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A METHOD FOR THE NONSPECIFIC ESTERASE STAINING OF T AND B LYMPHOCYTES AND MONOCYTES FOLLOWING IN VITRO MIGRATION UNDER AGAROSE

アガロース下試験管内遊走後のT及びBリンパ球
並びに単球の非特異性エステラーゼ染色法

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アガロース下試験管内遊走後のT及びBリンパ球並びに単球の非特異性エステラーゼ染色法

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SUMMARY

This report describes alterations in the agarose mononuclear leukocyte migration technique and the method of cell fixation which resulted in the satisfactory nonspecific esterase staining of human mononuclear leukocytes following 1 to 3 days of in vitro migration. Modifications in technique made it possible to properly fix the migrating cells on to glass slides without significant loss of enzyme activity. Differences in the nonspecific esterase staining characteristics of T lymphocytes, B lymphocytes, and monocytes during in vitro migration are described.

INTRODUCTION

This report describes a technique for the nonspecific esterase (NSE) staining of human peripheral blood monocytes and lymphocytes following in vitro random migration under agarose.[1,2] The method developed solves the difficult problem of the loss of leukocyte NSE activity which invariably occurs during the usual migration study fixation period of 30 minutes.[2] A satisfactory fixation period was achieved through reduction in the thickness of the layer of agarose which overlies the cells during migration. The desirability of making permanent preparations with Permount necessitated the use of a glass rather than a plastic surface for the migration studies. The NSE staining of these preparations was found to be very useful in the differential identification of several types of mononuclear leukocytes at any time during the usual study period of cell migration.

要 約

本報では，アガロース単核白血球遊走技法及び細胞固定法の修正により，試験管内遊走1～3日後のヒトの単核白血球の非特異性エステラーゼ染色に満足すべき結果を得たことを記述した．技法修正によって，酵素活性の有意な損失なく遊走細胞をスライドグラスに正しく固定させることが可能となった．試験管内遊走中のTリンパ球，Bリンパ球及び単球の非特異性エステラーゼ染色特徴にみられる相違点を述べた．

緒 言

本報では，試験管内アガロース下で不規則遊走した後のヒト末梢血液中の単球並びにリンパ球を非特異性エステラーゼ(NSE)染色する技法について述べる．[1,2] この技法により，通常の遊走検査法で30分の固定時間中に必然的に生ずる白血球NSE活性の損失という難題が解決される．[2] 遊走細胞の上にアガロースを薄く重層することによって，満足すべき固定時間が得られた．Permount 法によって永久標本を作製するためには，遊走検査にはプラスチック界面でなくガラス界面の使用が必要であった．通常の細胞遊走検査時間中，随時数種の単核白血球の鑑別同定を行うのに，NSE 染色が非常に有用なことが判明した．

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MATERIALS AND METHODS

The agarose technique for the study of lymphocyte migration has been described previously in some detail.[2] In order to stain for NSE activity it was necessary to modify the previously described procedure as follows: Heparinized venous blood from healthy adult volunteers was separated into granulocyte and mononuclear fractions by means of the Boyum technique.[3] The mononuclear cells then were separated into T and B cells by means of a modification of the method of Greaves and Brown.[4] The number of contaminating monocytes was estimated on smears made from each preparation by means of NSE staining. The monocytes in some B cell preparations were removed by means of glass adsorption. Repeat NSE stain preparations were used to evaluate the adequacy of the glass adsorption procedure in the removal of the monocytes. All cell preparations were resuspended in RPMI (Medium RPMI 1640, Nissui Seiyaku Co. Ltd., Tokyo, Japan) at a concentration of 1 X 10^6 cells per 10 μl. Viability of the cells following washing and suspension in RPMI was more than 97% as judged by means of trypan blue exclusion.

