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A STUDY OF THE 'ARTIFICIALLY-INDUCED' AND 'NATURAL' LUPUS ERYTHEMATOSUS CELL PHENOMENA

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A STUDY OF THE 'ARTIFICIALLY-INDUCED' AND 'NATURAL'

LUPUS ERYTHEMATOSUS CELL PHENOMENA

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CHAPTER I

INTRODUCTION

The discovery and description of the L.E. (Lupus Erythematosus) cell phenomenon by Hargraves (1) in 1948 has provided a valuable tool in the diagnosis of systemic lupus erythematosus. This phenomenon has come to be recognized as a distinctive manifestation of this disease. Although most investigators consider the production of this cell as a specific characteristic of systemic lupus erythematosus, there is evidence that similar cellular reactions may occur in various other conditions. Indeed, this preparation would appear to merit study as a basic cellular response to injury.

The L.E. phenomenon has been the subject of much investigation. Studies which have been concerned with the mechanisms of this phenomenon have clearly shown a protein factor in the blood of patients with systemic lupus erythematosus to be responsible for initiating the phenomenon. Several mechanisms have been postulated to explain the events of nuclear break-down preceding the L.E. cell formation; however, the serum factor that initiates the cellular mechanisms has been isolated and grossly identified.

Haserick (2), utilizing chemical and electrophoretic separation techniques, has demonstrated that a serum gamma globulin fraction from patients with systemic lupus erythematosus initiates the L.E. phenomenon in normal blood and is apparently the activating factor in the L.E. phenomenon. Since the action of this protein factor is to initiate processes inside of the cell which cause nuclear break-down, it would seem likely that other substances might be found that would initiate similar cellular processes.

Inderbitzen (3) described an ester of polyvinyl alcohol polysulfonic acid (PVAS) which induced the L.E. phenomenon artificially in normal blood. He found that when this compound was incubated with normal serum, a factor was liberated which, when added 'in vitro' to normal blood, would initiate the L.E. phenomenon. The cells produced in this artificial L.E. phenomenon were morphologically identical to the natural L.E. cells and the mechanisms by which they developed were apparently the same with the exception of the initiating factor. It was found that gamma globulin was in some way altered or combined with PVAS in the normal serum, when incubated, producing this artificial initiating factor.

In the present study a compound, cetyltrimethylammonium bromide (referred to hereafter as CTAB), was found which will precipitate portions of the beta and gamma globulin fractions when added to normal serum. This compound was injected subcutaneously into experimental animals to determine its 'in vivo' effect upon tissue glycoproteins. It was noted that CTAB produced extremely rapid necrosis at the site of injection. Microscopic examination of this tissue revealed massive

hyalnization with the production of hematoxylin-staining bodies. Since the hematoxylin-staining bodies and the inclusion bodies of L.E. cells have been shown by Lee (4) to be chemically and optically identical, it was decided to determine the effect of CTAB on white blood cells.

When CTAB was added 'in vitro' to normal blood it was found to provoke nuclear depolymerization in polymorphonuclear leukocytes, resulting in the formation of L.E.-like cells.

Study of this artificially-induced L.E. phenomenon which was produced in normal blood by CTAB and its similarities and relationships to the L.E. phenomenon of systemic lupus erythematosus, was proposed in amplification of the above findings.

CHAPTER II

HISTORY

Hargraves, Richmond and Morton (1) in 1948 first described an unusual cell which they found in heparinized bone marrow specimens from individuals with systemic lupus erythematosus. These cells developed when the bone marrow specimens were allowed to stand at room temperature for two hours. This cell, called the L.E. cell, was described as a mature polymorphonuclear neutrophil containing an amorphorus, structureless inclusion body. The inclusion body showed slightly altered nuclear staining characteristics and displaced the nuclei of the segmented neutrophil to the periphery of the cell. Since the discovery of this phenomenon inclusion bodies have been reported in various white blood cells, including band forms, eosinophils, monocytes, lymphocytes and myelocytes (4-8).

The L.E. cell inclusion body has been regarded as representing either phagocytized nuclear material from other cell nuclei, in which the chromatin structures have undergone depolymerization, or as the result of autolysis of one or more nuclear segments of the involved cell. Haserick (9) suggested that the inclusion bodies might consist of precipitated protein or that they might be of megakaryocytic or platelet origin.

Lupus erythematosus cell inclusion bodies are now generally accepted to be of nuclear origin. Differential staining techniques with Fuelgen and methyl green have shown them to be composed largely of depolymerized desoxyribose nucleic acid, (10). Berman et al (5) expressed doubt as to whether this material originates from the nuclei of lymphocytes or neutrophils; but that it was probable that they arose from both.

Neutrophils containing ingested red cells (11), precipitated protein material (12), or fungus forms (13) are not to be considered L.E. cells; yet they have been reported as a source of error (14).

When destructive changes occur in cellular nuclei, the cell, acting as a foreign object, may stimulate phagocytosis by normal phagocytic cells. As a result of such destructive changes, generally referred to as 'lysis', the cell nuclei lose their structural chromatin patterns and appear as relatively homogeneous masses. After the nuclear and cytoplasmic membranes rupture, the lysed nuclear mass is freed from other cellular components and will often exert a chemotactic attraction to wandering phagocytes. This phenomenon of lysis is referred to as nucleolysis or chromatolysis and the free masses have been called "amorphous masses" (14), "globs" (4,15) and "lysed nuclear masses" (16). The agent responsible for the lytic activity occurring in the L.E. phenomenon is known as the L.E. factor.

If a free mass of depolymerized nucleoprotein is chemotactic, it will attract phagocytic cells which agglutinate on the mass, and attempt to engulf it. In positive L.E. cell preparations these agglutinated clumps of cells are prominant. They appear characteristically as a central core of free nuclear material with a surrounding group of

phagocytes making up the "rosette" described by Haserick (17).

Klemperer (18) studying the tissue from patients with systemic lupus erythematosus, found that hematoxylin-staining bodies were a consistent histological finding in this disease. Subsequent cytochemical studies showed these hematoxylin-staining bodies to be composed of depolymerized desoxyribose nucleic acid (4).

Lee, Michael and Vural (4), employing the photometric methods used in the study of the hematoxylin-staining bodies, demonstrated that the inclusion body of the L.E. cell also contains depolymerized desoxyribose nucleic acid. They concluded that this lysed nuclear mass forming the L.E. cell inclusion body was optically and chemically identical to the hematoxylin-staining bodies.

The L.E. cell phenomenon has been the subject of much investigation. Investigations have been largely concerned with different methods of reproducing or enhancing the phenomenon, simplification of the procedure and the identification of factors involved in the phenomenon (6,8,19-32).

Snapper (27), Walsh (33) and Weiss (34) consider the L.E. cell phenomenon specific for acute disseminated lupus erythematosus, while Berman (5), Beerman (35) and Lee (36) maintain that it is a valuable confirmatory tool in the diagnosis but that the test is not an absolute diagnostic criterion of the disease. Both groups, however, admit that it cannot always be demonstrated in the acute and subacute forms of the disease and only rarely occurs in the chronic form.

Lupus erythematosus cells have been observed, though sometimes in small number in a variety of unrelated diseases, such as leukemia (37),

pernicious anemia in relapse (5), dermatitis hypertiformis (5), acquired hemolytic anemia (38), chronic hepatitis (39-41), penicillin reactions (42,43), multiple myloma (44), moniliasis (45), miliary tuberculosis (46), periarteritis nodosa (47) and scleroderma (48). Apart from disseminated lupus erythematosus perhaps the highest incidence of positive tests have been found in chronic rheumatoid arthritis (36,49,50). The phenomenon has also been noted to become positive during the severe relapse which often follows the withdrawal of cortisone therapy (50) and it has been suggested that systemic lupus erythematosus may have been coexistent in these patients (51).

Lee (38) has suggested that the phenomenon may represent an abnormal enzyme reaction but did not expound this postulation. It has been found to be positive in circumstances where there is active tissue destruction (1), disturbances of the antibody mechanisms (41) or the phenomenon may be related to hypersensitivity without the manifestations of lupus erythematosus (43).

The instigating factor for the L.E. phenomenon resides in the plasma, serum and probably all tissue fluids of the patient. Positive tests have been reported using joint effusions (14), pleural effusions (52), pericardial effusions (53), cerebro-spinal fluid (54) and urine (55,56).

Nucleolysis occurs as the result of the L.E. factor acting upon living leukocytes, independent of the source of these leukocytes. However, a difference in susceptability to lysis and phagocytosis of the cells from a variety of human and animal sources has been observed (57). The greatest species difference observed is that actual phagocytosis,

productive of the L.E. cell, is uncommon in dog bone-marrow preparations, while rosette formation is prominent and diagnostic. The L.E. factor may be transferred passively to laboratory animals (58), and will pass through the placental barrier (59), although it need not be associated with symptoms of lupus erythematosus in the infant.

Hargraves (16) suggested that the L.E. cell phenomenon reaction in blood may be initiated by the breakdown of platelets. This speculation was based on the observation that the phenomenon did not occur in vivo. Also, in three patients after splenectomy for hypersplenism (20) there developed symptoms of acute disseminated lupus erythematosus with positive L.E. tests. Further confirmation of the role of platelets in this phenomenon was reported, stressing the importance of blood coagulation in accentuating the reaction (60,61). Anticoagulants do not enhance the formation of the L.E. cells and may actually reduce the intensity of the reaction (38,60,61).

Although the phenomenon was thought to take place only in vitro, Chomet and his associates (62) found L.E. cells in peripheral blood smears from patients with advanced systemic lupus erythematosus. Haserick (2) had previously reported that the L.E. phenomenon was observed after 12-13 minutes of incubation in some instances. Friedman and his group (63) rationalized that hypoxia and stasis were probably present in the patient with advanced systemic lupus erythematosus and hypoxia was important in the activation of the L.E. factor. By constricting a finger of patients with systemic lupus erythematosus for 20 minutes and studying direct blood films made from blood of the constricted finger, they were able to demonstrate the L.E. phenomenon

'in vivo'.

On rare occassions L.E. cells have been found in freshly drawn blood smears (62,64) and in sections of tissue obtained at autopsy (8, 10). In a study of spleen obtained at autopsy from a patient with systemic lupus erythematosus, as diagnosed by the L.E. phenomenon, the nuclei of the spleen cells to a depth of three to four cell layers had lost their chromatin patterns, and homogenization with swelling was evident. Cells deeper in the splenic tissue contained normal-staining nuclei.

