COMPARISON BETWEEN LOCOMOTORY BEHAVIOR OF LYMPHOCYTES OF $\underline{WW}^{V}$ MUTANT AND NORMAL HOUSE-MOUSE.

by

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B. Sc. Nanyang University 1967

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in the Department

of

Zoology

We accept this thesis as conforming to the required standard.

The University of British Columbia

September, 1969

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Date September 15, 1969

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Acknowledgement

I am grateful to Dr. A. B. Acton for his assistance and advice during the course of this study and in the final thesis preparation. I wish to thank Dr. C. V. Finnegan and Dr. C. J. Anastasiou for allowing me to use the facilities in their laboratories and also for their advice. Thanks also extended to Dr. P. A. Larkin for his suggestion on the statistical analysis.

I am very much appreciate to the help given by Mrs. M. Douglas in the course of this study and the photographic assistance given by Mr. L. Veto.

ABSTRACT

The rate of motility of lymphocytes from anemic mice of the WW^V genotype and their normal ++ littermates was determined *in vitro* by time-lapse cinephotomicrography. A comparison of their speeds on an isogenic kidney monolayer or feeder layer suggests that the speed of locomotion of lymphocytes from the WW^V mice may be somewhat reduced. If subsequently verified, this would suggest that cells produced in the lymphatic tissue show, in addition to the well known abnormalities in the erythrocytes, pigmented cells of the neural crest and the primordial germ cells, a further defect caused by mutation at the W locus.

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INTRODUCTION

Mutations at the dominant-spotting (W) locus in the house mouse produce in the homozygous condition a triad of pleiotropic effects that are seen in the hematopoietic tissue, the coat color and the gonadal development (Russell, 1949, 1954). A number of alleles have been identified at this locus: these are W (Little, 1915); W^V (Little and Cloudman, 1937); W^j (Russell, et al., 1957); and W^b (Ballantyne, et al., 1962). Because of their superior viability, mice of the WW^V genotype have been the most extensively studied.

Substitution of the gene-pair WW^V for the normal ++ alleles produces a severe, though not lethal, macrocytic anemia. Mice having this WW^V genotype are the so-called black-eyed whites which are completely devoid of fur pigmentation and are infertile. However, though the severe anemia of these mice has been extensively studied morphologically (Russell and Fondal, 1951; Russell, et al., 1953: Russell, et al., 1963) and biochemically (Altman, et al., 1953; Singer and Russell, 1954; Altman and Russell, 1964), the precise nature of this mutation is still obscure, although some evidence has been accumulated regarding the action of this gene.

The gene-action leading to the hematopoietic defects has been shown to occur in the hematopoietic cells themselves, rather than the defects being imposed by the cellular environment or by the effects of other parts of the body

(McCulloch, et al., 1964; Lewis, et al., 1967; Russell, et al., 1956; Bernstein and Russell, 1959; Russell and Bernstein, 1968). This has been demonstrated repeatedly by successful implantation of hematopoietic cells from the adult marrow or the fetal liver of normal donors into adult WW^V, juvenile WW^V, and lethally anemic WW^V mice (Bernstein and Russell, 1959; Russell, et al., 1956; Bernstein, 1963). These workers concluded that the injected normal cells multiply in response to differentiating factors provided by the host and literally outgrow the indigenous hematopoietic cells of the anemic recipient. Tests of hemoglobin electrophoretic type, inherited independently of the W locus, have shown that implanted normal erythroid precursor cells produce all, or nearly all, of the circulating erythrocytes of WW^V mice, whose anemia has been cured following injections of coisogenic normal ++ hematopoietic cells. Thus, there is excellent evidence that the defect produced by the WW^V gene resides within the hematopoietic cells themselves and is not mediated by humoral or toxic factors. Furthermore, WW^V mice have been reported to respond very poorly to injections of large doses of exogenous erythropoietin, although normal littermate mice (++) respond strongly to very small doses of the same batch of erythropoietin (Keighley, et al., 1962; 1966). This defective capacity to respond to erythropoietin has proved to be an inherent character of WW^V blood-forming tissues, since $++/WW^V$ chimeras produced by transplantation of normal ++ bone marrow into WW^V mice respond well to the administration of this factor (Keighley, et al., 1962).

The pigment defect caused by the W mutation has been localized in the neural crest (Silvers, 1961) and recently has been confirmed to reside within the neural crest cells (Mayer and Green, 1968). Markert and Silvers (as reported by Silvers, 1961) transplanted embryonic tissue containing neural crest from mice destined to be completely white into the anterior chamber of the eye, an environment that is known to be favorable for melanoblast differentiation and melanin synthesis. However, they were unable to detect any pigment cells in the grafts; thus, a defect of the neural crest was suggested rather than a defect in their environment. Mayer and Green (1968) later investigated this problem by grafting normal ++ and WW mutant embryonic skin and neural crest in appropriate combinations into the coelom of host chick embryos. Grafts produced by combining ++ neural tubes with WW white skin resulted in 100% pigment production, through migration of pigmented cells from the neural crest, whereas only 39% of the WW neural tube and ++ skin combination grafts formed pigment. These results were interpreted as demonstrating a defect in the melanocytes of the black-eyed whites. Thus, the pigment-cell defect and anemia in the W mutants are the results of factors acting within the pigment- and blood-forming cells themselves. The deficiency in number of melanoblasts which underlies the lack of pigment is not a consequence of the anemia because this abnormality is already evident at the tenth day of gestation (Russell, 1963), that is, prior to the development of anemia.

Although it is not yet known how the dominant-spotting gene acts to produce the defect in the primordial germ cells, the sterility of the W homozygotes is evidently an inherent property of the gonad itself. Ovaries from isologous ++ mice were transplanted to the ovarian capsule of the homozygous mutants whose own ovaries had been removed; these anemic mice were able to support the transplanted ovaries and successfully concluded pregnancies: the offspring showed the genetic characters of the donors of the ovaries (Russell and Russell, 1948). Gonads removed from 12 to 16 day-old mutant embryos and grafted to the spleen of normal adult castrate mice (Russell, et al., 1956), or explantation of 12-day WW gonads to a favorable organ culture medium (Borghese, 1956), do not mitigate the germ cell defect which is fully expressed at 9 days. These experiments suggest that the defect in the primordial germ cells occurs within the cells themselves and is therefore unlikely to be secondary to anemia, since the defect in the gonads appears prior to the development of the first manifestation of disordered hematopoiesis (Russell, et al., 1956). This suggestion was confirmed by Borghese, who was able to distinguish in tissue culture between fertile and sterile gonads from 12-day embryos (Borghese, 1955; 1956; 1957). Further confirmation of the defective development of the germ cells and its occurrence before the defective hematopoiesis were reported by Mintz and Russell (Mintz, 1957; Mintz and

Russell, 1955, 1957; Russell, 1963), who showed that the number and location of the primordial germ cells was abnormal in the WW embryos.

Thus, it is quite evident from these three lines of evidence that the primary action of the W series of genes, that is, the site of the pertinent effect of W genes, is located within the affected cells themselves. However, the intrinsic nature of these cellular defects remains an unsolved problem, although many suggestions have been proposed in the past few years. It is known that there is impairment of cell differentiation and proliferation in the hematopoietic tissues (Russell and Bernstein, 1966), failure of multiplication during the migration of the primordial germ cells (Mintz, 1957, 1960), and failure of melanoblasts to arrive at the hair follicles (Markert and Silvers, 1956), but the relationship between these three defects produced by the W mutation appears to be obscure. It is noteworthy, however, that the three types of cells affected by this locus share the characteristics of being migratory and proliferative. It is, therefore, quite tempting to suggest that either, or both, of these functions is abnormal in the affected cells of the W mutant.

In addition to the three defective tissues, lymphocytes also have the characteristics of being migratory and proliferative, and it is the purpose of this investigation to find out whether the mutation at the W locus has any effect on these cells.

MATERIALS AND METHODS

For the study of the locomotory behavior of the lymphocytes, normal ++ and WW^V mutant mice of C57Bl background were used. The ++ mice were obtained from the offspring of an inbred strain of C57Bl. The ++ mice are characterized by a completely black hair coat, black eyes, and in general a pigmented tail and feet. The WW^V mice, which are sterile, were obtained by crossing the mice of W+ genotype to those with the W^V+ genotype. This cross gives about 25% black-eyed whites of WW^V genotype. The mice with W+ and W^V+ genotypes were maintained by backcrossing to the ++. W^V+ animals are grey with a white belly spot and W+ animals are black with a white belly spot and a white streak on their foreheads.

Kidneys of these mice were used to establish a uniform layer of cells, that is a so-called monolayer, on a coverslip. The monolayer acts both as a feeder layer, which conditions the culture for the survival of the lymphocytes, and to provide a substrate for the motile lymphocytes. At first, monolayers of ++ and WW^V genotypes were used to study the locomotory behavior of lymphocytes of the same genotype. However, a difference in genetic background of the monolayer from that of the lymphocytes may affect their ability to support the survival and locomotion of the lymphocytes. Therefore, four series of experiments (see Diagram A) were

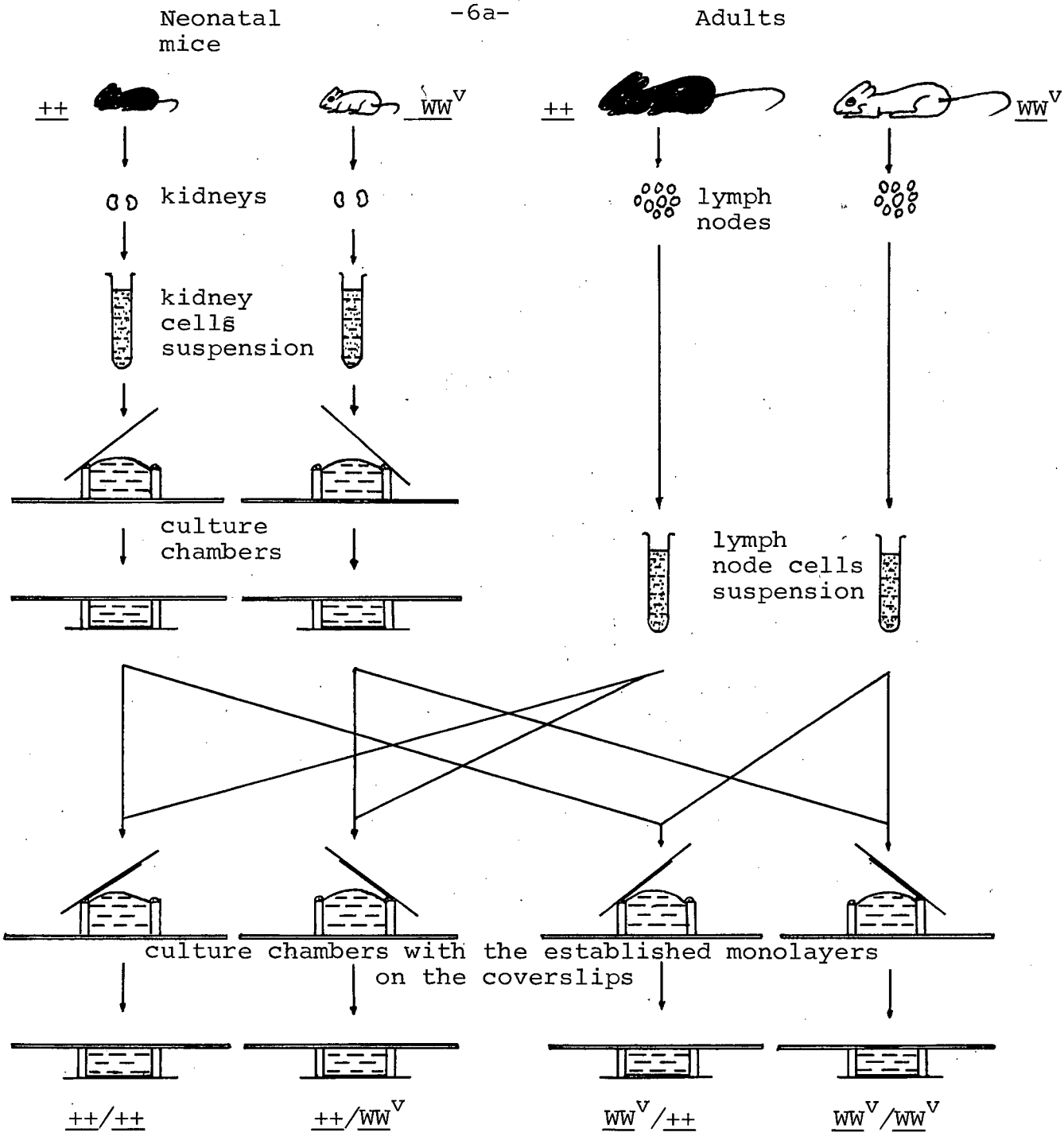

Diagram A. Experimental design for cinemicrographic study of the locomotion of ++ and WW^V lymphocytes on ++ and WW^V monolayers.

carried out to avoid the possibility of this bias. In the first series, both lymphocytes and monolayer were from ++ mice. In the second series, the lymphocytes were from ++ individuals and the monolayer from WW^V. In the third series, lymphocytes were obtained from ++ mice and the monolayer from WW^V mutants. In the fourth series, both lymphocytes and monolayer were from WW^V mice. In all cases, lymphocytes were obtained from the lymphnodes of fully matured animals and monolayers were cultured from the kidneys of neonatal mice.

