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DETERMINATION OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELL-MEDIATED CYTOTOXICITY BY AN AGAROSE PLATE METHOD

SPONTANEOUS RELEASE OF A SOLUBLE CYTOTOXIC FACTOR AGAINST SRBC TARGETS FROM MONOCYTES

アガロースプレート法によるヒト末梢血単核細胞の細胞障害能測定

単球によるSRBC標的に対する可溶性細胞障害因子のspontaneousな放出

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SUMMARY

The cell migration method using an agarose plate was applied to the cell-mediated cytotoxicity test. Human peripheral blood mononuclear cells (PBMC) as effector cells were separated into several cell populations, placed in the wells of agarose plates, and allowed to migrate. Nonsensitized or sensitized sheep red blood cells (SRBC) with anti-SRBC antibody were used as target cells and suspended in the agarose plates.

SRBC around the wells of agarose plates in which mononuclear cells were placed were hemolyzed after several days of incubation, and concentric hemolytic zones which could be grossly seen were formed regardless of anti-SRBC antibody sensitization. Since PBMC migrated only beneath the agarose plate and had minimal cell contact with SRBC targets, it was assumed that a soluble cytotoxic factor to hemolyze SRBC was released from PBMC.

No SRBC hemolytic zone formation was observed when phagocytic cells were eliminated

要 約

アガロースプレート法による細胞遊走能検査法を細胞障害能の測定に応用した．effector 細胞としてヒト末梢血単核細胞(PBMC)を用い，数種の単核細胞群に分離後アガロースプレートの試料孔に注入し遊走させた．標的細胞として抗 SRBC 抗体で感作したヒツジ赤血球(SRBC)や非感作 SRBC を用い，アガロースプレート中に浮遊させた．

恒温器で数日間培養することにより，単核細胞を注入したアガロースプレート試料孔周囲の SRBC が溶血し，肉眼で観察できる同心円状の溶血域が形成され，この溶血域は抗 SRBC 抗体感作の有無にかかわらず観察された．PBMC はアガロースプレートの下のみを遊走し，SRBC とはほとんど接触しないことから SRBC を溶血させるような可溶性の細胞障害因子が PBMC から放出されたものと思われた．

PBMC から食食細胞を除いても溶血域は全く形成

from PBMC and granulocytes also did not form a hemolytic zone. However, as adherent PBMC formed a zone of hemolysis, monocytes were considered to be the effector cells which released a soluble cytotoxic factor.

SRBC hemolytic zone formation was completely inhibited by mixing trypan blue in the agarose plate at a concentration that would not inhibit PBMC migration. Since trypan blue inhibits the activity of lysosomal enzymes, the cytotoxic factor released from PBMC is assumed to be a lysosomal enzyme.

Many monocytes or macrophages demonstrating extremely high acid phosphatase activity (a marker enzyme of lysosomes) were observed in the migrated PBMC.

INTRODUCTION

It has been elucidated that antibody dependent cellular cytotoxicity (ADCC) is mediated by direct contact with Fc receptors of effector cells and antibody-sensitized target cells,[1,2] but the mechanism involved in the destruction of target cells after contact is yet unknown.

We have been studying human PBMC migration using an agarose plate method[3] and, applying this method to cytotoxicity test, attempts were made to determine whether the soluble cytotoxic factor participates in the destruction of target cells in ADCC. In this assay, PBMC migrate only beneath the agarose plate and have minimal cell contact with the SRBC targets suspended in the agarose plate and therefore the SRBC are not hemolyzed if the soluble cytotoxic factor is not released from mononuclear cells, thus making this method suitable for the detection of the soluble cytotoxic factor.

As no SRBC hemolytic zone appeared after 3 to 16 hours of incubation which is the suitable response time for ADCC, participation of the soluble cytotoxic factor in ADCC was not suspected. However, by continued incubation SRBC hemolytic zones were formed around the wells of agarose plates with PBMC. The SRBC hemolytic zones were also formed in the non-sensitized SRBC-suspended agarose plate, thus it was assumed that the SRBC were hemolyzed directly by a soluble cytotoxic factor released from PBMC and not indirectly through anti-SRBC antibody.

されず，顆粒球も溶血域を形成しなかった．しかし，付着性のPBMCによって溶血域が形成されたことから，可溶性細胞障害因子を放出するeffector細胞は単球であると思われた．

SRBC溶血域の形成は，PBMCの遊走能を全く抑制しない濃度のトリパンブルーをアガロースプレート内に混入しておくことにより，完全に抑制された．トリパンブルーはリソゾーム酵素の活性を抑制するので，PBMCから放出された細胞障害因子はリソゾーム酵素であると思われる．

遊走させたヒトPBMC中には非常に高い酸性フォスファターゼ活性（リソゾームのマーカー酵素）を示す単球やマクロファージが多く見られた．

緒　言

抗体依存性細胞障害作用（ADCC）はeffector細胞のFcレセプターと，抗体感作標的細胞との直接的な接触によってもたらされることが明らかにされている．[1,2] しかし，接触後いかなる機序で標的細胞が破壊されるかについては明らかにされていない．

我々はアガロースプレート法によるヒト末梢血単核細胞（PBMC）の遊走能を検討しているが，[3] この方法を細胞障害能測定に応用し，ADCCにおける標的細胞の破壊に可溶性細胞障害因子が関与しているか否かを明らかにしようと試みた．この方法ではPBMCはアガロースプレートの下のみを遊走し，プレートに浮遊させた標的細胞としてのヒツジ赤血球（SRBC）とはほとんど接触しない．そのため可溶性細胞障害因子が単核細胞から放出されなければSRBCは溶血せず，可溶性細胞障害因子の有無を決定するのに適している．

ADCCの至適反応時間とされる3～16時間の培養では，SRBCの溶血域は全く形成されず，ADCCに可溶性細胞障害因子は関与していないと思われた．しかし，その後更に培養を続けることにより，PBMCを注入したアガロースプレート試料孔の周囲にSRBCの溶血域が形成された．このSRBC溶血域は非感作SRBCを浮遊させたアガロースプレートにも形成されたことから，SRBCの溶血は抗SRBC抗体を介して間接的におきているのではなく，PBMCから放出された可溶性細胞障害因子が直接SRBCに作用したものであると思われた．

This study will assess that the effector cells which release a soluble cytotoxic factor to hemolyze SRBC are monocytes and that the soluble cytotoxic factor may be a lysosomal enzyme.

本調査では，SRBCを溶血させる可溶性細胞障害因子を放出するeffector細胞が単球であり，可溶性細胞障害因子がリソゾーム酵素である可能性について述べる．

MATERIALS AND METHODS

Isolation of PBMC. A volume of 30 to 50 ml of heparinized (20 units/ml) venous blood was obtained from healthy adult volunteers and mixed with an equal volume of Ca^{++} and Mg^{++} free balanced salt solution [BSS(−)]. PBMC were isolated by the method of Böyum.[4] In brief, the diluted blood was centrifuged on a Ficoll-Conray density gradient at 4°C at 1,600 rpm for 20 minutes and PBMC recovered at the interface were collected, washed three times with BSS(−), and then used as PBMC with phagocytic cells.

材料及び方法

PBMCの分離． 健康成人有志1名につきヘパリン添加（20単位／ml）静脈血30ml～50mlを採取し，Ca^{++}及びMg^{++}を除いた平衡化食塩水（BSS(−)）を等量混合し，Böyumの方法[4]に準じてPBMCを分離した．すなわち希釈静脈血をFicoll-Conray液に重層し，4℃で1,600rpm20分間遠心分離し，中間層のPBMCを集め，BSS(−)で3回洗浄し食食細胞非除去PBMC群として用いた．

Elimination of Phagocytic Cells. Heparinized venous blood was mixed with KAC-2 (silica suspension, Japan Immunoresearch Labs., Takasaki, Japan) to 10% and was incubated at 37°C for 60 minutes with occasional agitation and KAC-2 was phagocytized. The KAC-2 treated venous blood was centrifuged on a Ficoll-Conray density gradient at 4°C at 2,500 rpm for 30 minutes and PBMC recovered at the interface were collected, washed three times with BSS(−), and then used as PBMC without phagocytic cells.

貪食細胞の除去． ヘパリン添加静脈血にKAC-2（silica suspension，日本抗体研究所，高崎市）を10%濃度になるよう加え，時々撹拌しながら37℃で60分間培養し，KAC-2を貪食させた．KAC-2で処理した静脈血をFicoll-Conray液に重層し，4℃で2,500rpm30分間遠心分離し，中間層のPBMCを集め，BSS(−)で3回洗浄して貪食細胞除去PBMC群として用いた．

Separation of PBMC by Rosette Formation with SRBC. The foregoing two PBMC populations were separated into rosette forming cells (RFC) with SRBC (E-RFC enriched cells) and non-RFC (E-RFC depleted cells) by a modification of the method of Greaves and Brown.[5] In brief, PBMC resuspended in BSS(−) at a concentration of 5×10^3 cells/μl were mixed with an equal volume of 1% suspension of packed SRBC in inactivated fetal calf serum (FCS - Grand Island Biological Co., New York, USA), which then was distributed into small test tubes, each containing 0.5 ml. After 15 minutes of incubation at room temperature, the tubes were spun at 4°C at 1,000 rpm for 5 minutes and then placed in an ice water bath for one hour. The pellets of all tubes were resuspended carefully by capillary pasteur pipette, layered over a Ficoll-Conray density gradient, and centrifuged again at 4°C at 1,600 rpm for 20 minutes. The pellet cells were used as E-RFC enriched cells and the interface

SRBCとのロゼット形成によるPBMCの分離． Greaves と Brownの方法[5]に準じて上記2種類のPBMC群を，それぞれSRBCとロゼットを形成する細胞群（E-RFC enriched細胞群）及びロゼットを形成しない細胞群（E-RFC depleted細胞群）に分離した．すなわち，5,000細胞／μlになるようBSS(−)に浮遊させたPBMCと，非働化ウシ胎児血清（FCS-Grand Island Biological Co., New York）に1%濃度になるよう再浮遊させた洗浄SRBCを等量混合し，0.5mlずつ小試験管に分注した．室温に15分間静置した後，4℃で1,000rpm 5分間遠心分離し，氷槽に1時間静置した．全試験管の沈渣を毛細管ピペットで注意深く再浮遊させ，再度Ficoll-Conray液に重層し，4℃で1,600rpm20分間遠心分離した．沈んだ細胞群をE-RFC enriched細胞群，中間層に浮いた細胞群

cells as E-RFC depleted cells. The contaminated SRBC were osmotically lysed quickly by distilled water and PBMC populations were washed three times with BSS(−).