Electrophoretic analysis and immunological studies of serum proteins from individuals with systemic lupus erythematosus indicate that the L.E. factor is contained in the gamma globulin fraction (2,5,9). Nucleophagocytosis as well as phagocytosis of various tissue cells have been produced by injecting this antigen into test animals and stimulating antibody formation. The serum of such animals will produce nucleophagocytosis when mixed with normal cells from another animal.

The L.E. factor is quite stable in sterile serum and may be preserved for a year or longer in the frozen state (14), but it is inactivated by heating to 65° C. or by bacterial contamination (2). The phenomenon can be inhibited by the addition of para-aminobenzoic acid and L.E. antibodies developed in rabbits against the specific gamma globulin, but it is not inhibited by cortisone, testosterone, estradiol, progesterone or control antibodies developed in rabbits against normal human plasma proteins (2,65).

Kuernick (15) has postulated that enzymatic activity of desoxyribonuclease may cause depolymerization of desoxyribose nucleic acid

due to the lack of an inhibitor of the enzyme. He suggested that the L.E. factor inactiviates an inhibitor of desoxyribonuclease which normally occurs in polymorphonuclear leukocytes. When the enzyme inhibitor is inactivated, depolymerization of nucleoproteins occurs. Attempts to demonstrate that serum desoxyribonuclease participates in the L.E. cell phenomenon have not been successful (66), although a nuclease inhibitor derived from normal human leukocytes inhibits the phenomenon (67).

Structures similar to L.E. cells have been produced with leukocytic antiserum (68), desoxyribonuclease (69), polyvinyl alcohol polysulfonic acid ester (3), virus cultures recovered from patients with lupus erythematosus (70). They have also been reported following prolonged administration of hydralazine in hypertension (71). Moolten and Clark (70) isolated a virus from each of six patients with systemic lupus erythematosus. In each case, after multiple successive incubations in chick embryos, they were able to reproduce the L.E. cell phenomenon by mixing the virus culture with normal blood cells.

Castello, Fernandez and Reamedois (58) reported the L.E. cell phenomenon in the blood of guinea pigs that had received repeated injections of 2 to 5 cc of whole blood from patients with systemic lupus erythematosus. Examination of the tissue from these experimental animals revealed changes similar to those found in material obtained from human patients with systemic lupus erythematosus and it is of interest that these changes could be induced only in female guinea pigs.

Since the L.E. phenomenon is the result of a biochemical reaction, it has been proposed that chemical and biological agents other than the L.E. factor might induce the phenomenon (1). Under experimental

conditions this was accomplished by Inderbitzen (3) who utilized the anticoagulant, polyvinyl alcohol polysulfonic acid ester (PVAS, Roche), as a nucleolytic agent. Experiments with PVAS yielded cells indistinguishable from the L.E. cell of systemic lupus erythematosus. A serum factor residing in the gamma globulin fraction was found to take part in the reaction. This artificial L.E. factor was produced by heating the gamma globulin fraction or whole serum with PVAS for about 30 minutes at 59° centigrade. If coagulation of the blood was inhibited by heparin before PVAS was added, the phenomenon did not occur. Although the cellular changes were morphologically identical to the genuine L.E. phenomenon, the serum had been subjected to physical processes and a foreign substance that does not occur physiologically. However, such an experimental approach might be fruitful for the study of fundamental mechanisms in the L.E. phenomenon of systemic lupus erythematosus.

CHAPTER III

METHODS

The animals used in this study were normal, healthy dogs, rabbits, mice and rats of both sexes. These animals, supplied from the animal quarters at the University of Oklahoma School of Medicine, were maintained as a source of normal blood for artificial L.E. preparations and as a source of donor cells and serum for the natural L.E. preparations. Blood from rats, mice and rabbits was drawn, under light ether anesthesia, by cardiac puncture; blood from dogs was obtained by venipuncture without anesthesia.

Patients included in this study were selected at random from the outpatient clinics of the University Hospitals, the Oklahoma County Farm Hospital, and McBride Bone and Joint Hospital, all of Oklahoma City, Oklahoma. This study group included both sexes and represented several age groups. They were further classified according to their medical diagnoses. Normal healthy individuals, used for controls, were also included. Stock L.E. serum was obtained from three female patients with systemic lupus erythematosus. Blood from these patients was drawn by venipuncture into a clean, dry syringe, transferred to a centrifuge tube and allowed to clot and separate. After centrifugation, the serum was decanted from the clot and preserved by freezing until needed.

L.E. Prep. (Lupus Erythematosus Preparation)

The technique for L.E. preparations was adapted from the method by Zimmer (61).

Blood for L.E. preparations was drawn by venipuncture or cardiac puncture into a clean, sterile, dry syringe through a 20 gauge needle. Anticoagulants were not used unless specified. After drawing the blood the needle was removed from the syringe and two ml of blood was placed into a clean 15 x 100 mm serological tube. The tube was then corked and allowed to stand at room temperature (28-30 degree C.) for two hours. Ordinarily at the end of this period of time the blood had clotted and satisfactory separation of the plasma had occurred. The clot was then broken loose from the wall of the tube with a wooden applicator stick, and with the other contents of the tube, poured onto a 40 gauge brass-wire sieve screen. A soft rubber stopper was used to mash the clot gently upon the screen, which allowed the serum and cells to pass through into a glass petri dish while the fibrin strands were left behind on the sieve. In the event that a clot had not formed at the end of two hours, the blood was gently stirred with a wooden applicator stick and the mixture regarded from this point on as if it had been defibrinated with the screen wire.

The serum-cell mixture was pipetted into a 1.5-2.0 x 100 mm capillary melting point tube until the latter was 3/4ths full. The tube was then heat-sealed at one end and centrifuged at maximum speed (approximately 1610 R.C.F.) for five minutes in an International Clinical Centrifuge. This treatment was usually sufficient to provide good separation of the buffy coat of white blood cells. Wire-cutting pliers were

used to break the capillary tube about 5-6 mm above the buffy coat and also at the end which had been heat sealed. The serum and buffy coat were gently smeared onto a clean glass microscope slide, and the remaining red cells were discarded. The buffy coat film was allowed to dry. The dry slide could then be stored, ready for staining whenever desired.

Capillary tubes were employed in this study, as described, in place of the more commonly used hematocrit tubes. They were considered more satisfactory because of convenience, low cost, and the relatively small amount of specimen required. Each tube contained a sufficient amount for one good slide.

In some experiments it was necessary to alter certain factors such as time, temperature, concentration, etc.; however, when pertinent alterations were employed they are described.

Stains

Four histological staining procedures were used in this study. Wright's and Giemsa's stains were utilized for identification of general cell morphology. Methyl green-pyrinon Y and the Feulgen reaction were employed for histochemical identification of desoxyribose and ribose nucleic acids.

Wright's Stain

A commercially-available Wright's stain was used (Banco). The dry slide was covered with staining solution for a period of two minutes. Two volumes of distilled water were then added to the slide and allowed to stand for an additional five minutes. The slide was then rinsed with fresh distilled water to remove excess stain, and allowed to drain dry.

Giemsa Stain

The Giemsa stain used was commercially-available as the stock solution (Harleco). Fresh dilutions of the stock solution were made immediately before the stain was to be used. For Giemsa staining the dried smears were fixed in 100% Methyl alcohol for five minutes, and allowed to drain dry before staining. They were immersed in the staining solution for one hour, rinsed in the buffer solution twice, drained, and allowed to dry.

Methyl Green-Pyronin Y

This staining reagent was prepared according to the method of Perry and Reynolds (72). It was used in attempts to differentiate desoxyribose and ribose nucleic acids in the cytoplasm, nuclei and cytoplasmic inclusion bodies of the cells. In preparation for this stain the slides were fixed in Carnoy's (73) fixative solution for ten minutes and stained according to the author's suggested procedure.

Feulgen

The Schiff reagent employed in this reaction was prepared by the "Cold Schiff" method and the procedure of the Feulgen reaction, as described by Lillie (74), was followed. This stain was used as a more specific indicator of desoxyribose nucleic acid.

All slides were dried after staining and stored for future microscopic examination. At the time of examination under the microscope, a cover glass was mounted on the slide with immersion oil to enhance cellular detail.

Addition of CTAB to Blood

Solutions of CTAB in absolute ethyl alcohol were freshly prepared. The usual concentration was one mgm per milliliter. To obtain dry material for testing, measured amounts of the solution were pipetted into clean serological tubes, evaporated to dryness at 80° centigrade, and stoppered. Accurate samples of small quantities of CTAB could thus be obtained in a stable dry form. The CTAB was reconstituted immediately before use by adding 0.2 ml of normal saline. Blood was added to the solution and the resultant mixture was gently stirred for a few seconds with a wooden applicator stick, thus insuring even distribution of CTAB throughout the serum. The latter procedure was found necessary in order to prevent gel formation which occurs within a short time with saline solutions of CTAB.

Methods Pertinent to Various Experiments

A Precision Model G-7 (sensitivity $\frac{1}{2}$° C) water bath was employed in all experiments in which critical control of the incubation temperature was essential.

A Beckman Model G pH meter with a calomel electrode (Beckman #270) was used to determine the effects of variations in pH on the phenomenon under study. A glass envelope, filled with saturated KCl solution was fitted over the end of the electrode to prevent fibrin deposits on the electrode tip. The tip of the glass envelope contained a small hole, plugged with cotton, which allowed free passage of the electrolyte solution but prevented the serum and cellular elements from clogging the electrode tip.

Experiments were designed to compare the naturally-occurring L.E. phenomenon to that produced with CTAB, and to determine if any antagonism or augmentation of the two phenomena existed.

The lupus factor was used either as the whole serum from patients with lupus erythematosus or was further purified. Purification was accomplished by separation of the gamma globulin fraction from serum by salt precipitation. The larger globulins were precipitated from the serum with the addition of ammonium sulfate until the mixture was 33.3 per cent saturated. The precipitate was centrifuged and dissolved in six ml of normal saline. The globulins were reprecipitated from solution at 33.3 per cent saturation with ammonium sulfate and again centrifuged. The precipitate was redissolved in eight ml of distilled water and dialyzed in a cellophane bag against tap water for one hour and then against distilled water for five hours. Some protein was denatured and precipitated from solution. The precipitate was centrifuged and discarded. The dialyzed solution containing the remaining proteins was dried by freeze-drying under vacuum, and constitutes the L.E. factor fraction referred to in this study.