(a) Preparation of mouse kidney monolayer

Primary monolayers were prepared by the method, with slight modifications, described by Dulbecco and Vogt (1954). Briefly, a neonatal mouse was killed by cervical dislocation and the two kidneys were excised aseptically. The organs were washed with Hanks' solution, finely minced with scissors, and then incubated with 0.25% trypsin solution. The first supernatant was usually discarded after incubating for 15 minutes and the second after a further hour of incubation. The third supernatant was obtained after pipetting up and down with a sterile pasteur pipette for 2-3 minutes and shaking on a rotator for 15 minutes. The cells in the supernatants were washed twice with Hanks' solution using low speed centrifugation and were finally suspended in the standard medium. The cell concentration was estimated using a hemocytometer and adjusted to a final concentration of about

$3x10^5$ cells per ml.

The culture chambers were prepared by sticking one rim of a glass ring (inner diameter 15 mm and height 5 mm) to a 75x25 mm glass slide with high-vacuum silicone grease. Approximately 1 ml. of the kidney-cell suspension was introduced into each chamber until it was full. A coverslip (diameter 22 or 25 mm) was used to cover the open face of the chamber by pressing it gently down, taking care to exclude air bubbles, to stick on the greased rim of the chamber. The chambers were inverted to allow the cells to settle onto the cover slip, and were incubated at 37°C for 3-4 days. After this time, a uniform primary monolayer was obtained which was then ready for the introduction of the lymphocytes.

In the course of monolayer cultivation, it was found that the age of the kidney and the type of medium used for establishing of the monolayer are very critical. A series of experiments was undertaken to test the effects of these variables. The results are tabulated (see Table I) and are summarized as follows: The preliminary observations and comparisons indicate that kidneys obtained from mice of ages varying from one to about twenty days are suitable for establishing monolayers, although the suitability decreases as the age of the mouse increases. Kidneys obtained from adult individuals, or from young mice of ages greater than thirty days, give very inconsistent results and usually are

Table I. Effects of culture medium and ages of mice in the establishing of uniform kidney-monolayers.

Medium \ Ages	1	5	10	15	20	25	30	Adult
Minimial Eagle's	++++	++++	++++	++++	+++	+++	+	poor and inconsistant
Waymouth MB 752/1		++		+++	+++	++	poor	
Dulbecco's modified Eagle's	+++	++		**	++	++	**	
TC 199	++	++		+	++	poor	poor	
MK		++	poor	++	++	+	poor	
MK with 0.5% yeastolate		**		+	poor	poor	poor	
MK with 0.1% yeastolate	+	**	poor	+	poor	+	poor	

Notes: ** means either cultures are contaminated or the medium is too alkaline.

unsuitable for monolayer cultivation, no matter what kind of medium is used. Five different kinds of medium were tried initially in the attempt to establish a uniform monolayer; they are (1) Minimal Eagle's Medium supplemented with 10% or 20% calf serum; (2) Waymouth's Medium supplemented with 10% fetal calf serum; (3) Dulbecco's modified Eagle's Medium supplemented with 10% or 20% fetal calf serum or horse serum; (4) TC 199 medium plus 10% or 20% fetal calf serum; and (5) MK medium supplemented with 10% fetal calf serum alone, or with the addition of 0.1% or 0.5% yeastolate. In addition, all the media include 100 units/ml. of penicillin G, 0.1 mg/ml. of streptomycin sulfate and 0.005 mg/ml. of phenol red as pH indicator.

Minimal Eagle's Medium was found to be the most suitable one for growing the neonatal kidneys, but unfortunately none of the five media was suitable for growing the adult kidneys. Consequently, all the monolayers used in the subsequent experiments were established using neonatal kidney tissues in Minimal Eagle's Medium. The neonatal kidney tissues have the further advantages of being easier to break down, digest, and separate into single cells, or clumps of cells, by either mechanical treatment or with trypsin solution, and the separated cells take a shorter time to adhere to and spread on the coverslip to form a uniform sheet. The preliminary observations seem to show that one kind of medium is more favorable for the growth and maintenance of certain cell types from the mouse kidneys.

For example, Minimal Eagle's Medium and Waymouth's Medium appear to support the growth of epithelioid-like cells better than they do the fibroblast-like cells under the present conditions. On the other hand, TC 199 medium seems to support the growth of fibroblast-like cells better than Minimal Eagle's Medium, although a uniform monolayer was never established with this medium. These results suggest that most of the mouse kidney cells in culture are epithelioid in nature and that they probably correspond to the epithelial cells lining the renal tubules. It is, therefore, tempting to suggest that the various classes of cells present in the mouse kidney may be isolated *in vitro* by repeated cultivation in a specific medium so that their physiological roles *in vivo* may be studied.

(b) Preparation of lymphocytes for behavioral studies

Lymphocyte suspensions were prepared from lymph nodes of both male and female ++ and WW^V adult mice. The mice were killed and the axillary, branchial, inguinal and mesenteric lymph nodes were removed with fine forceps to a Petri dish containing Hanks' solution. The fatty tissues surrounding the nodes were removed carefully and the nodes were then washed twice with Hanks' solution. The lymphocytes were released from the nodes by teasing in salt solution with two fine forceps. Two to three such cell suspensions were collected and washed twice with Hanks' solution using low speed centrifugation. The cell pellet was then suspended

in culture medium (Dulbecco's modified Eagle's medium supplemented with 20% horse serum). The cell concentration was finally adjusted to give a cell count of approximately $1\text{-}2 \times 10^6$ cells/ml.

The coverslip, when it was covered with a fully established monolayer, was removed from the culture chamber; the culture medium used to grow and maintain the monolayer was discarded and the culture chamber was refilled with the freshly prepared lymph-node cell suspension. The coverslip with its monolayer was then replaced and the culture inverted to allow the lymphocytes to settle so that they would penetrate the sheet of epithelioid cells to move between them and the coverslip. The cultures were incubated at 37°C and later used for microscopic observations and time-lapse cinephotomicrography.

(c) Staining of whole cultures

After each observation, or taking of photographs, the coverslip carrying the monolayer and moving lymphocytes was removed from the chamber and washed rapidly in warm (37°C) normal saline. The whole culture was then fixed rapidly in absolute methyl alcohol for about 10 minutes. The rapid washing and fixing are very critical in order to retain the original contour of the moving cells. The culture was stained with Wright's and Giemsa stains for 10 minutes, differentiated in acetone, dried by evaporation of the acetone in air, and mounted with Permount.

This technique has been found to preserve the shapes of the lymphocytes moving between coverslip and the monolayer very satisfactorily, although a slight shrinkage in size usually occurs as compared to the cells in the living culture. The different shapes of lymphocytes may conveniently be described as oval, round, worm-like or hand-mirror-like. The preservation of the contours of moving lymphocytes has been reported to be difficult (De Bruyn, 1945). However, Berman (1942) has preserved the amoeboid forms of rabbit lymphocytes in thin-film tissue cultures. The present finding seems to suggest that rapid fixation is necessary to preserve the amoeboid shape of the moving cells, since slow fixation usually causes the cells to round up.

(d) Photography

A time-lapse cinephotomicrographic apparatus was used to trace the movement of each individual lymphocyte. A Zeiss microscope equipped with phase-contrast objectives and a Nikon movie camera were used throughout the experiment. A time-lapse device was connected to the camera and the speed was adjusted to 30 frames/minute. The film (Double X Negative, Kodak Eastman) was taken at a magnification of 320 (Objective x40, Eyepiece x8 and Column factor x1). This figure was confirmed by measuring the magnified image of a known scale on the negative. A long-distance condenser was used because of the thick chambers. These chambers were maintained at $37^{o}C \pm 0.2^{o}C$ on the microscope stage with a

'Sage Curtain Incubator'. A thermister probe connected to the surface of the culture chamber was used to regulate the temperature of the culture.

The films were developed in Kodak D11 for 8-12 minutes at 20°C and fixed in high speed 'Amfix' for 5-10 minutes. The outlines of individual moving lymphocytes were traced on paper with the aid of a film editor. In this way the paths of different cells were constructed and the speed of each lymphocyte was calculated.

RESULTS

I. Morphological observations

Many studies have been reported in the literature concerning the locomotion of lymphocytes *in vitro*. However, a review of the literature has failed to disclose any qualitative or quantitative study of the motility of either normal or WW^V lymphocytes in mice, although some studies have been made on leukemic lymphocytes (De Bruyn, 1949; Pulvertaft, 1959). This is probably explained by the difficulty in getting mouse lymphocytes to survive and become active in tissue culture, unless they are cultured with some other type of cell as feeder layer (De Bruyn, 1949; Bichel, 1939;1952; Kieler and Kieler, 1954; Ginsburg, 1965). It is therefore necessary first to give a brief account of the modes of movement of mouse lymphocytes, though it may be found that their movements are somewhat similar to those of the other species reported. The following account applies equally well for the locomotion of normal and WW^V lymphocytes, since no differences were detected.

(a) Modes of movement

Cells of various sizes were observed to move between the coverslip and the cells of the kidney monolayer, even as early as the first hour of incubation of the lymphocytes in the culture chamber. The cells continue to move and can be seen still in active migration after four to five days of

cultivation, although the number, kind, and probably speed, of the cells in motion have changed by this time.

The locomotory behavior of lymphocytes *in vitro* has been described for man, rabbit, guinea pig and rat by a number of investigators (Lewis, 1921, 1931, 1933; De Bruyn, 1944, 1945, 1946; Rich, *et al.*, 1939; Robineaux, 1963) in both normal and pathological conditions. The present observations for ++ and WW^V mice agree very well with the classic descriptions. The locomotion of lymphocytes in tissue culture was described by Lewis (1931) as movement with a 'hand-mirror' shape. This shape is characterized by an active anterior pseudopodial region, a relatively inactive cell body containing the nucleus, and a posterior tail which usually appears to be passively dragged along. The locomotory phase, which is never continuous, was regarded by De Bruyn (1945) as being highly polarized, and is interrupted now and then by a non-locomotory phase which was described by Lewis as a 'period of rest' and by De Bruyn as a 'depolarized phase'. During the non-locomotory phase, the anterior pseudopodial region and the tail are withdrawn and the cell assumes a more or less spherical shape. However, the cells in their non-locomotory phase are not entirely inactive, but move around without showing any directional movement by pushing out temporary pseudopodia from all sides of the cell body. This is in contrast to the moving lymphocytes whose pseudopodia only arise from a limited area at the anterior part of the cell.

The locomotory behavior of mouse lymphocytes has never been studied in detail in tissue culture. The present studies show that mouse lymphocytes may exhibit, in addition to the so-called 'hand-mirror' shape (Figs. 1a,d,f; 2j; 3c, i,m), an oval (Figs. 6b,c; 7a,b,c), a somewhat rectangular (Figs. 1h,k; 2b; 3b,h), or sometimes even a very elongated shape (Figs. 4c,d,g; 5f-i) in their locomotory phase. These shapes may be seen, either in different lymphocytes, or even in the same individual at different times. Lymphocytes which show an elongated and a more or less cylindrical shape have been termed 'worm-like' in motion (Lewis, 1931; De Bruyn, 1945). This type of locomotion was regarded by Lewis as the natural movement of lymphocytes inside plasma-clot cultures. Figure 4 shows a moving lymphocyte with a 'hand-mirror' shape as it moves on a flat surface that is not delimited laterally by any obstacle. However, this hand-mirror form is later found to elongate and assumes a worm-like form as the cell squeezes its way through a limited space between the coverslip and the overlying cell. The degree of elongation varies from time to time depending on the space available; the nucleus is deformed by this 'gap' or 'tunnel' to give rise to one or more constrictions. The tail of the cell becomes less prominent owing to the elongation of the cell body as a whole. It is therefore quite evident that the locomotory behavior of lymphocytes in tissue culture depends very much on the type of substratum over which they move.