An agarose mixture containing antibiotics and other nutrients was prepared according to the method previously described.[2] A sterile 76 mm X 26 mm glass microscopic slide was placed on the bottom of a sterile glass petri dish. The warm agarose mixture then was layered over the surface of the glass slide to a thickness of about 2 mm by means of a sterile Pasteur pipette. The agarose was hardened by refrigeration at 4C for 30 minutes to 1 hour into a semisolid level gel which firmly adhered to the surface of the slide. Just prior to use, three circular wells, each 3.0 mm in diameter, 4.0 mm apart, and in a straight line were cut with a stainless steel template. The core of agarose in the center of each well was carefully removed by means of gentle suction through a Pasteur pipette, care being taken not to disturb the adherence of the adjacent agarose-slide interface.

A 10 μl aliquot (1 X 10^6 cells) of T, B, or monocyte-adsorbed B cells suspended in RPMI was carefully introduced into the wells by means of a capillary pipette. The petri dishes then were placed in a humidified, 5% CO_2 incubator at 37C for periods of 24-72 hours during which time the leukocytes migrated out from the wells as monolayers in the interface

材料並びに方法

リンパ球遊走検査を目的とするアガロース技法については前報で詳細に述べた.[2] NSE活性を染色するためには，前回の技法を次のように変更する必要があった：すなわちBoyum法[3]を用い，ヘパリン添加健康成人静脈血を顆粒球と単核細胞に分けた．次にGreaves及びBrownの変法[4]を使用して，単核細胞をT細胞とB細胞に分離した．NSE染色で各標本を塗抹し，それに基づいて混入単球数を推定した．ガラス吸着によってB細胞標本中の単球を除去した．ガラス吸着単球除去法が適切かどうかを評価するため，NSE染色標本を反復使用した．全細胞標本を10μl当たり細胞数1×10^6の濃度のRPMI（RPMI 1640培地，日水製薬，東京）に再懸濁した．RPMIで洗浄，懸濁後の細胞の生存力をトリパン・ブルー排除検査で測定したところ97%以上であった．

前報で述べた方法に従って，抗生物質液及び養液を含むアガロース混合液を用意した.[2] 滅菌ペトリ皿の底に76mm×26mmの滅菌顕微鏡用スライドグラスを置いた．次にパスツール・ピペットで，スライドグラスの表面に，加温アガロース混合液を約2mmの厚さに重層した．このアガロースを4℃で30分から1時間冷蔵し，半固体状のゲルとして固まらせ，スライドの表面に固着させた．使用直前にステンレス・スチールの型で，アガロース上に一直線に4.0mm間隔で直径3.0mmの試料孔を3個くりぬいた．周囲のアガロースとスライドの付着面を壊さないように注意しながら，パスツール・ピペットで，各試料孔中心部のアガロース栓状塊を静かに吸引して除去した．

毛細管ピペットで，RPMIに懸濁した10μlのT，B又は単球吸着B細胞（1×10^6細胞）を試料孔に注意深く注入した．次にペトリ皿を37℃の加湿5%CO_2恒温器に入れ，24—72時間培養した．その間，白血球は試料孔からアガロース・ゲルとスライド表面との

between the agarose gel and the glass surface. The petri dishes were removed from the incubator at appropriate intervals and covered with the NSE fixative solution at 4C for 10 minutes following which they were removed with forceps and dipped once in distilled water before the agarose gel was gently peeled off with the aid of a thin glass cover slip. The slides again were immediately dipped into distilled water, three times, and allowed to air dry before staining for NSE by the method previously described.[1] Unincubated fresh control smears from the T, B, and monocyte-adsorbed B cell preparations were also stained for comparison. Thin cover slips were mounted over the stained cells on each slide by means of Permount.