A control blood or serum sample was run in parallel with each experiment.

CHAPTER IV

RESULTS AND DISCUSSION

The morphological changes that occur in cells as the result of the L.E. phenomenon have been described by many investigators. The sequence of the events of this process has also been investigated. The L.E. factor, under suitable circumstances, induces nucleolysis in many white cells. The end-point of this reaction is considered to be the formation of the typical L.E. cell as described by Hargraves (1).

Nucleolysis may proceed in various ways and arrive at the same end-point. The entire nucleus of a cell may undergo uniform lysis. The nucleus swells, becomes a 'smokey-appearing', homogenous mass and the nuclear chromatin material disappears. Various amounts of chromatin material may remain if the process is not brought to completion. Cytolysis will then occur, which frees the depolymerized nuclear mass. Any remaining chromatin material may continue to depolymerize until it also becomes homogeneous. However, this does not always occur and the mass may retain small amounts of chromatin material throughout all of the events of the phenomenon. The free mass of altered nuclear material is thought to exhibit a chemotactic effect upon the remaining normal phagocytic cells, and may be phagocytized. Phagocytosis of the free nuclear mass by normal white blood cells completes the reaction. A normal phago-

cytic cell engulfs the free nuclear mass and becomes a characteristic L.E. cell. However, if depolymerization is not complete at the time of phagocytosis, i.e., if some chromatin material remains in the mass, the resultant cell is not considered a 'true' L.E. cell by most investigators. It appears that the formation of the L.E. cell is dependent on the concentration of the activating factor and the duration of its effect under optimal circumstances.

The action of the L.E. factor upon the nucleus of a cell is not necessarily upon the entire nucleus. In approximately five per cent of the affected cells, nucleolysis can be seen in only one or two nuclear segments of a polymorphonuclear neutrophil while the other segments retain their normal appearance. This segmented nucleolysis with depolymerization can continue until the affected nuclear segment enlarges and fills the cytoplasm. The remaining, normal-appearing nuclear segments are pushed to the periphery of the cell. This cell becomes morphologically indistinguishable from a cell that has phagocytized a free inclusion body. Both cells are identified as L.E. cells, although they are formed in different ways.

Various chemical and physical agents may produce nucleolysis, either total or segmental, but depolymerization is not usually evident under such circumstances. Such cytoplasmic bodies do not show the same staining characteristics as the depolymerized material in the L.E. preparations.

The criteria for identification of the L.E. cell are based upon cellular changes that occur as the result of the L.E. phenomenon. Progressive morphological changes that occur in the development of the

L.E. cell, were arbitrarily selected to represent a stage in the development of the L.E. phenomenon. The following four-point grading system was developed as an attempt to quantitate this phenomenon based upon these morphological changes.

Grade I (Color plate I, Figure 1). When the first signs of depolymerization, i.e., nuclear swelling, could be identified, whether segmental or total, the cell was considered to be a Grade I cell (designated by the arrows in Figure 1). This condition must be carefully distinguished from the nuclear swelling that normally occurs as blood cells age 'in vitro'.

Grade II (Color plate I, Figure 2). Nucleolysis may be followed by cytolysis which leaves the nuclear mass in a free state. Figure 2 illustrates four different degrees of such nucleolysis, although only one of the cells is considered to be Grade II. The normal lymphocyte in the figure is to be disregarded. The center segment of the upper cell in the figure is undergoing swelling while the other nuclear segments appear unaffected. This is another example of a Grade I cell. Directly below this cell is a nuclear mass, free of any cytoplasmic boundaries, which has been classified as a Grade II lupus cell. This cell reminant, or free mass, is to be differentiated from the bottom cell in the diagonal of cells. In the latter cell nucleolysis has occurred but not cytolysis, and the cytoplasmic boundaries are still evident. This cell is considered an intermediate stage between Grade I and Grade II.

The third cell from the top, in the diagonal, is another example of a Grade I cell with characteristic swelling evident in all nuclear segments.

COLOR PLATE I

Figure 1

Grade I L.E. (Lupus Erythematosus) cell designated by the arrow. The cytoplasmic and nuclear boundaries are evident but the nuclei are swollen and exhibits altered staining properties. Giemsa stain. 860x

Figure 2

Grade II L.E. (Lupus Erythematosus) cell designated by the arrow. Note that cytolysis and nucleolysis have occurred. Giemsa stain. 860x

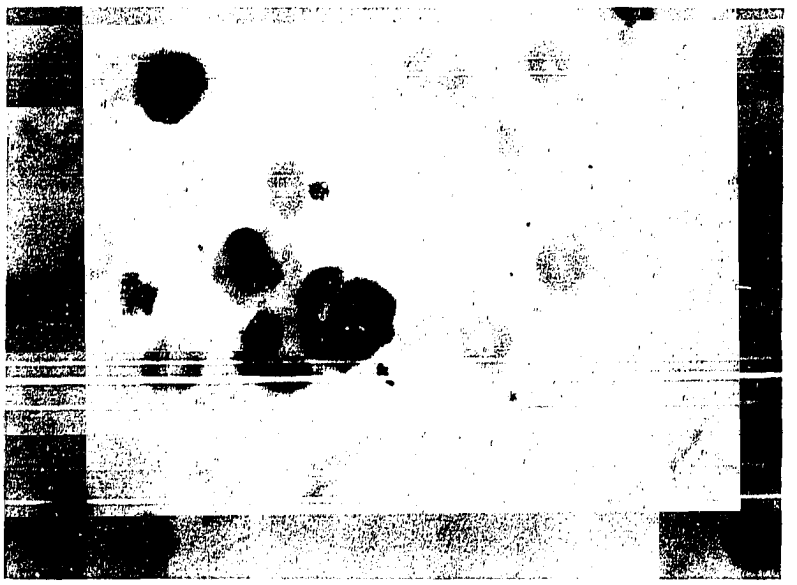


Figure 1

Figure 2

COLOR PLATE I

Grade III (Color Plate II, Figure 1). A free nuclear mass or Grade II cell is usually incompletely depolymerized and some chromatin material is recognizable. If this mass is phagocytized before the complete disappearance of chromatin material, the phagocyte with its inclusion body is classified as a Grade III cell (Figure 1, arrow). The chromatin material in such a cell is usually evident around the periphery of the included mass. This inclusion body may be either a phagocytized mass or the result of 'in-situ' depolymerization in a single nuclear segment, without phagocytosis.

Grade IV (Color Plate II, Figure 2). A Grade IV cell is designated by the arrow in this figure. It is characterized by the normal-staining nuclei compressed to the periphery and the large homogeneous body contained in the cytoplasm. This cell may have been produced by a normal phagocyte which has engulfed extraneous depolymerized nuclear material. The inclusion body may have become depolymerized after phagocytosis or the homogeneous cytoplasmic mass might have resulted from depolymerization of one nuclear segment.

Another intermediate stage of one pathway in the L.E. cell formation illustrates the chemotaxic properties exerted by the free nuclear depolymerized material. This phenomenon, known as 'rosette formation', (Color Plate III, Figure 1), is characterized by many phagocytic cells closely surrounding a free, hematoxylin-staining body. The rosette is of considerable interest in the survey of slides for evidence of the lupus phenomenon. However, the rosette is not always present in the L.E. preparations and, consequently, has been omitted in this grading system.

COLOR PLATE II

Figure 1

Grade III L.E. (Lupus Erythematosus) cell designated by the arrow. Note the chromatin-like material around the periphery of the inclusion body. Wright's stain. 860x

Figure 2

Grade IV L.E. (Lupus Erythematosus) cell designated by the arrow. The inclusion body appears relatively homogeneous and has displaced the normal-staining nuclei to the periphery of the cell. Giemsa stain. 860x