There are many factors which may account for the variations in shape seen in moving lymphocytes: (1) the heterogeneity of the lymphocyte population; (2) their speed; (3) the space in which they move that is available between the coverslip and the monolayer; (4) the age of the culture; and (5) the interaction between the moving cells themselves. However, the exact nature of these factors still cannot be determined at the present moment, although it seems that each has its effect on different occasions.

Tracings of the migratory pathways have revealed, in general, that lymphocytes move in a sinuous manner and frequently may repeat the same pattern, either in the same, or in the opposite direction. Though the direction of the movement of lymphocytes was regarded by De Bruyn (1945) as ramdon, the present observations seem to suggest a definite path for the moving lymphocytes. The difference between these two observations may be explained by the fact that the present observations were made of lymphocytes moving between the epithelioid cells and the coverslip so that they were constrained by a limited space. The lymphocytes usually move more or less rapidly for a few minutes in an approximately straight line, then come to rest, assume a rounded form, and then pass into a non-locomotory phase for a varying period of time. When the lymphocytes begin to move again, they do not always move off in the previous direction; they may move in any, including exactly the opposite direction. In the course of locomotion, a lymphocyte

may make a complete circle (Fig. 3), or come almost to rest and turn and twist about in a small space (Fig. 1); they may cross one another's paths without apparent deflection, or come into contact with one another without adhering. The bending and turning of a cell in locomotion is very common and is always associated with bending or 'constriction' of the nucleus.

Sometimes, a lymphocyte may be seen to show a rhythmic 'pulsation' of the cell body, giving the impression that the cell advances and retreats over a short distance that is a fraction of the length of the cell body. It may then seem as if the cell is in a non-locomotory phase, but the lymphocyte is actually advancing against an inpenetrable obstacle. During this period of constraint, the lymphocyte does not round up completely, but maintains its hand-mirror form with a somwhat compressed body and a prominent tail. After moving in this way for a short while, a lymphocyte usually stops moving in this direction and returns to its normal path. This suggests that normally a moving lymphocyte can only penetrate where a gap is available for it to squeeze through.

(b) Cell structures and their functional differentiation

The various components are very constant in their location in a moving lymphocyte. The nucleus is the most conspicuous of these, constituting almost the whole of the

body, and constantly changing in form during locomotion. This suggests that an important role is played by the nucleus during the period of active movement. The nucleus has been shown to play an active part in the locomotion of amoeba by nuclear transplantation between various strains (Jeon, 1968). The morphology of the cytoplasm of a moving lymphocyte is very consistent in its unequal distribution between the anterior pseudopodial region, the tail, and the region between the nucleus and the tail. A considerable amount of cytoplasm is located at the anterior end only during locomotion, and it is this part of the cell that is actually in close contact with the substratum on which it moves. The mitochondria within the cell are concentrated in the bulk of the cytoplasm which probably acts as the power house to supply energy required in active locomotion. The cytoplasm present in the tail is very scanty, but its rigidity is clearly seen as a cell changes its direction of motion. Though the tail can be of considerable length, it swings without showing any tendency to bend. Almost always the same part of the cytoplasm is observed to develop into the posterior tail as the mass of the cytoplasm is shifted during the process of directional change. However, the significance of this rigid or permanent tail is still not known, although McFarlane, et al. (1965, 1966) have suggested that this 'uropod' may function in immunological interchange of material between the lymphocyte and the

substratum, such as a fibroblast cell. Neither the present, nor the previous observations, (Lewis, 1931; De Bruyn, 1946) support this hypothesis because the tail of the moving lymphocyte is never observed to make close contact with the substratum, but appears to be simply a passive part of the moving cell.

(c) Transformation of lymphocytes

The above description of the locomotory behavior of lymphocytes in vitro applies equally well to small, medium, or large lymphocytes in the untransformed stage in either new or aged cultures (see Figs. 1, 2, 3 and 4). As the cultures become older, the locomotion of some lymphocytes appears to change (see Fig. 5), and this phenomenon was referred to by De Bruyn (1945) as hypertrophy. The present studies also show such transformation, but the time sequence and the final fate of the transformed cells do not appear to be the same as those reported by De Bruyn. Hypertrophy of lymphocytes appears to start at about 6-8 hours of incubation, by which time a few cells are seen to contain one or more vacuoles that are probably associated with this hypertrophy. most cells in this 6-8 hours period still show the typical motion of lymphocytes. Those that do not, differ only slightly in that the cytoplasm of the cell has become more mobile, and occasionally pseudopodia may be seen around the cell body and sometimes even on the tail. In the non-locomotory phase, the changes are even more conspicuous,

since the pseudopodia arising from the cell body are usually larger and more numerous, giving an impression of an 'undulating' movement. This transformation seems to involve a breakdown of the membrane surrounding the cells, which results in a more extensive spreading of the cytoplasm and an increase in the size of the cell. The pseudopodial activity of the hypertrophied lymphocytes is quite striking when contrasted to that of typical lymphocytes. The degree of this activity is directly related to the degree of hypertrophy of the cells. In the 6-8 hour culture, there are many cells which differ from the typical lymphocytes so slightly that it is impossible to draw a sharp line between them. At about 12 hours of incubation, there are more cells which exhibit the conspicuous pseudopodial activity in both phases, especially in the non-locomotory phase. They continually throw out large pseudopodia from all sides of the cell and the amount of cytoplasm is definitely greater than that of typical lymphocytes. The loss of firm consistency by the cytoplasm is even greater in certain hypertrophied cells whose cytoplasm becomes somewhat sticky and usually drags along, or sometimes even leaves cytoplasmic material behind on the substratum.

The moving lymphocytes, including both the typical and hypertrophied types, reach their maximal activity at about 12 hours of incubation, although the total number of cells actually moving between the monolayer and the coverslip

is less than 1% of the number of cells introduced (Table II). The number of cells in motion starts to decrease after about 24 hours of incubation. At this time, there are still a large number of typical lymphocytes and hypertrophied types still seen, but by now some wandering cells of highly hypertrophied type are encountered. At about 48 hours of incubation, almost all the cells are of the highly hypertrophied type, although some typical ones can be located. The movement of these highly hypertrophied wandering cells resembles very closely that of macrophages in that they continually throw out large pseudopodia from all sides of the body without showing any directional movement. However, it is doubtful whether this macrophage-like movement of the highly hypertrophied lymphocytes is actually that of transformed macrophages, as has been claimed by De Bruyn (1945). The morphology of the nucleus of certain transformed lymphocytes was found to be either a complete on incomplete ring, and this type of cell was found to increase in number as the cultures aged.

(d) Cellular associations

An association between normal lymphocytes and a variety of cells, among which are megakaryocytes, malignant cells, macrophages and cells in mitosis, has been recorded by several investigators (Humble, et al., 1956; Pulvertaft, 1959; Sharp and Burwell, 1960). The present observations do not reveal any sign of attraction of moving lymphocytes to cells

Table II. Cells count showing number of moving lymphocytes per culture chamber in cultures of different times.

Age of cultures in hours	Number of cells in movement	
	#1	#2
3	898	324
9	-	365
12	1177	-
24	650	456
48	-	138
Total cells introduced per chamber	6.1×10^5 cells/ml.	4.5×10^5 cells/ml.

Notes: The number of moving lymphocytes per culture chamber of 1 ml. capacity is always less than 1% of the total number of cells introduced.

undergoing mitosis, although many dividing cells were present in the cultures. In addition, lymphocytes themselves were never observed to undergo mitosis, or to associate with macrophages or other cell types. On the other hand, a close association between lymphocytes themselves was observed in certain cultures in which the lymphocytes were obtained from an animal that seemed to have been sensitized by a wound. In these cultures, lymphocytes appeared to attract one another and to move around each other in close contact. Such an association may consist of two or more cells (see Figs. 6 and 7). They show their normal motility during the association, but usually with an oval or even rounded outline. This may suggest a change in shape in relation to the phenomenon of immunity. The contiguous lymphocytes continue to move around one another for a considerable period of time, and even if they then move away from one another, they move back again in a short while. During most of this time, the contact between the lymphocytes appears to be of a surface-to-surface nature. Such prolonged contacts are not seen in cultures of lymphocytes obtained from an animal that has no sign of injury. Though random contact between normal or possibly transformed lymphocytes may be observed, such contacts never persisted for more than a few minutes. They sometimes appeared even to repel each other. The interaction or association between lymphocytes and macrophages has been termed 'peripolesis' by Sharp and Burwell (1960) and the movement of lymphocytes within a cell 'emperipolesis' by

Pulvertaft (1959). The present observation of the association of presumably 'sensitized' lymphocytes with each other is not an example of peripolesis since they do not interact with another type of cell. The same kind of association has been reported by Robinaux (1963) among immune lymphocytes from guinea-pig lymph nodes.

II. Quantitative analysis of the locomotion of lymphocytes

The locomotion of lymphocytes is intermittent, with alternating phases of locomotion and non-locomotion. Table III shows a number of measurements of the duration of these two phases in different lymphocytes. The locomotory phase is measured from the end of one non-locomotory phase to the beginning of the next; and the duration of the non-locomotory phase similarly is measured from the end of one locomotory phase to the beginning of the next. The data show that the durations of these phases vary a great deal from cell to cell, and even within the same individual observed during successive phases. For example, cell #3 was stationary for 10 seconds in one phase and moved for 1080 seconds in the next. However, cell #8 had 5 stationary phases of 58, 152, 42, 22 and 22 seconds successively and 4 locomotory phases of 378, 188, 254 and 256 seconds during a total time nearly equal to that of cell #3. Owing to the great variation among these measurements, an estimation of the average duration of these two phases is of doubtful value and an attempt to compare the durations of these two phases in ++

Table III. Measurements of times of locomotion and non-locomotion in different successive phases for selected lymphocytes.

Lymphocyte #	Non-locomotory phase		Locomotory phase	
	Time (seconds)	Average	Time (seconds)	Average
1	83	-	328	-
2	-	-	760	-
3	10	-	1080	-
4	16, 30	23	259, 292, 1494	682
5	58, 310, 174, 74, 14	126	84, 332, 388, 330, 62, 68	211
6	18, 280, 34	111	352, 906	649
7	18, 22	20	552, 294	423
8	58, 152, 42, 22, 22	59	378, 188, 254, 256	269

and WW^V lymphocytes was therefore abandoned.

An estimation of the locomotion of lymphocytes is more reliable, and the mean rate of locomotion has already been determined in various laboratories for the rat, rabbit and human (Lewis, 1933; De bruyn, 1945; McCutcheon, 1924; Schrek, 1963). Table IV presents the estimates of the mean speed of locomotion of 30 lymphocytes of different genotypes and sizes, in cultures of different ages, and with different combinations of lymphocyte and monolayer. The rate of locomotion was estimated from measurements of the distances traversed in a series of successive intervals of 20 seconds, that is for every 10 frames of the time-lapse film. The measurements were made from the posterior end of the cell, rather than from the anterior end, because the tail of the lymphocyte is more rigid and constant in form than the anterior transparent area. However, because the cell does not always move in a straight line, the estimate of the net distance covered during the 20 second interval is only a nearest approximation. An analysis of the locomotory behavior during this interval reveals that there is great variation in the instantaneous speed of the lymphocytes. The distance traversed in 20 seconds is different for different lymphocytes at approximately the same time in the same culture and even for the same individual in successive periods, and from moment to moment. The variation in the distances traversed may be attributed to the nature of lymphocyte movement; the non-linear movement of lymphocytes, the squeezing of

Table IV. The mean speed of locomotion of lymphocytes of various sizes in different combinations in cultures of different ages.