RESULTS

Differences in the NSE staining characteristics of T and B lymphocytes in separated T and B lymphocyte preparations which have migrated for 72 hours under agarose are readily distinguishable. The sparse, discrete, small areas of cytoplasmic stain of the T cells are in sharp contrast to the intense cytoplasmic staining of the monocytes and the virtual absence of cytoplasmic staining in the majority of B lymphocytes (Figures 1-3). These cell staining characteristics are reflected in the low power microscope appearance of each preparation (Figure 4). A large number of monocytes which are strongly positive for NSE are identified in the unadsorbed B cell preparations. The B cell preparations contain only an occasional cell with intense NSE staining characteristics following removal of the monocytes by means of glass adsorption (Figure 5). Occasional contaminating polymorphonuclear leukocytes are easily identified by their unique nuclear morphology in association with their negative NSE staining characteristics.[1]

DISCUSSION

A recently described adaptation of the agarose granulocyte migration technique has made it possible to define certain in vitro migration characteristics of human lymphocyte subpopulations.[2,5] The contamination of separated T and B cell preparations with monocytes and other cells, however, frequently complicates interpretation of microscopic observations. This is a particularly troublesome problem with the

界面に単一層を成して遊走した．適当な間隔でペトリ皿を恒温器から取り出し，NSE 固定液を注ぎ 4℃で10分間置いた後，鉗子で取り出しもう一度蒸留水に浸した．次に，薄いスライド用の覆いグラスを使ってアガロース・ゲルを静かに剝がした．スライドを直ちに 3 回蒸留水に浸し空気乾燥させた後，前述の技法[1]で NSE 染色を行った．比較のため T，B，及び単球吸着B 細胞の未培養の新鮮な塗抹対照標本も染色した．Permount 法によって，各スライドの染色細胞の上に薄い覆いグラスを載せた．

結　果

アガロース下で72時間遊走したT 及びB リンパ球の分離標本では，NSE 染色による両細胞の特徴に容易に判別できる程の差がある．細胞質が染色されたT 細胞は小部分に分離して散在しており，細胞質が濃染された単球及び細胞質の大部分が染色されていないB リンパ球の場合と著しく異なる（図１－３）．これら細胞の染色特徴は，低倍率の顕微鏡検査によって確認できる（図４）．NSE に対して強陽性を示す単球が，非吸着B 細胞標本に多数確認されている．グラス吸着で単球除去後のB 細胞標本では，強いNSE 染色特徴をもった細胞はまれに認められる程度である（図５）．時たま混入した多形核白血球は，NSE 染色が陰性の特性とともにその独特な核型から簡単に識別できる．[1]

考　察

最近記述した顆粒球のアガロース遊走技法の変法によって，ヒトリンパ球 subpopulation の試験管内遊走に関する一定の特性を明らかにすることが可能となった．[2,5] しかし，T 及びB 細胞の分離標本に単球その他の細胞が混入しているため，しばしば検微鏡所見の解釈が複雑となることがある．これは単球の

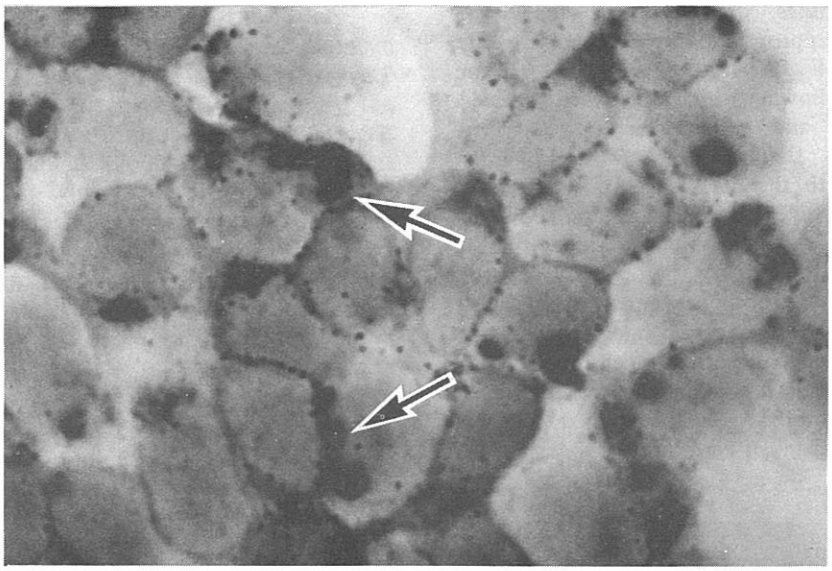

FIGURE 1. Discrete cytoplasmic sites of nonspecific esterase activity are stained in T lymphocytes following 72 hours of migration under agarose (× 1000).