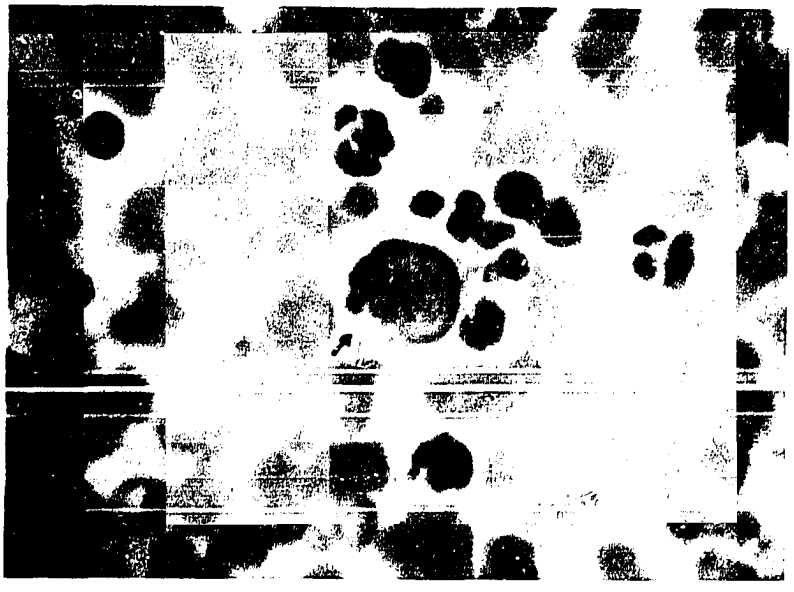


Figure 1

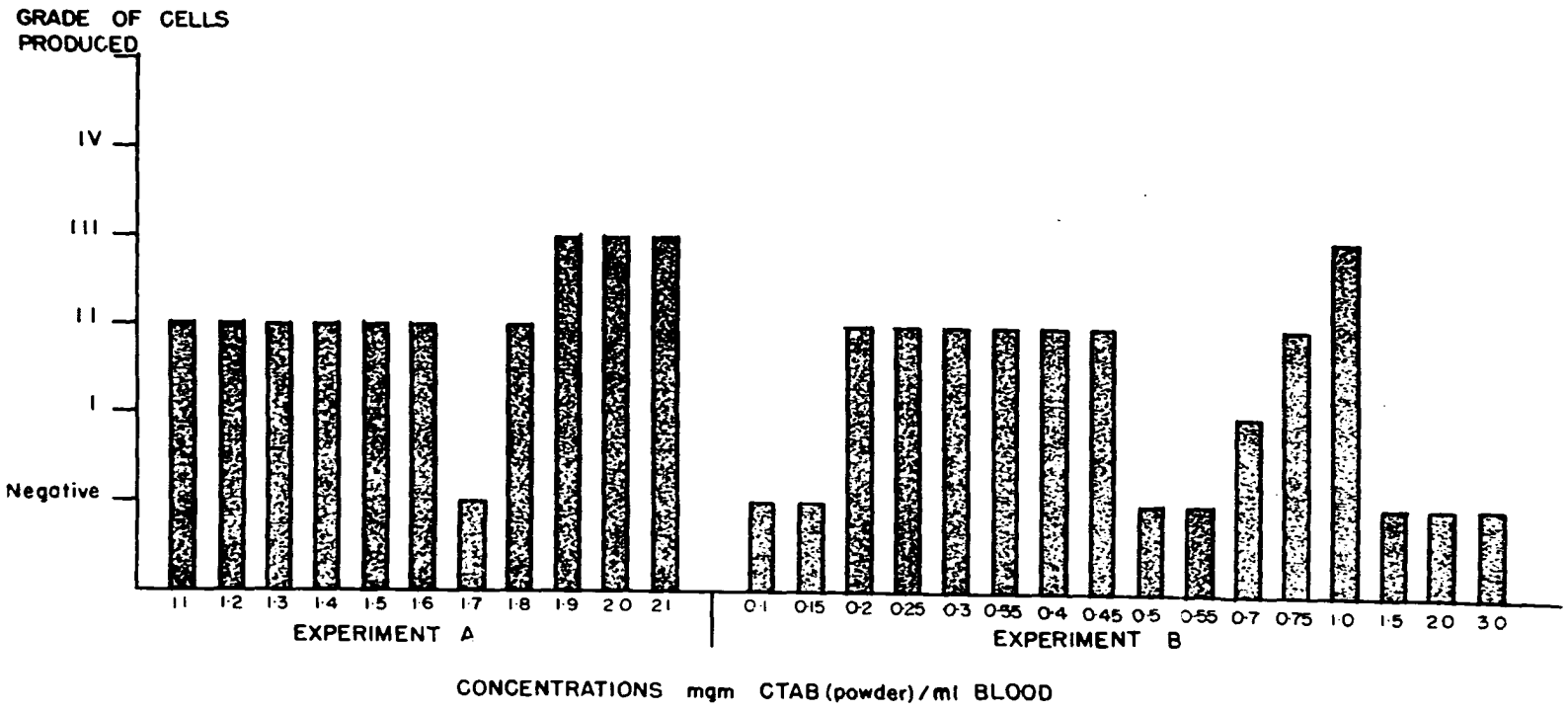
Figure 2

COLOR PLATE II

The aforementioned grading system was developed after considerable experience with the various manifestations of the lupus phenomenon and appears to express quantitative aspects of both the natural and CTAB-produced forms of the process.

The first attempt to induce the L.E.-like cells in normal blood by CTAB produced very promising results. Approximately one mgm of CTAB powder was placed into approximately two ml of fresh normal blood. After two hours at room temperature a L.E. preparation was made, and all cellular changes characteristic of a positive L.E. preparation were observed.

The optimal concentration of CTAB and methods for the introduction of this substance into the blood were first studied. The experiments, illustrated in Figure I, are examples of several attempts to determine the optimal concentration of powdered CTAB necessary to produce the lupus phenomenon in normal blood. Multiple Grade II cells, occasional Grade III, but no Grade IV cells were produced by these experiments. The erratic response to increasing concentrations of CTAB added in the powdered form instigated a search for other methods of introducing this agent. Since CTAB precipitates some plasma proteins, it is probably that serum proteins form a precipitated coat around the dry powder and interfere with solution of the compound. CTAB was therefore added by first dissolving the material in normal saline and adding the desired amount of the solution to the blood specimen (Figure II). This method was found to produce a more quantitative response and it was observed that concentrations of CTAB of 0.5 mgm/ml of blood to 1.5 mgm/ml of blood produced optimal results. However, a dilution factor had



RESULTS OF ADDING POWDERED CTAB TO NORMAL BLOOD

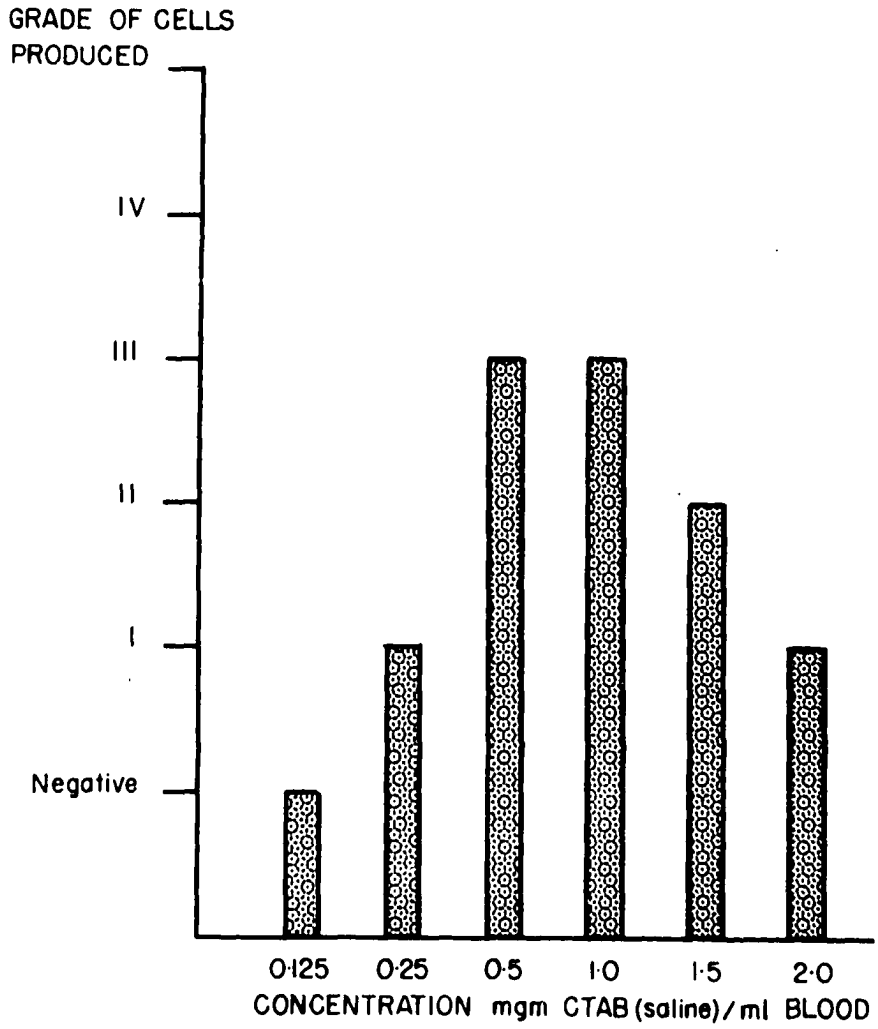
FIGURE I

been introduced, and it was also found that this solution was not stable. It was therefore decided to dissolve CTAB in absolute ethyl alcohol, pipette the desired concentration into a dry tube and evaporate off the alcohol. This facilitated a more rapid and accurate method for adding small amounts of the compound and it was stable indefinitely in the dry form. Immediately before the addition of blood, 0.2 ml of normal saline was introduced to redissolve the compound. The blood was then added and stirred rapidly to insure even mixing. This method proved to be satisfactory.

Concentrations of 0.5, 1.0, and 1.5 mgm CTAB/ml of blood were employed in all experiments to determine the optimal concentration for each blood sample. In some instances a wider range of concentrations were used. It was found that one mgm CTAB/ml of blood was usually optimal (Refer to Tables I and II).

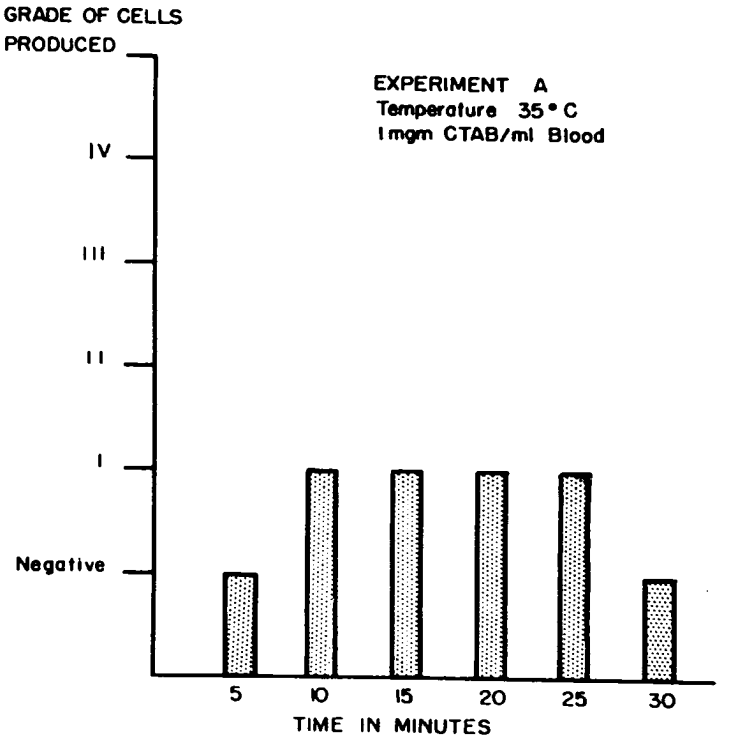
The two experiments illustrated in Figure III demonstrate the effects of time and temperature on the artificial L.E. cell phenomenon produced with CTAB in normal blood.

