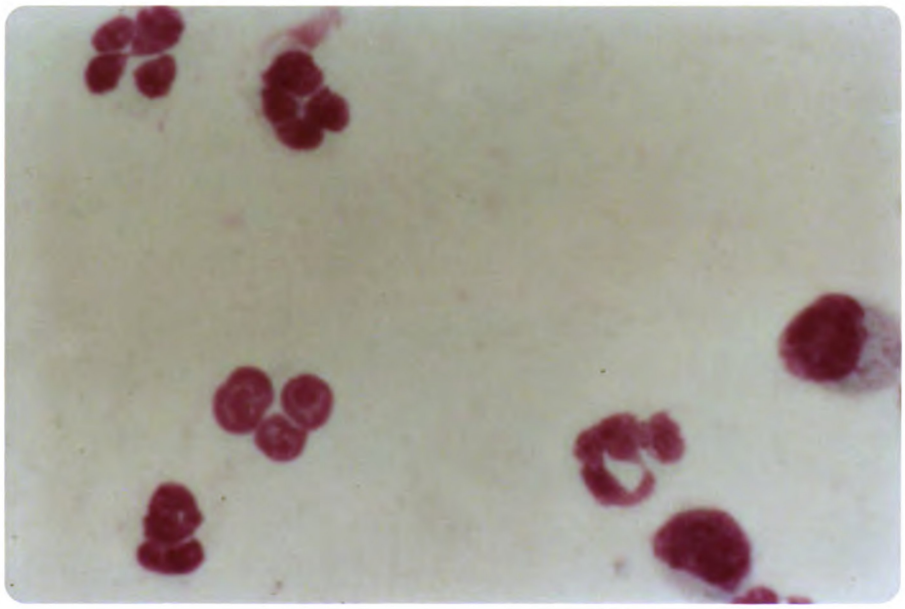


FIGURE 11

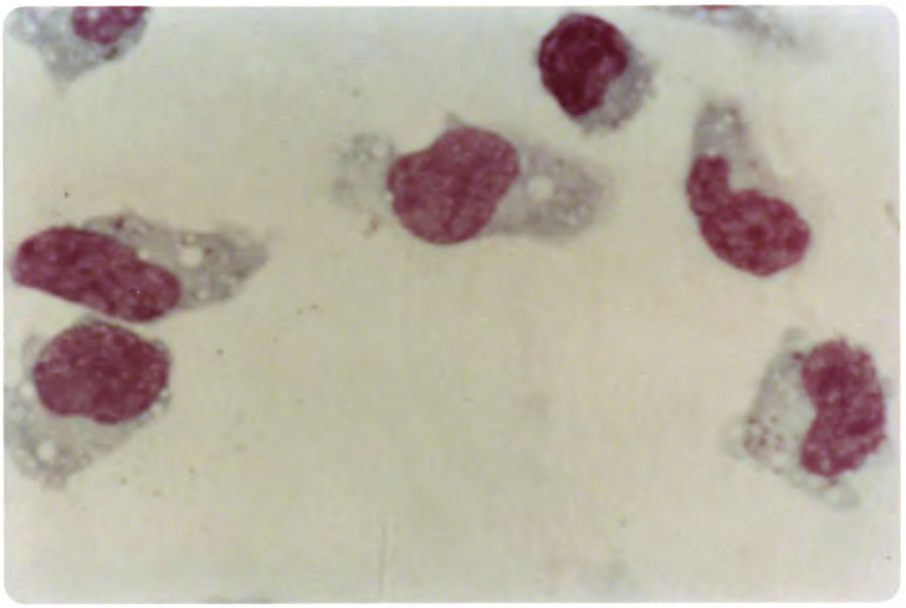
Six mononuclear cells in varying stages of enlargement with irregular elongated outlines. Eight hour stage.

Wright's-Leishman; x 1320.

FIGURE 12

Phagocytic mononuclear cell containing nuclear lobe of neutrophilic leukocyte. Ten hour stage.

Wright's-Leishman; x 1320.