図1．アガロース下で72時間遊走後のTリンパ球は分離した細胞質部位が染色され，非特異性活性を示す（1,000倍）．

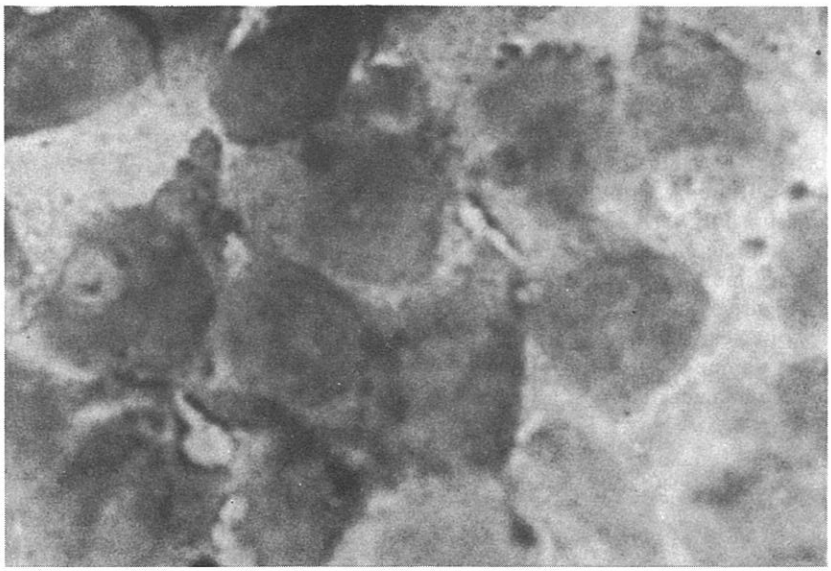

FIGURE 2. B lymphocytes following 72 hours of migration under agarose show only a diffuse trace of cytoplasmic nonspecific esterase activity (× 1000).

図2．アガロース下で72時間遊走後のBリンパ球では，細胞質の非特異性エステラーゼ活性は瀰漫性に痕跡程度が認められるのみである（1,000倍）．

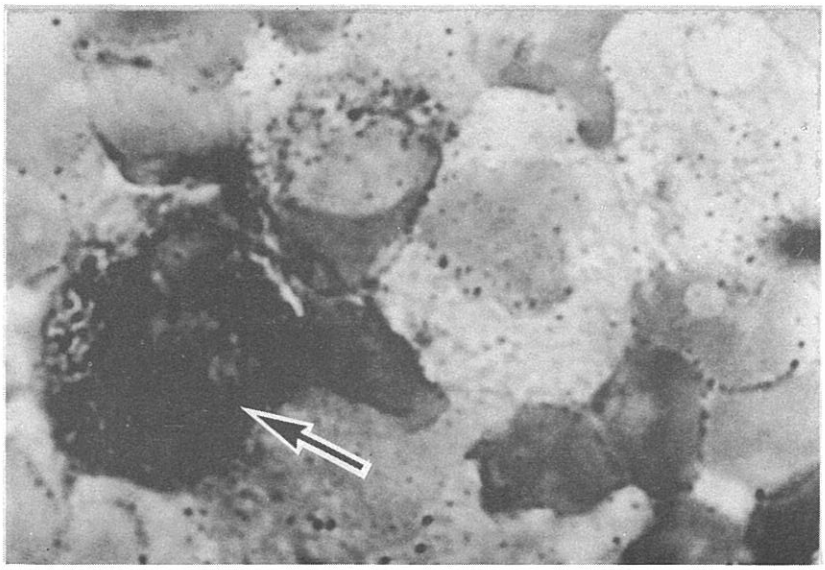

FIGURE 3. Intense cytoplasmic staining for nonspecific esterase activity is demonstrated in a monocyte in a B lymphocyte preparation following 72 hours of migration under agarose (× 1000).