It can be seen in Figure IIIa, that incubation of blood-CTAB mixtures at temperatures approximating the physiological temperature of the cells was unsatisfactory. Within limits, the duration of incubation was relatively unimportant, and after 25 minutes, all cells exhibited obvious signs of deterioration. In Figure IIIb, however, conducted at 28 °C. (approximately room temperature) Grade III cells were produced after two hours of incubation. After three hours only Grade II cells remained and there was considerable evidence of cell destruction. Consequently, all further experiments in this study were performed at room temperature,



RESULTS OF ADDING A SALINE SOLUTION OF CTAB TO BLOOD

FIGURE II



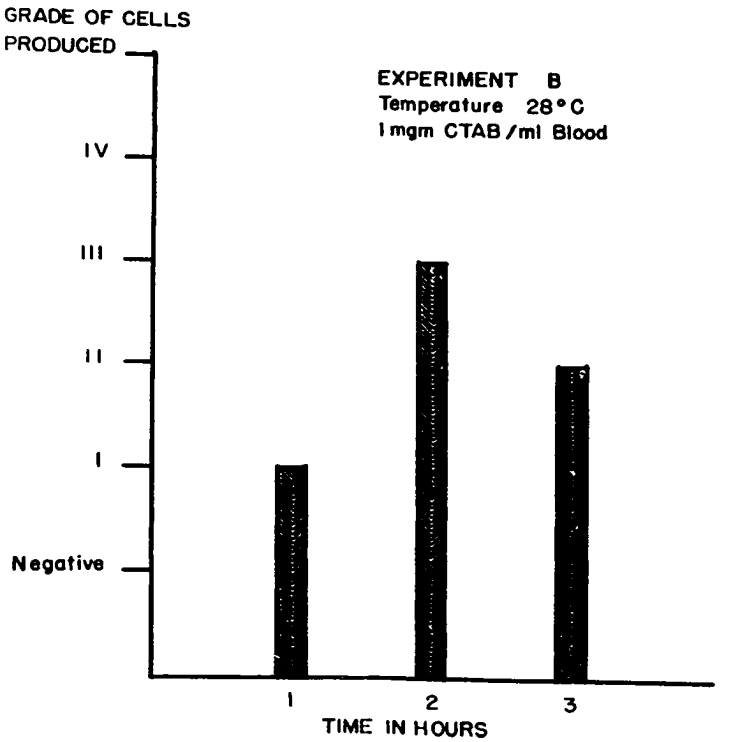


FIGURE IIIa

FIGURE IIIb

EFFECTS OF TIME AND TEMPERATURE ON THE

ARTIFICIAL CTAB-L.E. PHENOMENON

using a two hour incubation period.

The production of the artificial L.E. phenomenon with CTAB was attempted with freshly drawn blood from 25 individuals. None of the group had lupus erythematosus, and it included six normal individuals used as controls (Table I). A total of 102 L.E. preparations were made using concentrations of CTAB varying from 0.1 mgm to 3.0 mgm/ml of blood. The concentration of CTAB which provided optimal nucleolysis with minimal cellular destruction was determined for each blood specimen. The optimal concentration for the group was found to be 1.0 mgm/ml of blood. Above this concentration of CTAB, the majority of cells were lysed, and the more advanced cells of the lupus phenomenon had disappeared.

The highest grade of cell produced in the blood of each individual and the concentration at which it was produced is recorded in Table I. Grade II cells were the highest grade obtained in 40 per cent of these individuals, while Grade III cells appeared in 52 per cent of the group. Grade IV, the L.E.-like cell, occurred in two of the 25 individuals or eight per cent of the group. One of the individuals whose serum produced Grade IV cells was a normal male and the other a female with a diagnosis of degenerative arthritis. There was no apparent difference between the response of patients and normal controls with regard to the property of forming L.E. cells upon addition of CTAB.

Attempts were made to produce the artificial L.E. phenomenon by adding CTAB to blood drawn from various animals and to determine if there is a species difference in susceptability to nucleolysis induced by CTAB (Table II). A total of 42 animals were employed in this survey including: ten male and three female dogs, one male and 18 female rats,

TABLE I

Lupus Erythematosus Cell Production By Grades in Human Blood with Cetyltrimethylammonium Bromide (CTAB) Incubated for Two Hours at Room Temperature.

	Patient	Sex	Diagnosis	Number of Experiments	Concentration mgm CTAB/ml Blood Optimal Production	Grade Cell Produced
1.	L.I.	F	Normal	12	1.9	II
2.	L.B.	F	Normal	15	1.5	II
3.	C.F.	M	Normal	5	0.5	II
4.	L.G.	M	Normal	3	1.0	III
5.	J.H.	M	Normal	3	1.0	IV *
6.	B.P.	F	Normal	3	1.0	III
7.	A.J.	F	Deg. Arth.	5	1.0	III
8.	M.B.	F	Rheu. Arth.	5	0.1-2.0	II
9.	B.A.	F	Deg. Arth.	5	1.0	III
10.	L.F.	M	Rheu. Arth.	3	1.0	III
11.	V.D.	F	Deg. Arth.	3	1.0	IV *
12.	S.C.	F	Rheu. Arth.	3	1.0	II
13.	V.L.	F	Rheu. Arth.	3	1.0	III
14.	W.L.	M	Rheu. Arth.	3	1.0	III
15.	J.G.	F	Rheu. Arth.	3	1.0	III
16.	L.W.	F	Rheu. Arth.	3	0.5	II
17.	B.T.	F	Rheu. Arth.	3	1.0	III
18.	L.D.	F	Rheu. Arth.	3	0.5	II
19.	M.F.	F	Bronch. Asthma	2	1.0	III
20.	E.H.	F	Bronch. Asthma	2	1.0	II
21.	G.H.	M	Bronch. Asthma	2	1.0	II
22.	J.J.	M	Bronch. Asthma	2	1.0	II
23.	R.H.	M	Bronch. Asthma, Cardiac	3	1.0	III
24.	F.P.	M	Pulm. TBC (?)	3	1.0	III
25.	M.G.	F	Ref. Sh. Dyst.	5	1.0	II

* Grade IV L.E.-like Cells

two male and three female rabbits and five female mice with a total of 94 preparations made with blood from these animals. Grade IV cells were produced by CTAB in the blood of one female rat and one male rabbit. Grade III cells were produced in the blood of seven male dogs, while the remaining animals exhibited only Grade II cells with the exception of three dogs and one rabbit, in which CTAB was ineffective in the concentrations employed. No significant difference between species or sex was apparent and the results with CTAB were similar with all species (Tables I and II).

The relatively rare occurrence of Grade IV cells in the CTAB preparations, led to further attempts to increase the incidence of the typical L.E.-cell stage.

The possibility was considered that a change in pH of the blood subsequent to the addition of CTAB, might be an important factor in the phenomenon. Blood was collected, under ether anesthesia, from normal male rats and pooled for pH studies. The serum was removed from a sample of this blood and treated with various concentrations of CTAB. Accurate pH readings were recorded at 15 minute intervals over the two hour incubation period. The results illustrated in Figure IV show no significant variation between CTAB-treated and untreated sera.

When fresh normal blood was exposed to the atmosphere over a two hour period a decrease of 0.15 of a pH unit occurred (Figure V). When L.E. factor was introduced into a sample of this blood, the change in pH was slightly greater than that which occurred in the untreated sample, but this difference was insignificant. Likewise, when CTAB was added to a sample of this blood the pH change was not significantly

TABLE II

Lupus Erythematosus Cell Production By Grades in Normal Animal Blood with Cetyltrimethylammonium Bromide (CTAB) Incubated Two Hours at Room Temperature.

Exp. No.	No. of Animals Used	Specie	Sex	Number of Experiments	Concentration mgm CTAB/ml of Blood Optimal Production	Grade Cell Produced
32	1	Dog	M	2	0.0	Negative
33	1	Dog	M	4	0.0	Negative
37	1	Dog	F	8	1.0	II
40	7	Dog	F	7	1.0	III
48	1	Dog	F	5	0.5	II
52	1	Dog	F	2	0.0	Negative
58	1	Dog	M	7	1.0	II
34	2	Rat	F	5	1.0	III
53	1	Rat	M	4	1.0	II
54	1	Rat	F	4	0.5	IV *
57	14	Rat	F	14	1.0	II
72	1	Rat	F	4	1.0	III
36	1	Rabbit	m	4	1.0	II
41	1	Rabbit	F	8	2.0	II
42	1	Rabbit	F	4	1.0	II
44	1	Rabbit	F	4	0.0	Negative
71	1	Rabbit	F	3	1.0	IV *
38	5	Mice	F	5	2.0	II

* Grade IV L.E.-like Cells

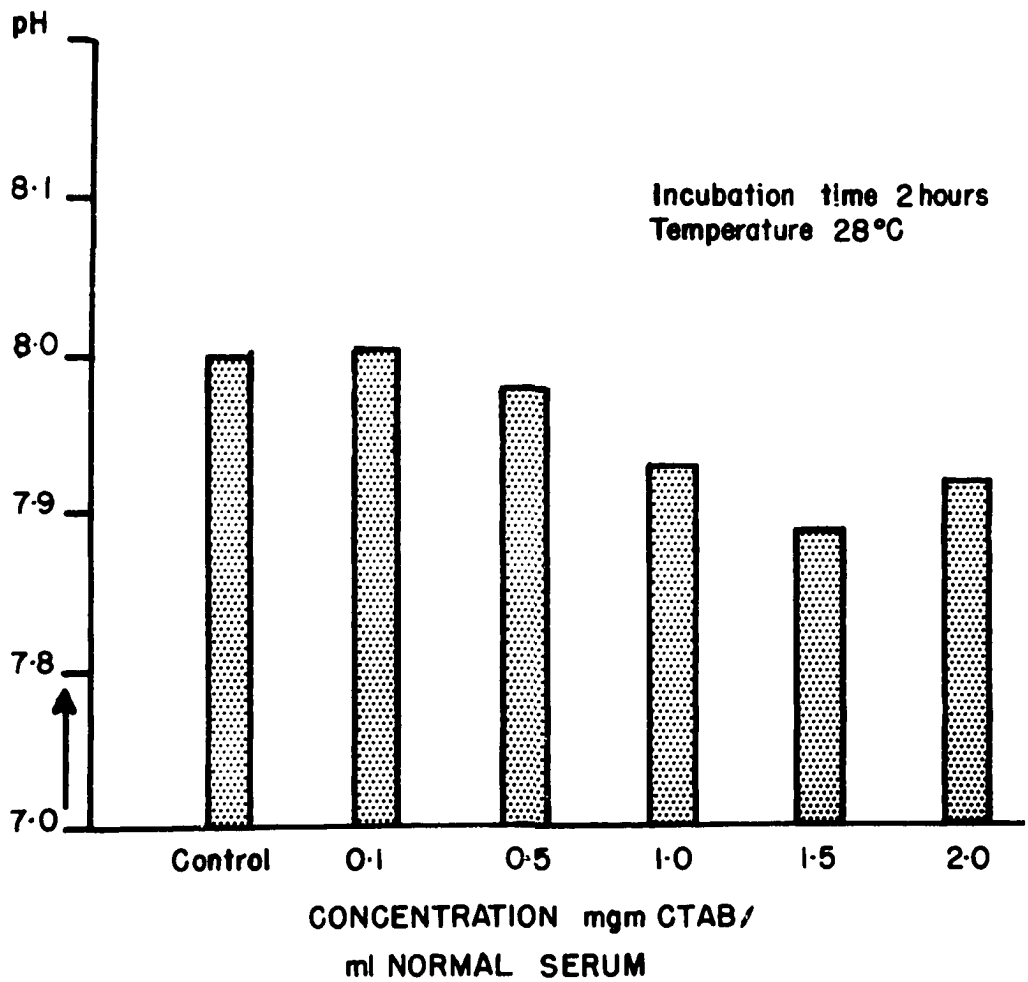
different from that of control blood. It was thus felt that pH changes could not be held accountable for the lupus-cell-promoting effect of this compound, and further studies along this line were abandoned.