Separation of PBMC by Nylon Wool Column. Nylon wool columns were prepared by the method of Danilovs et al.[6] Nylon wool (Wako Co., Osaka, Japan) was evenly packed into plastic drinking straws. PBMC with phagocytic cells suspended in 0.5 ml of RPMI 1640 culture solution with 5% inactivated FCS were added on the top of the column and incubated at 37°C for 30 minutes. The cells collected by allowing 10 ml of 37°C culture solution to drip through the column were utilized as nonadherent PBMC (by column) and the cells which were pressed through the column by adding 10 ml of 37°C culture solution and by repeatedly squeezing the straw vigorously were utilized as adherent PBMC (by column).

Isolation of Adherent PBMC by a Plastic Adherence Method. Adherent PBMC were collected by the method of Kumagai et al.[7] In brief, 1×10^7 PBMC suspended in 5 ml of RPMI 1640 with 10% FCS were poured into plastic petri dishes (Falcon 3002 tissue culture dish, Falcon Co., California, USA) pretreated with FCS for one night, and incubated at 37°C for one hour. Nonadherent PBMC were removed by washing with culture solution three to four times and then 2 ml of BSS(−) containing 0.2% ethylenediamine tetraacetate (EDTA - Wako Co., Osaka, Japan) and 5% FCS were poured into the dishes and incubated at 4°C for 15 minutes. Adherent PBMC were then collected by jetting the medium against the cells by pipette and washed three times with BSS(−). These cells were used as adherent PBMC (by dish). Cell viability of these several kinds of PBMC populations obtained by the above methods was assessed by trypan blue exclusion test and found consistently to be 95% or better.

Preparation of Agarose Plates. Culture plates of 1% agarose RPMI 1640 containing 20% heat-inactivated horse serum (HS) were prepared by the modified method of Pinkston et al.[3] In brief, 2.2 ml of distilled water was mixed with 0.8 ml of RPMI 1640 (×10 concentrated), 2.0 ml of HS (Grand Island Biological Co., New York, USA), 500 units of potassium penicillin G, 500 μg of streptomycin, and 10 μg of amphotericin B and was heated to 43°C. Forty

を E-RFC depleted細胞群とし，混入 SRBCを蒸留水で素早く浸透圧溶血させ，PBMC群を BSS (−) で3回洗浄して用いた．

ナイロンウールカラムによる PBMCの分離.
Danilovsらの方法[6]に準じてナイロンウールカラムを作成した．プラスチック製ストローにナイロンウール(和光純薬工業，大阪)を均等に詰めた．0.5mlの5%非働化 FCS添加 RPMI 1640培養液に浮遊させた貪食細胞非除去 PBMC群をカラムの先端から注入し，37℃で30分間培養した．37℃の培養液10 ml をカラムに流して集めた細胞を非付着 PBMC群，更に10 ml を流してカラムを数回強くもんで流出してきた細胞を付着 PBMC群として用いた．

プラスチック付着法による付着 PBMCの分離.
Kumagaiらの方法[7]に準じて付着 PBMCを集めた．すなわち，10% FCS添加 RPMI 1640培養液 5 ml に 1×10^7 個の PBMCを浮遊させ，あらかじめ FCSで1晩処理したプラスチック製ペトリ皿(Falcon 3002組織培養皿，Falcon Co., California)に注入し，37℃で1時間培養した．非付着 PBMCを培養液で3～4回洗い流し，0.2%の ethylenediamine tetraacetate (EDTA-和光純薬工業，大阪)と5%の FCSを含む BSS (−) 2 ml を注入し，4℃で15分間培養した．ピペットで付着 PBMCを培養皿からはがして集め，BSS (−) で3回洗浄した．この細胞群を付着 PBMC群として用いた．このようにして分離した種々の PBMC群の細胞の生存率はトリパンブルー排除検査ですべて95%以上であった．

アガロースプレートの作製法. Pinkstonらの方法[3]に準じて20%非働化ウマ血清(HS)添加1%アガロース RPMI 1640培養プレートを作製した．すなわち，蒸留水 2.2ml, 10倍濃縮 RPMI 1640培養液0.8ml, HS 2.0ml (Grand Island Biological Co., New York)，ペニシリンGカリウム500単位，ストレプトマイシン500 μg，アムホテリシンB 10μg を混合し，43℃まで加温した．

microliters of packed SRBC were added to the above solution to suspend SRBC in agarose plates as target cells. One hundred milligrams of agarose (Agarose A-45, Nakarai Co., Kyoto, Japan) were dissolved in 5 ml of distilled water, boiled and then cooled to 43°C. These two solutions were mixed quickly and 5 ml of the mixture was poured into each plastic petri dish. These agarose plates were kept at 4°C for about 30 minutes and wells 3.00 mm in diameter were cut after hardening. Three kinds of SRBC as target cells were suspended in agarose plates; nonsensitized SRBC, sensitized SRBC with IgG fraction of rabbit anti-SRBC antibody, and sensitized SRBC with IgM fraction (Japan Immunoresearch Labs., Takasaki, Japan). Agarose plates containing trypan blue at several concentrations were used to determine its inhibitory effects on cytotoxicity of PBMC.

Culture Conditions. Each PBMC population separated by various techniques was suspended in RPMI 1640 culture solution at a concentration of 1×10^5 cells/μl and 10 μl (1×10^6 cells) of these suspensions were placed in each agarose plate well. They were incubated at 37°C in a humidified 5% CO_2 incubator for 5 to 10 days and hemolysis of SRBC in agarose plates was observed daily.

Measurement of Hemolytic Zones and Migration Distance. The width of hemolytic zones was measured from the edge of the well to the end of hemolytic zone by micrometer-attached microscope. The migration distance of PBMC populations on fixed and stained preparation was measured with a table slide projector which provided 8- to 20-fold magnification.

Staining of Migrated Cells. After several days of incubation, 3 ml of buffered formalin acetone fixative solution were poured into each culture dish. After incubation at room temperature for 30 minutes, agarose layers were removed carefully and the culture dishes were washed and dried. Migrated cells were stained with May-Grünwald-Giemsa stain (M-G), and double staining of nonspecific esterase and chloroacetate esterase by the modified method of Li et al[8] and acid phosphatase staining by the modified method of Tomonaga and Hiwatashi[9] were performed.

RESULTS

Cytotoxicity of PBMC against Nonsensitized and

標的細胞としてのSRBCをアガロースプレートに浮遊させる場合は，この培養液にパックしたSRBCを40μl加えた．アガロース(アガロースA-45，半井化薬，京都) 100mgを5mlの蒸留水に溶解し，煮沸後43℃まで冷却し，上記培養液と手早く混合して5mlずつをプラスチック製ペトリ皿に注入した．アガロースプレートを4℃で30分間保存し，十分に固まった後，直径3㎜の試料孔を作った．アガロースプレートに浮遊させる標的細胞としてのSRBCは抗体を感作しないもの，家兎抗SRBC抗体のIgG成分で感作したもの，及びIgM成分(日本抗体研究所，高崎)で感作したものの3種を用いた．トリパンブルーによるPBMCの細胞障害能抑制テストを行う場合は，種々の濃度になるようトリパンブルーを混入したアガロースプレートを作製した．

培養法． 種々の方法で分離したそれぞれのPBMC群を1×10^5細胞／μlになるようRPMI1640培養液に浮遊させ，その10μl(1×10^6細胞)をアガロースプレートの各試料孔に注入した．これを37℃，加湿5％CO_2恒温器で5～10日間培養し，アガロースプレート内SRBCの溶血状態を毎日観察した．

溶血範囲及び遊走距離の測定． 溶血範囲は顕微鏡に接眼ミクロメーターを装着し，試料孔の辺縁から溶血した部分までの距離を測定した．固定し，染色したPBMC群の遊走距離は，卓上用スライドプロジェクターで8倍～20倍に拡大して測定した．

遊走細胞の染色． 数日間培養後，組織培養皿に緩衝ホルマリンアセトン固定液3mlを入れた．室温で30分間固定後，アガロースプレートを注意深くはがし，水洗して乾燥させた．遊走細胞はMay-Grünwald-Giemsa染色(M-G)，Liらの方法[8]に準じた非特異的エステラーゼとクロロアセテートエステラーゼの二重染色及び朝長と樋渡の方法[9]に準じた酸性フォスファターゼ染色を行った．

結　果

PBMCによるアガロースプレートに浮遊させた標的

TABLE 1 SRBC HEMOLYTIC ZONE FORMATION AROUND THE WELLS OF AGAROSE PLATES AND MIGRATION DISTANCE BY PBMC POPULATIONS

表1　PBMC群によるアガロースプレート試料孔周囲のSRBC溶血域形成及び遊走距離

PBMC Population	Hemolytic Zone Mean ± SD mm / Migration Distance Mean ± SD mm			
	Without Suspended SRBC	Suspended Nonsensitized SRBC	Suspended Anti-SRBC Antibody (IgG) Sensitized SRBC	Suspended Anti-SRBC Antibody (IgM) Sensitized SRBC
E-RFC Enriched Cells	– / 0.80 ± 0.22	1.15 ± 0.82 / 1.08 ± 0.25	0.96 ± 0.78 / 0.96 ± 0.22	1.21 ± 0.79 / 1.10 ± 0.21
E-RFC Depleted Cells	– / 2.58 ± 1.01	2.49 ± 1.21 / 3.54 ± 1.28	2.46 ± 1.02 / 3.26 ± 1.16	2.69 ± 1.08 / 3.53 ± 1.13

PBMC were separated into E-RFC enriched and depleted cells by spontaneous rosette formation with SRBC and were allowed to migrate. Four kinds of agarose plates were prepared; without suspended SRBC, with suspended nonsensitized SRBC, with suspended anti-SRBC antibody (IgG fraction) sensitized SRBC, and with suspended anti-SRBC antibody (IgM fraction) sensitized SRBC. The width of SRBC hemolytic zones and migration distance by PBMC populations were measured after five days of incubation at 37° C in a humidified 5% CO_2 incubator.