Type of combination	Age of culture (hour)	Sizes of lymphocytes	Number of observed intervals (20 seconds each)	Mean speed (μ/min.)
normal ++ lymphocytes on normal ++ monolayer (++/++)	5	small	39	9.1
	6	small	36	13.9
	24	small-medium	52	9.1
	25	small-medium	31	11.7
	26	small-medium	39	13.4
	48	medium-big	43	11.2
	49	small-medium	95	10.9
	8	medium-big	66	10.9
	7½	small-medium	39	7.4
Average mean speed for ++/++ combination: 10.8 μ/min.				
normal ++ lymphocytes on WW^V mutant monolayer (++/WW^V)	4	small-medium	40	9.5
	5	medium-big	75	13.1
	8	small	38	8.2
	4½	small	80	11.5
	8½	medium	64	9.7
Average mean speed for ++/WW^V combination: 10.4 μ/min.				
WW^V mutant lymphocytes on normal ++ monolayer (WW^V/++)	8½	small-medium	90	9.9
	9	big	36	7.1
	9½	small-medium	106	12.1
	8	small	63	10.5
	4½	small-medium	119	10.9
	5	small	37	11.2
	4½	medium	44	12.2
	5	medium	36	11.3
	9	medium	34	8.5
	9½	medium-big	45	9.7
Average mean speed for WW^V/++ combination: 10.3 μ/min.				
WW^V mutant lymphocytes on mutant WW^V monolayer (WW^V/WW^V)	4	medium-big	36	8.6
	4½	small-medium	67	7.8
	24	medium	59	8.4
	11	small	101	9.9
	6	medium-big	60	6.8
	5	small	74	10.5
Average mean speed for WW^V/WW^V combination: 8.7 μ/min.				

lymphocytes through gaps, and the change of speed just after movement starts, or before it ceases, all contribute to this variation. However, the mean speed calculated from many such measurements can reasonably be used as a measure for purposes of comparison. In this study, the mean speed was obtained from at least 30 measurements. Taken overall, the average speed of the 30 lymphocytes that were studied, taking no account of genotype is 10.1 $\pm$ 0.3 μ/min. The range is from 6.8 μ/min. to 13.9 μ/min (see Table IV). If a comparison is made of the rate of locomotion of lymphocytes in mice with others, it is quite evident that mouse lymphocytes move much more slowly than do those of man, rat or rabbit. The average rate of movement has been found to be 16.5 μ/min. for rat lymphocytes (Lewis, 1933), 33μ/min. for rabbit lymphocytes (De Bruyn, 1945), and from 6 to 20 μ/min., with an average of about 15 μ/min., for human lymphocytes (Lewis and Webster, 1921; McCutcheon, 1924; Henderson, 1928; Schrek, 1963). Since the culture systems used in the above determination are not identical, differences in rate of locomotion of lymphocytes from various species must be accepted with caution.

For the analysis and evaluation of the effect of the WW^V gene-pair on the locomotory behavior of lymphocytes *in vitro*, the data presented in Table IV are subdivided into 4 subsets, each with a different lymphocyte-monolayer combination. Table V shows the biometric data and Diagram B summarizes the mean speeds of lymphocytes from ++ and WW^V lymph nodes moving

Table V. Biometric constants of the rate of locomotion for the 4 types of lymphocyte-monolayer combinations.

Item	Kinds of combinations	++/++	++/WW^V	WW^V/++	WW^V/WW^V	Total
Range in μ/minute	Fastest rate	13.9	13.1	12.2	10.5	13.9
	Slowest rate	7.4	8.2	7.1	6.8	6.8
Mean rate of locomotion in μ/minute ± standard error		10.8±0.7	10.4±0.8	10.3±0.5	8.7±0.5	10.1±0.3
Standard deviation in μ/minute		2.1	1.9	1.6	1.4	1.9
Coefficient of variation (percent)		19.3	18.4	15.5	15.5	18.3

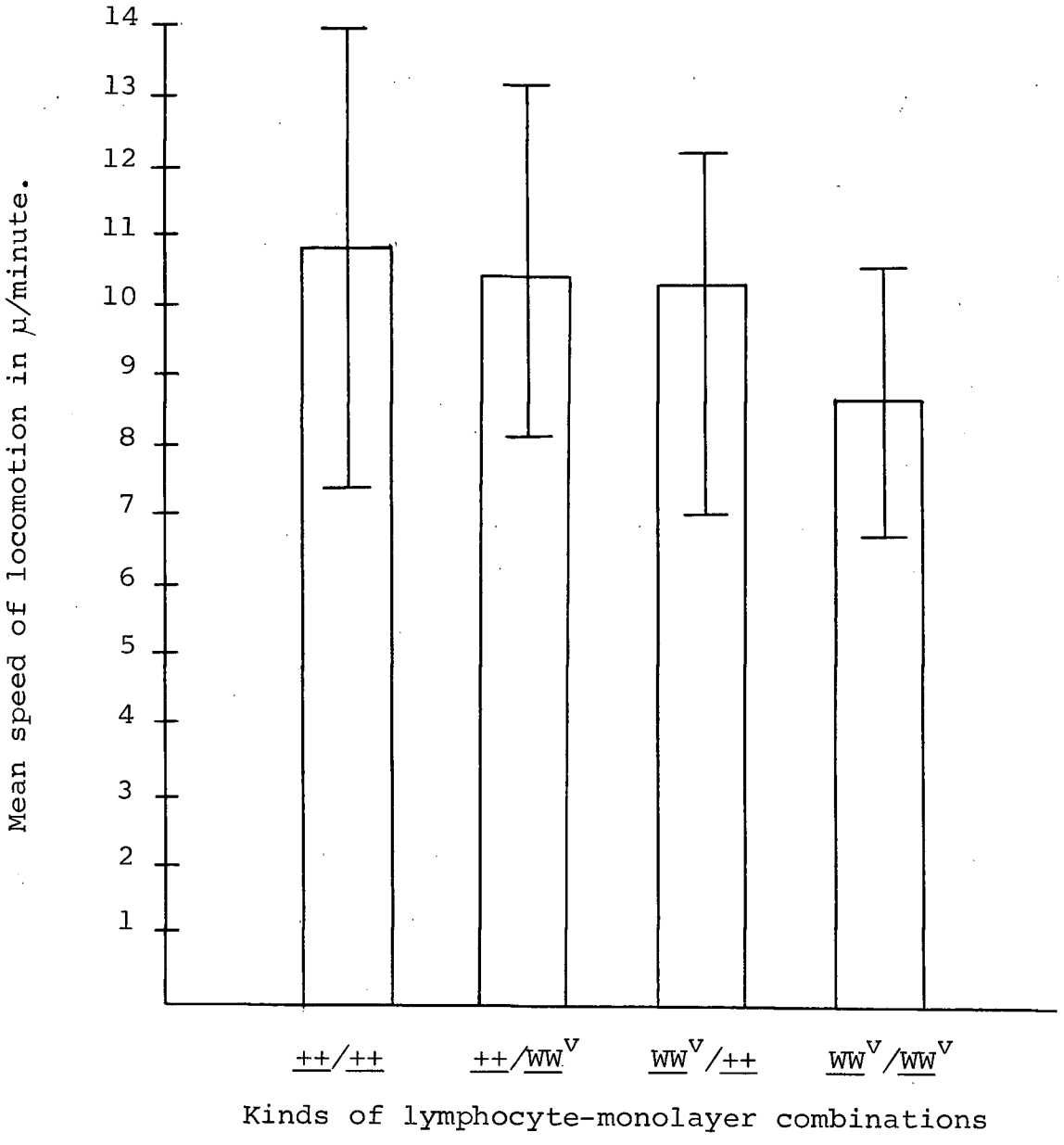

Diagram B. The mean speed, the maximum and the minimum speed of the locomotion of ++ and WW^V lymphocytes on the ++ and WW^V monolayers.

on ++ and WW^V renal monolayers. The mean speed in the ++/++ combination is found to be 10.9 $\pm$ 0.7 μ/min., with a variation from 7.4 μ/min. to 13.9 μ/min. The mean speed in the ++/WW^V combination varies from 8.2 μ/min. to 13.2 μ/min., with an average of 10.4 $\pm$ 0.8 μ/min. Similarly, the mean speed in the WW^V/++ combination is determined to be 10.3 $\pm$ 0.5 μ/min., with a minimum value of 7.1 μ/min. and a maximum of 12.2 μ/min. Finally, the mean speed in the WW^V/WW^V combination is 8.7 $\pm$ 0.5 μ/min. with a range from 6.8 μ/min. to 10.5 μ/min. These results suggest that both the mean rate of locomotion and the maximum rate of locomotion may be highest for the ++ lymphocytes moving on the ++ monolayer, and the lowest for the WW^V lymphocytes moving on the WW^V monolayer. This means that the motility of lymphocytes of WW^V mice is somewhat impaired when compared to the control groups. The difference in their mean speeds is statistically significant if a t-test is used ($P < 0.025$; see Appendix A), but the data are unfortunately too few to allow conclusions to be drawn from any of the other comparisons.

Since the term 'WW^V lymphocytes' has been used to cover all lymphocytes from the WW^V mice that have a similar morphology, a defect in the lymphocytes with respect to their rate of locomotion may therefore not apply to all. Morphologically similar lymphocytes have been shown to be heterogeneous with respect to their function and life cycle (Gowans and McGregor, 1965). 'WW^V lymphocyte' is used merely

for descriptive purposes and does not imply any qualitative or quantitative abnormality in all, or even in any, of the lymphocytes of the mutant mice. Indeed, heterogeneity among morphologically similar lymphocytes from the same source has been found to exist in the present study by statistical analysis of rates of locomotion. When an analysis of variance for the four combinations of lymphocyte and monolayer is made in a one-way classification of hierachical design with unequal sample size (for calculation, see Appendix B), a significant F-value was obtained for variance between the individual lymphocytes tested. This leads to the conclusion that lymphocytes from a single mouse are heterogeneous with respect to their rate of locomotion, and therefore are derived from different sub-populations. Recognition of the particular cell with the impaired motility is not possible.

Furthermore, a close examination of the data for the movement of lymphocytes on a coisogenic renal monolayer shows that the WW^V renal monolayer is somewhat less able than the isogenic ++ renal monolayer to support growth and activity of the ++ lymphocytes and, as might be expected from this, a ++ monolayer supports the activity of WW^V lymphocytes better than the isogenic WW^V monolayer (see Diagram B). Thus, the mean rate of locomotion of ++ lymphocytes on an isogenic monolayer was found to be 10.8 $\pm$ 0.7 μ/min., with a maximum of 13.9 μ/min., whereas the rate recorded for their movement on the coisogenic WW^V monolayer was only 10.4 $\pm$ 0.8 μ/min., with a

maximum value of 13.1 μ/min. This indicates that both the mean and the maximum speed are somewhat lower for the ++ cells moving on a WW^V mutant monolayer. Similarly, the WW^V lymphocytes move faster on the ++ monolayer, with a mean rate of 10.3 ± 0.5 μ/min., and a maximum of 12.2 μ/min. Although these values are somewhat smaller than those for ++ lymphocytes moving on the WW^V monolayer, they are very much greater than the rates recorded for the WW^V lymphocytes moving on the isogenic WW^V monolayer which have a mean rate of 8.7 ± 0.5 μ/min., and a maximum value of only 10.5 μ/min.

The best explanation of this variation is that the WW^V renal monolayer has a somewhat impaired ability to support the activity of either normal ++ or WW^V lymphocytes, though the sample sizes are too small to give statistically significant probabilities (see Appendix B).

DISCUSSION

There have previously been no descriptions of the locomotory behavior of mouse lymphocytes. It was therefore necessary first to find out to what extent their movement resembles, or differs from, those of other mammals. The motile lymphocytes in the present culture system are readily recognized when they move on a flat surface by their 'hand-mirror' shape. However, they may change to the so-called 'worm-like' shape when they squeeze through gaps between the cells composing the substratum. The 'worm-like' and 'hand-mirror' configurations of locomotion were described in other species by both Lewis (Lewis and Webster, 1921; Lewis, 1931, 1933) and Rich, et al., (1939) as being the normal shapes of lymphocytes moving in vitro, and in this respect the lymphocytes of the mouse resemble those of the other species that have been described.