The addition of certain cations (calcium, potassium, magnesium and zinc) up to one meq/ liter to blood-CTAB mixtures, and the removal of calcium with sodium oxalate, did not change the artificial L.E. cell phenomenon produced by CTAB.

Clotting is usually retarded by CTAB and in some blood specimens is inhibited entirely; however, the nucleolytic activity of CTAB was not altered by the addition of heparin.

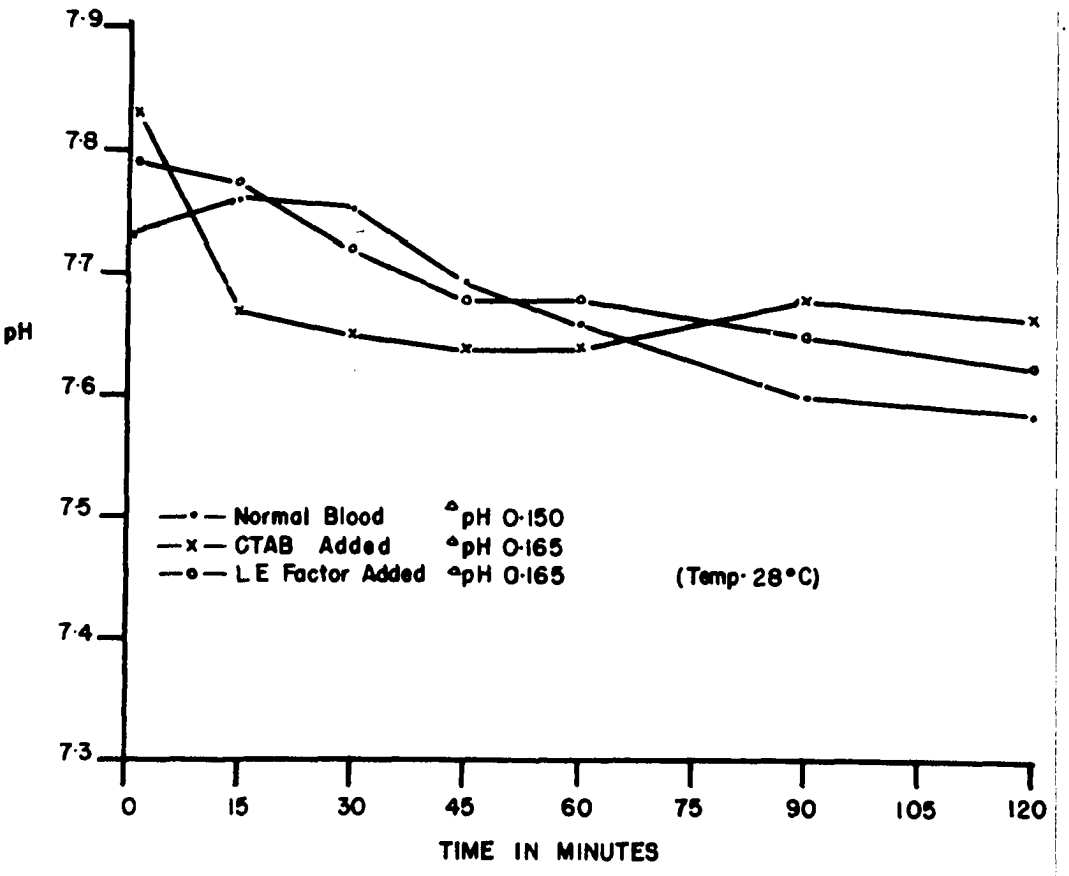
Inderbitzen (3) found that the sodium salt of polyvinyl alcohol polysulfonic acid ester would induce the artificial L.E. cell phenomenon in normal blood only after pre-incubation of this compound with normal serum in order to produce or release the active inducing factor. CTAB produced the artificial phenomenon without this pre-incubation with serum, but in a further attempt to enhance the phenomenon an additional factor was introduced (Table III). Serum and CTAB (one mgm/ml of serum) were incubated together at varying intervals of time and at different temperatures. The serum-CTAB incubated mixtures were rapidly cooled to room temperature and added to normal blood ($\frac{1}{2}$ ml mixture/ml blood) for the artificial L.E. cell preparations. Grade IV cells were produced by serum-CTAB mixtures from tubes seven and eight, 50° C for 20 min. and 50° C. for 30 min. respectively. However, this encouraging result was not reproducable, and in three repeated attempts only Grade II and III cells were produced in any of the tubes.

In preliminary studies it was found that CTAB will precipitate



CHANGES IN SERUM pH RESULTING FROM THE INCUBATION OF INCREASING CONCENTRATIONS OF CTAB WITH NORMAL BLOOD

FIGURE IV



CHANGES IN pH OF NORMAL BLOOD RESULTING FROM THE ADDITION OF CTAB AND L.E. FACTOR

FIGURE V

some of the plasma proteins and it was also noted here that with increasing temperature the amount of protein precipitated also increased. The precipitated protein was of a gummy consistancy and insoluble in most protein solvents. When this protein material was added to normal blood it did not exhibit nucleolytic activity, and chemotaxis was lacking. It was also noted in preliminary studies that CTAB caused rapid destruction of all cells in the absence of serum, and it seemed probably that some factor present in serum was necessary to prevent this rapid destruction. The possibility thus exists that serum proteins act as protective agents which inhibit the surface acting cytolytic property of CTAB and allow the compound to exert its effect upon the cell nucleus instead.

An attempt was made to determine if a portion of the CTAB molecule was responsible for its nucleolytic activity, or if the molecule functioned as a whole in the production of this effect. The structural formula of CTAB is shown in Figure VI along with the formulae of other compounds compared to this agent.

Active constituent groups of the CTAB molecule are illustrated and numbered. Other compounds representing these active groups were tested. The compounds (Hexamethonium bromide, cetyl alcohol, cetyltrimethyl ammonium chloride, sodium bromide) were added to normal blood in concentrations of 0.5, 1.5, 2.0, 2.5, and 3.0 mgm/ml of blood, and L.E. preparations made after two hours incubation at room temperature. Hexamethonium bromide and cetyl alcohol produced minor cell destruction but no other significant results. It was surprising to find that cetyltrimethylammonium chloride produced only Grade II cells in the same blood

TABLE III

Effect of Pre-incubating CTAB With Normal Serum on Producing the Artificial L.E. Cell Phenomenon in Normal Blood.

(Concentration - 1 mgm CTAB/ml of Serum)

Tube	Temperature °C	Minutes Incubation Time of Serum	Grade Cell Produced in Blood
1	40	0 /	I
2	40	10	II
3	40	20	II
4	40	30	III
5	50	0 /	II
6	50	10	I
7	50	20	IV *
8	50	30	IV *
9	60	0 /	II
10	60	10	II
11	60	20	II
12	60	30	II

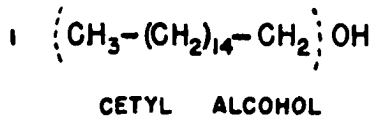
*Grade IV L.E.-like Cell

/ This serum was preheated to the designated temperature, CTAB added and then rapidly cooled to room temperature before the addition of blood.

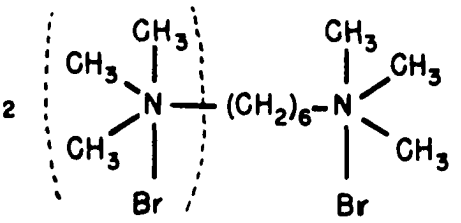
and in the same concentration in which Grade III cells were produced by CTAB. Sodium bromide in the concentrations used caused rapid cell destruction. Present evidence indicates that CTAB is specific among the compounds tested in inducing the artificial L.E. phenomenon.

The serum from patients with systemic lupus erythematosus induced the L.E. phenomenon when added to normal blood in proportions of ½ ml of L.E. serum to 1 ml of fresh blood. With such natural L.E. cell preparations all grades of lupus cells, including rosettes, were found. Typical rosettes and L.E. cells produced by natural and artificial means are shown in Color Plate III and IV. Figure 1, Color Plate III, illustrates a rosette formed in a natural L.E. cell preparation and Figure 2, Color Plate III, presents a rosette found in a CTAB-L.E. cell preparation. In Figure 1, Color Plate IV, numerous Grade III and IV cells from a natural L.E. cell preparation are shown and Figure 2, Color Plate IV, shows a single Grade IV cell produced by CTAB in normal blood.