PBMCはSRBCとのspontaneousなロゼット形成によりE-RFC enriched細胞群及びE-RFC depleted細胞群に分離して遊走させた．アガロースプレートはSRBCを浮遊させないもの，非感作SRBCを浮遊させたもの，SRBC抗体のIgG成分又はIgM成分で感作したSRBCを浮遊させたものの4種を用いた．37℃加湿5％ CO_2 恒温器で5日間培養後に，それぞれのPBMC群によるSRBC溶血範囲及び遊走距離を測定した．

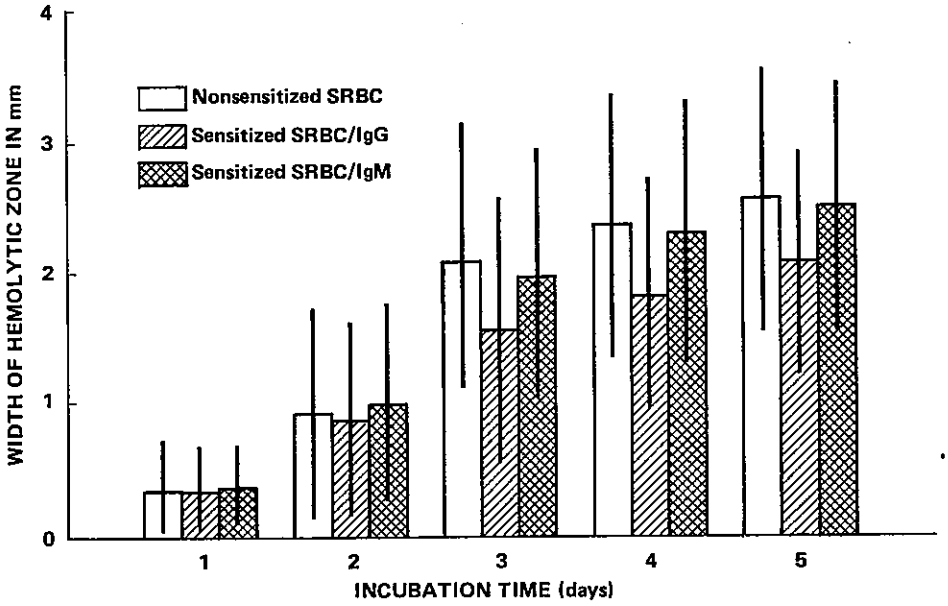
Anti-SRBC Antibody-Sensitized SRBC Targets Suspended in Agarose Plates. Using PBMC of 20 healthy adult donors, a study was made to determine whether a soluble cytotoxic factor participates in the destruction of target cells in ADCC. PBMC were separated into E-RFC enriched and depleted cells, which were allowed to migrate. Three kinds of SRBC; nonsensitized SRBC, sensitized SRBC with IgG fraction of rabbit anti-SRBC antibody, and sensitized SRBC with IgM fraction, were suspended in each agarose plate as target cells. No SRBC hemolysis was observed in agarose plates after 3 to 16 hours of incubation which is the suitable response time of ADCC. However, SRBC around the wells of agarose plates where PBMC were placed began to be hemolyzed after 24 to 48 hours of incubation and clear and concentric hemolytic zones which could be grossly seen were formed. These hemolytic zones were formed not only in the agarose plates in which antibody-sensitized SRBC were suspended but also in the agarose plates in which nonsensitized SRBC were suspended.

The width of hemolytic zone and PBMC migration distance were measured after five days of incubation (Table 1). Both E-RFC enriched and

非感作SRBC及び抗SRBC抗体感作SRBCに対する細胞障害能． ADCCにおける標的細胞破壊の過程に，可溶性細胞障害因子が関与しているか否かについて，20名の健康成人PBMCを用いて検討した．PBMCはE-RFC enriched細胞群及びE-RFC depleted細胞群に分離して遊走させた．標的細胞としてのSRBCは家兎抗SRBC抗体のIgG成分又はIgM成分で感作したもの及び非感作SRBCの3種をそれぞれのアガロースプレートに浮遊させた．ADCCの反応時間とされる3～16時間の培養ではアガロースプレート内のSRBCは全く溶血しなかった．しかし24～48時間後ころよりPBMCを注入したアガロースプレート試料孔周囲のSRBCが溶血し，肉眼で観察できる同心円状の溶血域が形成された．この溶血域は，抗体で感作したSRBCを浮遊させたアガロースプレートのみならず，非感作SRBCを浮遊させたアガロースプレートにも観察された．

培養5日後の溶血範囲及びPBMCの遊走距離を測定した（表1）．E-RFC enriched細胞群もE-RFC depleted細胞群も，アガロースプレート内に浮遊した

FIGURE 1 TIME COURSE OF THE WIDTH OF SRBC HEMOLYTIC ZONES BY E-RFC DEPLETED CELLS WITH PHAGOCYTIC CELLS
図1　貪食細胞非除去 E-RFC depleted 細胞群による SRBC 溶血範囲の経時的変化



Three kinds of SRBC (nonsensitized SRBC, sensitized SRBC with IgG fraction of anti-SRBC antibody, and sensitized SRBC with IgM fraction) were suspended in each agarose plate, and the width of hemolytic zones around the wells was measured daily for five days using a microscope with ocular micrometer.
アガロースプレートは非感作 SRBC を浮遊させたもの，抗 SRBC 抗体の IgG 成分及び IgM 成分で感作した SRBC を浮遊させたものの3種を用い，試料孔周囲の溶血範囲を毎日，5日目まで接眼ミクロメーターを装着した顕微鏡を用いて測定した．

depleted cells hemolyzed SRBC suspended in agarose plates regardless of anti-SRBC antibody sensitization, and no enlargement of hemolytic zones by antibody sensitization could be observed. The width of hemolytic zones by E-RFC depleted cells was about two times greater than that by E-RFC enriched cells. The migration distance of E-RFC depleted cells was also about three times greater than that of E-RFC enriched cells. The migration distance of both cell populations was extended by suspending SRBC in agarose plates. The time course of the hemolytic zone formation by E-RFC depleted cells was followed for 13 healthy adult donors for periods up to five days (Figure 1). The hemolytic zone appeared after 24 to 48 hours of incubation, enlarged rapidly for 72 hours of incubation and thereafter expanded gradually. A similar pattern was also observed with E-RFC enriched cells.

Since it has been shown that PBMC releases a soluble cytotoxic factor by culture to hemolyze

SRBC を抗 SRBC 抗体感作の有無にかかわらず溶血させ，感作 SRBC を使用することにより溶血範囲が広くなるようなことはなかった．E-RFC depleted 細胞群による溶血範囲は E-RFC enriched 細胞群の約2倍であった．遊走距離も E-RFC depleted 細胞群が E-RFC enriched 細胞群の約3倍であった．また，いずれの細胞群においても，アガロースプレート内に SRBC を混入しておくことにより遊走距離が長くなった．13名の健康成人の E-RFC depleted 細胞群による溶血域形成の経時的変化を5日間まで観察した（図1）．溶血域は培養24～48時間後に出現し，72時間培養時に急速に広がり，以後徐々に拡大していった．同じような傾向が E-RFC enriched 細胞群においても観察された．

PBMC は，培養により抗 SRBC 抗体感作の有無に

TABLE 2 SRBC HEMOLYTIC ZONE FORMATION AND MIGRATION DISTANCE BY SEVERAL PBMC POPULATIONS

表2　種々の PBMC 群による SRBC 溶血域形成及び遊走距離

PBMC Population	Monocyte Contamination Mean ± SD %	Hemolytic Zone Mean ± SD mm / Migration Distance Mean ± SD mm
With phagocytic cells		
E-RFC enriched cells	7.8 ± 4.6	0.61 ± 0.38 / 1.25 ± 0.43
E-RFC depleted cells	32.1 ± 11.5	1.77 ± 0.80 / 3.09 ± 0.91
Without phagocytic cells		
E-RFC enriched cells	1.2 ± 1.0	0 / 0.80 ± 0.22
E-RFC depleted cells	3.8 ± 3.6	0.14 ± 0.26 / 0.78 ± 0.22
Nonadherent PBMC (by column)	1.3 ± 1.1	0 / 1.10 ± 0.22
Adherent PBMC (by column)	18.5 ± 7.8	1.07 ± 0.21 / 2.40 ± 1.06

PBMC were separated into PBMC with and without phagocytic cells by KAC-2, and each PBMC population was then divided into E-RFC enriched and depleted cells. PBMC were also separated into nonadherent and adherent PBMC by nylon wool column. The rates of monocyte contamination in these PBMC populations were determined by nonspecific esterase stain before migration. Nonsensitized SRBC-suspended agarose plates were used, and the width of hemolytic zones and migration distance by PBMC populations were measured after five days of incubation at 37°C in a humidified 5% CO_2 incubator.

PBMC はあらかじめ KAC-2 によって貪食細胞を除去した PBMC 群及び非除去 PBMC 群に分離し，更に E-RFC enriched 細胞群及び E-RFC depleted 細胞群に分離した．また，ナイロンウールカラムを用いて非付着 PBMC 群及び付着 PBMC 群に分離して遊走させた．遊走前のそれぞれの細胞群における単球混入率は非特異的エステラーゼ染色することにより算出した．アガロースプレートは非感作 SRBC を浮遊させたものを用い，37℃加湿 5 % CO_2 恒温器で 5 日間培養後のそれぞれの PBMC 群別の溶血範囲及び遊走距離を測定した．

SRBC regardless of sensitization with anti-SRBC antibody, the subsequent experiments were performed using nonsensitized SRBC as target cells. We investigated the nature of effector cells which release a soluble cytotoxic factor against SRBC.