図３．　アガロース下で72時間遊走後のＢリンパ球標本中の単球に，非特異性エステラーゼ活性の強い濃染細胞質が認められる（1,000倍）．

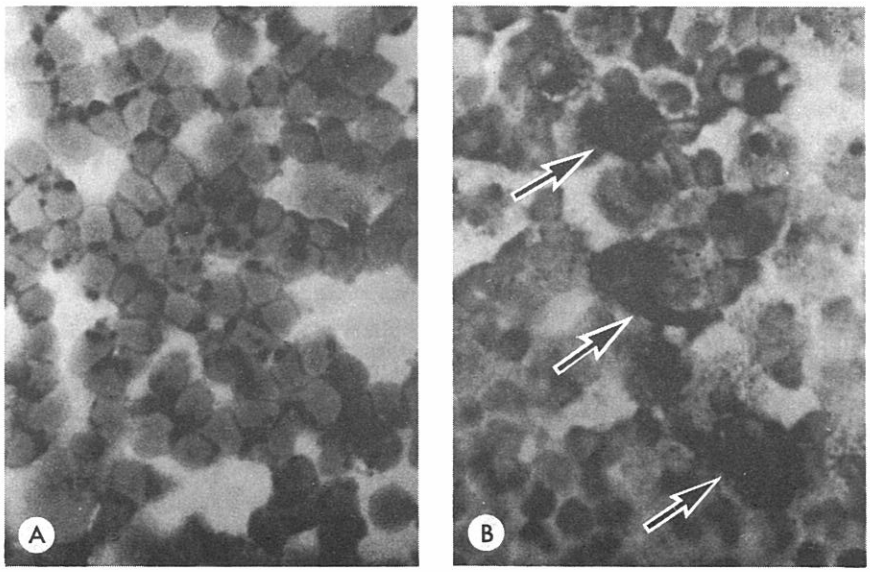

FIGURE 4. A: The discrete cytoplasmic areas of nonspecific esterase activity in T lymphocytes are easily demonstrated following 72 hours of migration under agarose (× 400). B: Cluster of monocytes which have stained intensely for nonspecific esterase are easily identified in this B lymphocyte preparation following 72 hours of migration under agarose (× 400).

図４．　Ａ：Ｔリンパ球の非特異性エステラーゼ活性を示す分離した細胞質部分はアガロース下で72時間遊走後容易に認められる（400倍）．Ｂ：非特異性エステラーゼの強い濃染単球集団が，アガロース下で72時間遊走後のＢリンパ球標本に容易に認められる（400倍）．