In the study of migration from a lymph node fragment into a thick plasma clot, Lewis and Webster (1921) noted that each lymphocyte as it moved through a plasma clot gave the impression that it was being squeezed through a ring, with alternating phases of rest and movement, though even in the resting phase numerous small pseudopodia were continually thrown out and retracted on all sides of the cell. Locomotion began with the extrusion of a single, large, sausage-shaped, pseudopodium from what now became the anterior end of the

moving cell. A groove, which was termed 'constriction ring', then developed at the base of the pseudopodium where it joined the cell body. The 'constriction ring' remained fixed in space and the rest of the cell now flowed through it into the pseudopodium. In the process, the nucleus became very deformed and might even be twisted during its passage through the 'ring'; this was the so-called 'worm-like' configuration. Once the nucleus had passed through the 'ring' it regained its 'hand-mirror' configuration, with the round nucleus in front and a tail of cytoplasm behind. The tail was then slowly taken up and the cell returned to its rounded resting configuration, and the whole 'ring' cycle was completed. The hand-mirror configuration was described by them as only one phase in the cycle of locomotion. Rich, et al. (1939) also studied the the movement of lymph node lymphocytes in plasma clot cultures, but claimed that all the lymphocytes, regardless of their sizes, always moved with the hand-mirror configuration. The round anterior end of the cell displayed a fringe of small pseudopodia, while most of the cytoplasm formed a tail trailing at the posterior end. The whole cell glided forwards steadily without the formation of any 'constriction rings' as described by Lewis. This apparent discrepancy between the two accounts was later resolved by De Bruyn (1944), who also studied lymph node cells in a plasma clot. He found that the lymphocytes moving within the clot assumed a

'worm-like' configuration, as claimed by Lewis, but that all those moving between the coverglass and the plasma clot exhibited the slow gliding and constant hand-mirror form described by Rich *et al*. De Bruyn thought the 'constriction rings' to be external, and in fact to be holes in the fibrin network. The present observations agree very well with De Bruyn's, and further speak against Lewis's conclusion that 'since lymphocytes have been so accustomed, for thousands of generations, to squeezing through tight places they go on acting in this way when there are no small holes to go through' since many lymphocytes have been seen in the present study to stop and change their direction of locomotion if there is no space available between coverslip and the monolayer, although they may push their way through for a short distance. Harris (1953), in his study of the movements of thoracic duct lymphocytes in a dilute plasma clot, claimed that the type of movement in his culture system was very variable: one and same lymphocyte might move, at different times, with the hand-mirror configuration, or worm-like configuration, or in a non-polarized amoeboid fashion. This description agrees well with what has been observed in the present study. Since Harris's culture system was somewhat intermediate between those of Lewis and Rich, et al., it is not surprising that he found such variation.

Almost all the early studies concerning the locomotion of lymphocytes *in vitro* were made using frames of a cinemicro-

graphic film of individual cells migrating through a plasma clot. More recently, the cells were trapped between two coverslips, or between coverslip and agar, and it is doubtful whether their movements in these experimental systems are altogether normal. Previous findings showed that the optical conditions for the study of the movement of lymphocytes in plasma clots were very poor and the situation could be improved if cells were allowed to move on a plane surface such as a glass coverslip. Unfortunately, lymphocytes will not adhere to a glass surface and crawl on it (Fichtelius, 1951; Harris, 1953), but they are capable of moving between two closely apposed surfaces. The culture system used in the work reported here retains the indispensible conditions, provided by a plasma clot, namely the contribution to the survival of the lymphocytes by a feeder layer of other cells, while at the same time dispensing with its poor optical properties.

Both Lewis and De Bruyn have claimed that the movement of lymphocytes is a purposeless wandering. However, since lymphocytes have the ability to move towards certain cell types *in vitro* (Humble, *et al.*, 1956; Pulvertaft, 1959) and to regions of inflammation (Conheim,1867; Trowell, 1965) *in vivo*, and to aggregate in regions of homograft rejection (Wilson, 1965; Mitchison, 1954; Billingham, *et al.*, 1954; Gowans, 1965), it is doubtful whether the motility of these cells, either *in vitro* or *in vivo*, can be a purposeless wandering. Nevertheless, no one specific stimulus has been

reported in the literature (McCutcheon, 1946, 1955; Coman, 1940; Harris, 1953; Kass and De Bruyn, 1967). Although there is still no evidence of chemotaxis by lymphocytes, the close association of the lymphocytes with other cell types in the phenomena of 'peripolesis' (Sharp and Burwell, 1960) and 'emperipolesis' (Pulvertaft, 1959) has been fully established, though their exact nature is still obscure. The phenomenon of 'peripolesis' was noted earlier by Pulvertaft and Jayne (1953) in the association between small lymphocytes or megakaryocytes and certain tumor cells. The latter association was also described by Humble, et al. (1956) who concluded that lymphocytes may actually facilitate the spread of cancer. However, the experiment of Sharp and Burwell (1960) suggested that lymphocytes are attracted to certain cells, such as macrophages or reticulum cells, in the course of immune reactions. The process of 'emperipolesis' was first noted by Humble, et al. (1956) when lymphocytes penetrate certain megakaryocytes or tumor cells and move freely round within the host cytoplasm. Later, Pulvertaft (1959) reported the same phenomenon by leukemic lymphocytes of man and mouse within chick fibroblasts. Emperipolesis has been observed in vitro within macrophages (Lewis, 1925), thymus epithelial cells (Trowell, 1949; Klein, 1958), fibroblasts (Fischer and Dolschansky, 1929; Trowell, 1949; Bichel, 1939), and sarcoma cells (Koller and Waymouth, 1953), and by leukemic lymphocytes within histocytes (Shelton and Rice, 1958).

Though emperipolesis was not seen, the present observations reveal a third type of association between the presumably sensitized lymphocytes themselves. A similar type of association has been shown to occur between small and medium lymphocytes by Robineaux (1963) in his study of movements of cells involved in inflammation and immunity. These results seem to suggest an alteration of the cell membrance of lymphocytes associated with disease, inflammation or immune reactions in the animals concerned.

Hypertrophy of lymphocytes, or possibly the transformation of lymphocytes into macrophages, was observed in the present culture system. These investigations show that forms of locomotion exist that are intermediate between those of typical lymphocytes and macrophages. As the degree of hypertrophy increased with time, the mode of locomotion of the cells gradually came to resemble more closely that of macrophages. The same sequence of hypertrophy has also been reported by De Bruyn (1945) in his studies of the migration of lymphocytes in plasma-clot cultures. Trowell (1965) has reviewed reports presented in the literature concerning the ability of lymphocytes to transform into macrophages. He listed 53 publications, of which 23 claimed that the macrophages developed exclusively from lymphocytes.

The ability of the monolayer to maintain and prolong the survival and activity of many types of cells, including the lymphocytes, has led many investigators to suggest that

the monolayer may supply necessary metabolites for the coexisting cells, or that it may remove toxic metabolic products, or that it may act mechanically (Ginsburg, 1965; Bichel, 1939, 1952; De Bruyn, 1949; Kieler and Kieler, 1954; Puck, et al., 1956; Brooke and Osgood, 1959; Woodliff, 1964). Therefore the decreased ability of the WW^V monolayer to support normal activity, as judged by the reduced mean rate of locomotion on the WW^V monolayer, may be due either to lack of certain metabolites necessary for the maintenance of a balanced condition in vitro, or an alteration in the surfaces of the cells in the monolayer so that the locomotion is impeded mechanically. Unfortunately, the difference between the ability of normal and WW^V monolayer to support the activity of lymphocytes in vitro may not be statistically significant, owing to the small sample sizes.

In this investigation, it was found that lymphocytes obtained from the lymph nodes of WW^V mutant mice do not differ in vitro in any way from those of ++ mice in the manner in which they move. Their movements are intermittent with alternating locomotory and non-locomotory phases, and they move in a sinuous manner, without abrupt changes in direction. However, a quantitative analysis of the reates of locomotion suggests that the cells are heterogeneous in this respect, though their modes of locomotion are similar. Cells obtained from lymph nodes are a heterogeneous population with respect to their functional potential (Gowans, 1965; Everett, et al., 1964; Oort, et al., 1965; Good and Finstard,

1967; Lance and Taub, 1969). This probably explains why the cells that are randomly selected for experiments show such a great variation. However, assuming that the measured rates of locomotion are in each case of randomly selected samples from the lymphoid populations of ++ and WW^V mice, a comparison of the mean speed using a t-test is possible. The result of the comparison suggests, though it is not conclusive, that a difference exists in the rate of locomotion between lymph-node populations obtained from normal and mutant mice, when the lymphocytes are moving through an isogenic monolayer. These are just the combinations that most closely resemble the natural situation in the mice. However, the results may also be interpreted to indicate that the motility of the lymphocytes is also influenced by the genotype of the monolayer through which they are moving. More measurements are needed before any assertions can be made with confidence.

It therefore seems possible that the mutation at the W locus in the house mouse, in addition to affecting the erythroid and myeloid tissue of the blood-forming tissue, also affects the cells in the lymphoid tissues. Although the exact nature of the defect in the hematopoietic tissue in WW^V mutant mice is still unknown, it has, however, been localized to an abnormality in the hematopoietic colony-forming stem cells (CFU), that may be the precursor cells of the erythroid, myeloid or even lymphoid tissues (Wu, et al., 1968). Whether a qualitative defect in the CFU cell, or a

decrease in total pool size, is primarily responsible, remains to be discovered.

The defect in erythropoiesis giving rise to the anemia has been shown by spleen colony studies (McCulloch, et al., 1964; Lewis, et al., 1967) to reside in an erythroid stem cell. Transplantation of marrow cells from $W^{V}W^{V}$ mice into normal ++ irradiated hosts produces colonies that contain almost no erythroid cells and that are fewer in number and smaller in size than those which occur after transplantation of normal tissue. ++ marrow transplanted into either irradiated or non-irradiated $W^{V}W^{V}$ mice results in apparently normal macroscopic colonies and the anemia and radiosensitivity of the $W^{V}W^{V}$ mice are eliminated (McCulloch, et al., 1964; Lewis, 1967; Russell, et al., 1956; Bernstein and Russell, 1959; Russell and Bernstein, 1968). These findings suggest a qualitative defect in the colony-forming cell, or the erythroid precursor cell, that results in an impairment of their differentiation into erythroid cells, and perhaps at the same time in a decrease in pool size of this precursor cell. Increased cell production resulting in improvement in the anemia, following stimulation of the CFU or the erythroid precursor cells by exposure to hypoxia (Keighley, et al., 1966; Fried, et al., 1967), or after administration of androgens (Fried, et al., 1967), together with the capability to respond to high doses or erythropoietin (Keighley, et al., 1966) in the $W^{V}W^{V}$ mice, suggest that the

CFU or the erythroid precursor cell of the mutant responds to normal stimulation, but that the sensitivity is lowered. Studies by Lewis, et al. (1967) reveal that spleen colonies of all cell types arising from transplanted WW^V marrow are smaller and fewer in number than occurs with transplanted normal cells. This suggests a small CFU pool, but on the other hand, the marrow and spleen may not reflect the composition of the total body stem-cell pool (Boggs, et al., 1968).

Mouse hematopoietic colony-forming stem cells have been shown capable of differentiating along both erythropoietic and granulocytic lines (Wu, et al., 1967). Studies of the incorporation of ^{59}Fe and $^{131}IUDR$ into the spleen colony have suggested that the production of both non-erythroid and erythroid cells is defective (Bennett and Cudkowicz, 1966). Adult mice of the WW^V genotype, in addition to having a defect in erythropoiesis, also have a defect in their myeloid and megakaryocytic tissue (Chervenick and Boggs, 1969). This defect is manifested by a decreased total nucleated-cell count, as determined in the humerus, as well as by a decreased number of neutrophils and megakaryocytes in the marrow. However, other studies have shown a normal number of leukocytes and platelets in the blood of WW^V mice (Lewis, et al., 1967), though there has been a report of a slight decrease in both elements (Gruneberg, 1939). The defect in the myeloid tissue may be due to delayed and reduced proliferation and differentiation, as in the erythropoietic

tissue, but it is less severe (Bennett, et al., 1968). Evidence has been presented (Till, et al., 1967; Fowler, et al., 1967; O'Grady, et al., 1968) for the existence of stem-cell pool more mature than the CFU. There is indirect evidence to suggest that a more differentiated neutrophil stem-cell pool may also exist. Studies by Bennett, et al., (1968) of colony formation in vitro indicate that the marrow of WW^V mice produces colonies similar in size and number to those observed when marrow from normal mice is used. Colonies in this in vitro system are composed primarily of neutrophils and macrophages. These observations, along with the decrease in total neutrophil mass reported by Chervenick and Boggs (1969), suggest that neutrophils are normally produced beyond the CFU cell stage and that either proliferation or differentiation of the CFU cell is abnormal.

Furthermore, the CFU cell present in the normal bone marrow have been reported to include cells capable of re-populating not only the myeloid tissues by also the lymphoid tissues of irradiated mice (Ford, et al., 1966; Mickle, et al., 1966). While it seems probable that the CFU cells of marrow might be responsible for repopulating both tissues (Trentin and Fahlberg, 1963; Till, et al., 1967; Wu, et al., 1968), present cytological evidence shows that single clones, whose members are identified by the presence of unique chromosomal markers, may contain not only thymus and lymph node cells, but also hematopoietic colony-forming cells. These findings mean, either that lymphoid cells are descended from CFU cells,

or that both classes have a common progenitor. Thus the CFU, erythroblasts, granulocytes, thymic cells, and the cells of the lymph nodes may all belong to the same clone. In addition, Shearer and Cudkowicz (1967) have reported that the W mutations also affect the immune system, since the number of anti-sheep hemolysin-forming cells generated by spleen of mutant mice was only one-third to one-half the number of anti-sheep hemolysin-forming cells generated by spleens of normal littermates. This evidence, together with that mentioned above, strongly indicates that antibody-producing cells and CFU cells may belong to the same clone. Thus the primary target of the genetic defects induced by the W mutations may be either a pluripotent stem cell, or a more differentiated precursor cell pool.