SRBC Hemolytic Zone Formation and Migration Distance by Several PBMC Populations. The changes in width of SRBC hemolytic zones and in migration distance of PBMC through removal of phagocytic cells from PBMC were investigated using nine healthy adult donors (Table 2). PBMC were divided into PBMC with and without phagocytic cells by KAC-2 and then these PBMC populations were divided into E-RFC enriched and depleted cells, which were allowed to migrate. The rate of monocyte contamination in each PBMC population markedly decreased by eliminating phagocytic cells.

The hemolytic zone formation was most prominent when E-RFC depleted cells with

かかわらず SRBC を溶血させるような細胞障害因子を放出することがわかったため，以後の実験はすべて非感作 SRBC を標的細胞として用いた．そして，この SRBC を溶血させる可溶性細胞障害因子を放出する effector 細胞が何であるかについて検討した．

種々の PBMC 群による SRBC 溶血域形成及び遊走距離．PBMC から貪食細胞を除去することによって SRBC の溶血範囲，及び PBMC の遊走距離がどのように変化するかを 9 名の健康成人について検討した（表2）．まず，KAC-2 によって貪食細胞を除去した PBMC 群及び非除去 PBMC 群に分離し，更にこれらの PBMC 群をそれぞれ E-RFC enriched 細胞群及び E-RFC depleted 細胞群に分離して遊走させた．各 PBMC 群の単球混入率は貪食細胞を除去することにより著減した．

溶血域形成は，単球混入率の最も高かった貪食細胞

phagocytic cells showing the highest monocyte contamination rate were allowed to migrate (Table 2 and Figure 2). The formation of hemolytic zone was almost completely abolished by eliminating phagocytic cells in all PBMC populations. The migration distance was also decreased by eliminating phagocytic cells especially in E-RFC depleted cells (Figure 3). The difference in migration distance between E-RFC enriched and depleted cells almost completely disappeared after phagocytic cell elimination. Though granulocytes migrated quite well, no hemolytic zone was formed. There was no zone formation around the wells in which only culture solution was placed as control.

非除去の E-RFC depleted細胞群において最も明らかであった（表2及び図2）．貪食細胞を除去することにより，いずれの PBMC群においても溶血域はほとんど形成されなくなった．遊走距離も貪食細胞を除去することにより，特に E-RFC depleted細胞群で減少した（図3）．貪食細胞除去後は E-RFC enriched細胞群と E-RFC depleted細胞群との間の遊走距離の差がほとんどなくなった．顆粒球は非常によく遊走したが，溶血域は全く形成されず，コントロールとして培養液のみを注入した試料孔周囲にも形成されなかった．

PBMC were also divided into adherent and nonadherent PBMC (by column) by nylon wool column and the differences in width of hemolytic zones and in migration distance by each PBMC population were examined using 16 healthy adult donors (Table 2). Hardly any hemolytic zone was formed when nonadherent PBMC (by column) which contained few monocytes were allowed to migrate. However, adherent PBMC (by column) containing about 20% monocytes formed clear hemolytic zones around the well of agarose plates. The migration distance of adherent PBMC (by column) was about two times greater than that of nonadherent PBMC (by column).

ナイロンウールカラムによって PBMC を付着 PBMC群及び非付着 PBMC 群に分離し，それぞれの PBMC 群による溶血範囲及び遊走距離がどのように異なるかを16名の健康成人について検討した（表2）．ごく少量の単球しか混入していない非付着 PBMC 群を遊走させた場合，溶血域はほとんど形成されなかった．しかし，約20%の単球が混入した付着 PBMC 群では，アガロースプレートの試料孔周囲に明瞭な溶血域が形成された．付着 PBMC 群の遊走距離は非付着 PBMC 群の約2倍であった．

SRBC Hemolytic Zone Formation and Migration Distance by Cells Adherent to Plastic Petri Dishes. The width of hemolytic zones and migration distance by cells adherent to FCS-treated plastic petri dishes were determined using 10 healthy adult donors. The cell components of the adherent PBMC (by dish) before culture were as follows: monocytes 84.6% ± 8.0%, lymphocytes 6.1% ± 3.0%, and granulocytes 9.3% ± 7.4%. Since hemolytic zone by this adherent PBMC (by dish) was not formed until after 5 days but appeared after 7 days of incubation, it was continued for only 10 days in this experiment. The width of hemolytic zones and migration distance by adherent PBMC (by dish) were determined after 7 to 10 days of incubation (Table 3). The hemolytic zone after 7 days of incubation was narrow, but it enlarged rapidly after 10 days of incubation. The migration distance by this adherent PBMC (by dish) was the greatest among several kinds of PBMC populations subjected to migration.

プラスチック製ペトリ皿に付着する細胞による SRBC 溶血域形成及び遊走距離． FCS 処理プラスチック製ペトリ皿に付着する細胞の溶血範囲及び遊走距離を10名の健康成人について検討した．培養前の付着 PBMC 群の細胞構成は，単球84.6%±8.0%，リンパ球6.1%±3.0%，顆粒球9.3%±7.4%であった．この付着 PBMC 群による溶血域の形成は培養5日後まではほとんど行われず，培養7日後ころから出現したため，この実験のみ10日間まで培養を続けた．培養7日後から10日後の付着 PBMC 群による溶血範囲及び遊走距離を測定した（表3）．培養7日後の溶血域は小さく，その後10日目までに急速に拡大した．遊走距離はこれまで行った PBMC 群の中で最大であった．

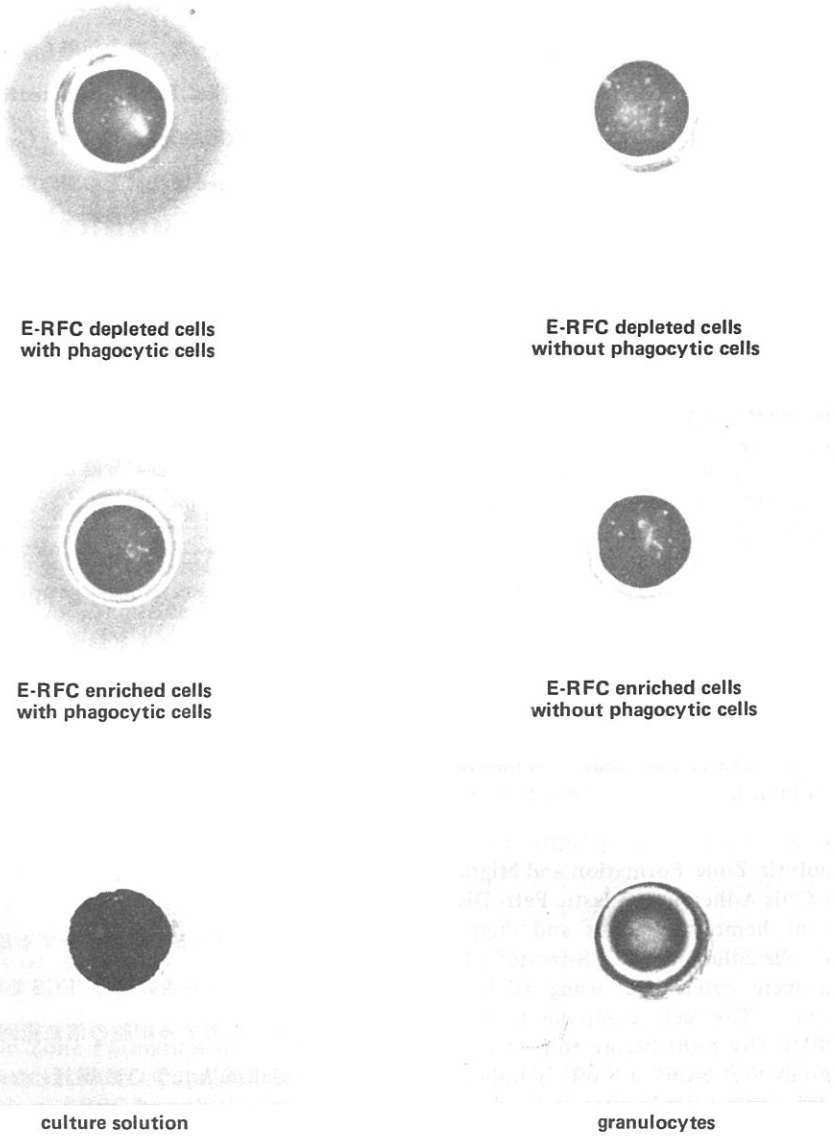


Figure 2. Hemolytic zone formation around the wells of agarose plate after five days of incubation by several kinds of PBMC populations and granulocytes

Clear and circular hemolytic zones were formed around each well in which E-RFC enriched or depleted cells with phagocytic cells were placed. No hemolytic zones were formed around wells containing E-RFC enriched or depleted cells without phagocytic cells and no hemolytic zones were observed around wells containing granulocytes or only culture fluid.

図2　種々のPBMC群及び顆粒球による5日間培養後のアガロースプレート試料孔周囲の溶血域形成

貪食細胞非除去 E-RFC depleted 又は E-RFC enriched 細胞群を注入したアガロースプレート試料孔周囲に透明で円形の溶血域が形成された．両細胞群から貪食細胞を除いて遊走させると溶血域は全く形成されなくなった．また顆粒球や培養液のみを注入した試料孔周囲にも溶血域は形成されなかった．