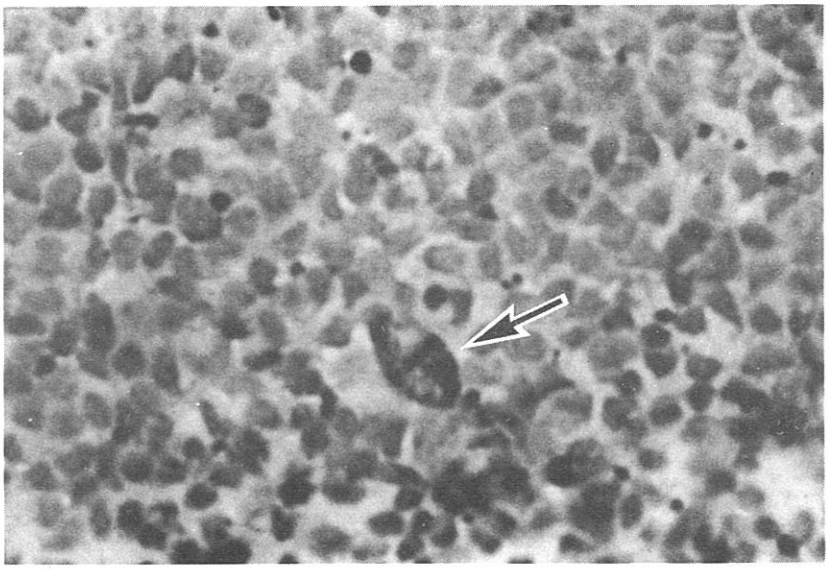

FIGURE 5. Only one monocyte is identified in this B lymphocyte preparation by means of nonspecific esterase stain following glass adsorption and migration under agarose for 72 hours (× 400).

図5．アガロース下で72時間遊走後のグラス吸着Bリンパ球標本中，非特異性エステラーゼ染色によって確認される単球は1個だけである（400倍）．

B cell preparations which usually are quite heavily contaminated with monocytes. B lymphocytes frequently assume a somewhat monocytoid appearance during random migration under agarose which adds to the difficulty in their morphologic identification. Monocyte adsorption techniques are not a satisfactory solution to this problem since they usually are associated with heavy losses of B lymphocytes.

The precise histochemical identification of all lymphocyte subpopulations and monocytes in the peripheral blood is not possible at this time, but differences in NSE activity of these cells contribute to their identification. Recognition of the abundant NSE activity in monocytes has made the histochemical staining technique for this enzyme of value in research and in the histologic classification of certain leukemias.[6,7] Recently, the NSE staining characteristics of T and B lymphocytes has been defined.[8]

Adaptation of NSE staining techniques to the study of lymphocyte subpopulations during various stages of random migration by means of the agarose plate technique was associated with

混入が極めて多いB細胞標本の場合に特にやっかいな問題である．Bリンパ球はアガロース下で不規則遊走中，幾分単球様の形態を示すことが多いが，これによってますますBリンパ球の形態的確認が困難となる．単球吸着技法は通常多数のBリンパ球の損失を伴うので，この問題解決には良い方法とはいえない．

末梢血液中の全リンパ球subpopulationと単球の正確な組織化学的確認は，現時点では不可能であるが，これら細胞のNSE活性の差は，それぞれの確認に役立つ．単球に豊富なNSE活性が注目されてから，この酵素の組織化学的染色技法が，研究並びに特定の白血病の組織学的分類に重要な価値をもつようになった．[6,7]また最近になって，T及びBリンパ球のNSE染色特性が明らかにされた．[8]

アガロース平板法により不規則遊走の諸段階におけるリンパ球subpopulationを研究するため，NSE染色

several problems. Preliminary studies with fresh blood smears revealed that virtually all NSE activity disappeared from the cells following fixation for the usual period of 30 minutes. It was observed that after 4-5 minutes of fixation there was partial loss of NSE activity and virtually complete loss occurred if the period of fixation exceeded 8-10 minutes. On the other hand, fixation of the cells under agarose gels in petri dishes for shorter periods of time was inadequate and resulted in partial or complete loss of surface adherent cells during the process of removing the agarose. Another minor problem was that it was not possible to use plastic petri dishes to make permanent preparations with Permount.

A satisfactory solution to these problems was achieved through the use of a thin layer of agar on glass microscopic slides. This permitted reduction of the fixation time to 10 minutes, a duration for which NSE activity appears unchanged and cell loss is minimal. The migration of T and B lymphocytes on glass was observed to be comparable to migration on plastic so that the use of acetone during the staining process was no longer a problem.

Another point that deserves emphasis is that the fixative solution should not be reused as this results in poor fixation of postmigratory mononuclear leukocytes. Reuse appears to result in dilution or other alteration of the fixative by substances in the agarose gel.