If the conclusion that has been made from the present study, that there is a defect in the locomotory behavior of the small lymphocytes of the WW^V mouse, is confirmed, there will be one more piece of evidence, along with all those described above, to show that the W mutations of the mouse have an even more far-reaching effect than was at first supposed. Whether this evidence will throw light on the possible origin of the bloodcell lines from a single stem cell remains to be seen.

SUMMARY AND CONCLUSION

Time-lapse cinephotomicrography was used to study the effect *in vitro* of the W mutations in the house mouse on the locomotory behavior of their lymphocytes on isogenic and coisogenic monolayers of kidney cells. No morphologically detectable difference in the modes of movement was observed between WW^V and ++ lymphocytes. They both showed polarized movement with occasional change in direction. They move in a sinuous manner, their shape changing alternately from the round resting form to the hand-mirror or worm-like form of their locomotory phase. Many intermediate forms were also observed. Association between presumably sensitized lymphocytes, transformation of lymphocytes into macrophage-like cells, and functional differences between various parts of the moving lymphocytes were also noted.

The movements of selected lymphocytes were traced from cinemicrographic films, and a measurement of the rate of locomotion showed that mouse lymphocytes move at approximately 10 μ/minute. A comparison of the mean rates of locomotion through isogenic monolayers between the WW^V and ++ lymphocytes suggests that the motility of the WW^V lymphocytes is somewhat reduced. However, heterogeneity among morphologically similar lymphocytes of the same genotype with respect to their rate of locomotion makes it difficult

to draw satisfactory conclusions. A decrease in the ability of a WW^v kidney monolayer to support the activity of either ++ or WW^v lymphocytes was also found. It is tempting to conclude that the lymphocytes, which share with three other known defective tissues the defects in proliferation and migration, caused by the W mutations are similarly defective, as shown by their impaired motility *in vitro*.

Appendix A. Computation of t: Test of the hypothesis that the difference between the mean speeds of ++ and $\underline{WW}^V$ lymphocytes on their isologous monolayer is zero.

Item	Speed (u/min.)		
	++/++	$\underline{WW}^V/\underline{WW}^V$	
Observations (y)	9.1 13.9 9.1 11.7 13.4 11.2 10.9 10.9 7.4	8.6 7.8 8.4 9.9 6.8 10.5	
Sums of y	97.6	52.0	
Sample size(n)	9	6	
Mean of y ($\bar{y}$)	10.8	8.7	$\bar{y}_1 - \bar{y}_2 = 2.1$
(Sums of y)^2	9525.8	2704.0	
(Sums of y)^2/n	1058.4	450.7	
Sums of y^2	1092.5	459.8	
SS	34.1	9.1	Pooled sums of squares= 43.2
DF	8	5	Pooled degrees of freedom= 13
Variance of sample (s^2)			Pooled estimate of population variance (S_p^2)= 3.3231
1/n			$1/n_1 + 1/n_2 = 0.2778$
			$S_p^2 (1/n_1+1/n_2) = 0.9232$
			Square root of $S_p^2 (1/n_1+1/n_2)$= 0.9608
			$t_{cal.} = 2.1857$
			$t_{tab.\ p=0.025,\ df=13} = 2.160$

The probability that the hypothesis is valid is therefore less than 0.025.

Appendix B. Analysis of variance: Nested (hierachical) design in one-way classification with unequal sample sizes.

Kind of lymphocyte-monolayer combination	Lymphocyte #	Total distance traversed per lymphocyte (T)	Number of observed intervals (n)	T^2/n
++ lymphocyte on ++ monolayer	1	34.60	43	27.84
	2	35.95	36	35.90
	3	25.45	39	16.61
	4	33.85	52	22.04
	5	37.50	39	36.06
	6	74.50	95	58.42
	7	25.95	31	21.72
	8	51.45	66	44.92
	9	20.65	39	10.93
		T_m: 339.90	n_m: 440	T_m^2/n_m:262.57
++ lymphocyte on WW^v monolayer	1	27.25	40	18.56
	2	70.70	75	66.65
	3	22.20	38	12.97
	4	65.85	80	54.20
	5	44.50	64	30.94
		T_m: 230.50	n_m: 297	T_m^2/n_m:178.90
WW^v lymphocyte on ++ monolayer	1	38.45	44	33.60
	2	29.15	36	23.60
	3	20.65	34	12.54
	4	31.20	44	22.12
	5	47.50	63	35.81
	6	63.80	90	45.23
	7	18.35	36	9.35
	8	92.30	106	80.37
	9	92.95	119	72.60
	10	29.80	37	24.00
		T_m: 464.15	n_m: 609	T_m^2/n_m:353.75
WW^v lymphocyte on WW^v monolayer	1	71.75	101	50.97
	2	36.10	58	22.47
	3	35.50	59	21.36
	4	22.25	36	13.75
	5	55.80	74	42.08
	6	29.10	60	14.11
		T_m: 250.50	n_m: 388	T_m^2/n_m:161.73

PRELIMINARY CALCULATIONS

Type of total	Total of squares	Number of items squared	Number of observations per squared item	Total of squares per observation
Grand	$(1285.05)^2$	1	1734	952.34
Combinations	-	-	-	956.95
Individual cells	-	-	-	981.72
Observations	1190.35	1734	1	1190.35

Grand total of square: $(339.90 + 230.50 + 464.15 + 250.50)^2$
$= (1285.05)^2$

Grand total of square per observation: $(1285.05)^2/1734 = 952.34$

Combinations total of square per observation: $(262.57 + 178.90 + 353.75 + 161.73) = 956.95$

Individual cells total of square per observation is obtained by adding all the values in the column T^2/n of the four combinations.

Observations total of square per observation is determined by adding all the squared values of the original measurements, that is the distance traversed per 20 seconds interval, and then divided by one.

ANALYSIS OF VARIANCE

Source	SS	DF	MS
Combinations	(956.95-952.34)=4.16	3	1.5367
Individual cells within combinations	(981.72-956.95)=24.77	26	0.9527
Error within individual cells	(1190.35-981.72)=208.63	1704	0.1224
Total	(1190.35-952.34)=238.01	1733	

In a hierachical classification the appropriate F-ratio is the variance estimate (MS) of successive tiers. Thus,

$$F\left(\frac{\text{Combinations}}{\text{Individual cells within combinations}}\right) = \frac{1.5367}{0.9527} = 1.6130$$

(DF= 3;4)

Not significant

$$F\left(\frac{\text{Individual cells within combinations}}{\text{Error within individual cells}}\right) = \frac{0.9527}{0.1224} = 7.7835$$

(DF= 26;1704)

Significant, $P<0.05$

The conclusion of the experiment is therefore that individual cells differ significantly but that combinations do not, a result that suggests heterogeneity in the rate of locomotion of individual lymphocytes. Further investigation is necessary to ascertain the causes of the variation among the individual cells.

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Explanation of Figures

Tissue culture of lymph nodes from either $\underline{++}$ or $\underline{WW^V}$ individuals. The line drawings are tracing of the cell outlines obtained by projecting the time-lapse cinemicrographic film on paper. The interval between each tracing is 20 seconds. The total magnifications of the traced pathways and the photographs are 2150x and 2650x respectively. The letters on each photograph correspond to those alongside the tracing of the path of the lymphocyte.

Figure 1

Photographs of a small lymphocyte and trace of its path in a culture in which $\underline{WW^V}$ lymphocytes are combined with a $\underline{WW^V}$ monolayer. Two depolarized phases (dp) and probably two regions of 'constriction' (cn) are located in this tracing.

Figures 1a, b, d, f, i, and j show the movement with a 'hand-mirror' shape.

Figures 1b and c show regions of 'constriction' with deformation of the nucleus on one side of the moving cell.

Figures 1e and g show the round shape of the cell in its non-locomotory phase. Pseudopodial activity can be seen on all sides of the cell in Figure 1e.

Figures 1h and k show a somewhat rectangular shape of the lymphocyte at a stage when it has just begun to move.

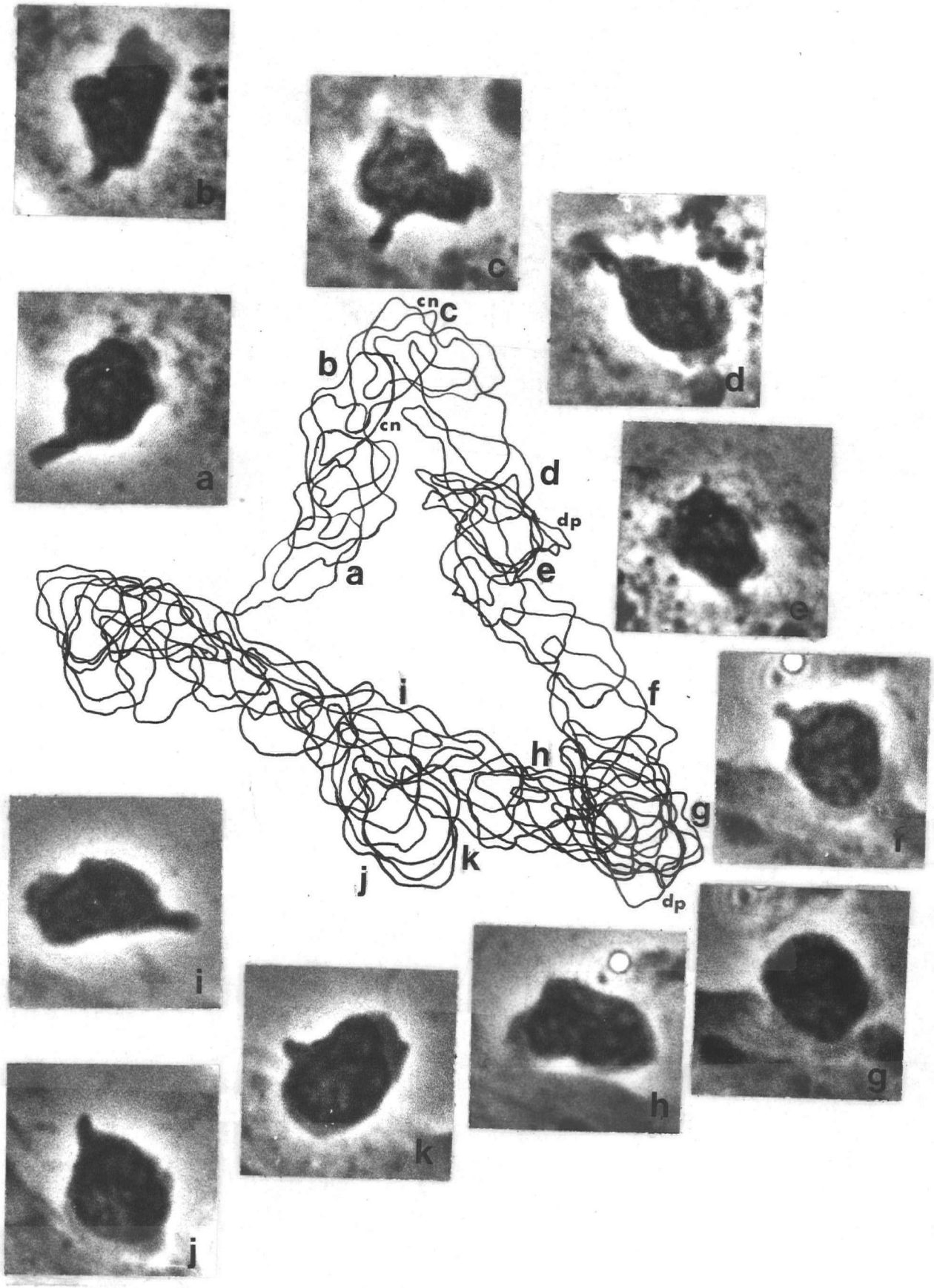
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Figure 2

$\underline{WW}^{V}/\underline{WW}^{V}$ combination as in Figure 1. 'Constriction' of the nucleus are frequently observed. There is probably one depolarized phase (dp).

Figures 2e-i show a series of 'constrictions' as the cell moves through obstacles.

Figures 2a, d, and j show the 'hand-mirror' form of locomotion.

Figure 2c shows a somewhat compressed form of 'hand-mirror' shape.

Figure 2b shows a 'rectangular' form which is assumed after a depolarized phase.