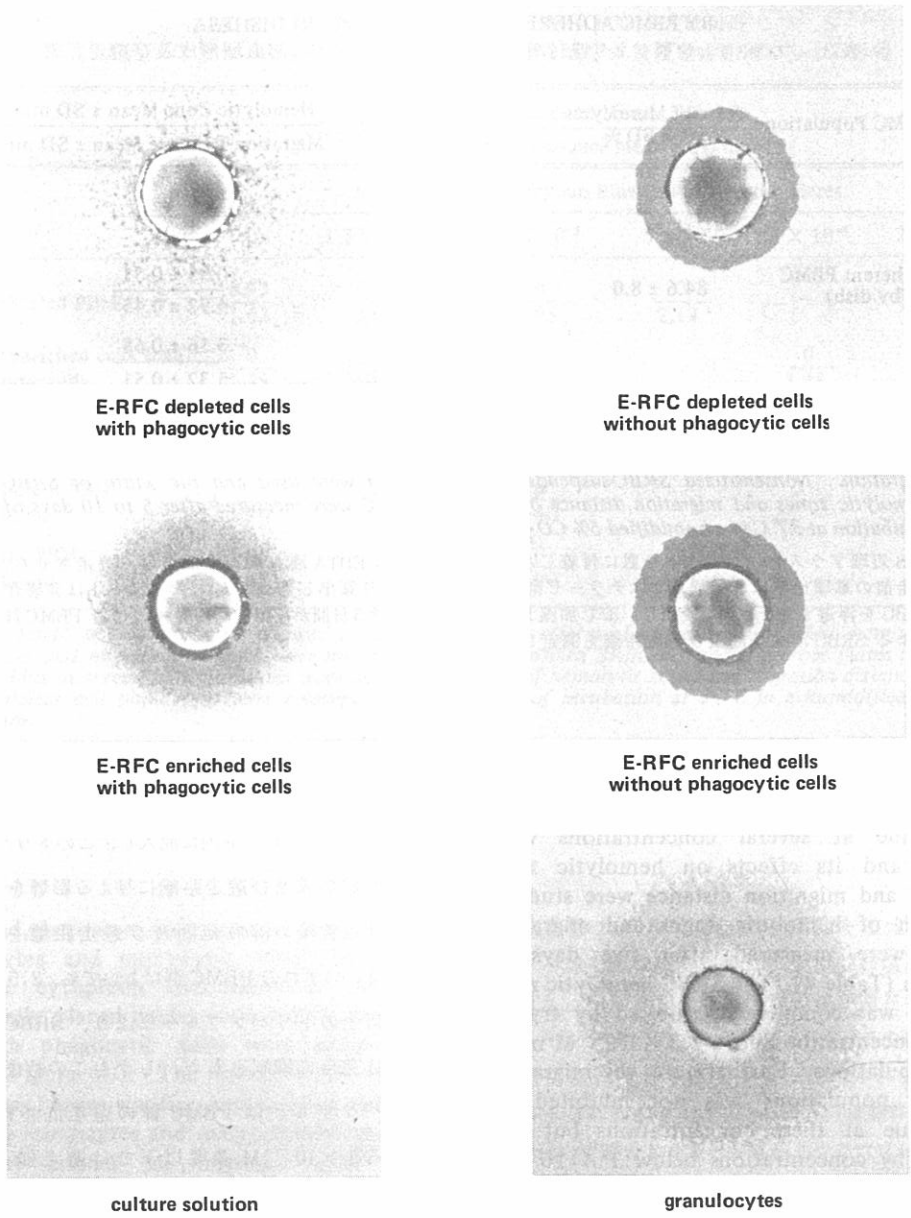


Figure 3. Characteristics of migrated PBMC populations after five days of incubation

The agarose layer in Figure 2 was removed, and migrated cells were stained. E-RFC depleted cells with phagocytic cells migrated best. The migration distance of each cell population decreased by elimination of phagocytic cells, especially in E-RFC depleted cell preparation. Granulocytes migrated fairly well.

図 3　5日間培養後遊走 PBMC 群の特性

図2のアガロース層を除去し，遊走細胞を染色した．貪食細胞非除去 E-RFC depleted 細胞が最もよく遊走した．貪食細胞の除去により各細胞群の遊走距離が減少し，特に E-RFC depleted 細胞において顕著であった．顆粒球はかなりよく遊走した．

TABLE 3 SRBC HEMOLYTIC ZONE FORMATION AND MIGRATION DISTANCE BY PBMC ADHERENT TO PLASTIC PETRI DISHES

表3　プラスチック製ペトリ皿付着 PBMC 群による SRBC 溶血域形成及び遊走距離

PBMC Population	Rate of Monocytes Mean ± SD %	Incubation Time (days)	Hemolytic Zone Mean ± SD mm / Migration Distance Mean ± SD mm
Adherent PBMC (by dish)	84.6 ± 8.0	5	0 / ND
		7	0.95 ± 0.51 / 4.92 ± 0.45
		10	3.56 ± 0.68 / 5.32 ± 0.51

PBMC adherent to FCS-treated plastic petri dishes were stripped by 0.2% EDTA solution and were allowed to migrate. The rate of monocytes was determined by nonspecific esterase stain before migration. Nonsensitized SRBC-suspended agarose plates were used and the width of SRBC hemolytic zones and migration distance by adherent PBMC were measured after 5 to 10 days of incubation at 37°C in a humidified 5% CO_2 incubator.

FCS 処理プラスチック製ペトリ皿に付着した PBMC を0.2% EDTA 液を用いてはがし，遊走させた．遊走前の単球比率は非特異的エステラーゼ染色することにより算出した．アガロースプレートは非感作 SRBC を浮遊させたものを使用し，37℃ 加湿 5 % CO_2 恒温器で 5 日間から10日間培養後の付着 PBMC 群による SRBC 溶血範囲及び遊走距離を測定した．

Effects of Trypan Blue on SRBC Hemolytic Zone Formation and Migration Distance by PBMC Populations. Agarose plates containing trypan blue at several concentrations were prepared and its effects on hemolytic zone formation and migration distance were studied. The width of hemolytic zones and migration distance were measured after five days of incubation (Table 4). The SRBC hemolytic zone formation was completely inhibited by trypan blue at concentration above 2.5×10^{-5} M in all PBMC populations. Furthermore, the migration of PBMC populations was not inhibited by trypan blue at these concentrations but was enhanced by concentrations below 1×10^{-4} M and the migration distance was increased. However, the migration distance was decreased at concentrations above 2×10^{-4} M.

トリパンブルーの SRBC 溶血域形成及び PBMC 群の遊走距離に与える影響． 種々の濃度のトリパンブルーをアガロースプレート内に混入し，このトリパンブルーの溶血域形成及び遊走距離に与える影響を検討した．5日間培養後の溶血範囲及び遊走距離を測定した（表4）．いずれの PBMC 群においても，2.5×10^{-5}M 濃度以上のトリパンブルーにより，SRBC の溶血域形成は完全に抑制された．しかもこの程度の濃度のトリパンブルーは PBMC 群の遊走能を抑制せず，むしろ 1×10^{-4}M 濃度以下では遊走能を刺激し，遊走距離が長くなった．しかし，2×10^{-4}M 濃度以上では遊走距離は短かくなった．

Microscopic Findings of Migrated PBMC Populations. The migrated PBMC populations were stained with M-G after five days of incubation (Figure 4). Most of the migrated cells, when E-RFC enriched cells with phagocytic cells were allowed to migrate, were lymphocytes but a few lymphoblastoid transformed cells were seen (Figure 4A). Some monocytes having elongated and eccentrically placed nuclei and phagocytized small and pyknotic cells were also seen. On the

遊走 PBMC 群の顕微鏡的所見． PBMC 群を 5 日間遊走後 M-G で染色した（図4）．貪食細胞非除去 E-RFC enriched 細胞群を遊走させた場合の遊走細胞のほとんどはリンパ球であり，一部に幼若化した細胞が見られた（図4A）．また，細長い核が偏在し，小さな核濃縮細胞を貪食した単球が見られた．一方，貪食

TABLE 4 EFFECTS OF TRYPAN BLUE ON SRBC HEMOLYTIC ZONE FORMATION AND MIGRATION DISTANCE BY PBMC POPULATIONS

表4　トリパンブルーの PBMC 群による SRBC 溶血域形成及び遊走距離に与える影響

PBMC Population	Hemolytic Zone Mean ± SD mm / Migration Distance Mean ± SD mm Concentration of Trypan Blue (M) of Agarose Plates					
	0	1.3×10^{-5}	2.5×10^{-5}	5×10^{-5}	1×10^{-4}	2×10^{-4}
Nonseparated PBMC	0.93 / 1.52	0.87 / 1.97	0 / 1.95	0 / 2.12		
E-RFC enriched cells with Phagocytic cells	0 / 1.23				0 / 1.38	0 / 0.83
E-RFC depleted cells with phagocytic cells	1.50 / 4.50				0 / 5.00	0 / 2.30
Nonadherent PBMC (by column)	0 / 1.10		0 / 1.40			0 / 0.95
Adherent PBMC (by column)	1.05 / 4.65		0 / 5.90			0 / 4.15

Several PBMC populations were allowed to migrate, and the effects of trypan blue on SRBC hemolytic zone formation and migration distance were investigated. Nonsensitized SRBC-suspended agarose plates containing trypan blue at several concentrations were used. The width of hemolytic zones and migration distance by each mononuclear cell population were measured after five days of incubation at 37°C in a humidified 5% CO_2 incubator.

種々の PBMC 群を遊走させ，トリパンブルーの SRBC 溶血域形成及び遊走距離に与える影響を調べた．アガロースプレートは種々の濃度になるようにトリパンブルーを混入し，非感作 SRBC を浮遊させたものを用いた．37℃ 加湿 5 % CO_2 恒温器で 5 日間培養後のそれぞれの単核細胞群による溶血範囲及び遊走距離を測定した．

other hand, there were many macrophages besides lymphocytes and monocytes which had large basophilic cytoplasm and round or elliptical eccentrically placed nuclei when E-RFC depleted cells with phagocytic cells were allowed to migrate (Figure 4B). The nuclear chromatin of these macrophages was fine and nucleoli could be seen. No monocytes and macrophages could be seen and almost all migrated cells were lymphocytes when E-RFC enriched or depleted cells without phagocytic cells were allowed to migrate, and pyknotic cells and cell debris were increased (Figure 4C, D).