The use of multiple wells on a single glass slide makes it possible to compare the histochemical characteristics of different cell types with the reassurance that variations in stain technique are not a problem. The use of duplicate preparations on the same slide may be of value for comparing the effects of various substances on certain types of cells. Studies of enzyme generation or loss during migration also are possible through the utilization of this slide technique.

The NSE staining characteristics of the migrating lymphocytes and monocytes appear stable without significant change in NSE activity for periods of incubation up to 72 hours when compared with control fresh smears. The method described in this report should prove to be quite useful in the identification of T and B lymphocytes and monocytes in a mixed population of migrating mononuclear cells.

技法を応用することについては幾つか問題があった．塗抹標本を用いた最初の研究では平板を通常時間の30分間固定すると，細胞のNSE活性はほとんど全部消失することが分かった．固定開始後４－５分でNSE活性の一部消失が始まり，８－10分を超えるとほぼ完全消失した．他方，ペトリ皿アガロース・ゲル下の細胞の固定時間を短縮すれば固定は不十分であり，アガロース除去の段階で表面に付着した細胞の一部又は全部に損失が生じる結果になる．別に，小さな問題ではあるが，プラスチック・ペトリ皿ではPermountが使えないので，永久保存のためにプラスチックのペトリ皿を使用することができなかった．

上記の問題に対して，顕微鏡用スライドグラス上のアガロース層を薄くすることにより，満足すべき解決を得た．この結果固定時間は10分間に短縮され，NSE活性に変化は認められず，細胞の損失は最小限にとどまった．グラス上のT及びBリンパ球の遊走はプラスチック上の遊走と同様なことが観察されたので，染色過程におけるアセトンの使用は問題ではなくなった．

今一つ重要な点は，固定液を再使用すべきではないことである．これは，遊走後の単核白血球の固定が不良となるためである．再使用すると，アガロース・ゲル中の物質によって，固定液が希釈されたり変化したりするようである．

１枚のスライドグラス上に多数の試料孔を作ることによって，染色技法に関係なく，種々の細胞型の組織化学的特徴を比較することが可能となる．同一スライド上に同種の細胞標本を使用すれば，種々の物質が特定の細胞に及ぼす影響を比較するのに役立つかもしれない．このスライド技法を利用すれば，遊走過程で酵素の発生又は消失を研究することも可能である．

新鮮な塗抹対照標本と比較すると，培養時間が72時間までは遊走リンパ球並びに単球のNSE染色特徴は安定していて，NSE活性に有意な変化はないようである．本報で述べた技法は，遊走単核細胞の混合細胞集団中においてT及びBリンパ球並びに単球を確認する上に極めて有用なものと考える．

REFERENCES
参考文献

1. YAM LT, LI CY, CROSBY WH: Cytochemical identification of monocytes and granulocytes. Am J Clin Pathol 55:283-90, 1971

2. PINKSTON JA, FINCH SC, IIDA S, CAPLAN R: An agarose plate technique for the study of human T and B lymphocyte migration. Jpn J Exp Med 48:279-82, 1978 (RERF TR 1-78)

3. BOYUM A: Separation of blood leucocytes, granulocytes and lymphocytes. Tissue Antigens 4:269-74, 1974

4. GREAVES MF, BROWN G: Purification of human T and B lymphocytes. J Immunol 112:420-3, 1974

5. FINCH SC, PINKSTON JA: Unpublished observations, 1978

6. GORDON DS, HUBBARD M: Surface membrane characteristics and cytochemistry of the abnormal cells in adult acute leukemia. Blood 51:681-92, 1978

7. SCHMALZL F, BRAUNSTEINER H: The application of cytochemical methods to the study of acute leukemia. A review. Acta Haematol 45:209-17, 1971

8. KULENKAMPFF J, JANOSSY G, GREAVES MF: Acid esterase in human lymphoid cells and leukaemic blasts: a marker for T lymphocytes. Br J Haematol 36:231-9, 1977