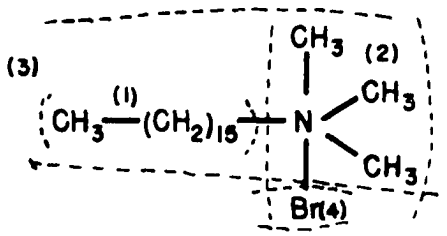
The methyl green-pyronin Y (63) staining reagent produced a positive reaction in the inclusion bodies of both the natural and artificially induced Grade IV cells. The authors of the method attributed this reaction to the presence of desoxyribose nucleic acid. Ribose nucleic acid was found in minute quantities in the cytoplasm of these cells by the same reagent. The Feulgen reaction is generally accepted as a specific indicator for desoxyribose nucleic acid. The inclusion bodies, in the Grade IV cells, were found to be Feulgen positive and this correlates with the methyl green-pyronin reaction. No experiments were designed to test the hypothesis of Kuernick (57) that the L.E. factor inactivates an inhibitor of desoxyribonuclease and permits depoly-



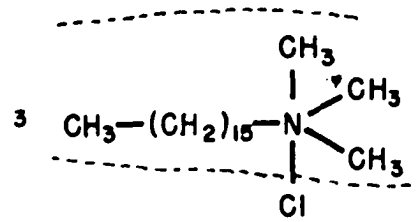
CETYL ALCOHOL



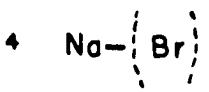
HEXAMETHONIUM BROMIDE



CETYLTRIMETHYLAMMONIUM BROMIDE



CETYLTRIMETHYLAMMONIUM CHLORIDE



SODIUM BROMIDE

STRUCTURAL FORMULAE OF COMPOUNDS REPRESENTATIVE OF ACTIVE CHEMICAL GROUPS OF THE CTAB MOLECULE WHICH WERE TESTED FOR L.E. INDUCING ACTIVITY

FIGURE VI

merization of desoxyribose nucleic acid by this enzyme.

It was found that when CTAB and L.E. factor were added together to normal blood the L.E. cell phenomenon was partially or completely inhibited. Figure 1, Color Plate V shows the results provided by addition of natural L.E. factor to normal blood, and Figure 2 demonstrates the inhibition in a sample of the same blood when CTAB and L.E. factor are added together. The process by which this inactivation occurs was not investigated but it is likely that CTAB denatures the L.E. factor and is likewise consumed in the reaction. This possibility is advanced because of evidence that CTAB denatures most of the globulins in serum, and the L.E. factor has been shown to reside in the gamma globulin fraction. Color Plate VI further illustrates two cell types with cytoplasmic inclusions that are sometimes produced by CTAB but are not significant of the L.E. phenomenon. Figure 1 is an example of nucleophagocytosis in which the inclusion body is a normal lymphocyte and Figure 2 illustrates erythrophagocytosis, in which an erythrocyte is the included object.

Nucleophagocytosis and erythrophagocytosis of normal cellular elements are thought to indicate stimulation of phagocytosis. These phenomena are often found in L.E. preparations, and should not be confused with the Grade IV cell in interpreting the L.E. phenomenon. Although these examples were produced in a CTAB-artificial L.E. cell phenomenon, the experimental findings presented in this study suggest that phagocytosis is not stimulated and that the primary action of CTAB upon a cell is the induction of nuclear break-down. The inability of CTAB to produce a greater incidence of Grade IV cells is probably due to

COLOR PLATE III

Figure 1

A "Rosette" found in a natural L.E. (Lupus Erythematosus) preparation. Giemsa stain. 860x

Figure 2

A "Rosette" induced by the addition of CTAB to normal blood. Wright's stain. 860x

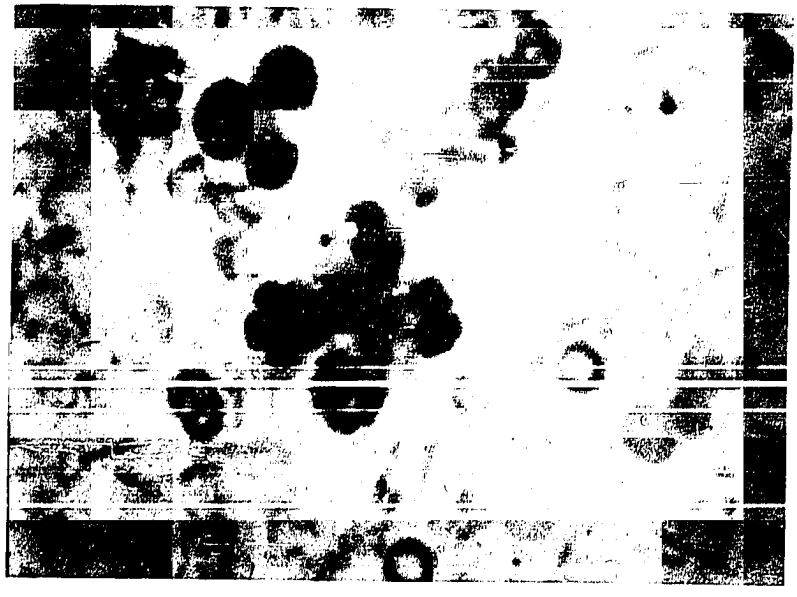


Figure 1

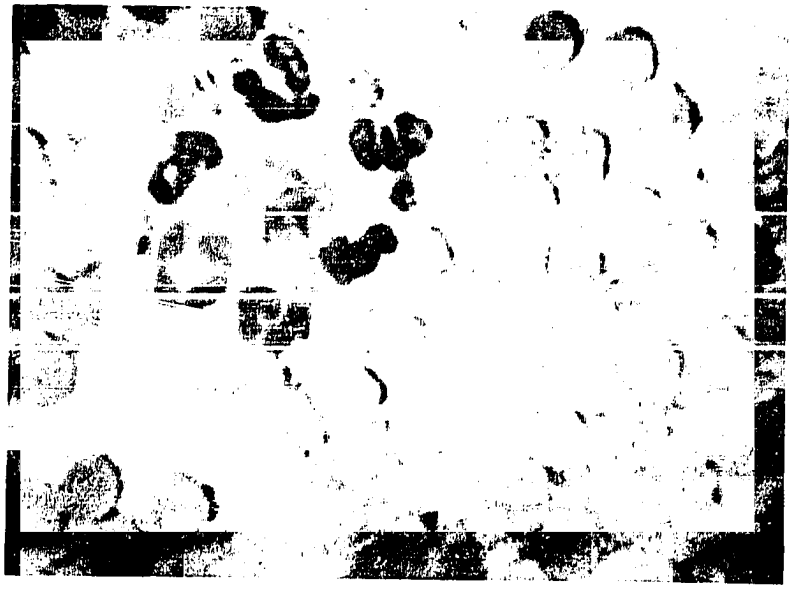


Figure 2

COLOR PLATE III

COLOR PLATE IV

Figure 1

L.E. (Lupus Erythematosus) cell (Grade IV) found in a natural L.E. preparation. Note also numerous Grade II and occassional Grade III cells present. Wright's stain. 860x

Figure 2

A Grade IV (L.E.-like Cell) artificially produced by CTAB in normal blood. Wright's stain. 860x

Figure 1

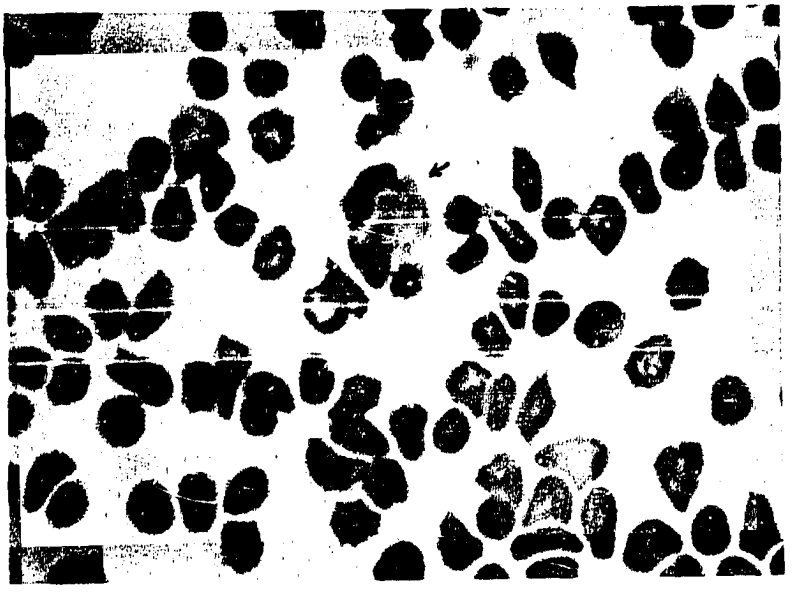


Figure 2

COLOR PLATE IV

COLOR PLATE V

Figure 1

Nucleolysis induced by the addition of L.E. Factor to normal blood. Wright's stain. 860x

Figure 2

The results of adding L.E. Factor and CTAB together to normal blood. Note that nucleolysis is minor and insignificant. Wright's stain. 860x