Double staining of nonspecific esterase and chloroacetate esterase, and acid phosphatase staining were performed on E-RFC enriched and depleted cells with phagocytic cells after five days of incubation (Figure 5). Some monocytes with eccentrically located nuclei and phagocytized pyknotic cells appeared when E-RFC enriched cells with phagocytic cells were allowed to migrate and these cells showed high nonspecific

細胞非除去 E-RFC depleted細胞を遊走させると，リンパ球や単球細胞以外に，大型で好塩基性の胞体を有し，円形若しくは楕円形の核が偏在するマクロファージが多く存在した（図 4 B）．この細胞の核網構造は繊細であり，核小体が認められる．E-RFC enriched細胞群や E-RFC depleted細胞群から貪食細胞を除いて遊走させると，単球やマクロファージは全く観察されなくなり，遊走細胞のほとんどはリンパ球であり，濃染した核を有する細胞や細胞屑が増加している（図 4 C，D）．

貪食細胞非除去 E-RFC enriched細胞群及び E-RFC depleted細胞群の 5 日間遊走後の細胞に，非特異的エステラーゼとクロロアセテートエステラーゼの二重染色及び酸性フォスファターゼ染色をほどこした（図 5）．貪食細胞非除去 E-RFC enriched細胞群を遊走させた場合，核が偏在し，核濃縮細胞を貪食した単球が出現したが，この細胞は非特異的エステラーゼ

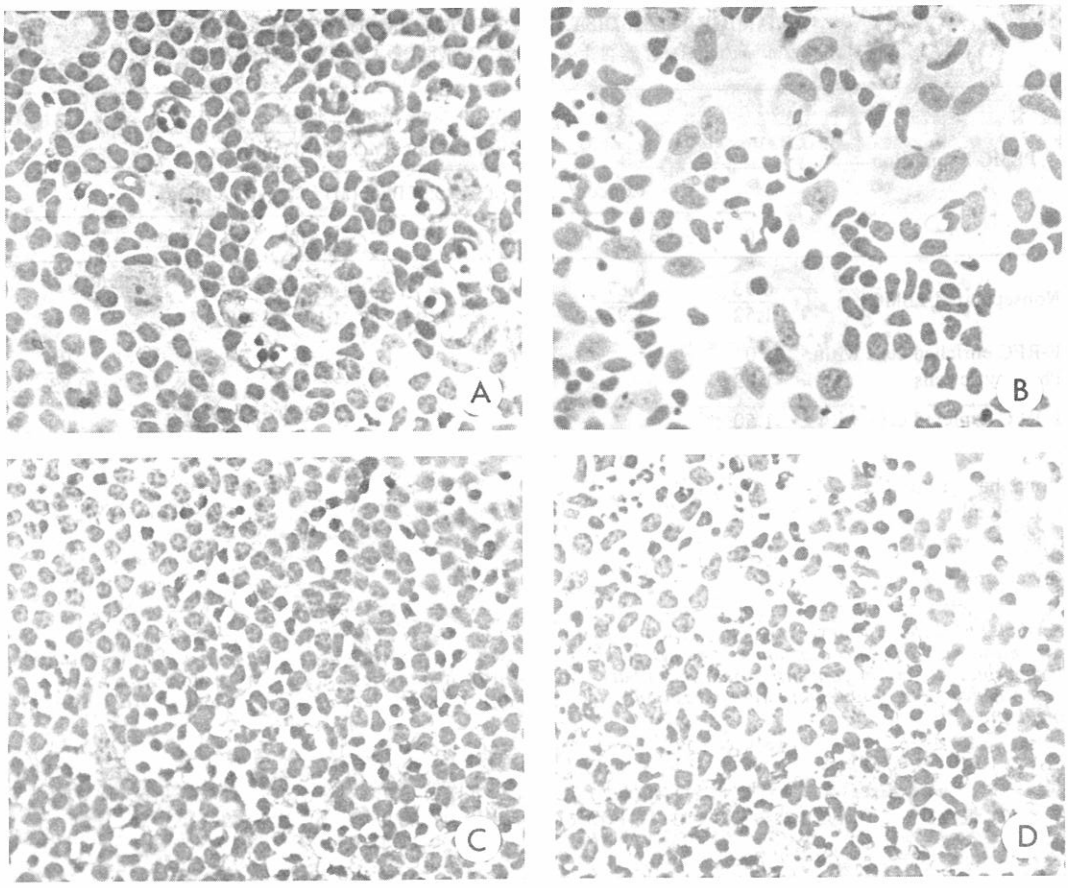


Figure 4. Microscopic findings of migrated PBMC populations

Nonsensitized SRBC-suspended agarose plates were used, and migrated cells were fixed with buffered formalin acetone after five days of incubation. M-G stain. ×200. A:E-RFC enriched cells with phagocytic cells. The migrated cells are mostly lymphocytes with a few blastoid transformed cells. Some monocytes which have elongated and eccentrically placed nuclei with phagocytized pyknotic cells considered to be degenerated lymphocytes can also be seen. B: E-RFC depleted cells with phagocytic cells. There are many macrophages besides lymphocytes and monocytes, which have wide and basophilic cytoplasm with round or elliptical eccentrically placed nuclei. The unclear chromatin of these macrophages are comparatively fine and have nucleoli. C: E-RFC enriched cells without phagocytic cells. Almost all migrated cells are lymphocytes and no monocytes or macrophages can be seen. There are many pyknotic cells considered to be degenerated lymphocytes. D:E-RFC depleted cells without phagocytic cells. Almost all migrated cells are lymphocytes and no monocytes or macrophages can be observed. There are a large number of pyknotic cells and cell debris.

図 4　遊走した PBMC 群の顕微鏡所見

非感作 SRBC 浮遊アガロースプレートを使用し，5日間培養後に遊走細胞を緩衝ホルマリンアセトンで固定した．M-G 染色．×200．A：貪食細胞非除法 E-RFC enriched 細胞群．リンパ球が遊走細胞のほとんどを占め，少数の幼若化したリンパ球が見られる．細長い核が偏在し，濃染した核を有する変性リンパ球らしき物を貪食した単球と思われる細胞も見られる．B：貪食細胞非除去 E-RFC depleted 細胞群．リンパ球や単球様細胞以外に，胞体が好塩基性で広く，円形若しくは楕円形の核が偏在するマクロファージが多数見られる．このマクロファージの核網構造は比較的繊細であり，核小体を有している．C：貪食細胞除去 E-RFC enriched 細胞群．リンパ球が遊走細胞のほとんどを占め，単球やマクロファージは見られない．濃染した核を有する変性リンパ球らしき物が多い．D：貪食細胞除去 E-RFC depleted 細胞群．遊走細胞のほとんどはリンパ球であり，単球やマクロファージは見られない．濃染した核を有する細胞や細胞屑が極めて多い．

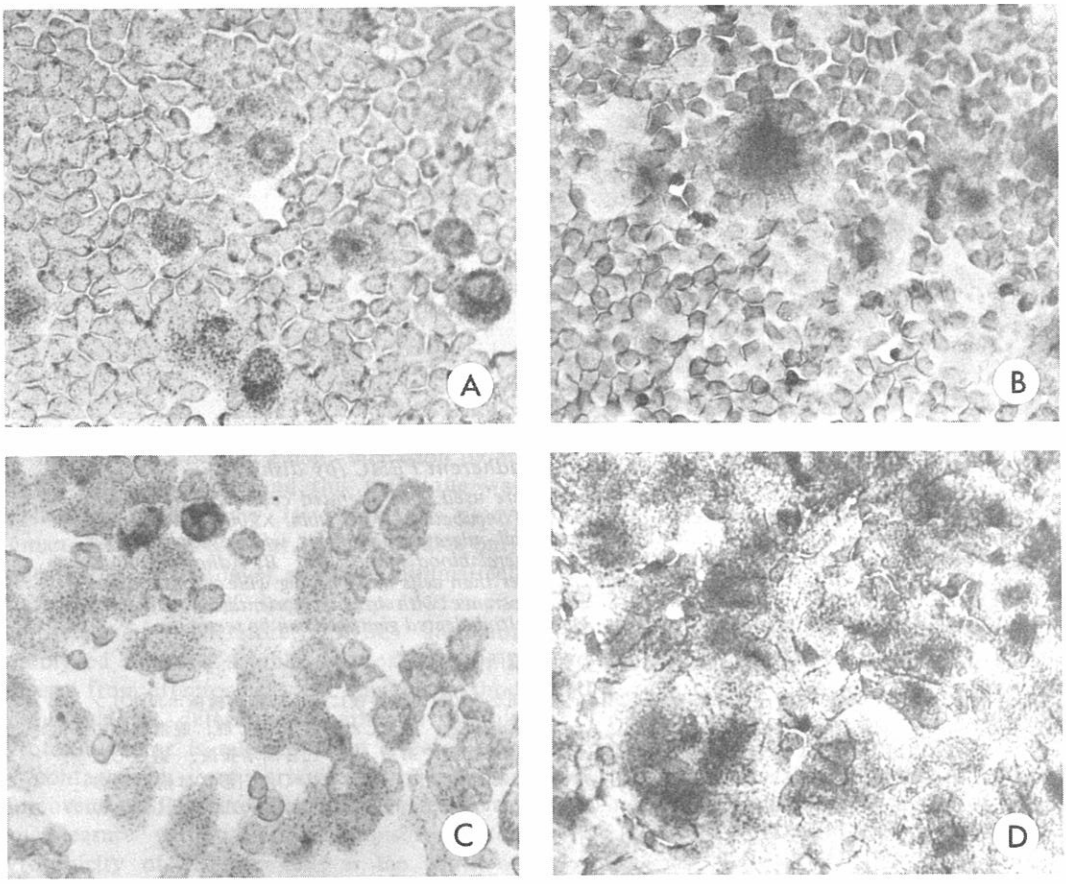


Figure 5. Microscopic findings of migrated PBMC population

Nonsensitized SRBC-suspended agarose plates were used, and migrated cells were fixed with buffered formalin acetone after five days of incubation. Double stains with nonspecific esterase and chloroacetate esterase (A, B) and acid phosphatase stain (C, D). ×200. A:E-RFC enriched cells with phagocytic cells. There are some monocytes which show high nonspecific esterase activity mixed with many lymphocytes. B: E-RFC depleted cells with phagocytic cells. Macrophages also show high nonspecific esterase activity, indicating that macrophages are derived from monocytes. There are a few granulocytes having a high chloroacetate esterase activity. C: E-RFC enriched cells with phagocytic cells. Monocytes show high acid phosphatase activity. D:E-RFC depleted cells with phagocytic cells. The acid phosphatase activity of macrophages is higher than that of monocytes and its activity is also demonstrated outside the cells.