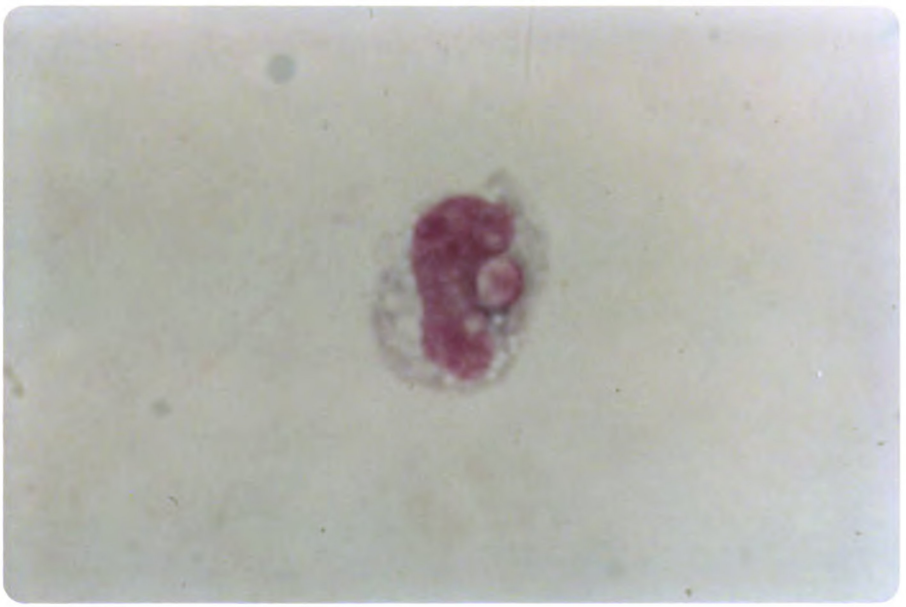


FIGURE 13

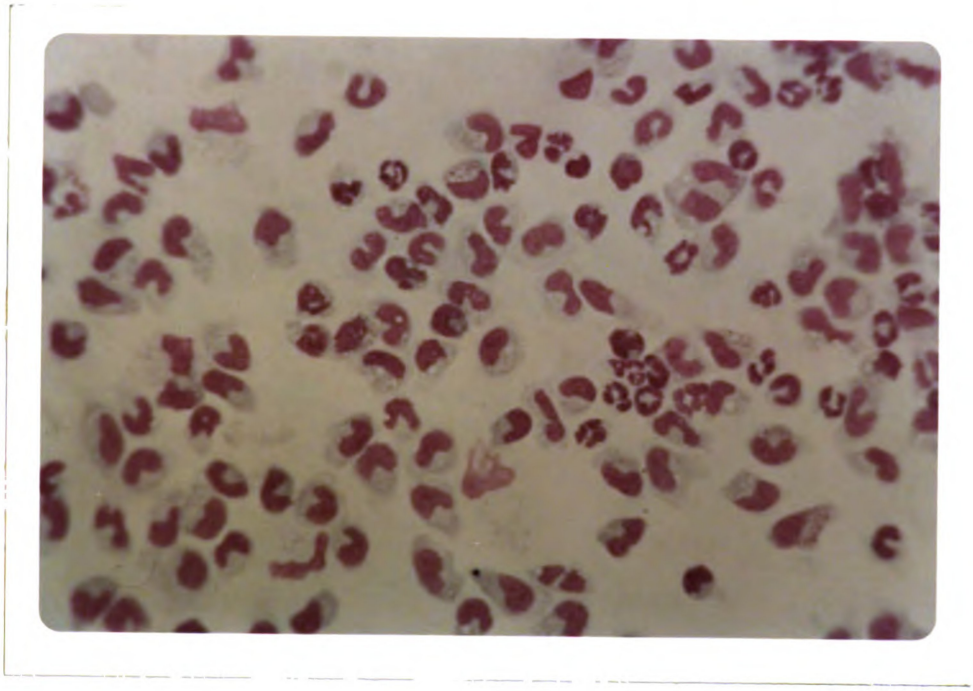
Low power view. Fourteen hour stage. Note predominance of mononuclear cells.

Wright's-Leishman; x 330.

FIGURE 14

Eight enlarged mononuclear cells. Sixteen hour stage. Note azurophilic granules, fine chromatin pattern and intracytoplasmic vacuoles.