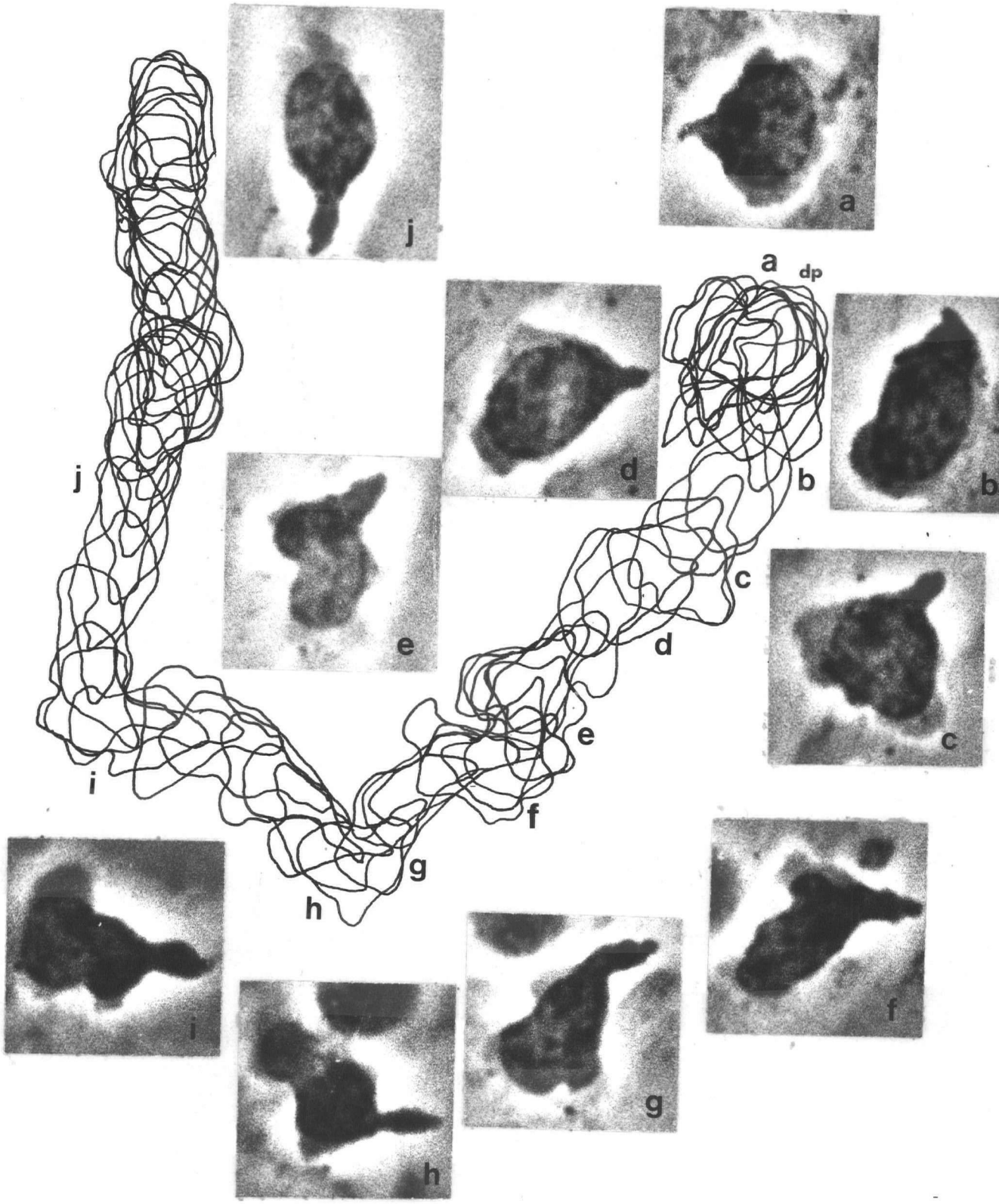
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Figure 3

$\underline{WW}^{v}/\underline{WW}^{v}$ combination as in Figure 1. The lymphocyte makes a complete circle which it repeats later (not shown in the tracing). Only one depolarized phase (dp) is seen in the entire period of locomotion.

Figure 3a shows the lymphocyte in the rounded configuration of the non-locomotory phase.

Figure 3b shows a somewhat rectangular form.

Figures 3c-i show a series of changes from the 'hand-mirror' form to one which is constricted as the lymphocyte moves through a limited space.

Figures 3j-m show inclination of the lymphocyte from side to side as it moves in a circular path.

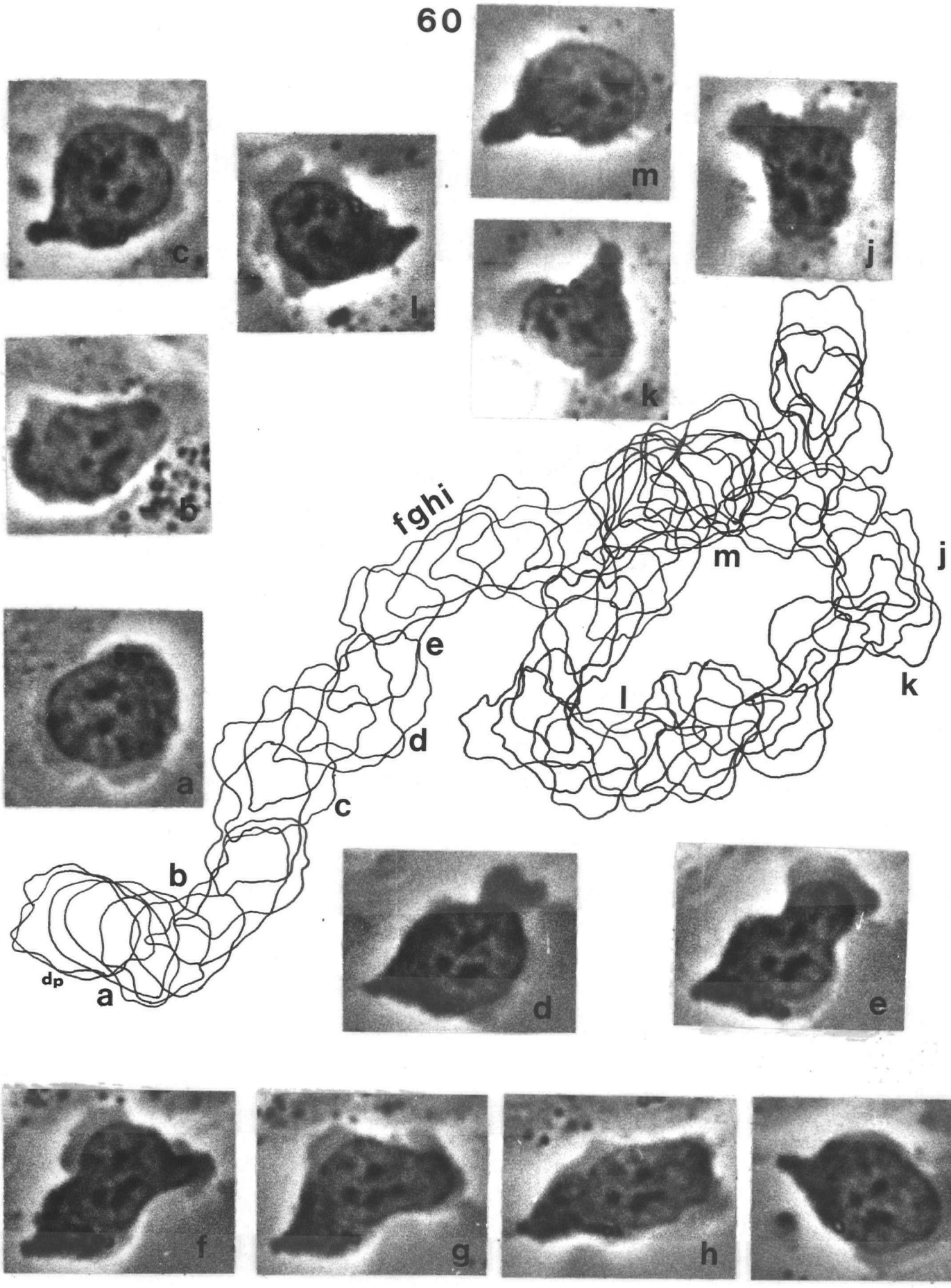
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Figure 4

Large lymphocyte in a $\underline{WW}^{V}/\underline{WW}^{V}$ combination as in Figure 1. The lymphocyte changes from the 'hand-mirror' form to the 'worm-like' form as it moves through a 'tunnel-like' space, and returns to the 'hand-mirror' form afterwards. Figures 4b-i show two series of such changes.

Figure 4a shows the lymphocyte as it starts to move after a period of rest.

Figures 4b and f show a lymphocyte with a bean-shaped nucleus as it begins to squeeze through the 'tunnel-like' space.

Figures 4c and g show the lymphocyte with most of its nucleus pushed into this limited space.

Figure 4d shows a 'worm-like' form with a somewhat twisted and cylindrical nucleus.

Figure 4e shows that the lymphocyte has returned to its 'hand-mirror' form.

Figure 4h indicates that the lymphocyte has started to incline to one side after passing through the limited space.

Figure 4i shows the movement of the lymphocyte within a limited space. Note the somewhat elongated shape and deformation of the nucleus.

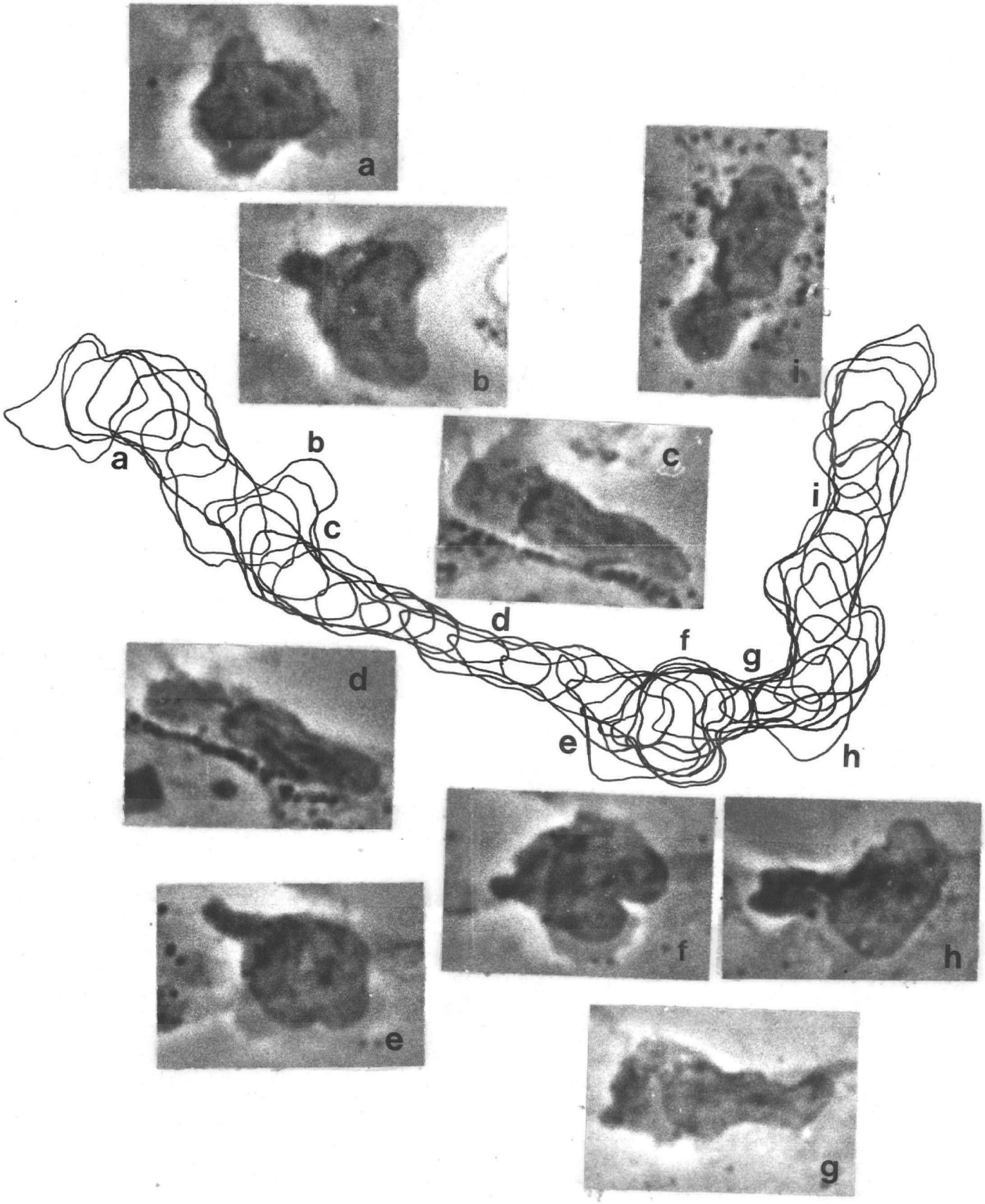
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Figure 5

A 'hypertrophied' ++ lymphocyte in a ++ monolayer. The lymphocyte becomes 'worm-like' as it squeezes through a limited space. The vacuoles and the large size of the lymphocyte are very prominent features of the hypertrophied condition.