図 5　遊走した PBMC 群の顕微鏡所見

非感作 SRBC 浮遊アガロースプレートを使用し，5日間培養後に遊走細胞を緩衝ホルマリンアセトンで固定した．非特異的エステラーゼとクロロアセテートエステラーゼの二重染色（A, B）及び酸性フォスファターゼ染色（C, D）．×200．A：貪食細胞非除去 E-RFC enriched 細胞群．非特異的エステラーゼ活性の高い単球が多くのリンパ球にまじって観察される．B：貪食細胞非除去 E-RFC depleted 細胞群．マクロファージの非特異的エステラーゼ活性も高く，単球由来の細胞であることを示す．クロロアセテートエステラーゼ活性の高い顆粒球が少数見られる．C：貪食細胞非除去 E-RFC enriched 細胞群．単球の酸性フォスファターゼ活性は高い．D：貪食細胞非除去 E-RFC depleted 細胞群．マクロファージの酸性フォスファターゼ活性は，単球の活性より更に高く，胞体の外側にも活性が見られる．

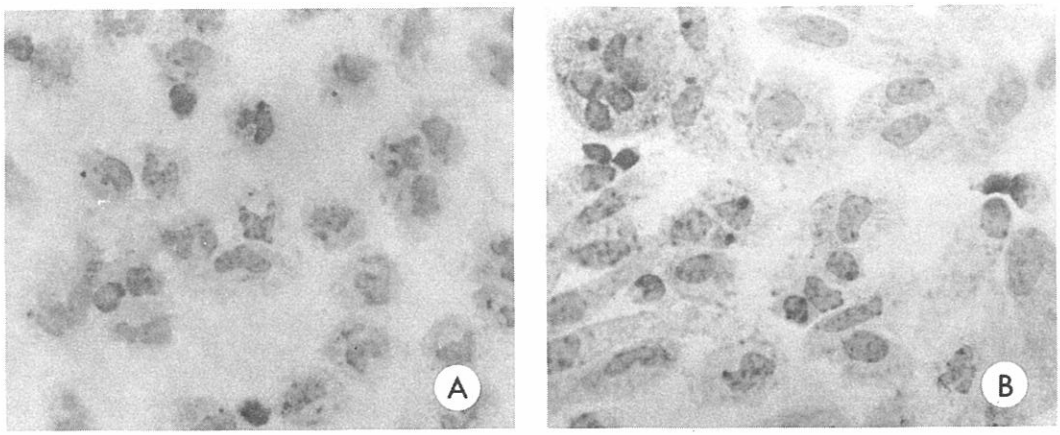


Figure 6. Microscopic findings of migrated adherent PBMC (by dish).

Nonsensitized SRBC-suspended agarose plates were used, and migrated cells were fixed with buffered formalin acetone after 5 to 10 days of incubation. M-G stain. ×200. A: Adherent PBMC after five days of incubation. Almost all migrated cells have wide cytoplasm and irregular-shaped nuclei, and are similar to peripheral blood monocytes. B: Adherent PBMC after 10 days of incubation. These cells are larger than cells in A, having wide cytoplasm and round or elliptical nuclei showing a tailed appearance with irregular orientation and were thought to have changed to macrophages. One multinucleated giant cell can be seen.

図 6　遊走したペトリ皿付着 PBMC 群の顕微鏡所見

非感作 SRBC 浮遊アガロースプレートを使用し，5日間から10日間培養後に遊走細胞を緩衝ホルマリンアセトンで固定した．M-G 染色．×200．A：5日間培養後，遊走した付着 PBMC 群．遊走細胞のほとんどは胞体が広く不整形の核を有した末梢血単球様細胞である．B：10日間培養後，遊走した付着 PBMC 群．これらの細胞は A の細胞より大きな細胞であり，胞体は広く，円形又は楕円形の核を有し，不規則指向性を示した尾部を有している．このような細胞はマクロファージに変化したものと思われる．多核巨細胞が1個観察される．

esterase activity (Figure 5A). Macrophages observed in migrated E-RFC depleted cells with phagocytic cells of Figure 4B also showed high nonspecific esterase activity, indicating that these cells were of monocytic origin (Figure 5B). The acid phosphatase activity of these migrated monocytes and macrophages was very high, the activity of macrophages being extremely high (Figure 5C, D). Since the acid phosphatase activity was also found outside the cells, lysosomal enzymes may have been released from these cells.

Adherent PBMC (by dish) were stained with M-G after 5 to 10 days of incubation (Figure 6). Adherent PBMC (by dish) incubated for five days were small, had irregular shaped nuclei, and were similar to peripheral blood monocytes (Figure 6A). In contrast, adherent PBMC (by dish) incubated for 10 days were larger, had a tailed appearance with random orientation, and were thought to have changed to macrophages (Figure 6B).

活性が高かった（図 5A）．また，図 4B の遊走した貪食細胞非除去 E-RFC depleted 細胞群にみられるマクロファージも同じように非特異的エステラーゼ活性が高く，単球由来の細胞であることを示す（図 5B）．これらの単球及びマクロファージの酸性フォスファターゼ活性は極めて高く，特にマクロファージの活性は著しく高い（図 5C, D）．酸性フォスファターゼ活性は，これらの細胞の外側にも見られ，リソゾーム酵素がこれらの細胞から分泌されたことを示すものと思われる．

ペトリ皿付着 PBMC 群の 5 日及び10日間培養後 M-G 染色をした（図 6）．5 日間培養後の付着 PBMC は小型であり，不整形の核を有し，末梢血の単球とよく似ている（図 6A）．これに対し10日間培養した付着 PBMC 群は大きく，不規則指向性を示した尾部を有し，マクロファージに変化したと考えられる（図 6B）．

DISCUSSION

Radioisotope release assay is the most frequently used method to determine cytotoxicity of PBMC.[10-19] Several other methods are used such as determination of target cell viability after assay by eosin Y and trypan blue,[20-22] measurement of target cell proliferation after assay by counting,[23] and staining.[24,25] However, almost all these assays are conducted in liquid culture medium with effector cells and target cells in direct contact with each other. Therefore, cytotoxicity test of culture supernatant of these assays must be repeated to determine whether the soluble cytotoxic factor participates in the destruction of target cells.

We have studied human PBMC migration by an agarose plate method, and this technique was applied to cytotoxicity test. In this assay, human PBMC as effector cells migrate only beneath the agarose layer and have minimal cell contact with the SRBC targets suspended in the agarose layer. Therefore, SRBC are not hemolyzed unless a soluble cytotoxic factor is released from effector cells and thus this method is suitable to determine whether a soluble cytotoxic factor is released from effector cells by contact with some parts of the target cells. Moreover, as SRBC are used as target cells, we can measure without using radioisotopes the cytotoxicity of effector cells as the width of hemolytic zones around the wells of agarose plates. Since human PBMC as effector cells migrate on the surface of plastic petri dishes, the morphology and enzyme activity of effector cells after assay can be adequately examined by staining.

This assay was used to determine whether the destruction of target cells in ADCC was mediated by a soluble cytotoxic factor. Since ADCC is mediated not only by IgG fraction but also by IgM fraction of antitarget cell antibody,[26] IgG and IgM fractions of anti-SRBC antibody-sensitized SRBC were suspended in agarose plates. Nonsensitized SRBC-suspended agarose plates were also used as control. As no SRBC hemolysis could be observed after 3 to 16 hours of incubation which is the suitable response time of ADCC,[2,26] the participation of a soluble cytotoxic factor in target cell destruction in ADCC could not be suspected. However, SRBC close to the wells of agarose plates in which PBMC populations were placed began to be hemolyzed after 24 to 48 hours of incubation,

考　察

PBMC による細胞障害能を測定する方法として，現在最もよく利用されているのはラジオアイソトープ放出法である.[10-19] その他，エオジンYやトリパンブルーによって assay 後の標的細胞の生存率をみる方法,[20-22] 標的細胞の増殖数を数える方法,[23] 染色して標的細胞の増殖の度合いを見る方法[24,25] などがある．しかし，これらの方法はすべて液体培地を用いて行われており，標的細胞と effector 細胞を直接接触させてある．そのため，標的細胞破壊の過程に可溶性細胞障害因子が関与しているか否かを明らかにするためには，再度その上清を用いて細胞障害能の測定を行う必要がある．

我々はヒト PBMC の遊走能をアガロースプレート法を用いて検討しているが，この方法を細胞障害能の測定に応用した．この方法では，effector 細胞であるヒト PBMC はアガロース層の下を遊走するため，アガロース層内に浮遊している SRBC 標的細胞とはごく一部分としか接触しない．そのため可溶性細胞障害因子が effector 細胞から放出されなければSRBC は溶血せず，一部の標的細胞との接触によって effector 細胞から可溶性細胞障害因子が放出されるか否かを決定するのに適している．また，標的細胞として SRBC を使用しているため，ラジオアイソトープを使用せず細胞障害能をアガロースプレート試料孔周囲の溶血範囲として測定することができる．effector 細胞であるヒト PBMC はプラスチック製ペトリ皿の表面を遊走するため，染色することにより assay 後の effector 細胞の形態や酵素活性を十分検討することができる．

我々はこの方法を用いて，ADCC における標的細胞の破壊が可溶性細胞障害因子によってなされているかどうかを明らかにしようと試みた．ADCC は抗標的細胞抗体の IgG 成分のみならず，IgM 成分によってももたらされるため,[26] 抗 SRBC 抗体の IgG 成分又は IgM 成分で感作した SRBC をアガロースプレート内に浮遊させた．また，コントロールとして非感作 SRBC を浮遊させたアガロースプレートも用いた．ADCC の反応時間とされる 3～16時間の培養では SRBC の溶血は全く観察されず,[2,26] ADCC における標的細胞の破壊には可溶性細胞障害因子は関与していないように思われた．しかし，培養24～48時間目ころから PBMC 群を注入したアガロースプレート試料孔周囲の

and formation of clear and concentric hemolytic zones could be grossly observed. This phenomenon was observed also in agarose plates with suspended nonsensitized SRBC as control. These results demonstrate that the hemolysis of SRBC occurred by the action of a soluble cytotoxic factor released from PBMC directly against SRBC, and not indirectly through anti-SRBC antibody.