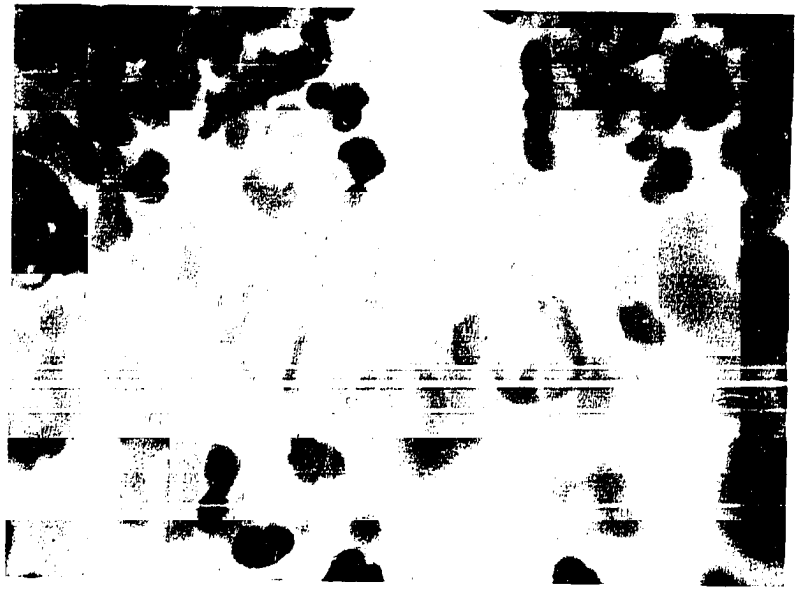


Figure 1

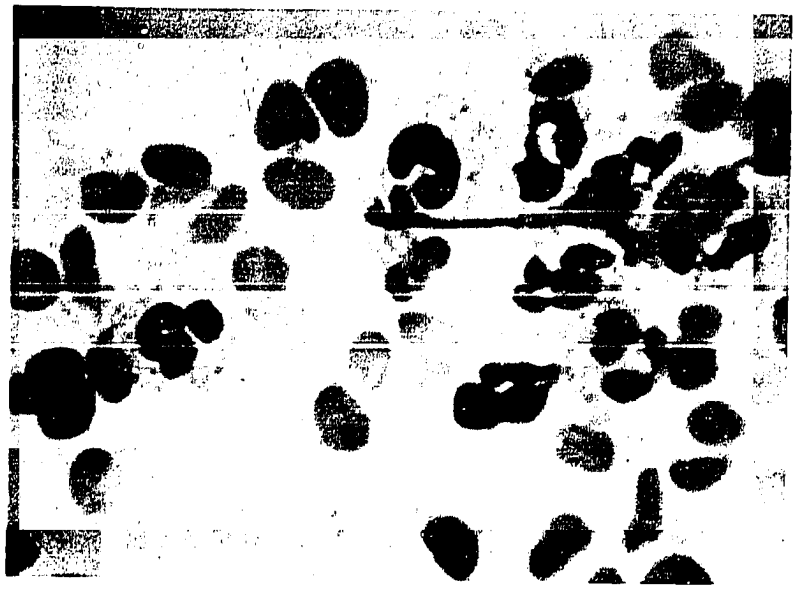


Figure 2

COLOR PLATE V

its generalized detrimental effect on all blood cells, only a few surviving which are capable of the dynamic power of phagocytosis. The L.E. factor appears to produce nucleolysis in only selected cells of the population and to stimulate phagocytosis in other cells.

COLOR PLATE VI

Figure 1

Nucleophagocytosis designated by the arrow. The included body appears to be a normal lymphocyte. Giemsa stain. 860x

Figure 2

Erythrophagocytosis designated by the arrow. The cell has phagocytized a normal-appearing erythrocyte. Wright's stain. 860x

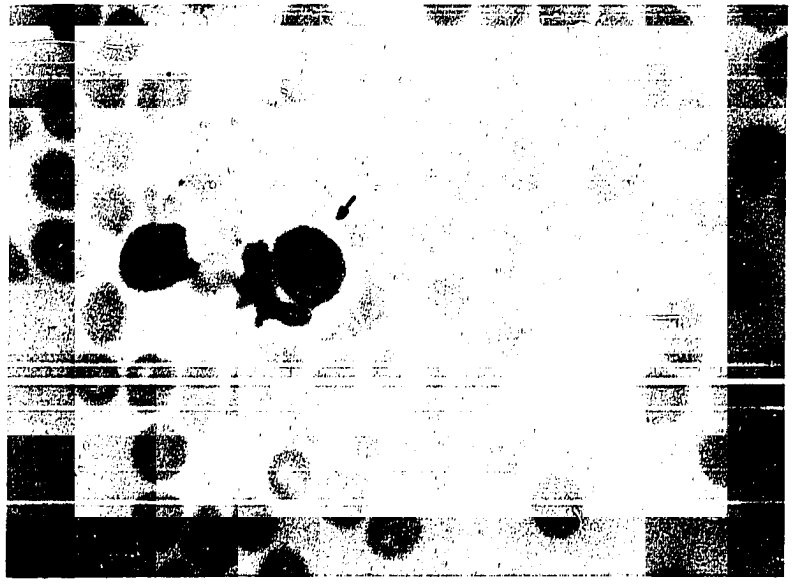


Figure 1

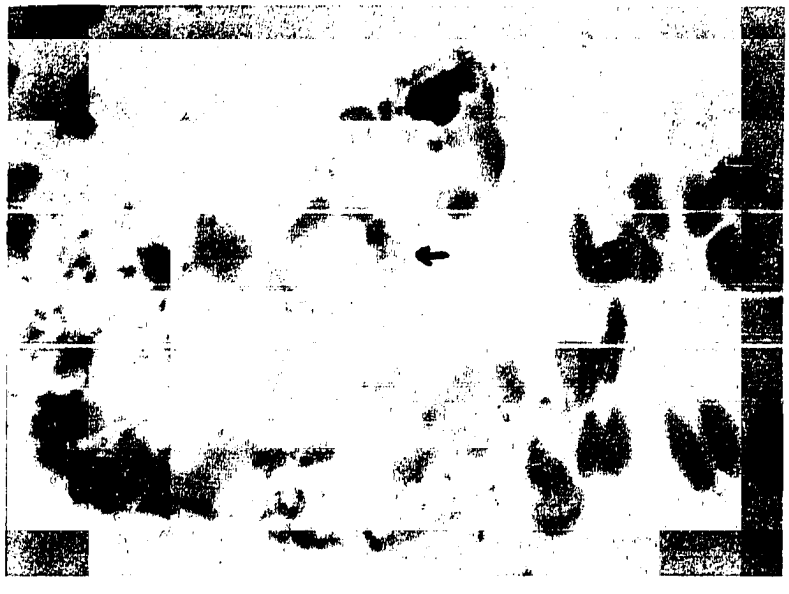


Figure 2

COLOR PLATE VI

CHAPTER V

SUMMARY

The L.E. phenomenon is considered a valuable laboratory aid for the diagnosis of systemic lupus erythematosus. The discovery of this phenomenon provides considerable impetus to basic and clinical research in systemic lupus erythematosus and allied diseases. The phenomenon has subsequently been reported in other diseases of ground substance, hypersensitivity states and in various other disease states. This abnormal cellular reaction would appear to represent a basic aspect of cell pathology. Investigations have resulted in the postulation of several interesting mechanisms and pathways leading to the production of the phenomenon. Various means of inducing the phenomenon have been reported. Production of this phenomenon in normal blood by chemical means was reported by Inderbitzen (3). The present study has been concerned with the investigation of conditions necessary for production of the phenomenon and analysis of the cellular changes incidental to it.

The finding that a long chain aliphatic quaternary ammonium compound, cetyltrimethylammonium bromide (CTAB), will artificially induce a L.E.-like phenomenon in normal blood has further extended this study. The occasional production of true lupus cells with CTAB in normal human and animal blood was observed. Lesser manifestations of the

phenomenon were regularly produced. The typical L.E. cell represents the end-point of the L.E. cell reaction, and many recognizable stages occur in the development of this end-point. A four-point system of grading, based upon changes in cellular morphology, was designed to quantitate the stage of advancement of this phenomenon. The first recognizable deviation from normal was designated Grade I and Grade IV, the typical L.E. cell, as the end-point of the reaction. Grades II and III are intermediate stages in the cellular phenomenon. The rosette, another intermediate cellular phenomenon, was not considered in the grading system because it was not always found to be present. It was determined that a concentration of one mgm of CTAB/ml of blood was optimal for the production of this phenomenon. The most satisfactory results were obtained after an incubation period of two hours at room temperature (approximately 28° C.).

Experiments were designed to determine the most favorable conditions for the action of CTAB and to elucidate the basic mechanism of its action.

On the possibility that a shift in acid-base balance of normal blood might be induced by CTAB and thus account for its action, pH determinations were made on normal serum and blood, with and without added CTAB. The pH changes produced by CTAB and L.E. factor were almost identical, and so minor that they did not warrant further investigation.

Positive (Grade IV) CTAB-L.E. cells were produced with the blood of two out of 25 human subjects, one normal and one diagnosed as degenerative arthritis. Cetyltrimethylammonium bromide produced L.E.-like cells in blood from one rabbit and one rat, among the several

animals tested.

The addition of calcium, magnesium, potassium or zinc did not enhance the action of CTAB. The removal of calcium with sodium oxalate or the addition of heparin did not alter the reaction.

Preincubation of serum with CTAB, utilizing the technique of Inderbitzen, did not enhance production of the lupus phenomenon.

Certain chemical agents, representing component groups of the CTAB molecule, were without effect in initiating the lupus phenomenon which indicates that the activity is probably dependent upon the intact molecule.

The inclusion bodies of both the CTAB-L.E.-like cell and the L.E. cell produced with L.E. factor were methyl green-pyronin Y and Feulgen positive which indicates the presence of desoxyribose nucleic acid and suggests that the two are chemically similar.

When CTAB and the L.E. factor were added together to normal blood, the L.E. phenomenon was inhibited. A probable explanation is that CTAB denatures most of the globulins in serum and the L.E. factor is thought to be a gamma globulin.

Nucleophagocytosis and erythrophagocytosis are sometimes produced by CTAB in the artificial L.E. phenomenon, but in general it would appear that CTAB is injurious to all cells and probably does not stimulate phagocytosis.

CHAPTER VI

CONCLUSIONS

1. An artificial L.E. cell phenomenon may be produced in normal human and animal blood with CTAB.
2. A system of quantitating the progression of the phenomenon was developed and used to evaluate the cellular effects produced by CTAB under various experimental conditions.
3. Optimal results were obtained at a concentration of one mgm CTAB/ml of blood incubated for two hours at approximately 28° centigrade.
4. Shifts in pH were found to be insignificant in the L.E. phenomena produced by the L.E. factor as well as that produced by cetyltrimethylammonium bromide.
5. Neither the addition of calcium, magnesium, potassium or zinc nor the removal of calcium altered the action of CTAB.
6. The inhibition of coagulation by heparin or oxalate did not alter the reaction.
7. Preincubation of normal serum with CTAB does not significantly alter its activity.
8. Chemical agents chosen as representative of the constituent chemical groups of the CTAB molecule were tested and found incapable of producing the phenomenon, which indicates that the CTAB molecule as

such is specific in function.

9. The inclusion bodies of the CTAB-L.E.-like cell and the natural L.E. cell were methyl green-pyronin Y and Feulgen positive which indicates the presence of desoxyribose nucleic acid.

10. When CTAB and L.E. factors were mixed together in blood the L.E. phenomenon is partially or completely inhibited.

11. Indications were found that CTAB is injurious to all cells and does not stimulate phagocytosis.

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