Figure 5a shows an oval configuration for the lymphocyte as it just starts to move.

Figures 5b-e show the movement of the lymphocyte through a very small space. The deformation of the nucleus is very prominent; the resumption of the 'hand-mirror' form is seen in Figure 5e.

Figures 5f-j show the movement of the lymphocyte through a slightly limited space. The elongation of the cell is evident, as are the constrictions and twisting of the cell body owing to the obstacles alongside the path. Figures 5f and g show the 'worm-like' configuration with a twisted and cylindrical body. Figures 5h and i show a elongated form of 'hand-mirror' shape and Figure 5j shows the usual 'hand-mirror' form.

Figure 5k shows the round configuration of the resting lymphocyte.

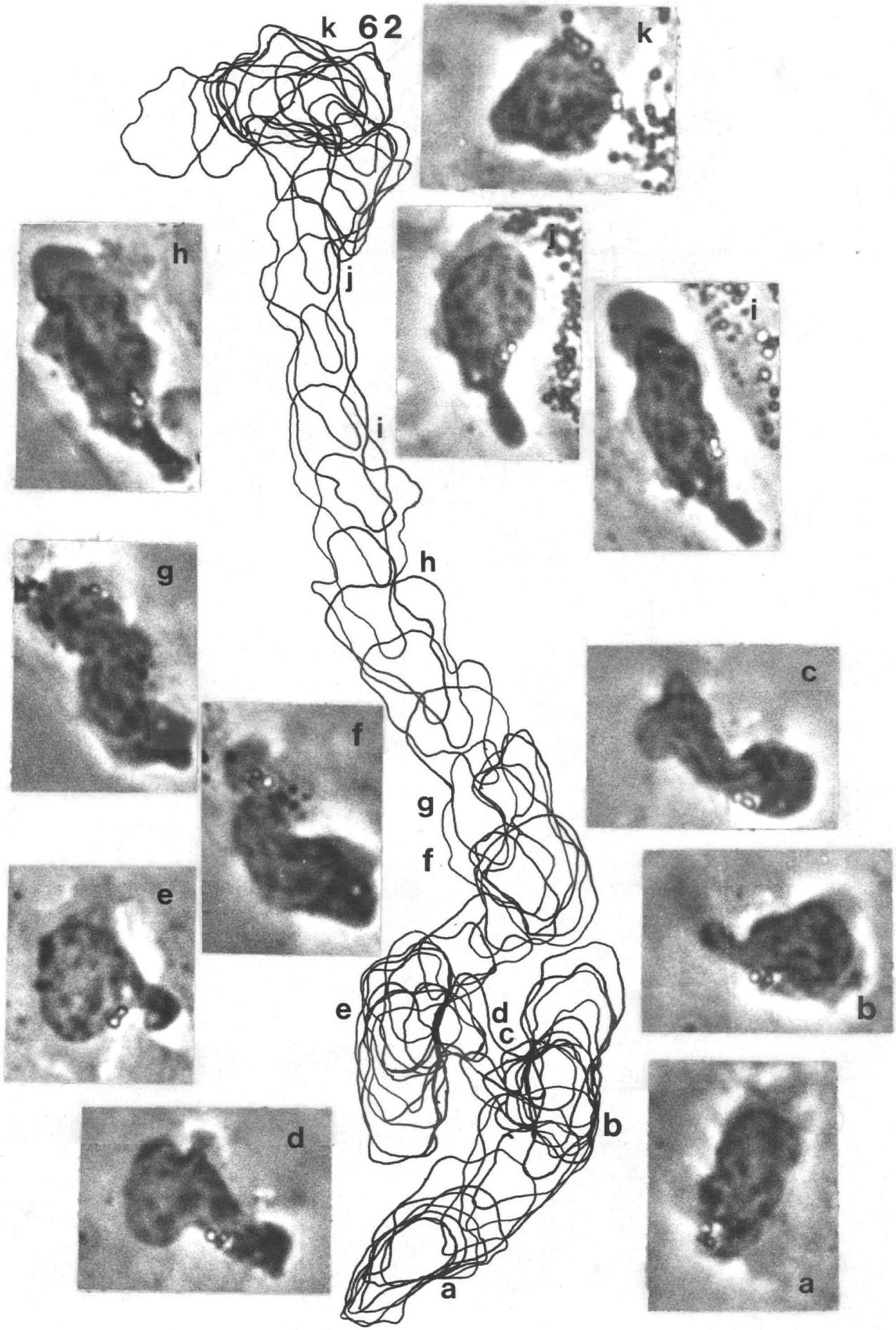
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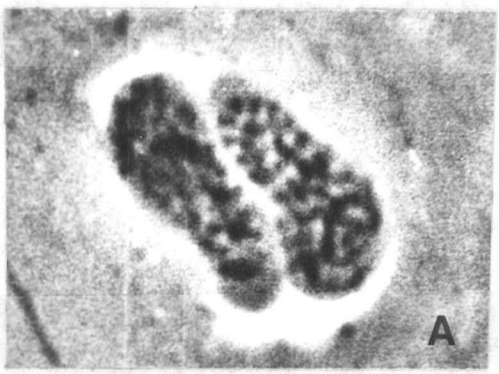
Figure 6

Photographs showing close association of two $\underline{WW}^{V}$ lymphocytes, or possibly plasmacytes, on a $\underline{WW}^{V}$ monolayer. The nuclei of these two cells show a striking 'spoke-like' appearance. The cells usually have an oval form when either in close contact (Figure 6b) or separated (Figure 6c). They may twist against each other to form a somewhat elongated shape (Figure 6a). Figure 6d shows the two cells making contact in their anterior lateral regions.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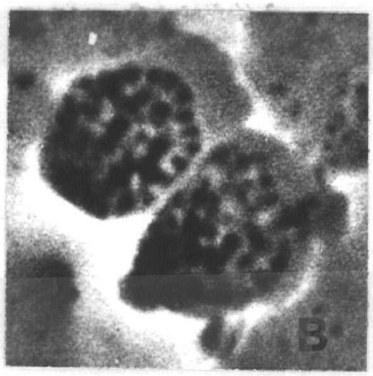
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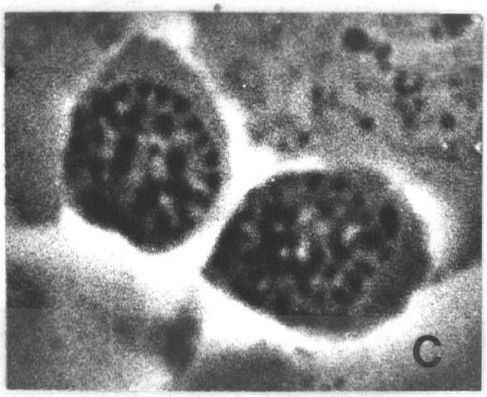

C

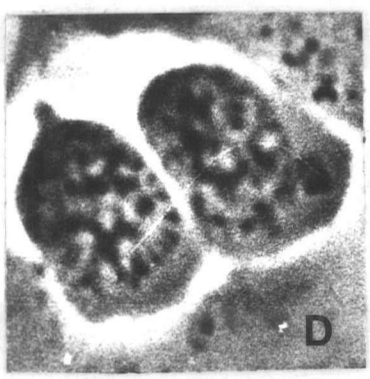
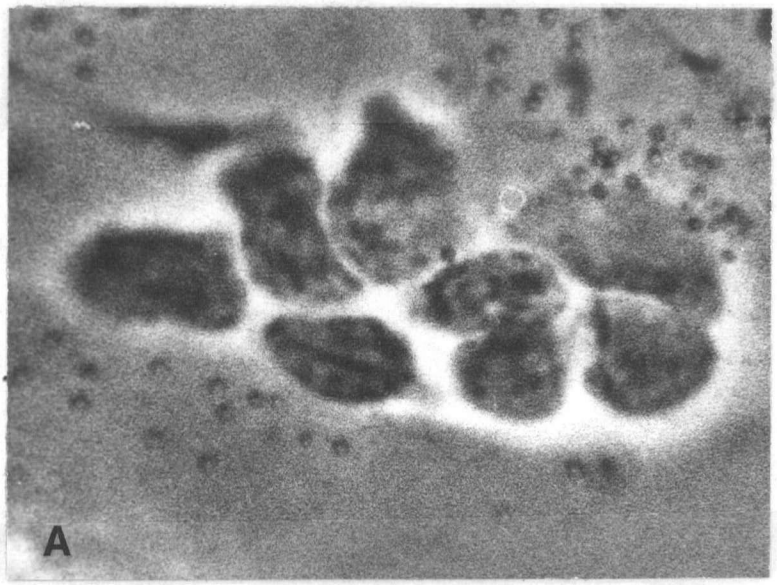
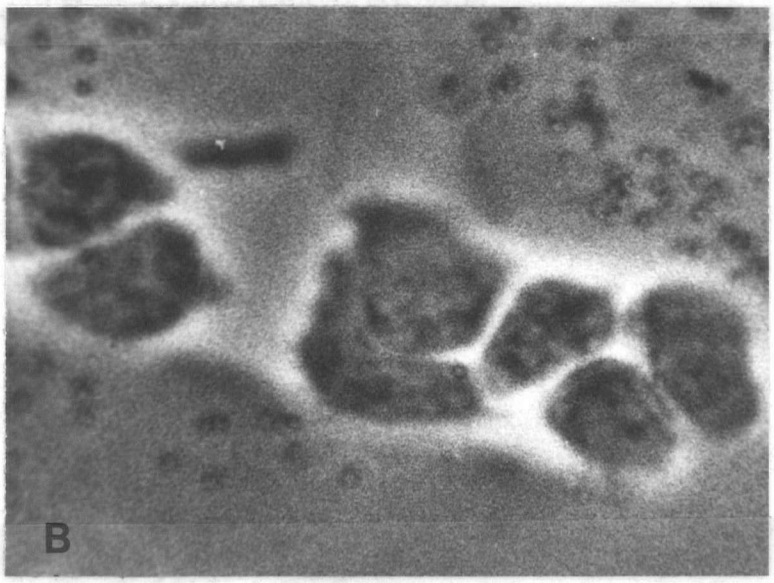
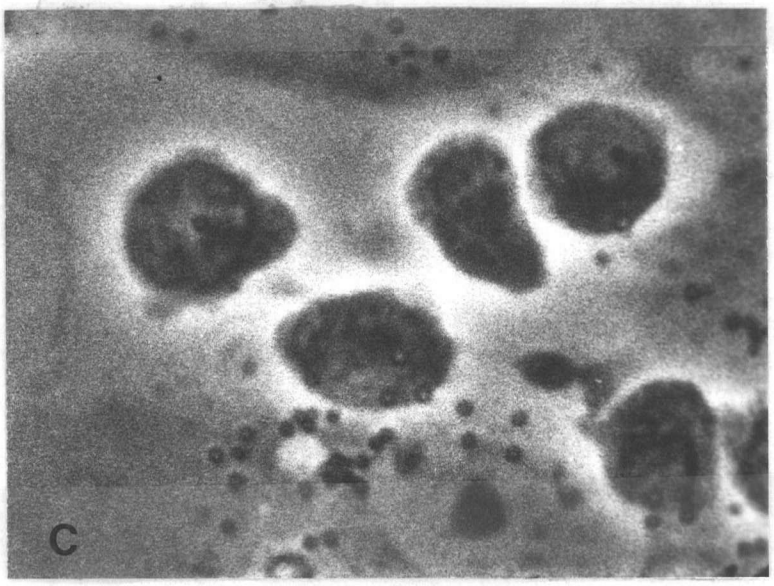

D

Figure 7

Photographs showing the close association and separation of 8 $\underline{WW}^{V}$ presumably sensitized lymphocytes on a $\underline{++}$ monolayer. The cells in close contact are small and medium-sized lymphocytes. They may move around one another in close contact (Figure 7a); they may separate into two groups of associated cells (Figure 7b); or they may separate from one another (Figure 7c), but they eventually move back to meet one another again.


A


B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