Wright's-Leishman; x 1320.



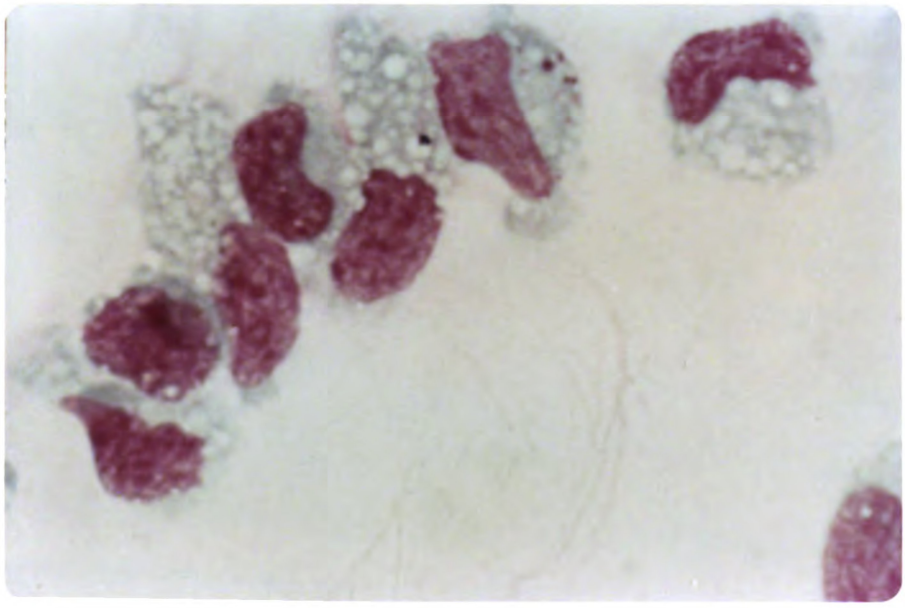
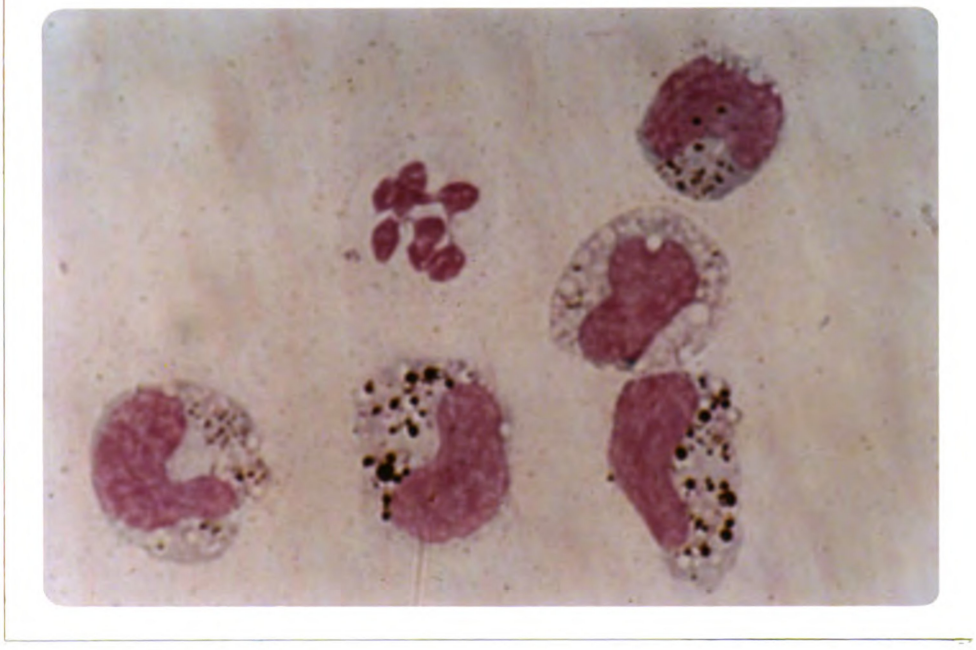


FIGURE 15

One neutrophilic leukocyte and five large mononuclear cells, four having phagocytized Pelikan ink. Twenty hour stage.

Wright's-Leishman; x 1320.



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