PBMC populations without phagocytic cells were used as effector cells to determine the origin of effector cells which release a soluble factor having cytotoxic activity against SRBC. Though hemolytic zones were formed by E-RFC enriched cells with phagocytic cells (monocytes 7.8%), no hemolytic zones were formed by E-RFC enriched cells without phagocytic cells (monocytes 1.2%). In the same manner, large hemolytic zones were formed by E-RFC depleted cells with phagocytic cells (monocytes 32.1%), but hardly any hemolytic zones were formed by E-RFC depleted cells without phagocytic cells (monocytes 3.8%). In the experiment using PBMC populations separated by nylon wool column, no hemolytic zones were formed by nonadherent PBMC (by column, monocytes 1.3%), but evident hemolytic zones were formed by adherent PBMC (by column, monocytes 18.5%). These results indicate that the effector cells which release a soluble cytotoxic factor against SRBC are phagocytic and adherent PBMC and are monocytes. However, there is a possibility that true effector cells are PBMC other than monocytes with monocytes serving merely as helpers. Then, we harvested PBMC adherent to FCS-treated plastic petri dishes by stripping with 0.2% EDTA and these adherent PBMC (by dish) were used as effector cells. They were composed of monocytes 84.6%, lymphocytes 6.1%, and granulocytes 9.3%, showing a fairly high percentage of monocytes. The migration activity of these adherent PBMC was extremely good with their migration distance being the longest among several PBMC populations. However, no SRBC hemolysis was observed after five days of incubation when these adherent PBMC were allowed to migrate, and large and clear hemolytic zones were formed following 2 to 5 additional days of incubation. The formation of hemolytic zones by adherent PBMC composed of a fairly high percentage of monocytes supports the view that true effector cells are monocytes. We have confirmed that guinea pig peritoneal macrophages

SRBCも溶血し始め，肉眼で観察できる明瞭な同心円状の溶血域が形成された．この現象は，コントロールとして用いた非感作 SRBC を浮遊させたアガロースプレートにおいても観察された．このことから，SRBC の溶血は抗 SRBC 抗体を介してではなく，PBMC から放出される可溶性細胞障害因子が直接 SRBC に作用して起こったものと思われる．

この SRBC に対し，細胞障害作用を有する可溶性因子を放出する effector 細胞が何であるかを明らかにするため，PBMC 群から貪食細胞を除いたものを effector 細胞として検討した．貪食細胞を除く前の E-RFC enriched 細胞群（単球混入率7.8%）では溶血域が形成されたが，貪食細胞を除いた後の E-RFC enriched 細胞群（単球混入率1.2%）では全く形成されなくなった．同じように貪食細胞を除く前の E-RFC depleted 細胞群（単球混入率32.1%）では大きな溶血域が形成されたが，貪食細胞を除いた後の E-RFC depleted 細胞群（単球混入率3.8%）では溶血域がほとんど形成されなくなった．

またナイロンウールカラムで分離した PBMC 群を用いた実験では，非付着 PBMC 群（単球混入率1.3%）では全く溶血域が形成されず，付着 PBMC 群（単球混入率18.5%）では明瞭な溶血域が形成された．このような結果から，SRBC を溶血させる可溶性細胞障害因子を放出する effector 細胞は貪食能を有し，付着性のある PBMC，つまり単球であると思われた．しかし，単球は補助的な役割を果たしているのみで，真の effector 細胞は別の PBMC である可能性が残る．そこで，FCS 処理プラスチック製ペトリ皿に付着する PBMC を 0.2% EDTA を用いてはがし，この付着 PBMC 群を effector 細胞として用いた．この付着 PBMC 群の細胞構成は，単球84.6%，リンパ球6.1%，顆粒球9.3%であり，かなり高率に単球を集めることができた．この付着 PBMC 群の遊走能は極めて良好であり，種々の PBMC 群の中では最も遊走距離が長かった．しかしこの付着 PBMC 群を遊走させた場合 5 日間の培養期間では SRBC は全く溶血せず，更に引き続き 2 日～5 日間の培養の後に大きく明瞭な溶血域が形成された．このように単球がかなり高率な付着 PBMC 群においても溶血域が形成されたことは，真の effector 細胞が単球であることを支持するものと思われる．我々はまた，モルモットの腹腔マクロファージも SRBC を溶血させる可溶性の

also release a soluble cytotoxic factor to hemolyze SRBC.

There are many reports on the cytotoxicity of monocytes and macrophages, but most of these use animal peritoneal macrophages as effector cells with only a few using human peripheral blood monocytes as effector cells. Moreover, in most of these studies cytotoxicity tests have been performed after stimulation or activation of monocytes and macrophages with lipopolysaccharide (LPS),[12,19,27] BCG,[27,28] *Corynebacterium liquefaciens*,[22] interferon,[29,30] and macrophage activating factor (MAF) containing lymphokine,[30,31] and the cytotoxic activity of these cells before activation is reported to be very weak or negative. These stimulants were not added in our assay, but human peripheral blood monocytes released a soluble cytotoxic factor to hemolyze SRBC spontaneously.

There are reports that cytotoxic activity of monocytes and macrophages have been observed without adding any stimulants. Human PBMC become spontaneously cytotoxic against a broad range of erythrocytes after 6 to 7 days of culture and effector cells are adherent and have phagocytic activity, suggesting that they are monocytes.[14] Peritoneal macrophages of mice and rats also become cytotoxic against tumor cells without stimulation after 48 hours of incubation[32] or after a latent period of 12 to 20 hours.[33] It thus appears that a latent period is needed for monocytes and macrophages to adequately exhibit cytotoxic activity without stimulants. In our experiment it took 24 to 48 hours of incubation to form hemolytic zones when PBMC populations containing many lymphocytes and some monocytes were allowed to migrate and more than five days of incubation when adherent PBMC (by dish) containing few lymphocytes and many monocytes were allowed to migrate. The reason why SRBC were hemolyzed after a shorter latent period when PBMC containing many lymphocytes were allowed to migrate may be that monocytes were probably stimulated or activated by MAF released from lymphocytes. However, MAF is hardly released when adherent PBMC (by dish) containing few lymphocytes are allowed to migrate and this may result in taking a long time to hemolyze SRBC. It has been shown recently that serum[34] and reagents[25] are contaminated by LPS. Since we used HS to prepare agarose culture plates, contamination of LPS can be

細胞障害因子を放出することを確認している.

単球，マクロファージ系の細胞障害能の研究については多くの論文があるが，その多くは実験動物の腹腔マクロファージを effector 細胞として用いており，ヒト末梢血単球を用いて実験を行った論文は少ない．また，多くの研究は lipopolysaccharide (LPS)[12,19,27] や BCG,[27,28] *Corynebacterium liquefaciens*,[22] インターフェロン,[29,30] リンホカインを含むマクロファージ activating factor (MAF)[30,31] 等で単球又はマクロファージを刺激したり活性化して細胞障害能を検討しており，活性化されていないものは細胞障害能がないか極めて弱いとされている．我々はこのような刺激物を全く添加しなかったが，ヒト末梢血単球は spontaneous に SRBC を溶血させる可溶性の細胞障害因子を放出した．

このように刺激物の添加なしでも，単球又はマクロファージに細胞障害能があるとする報告はほかにもある．ヒト PBMC は 6～7 日間培養することにより，多くの種類の赤血球に対し spontaneous に細胞障害性を有するようになり，これらの細胞は付着性で貪食能を有していることから単球であると考えられている.[14] また，マウスやラットの腹腔マクロファージも無刺激で48時間培養後に腫瘍細胞に対し細胞障害性を示すようになることや,[32] 12～20時間の潜伏期の後に細胞障害性を示すようになることが報告されている.[33] このように，無刺激で単球又はマクロファージが細胞障害性を十分に発揮するためには，幾らかの潜伏期が必要であるようである．我々の実験においても，多くのリンパ球と多少の単球を含む PBMC 群の遊走では 24～48時間の培養後に溶血域が形成され，ごく少量のリンパ球と多くの単球を含むペトリ皿付着 PBMC 群の遊走では，5 日間以上の培養後に溶血域が形成された．リンパ球の混入が多い PBMC 群を遊走させた場合，短時間の潜伏期の後に SRBC が溶血した理由として，リンパ球より放出されたかもしれない MAF によって単球が刺激又は活性化された可能性がある．しかし，少量のリンパ球を含むペトリ皿付着 PBMC 群を遊走させた場合，MAF はほとんど放出されず，このために SRBC の溶血に長時間を要したのかもしれない．近年，血清[34] や試薬[25] 内に LPS が混入していることが明らかにされている．我々は培養液の添加血清として HS を使用しているため，LPS の混入は十分に

expected. This contaminated LPS and mechanical stimulation permitting migration on the surface of plastic petri dishes may play an important role in the stimulation or activation of monocytes, and consequently in release of a cytotoxic factor from monocytes.

考えられる．このような混入したLPSやプラスチック製ペトリ皿に付着して遊走するという物理的な刺激も，単球の刺激又は活性化，ひいては単球からの可溶性障害因子の放出に重要な役割を果たすかもしれない．

A great deal of discussion has been made on the question of whether the soluble cytotoxic factor participates in target cell destruction by monocytes or macrophages. In the experiment using mice peritoneal macrophages as effector cells and mice red blood cells as target cells, loss of cytotoxic activity by separating effector cells and target cells with a millipore filter or dialysis membrane was reported.[15,16] Lack of cytotoxic activities in culture supernatants of activated mouse peritoneal macrophages[19,29] and human peripheral blood monocytes[35] has also been reported, and it has been accepted that a soluble cytotoxic factor does not participate in target cell destruction by monocytes and macrophages.[36] However, contrary to the foregoing, there are some studies which report of release of a soluble cytotoxic factor from monocytes or macrophages consistent with our results. Release of a soluble cytotoxic factor against mice red blood cells was observed in an experiment using syngenic peritoneal macrophages as effector cells.[10] Such a soluble cytotoxic factor has also been demonstrated in experiments using peritoneal macrophage of guinea pigs[20,21] and rats.[11,14] Furthermore, cytotoxic activity in culture supernatant of established macrophage cell lines has also been reported.[13] There is hardly any report on the release of a soluble cytotoxic factor from human peripheral blood monocytes.

単球，マクロファージによる標的細胞破壊に可溶性細胞障害因子が関与しているか否かについては，数多くの論議がなされてきた．マウスの腹腔マクロファージをeffector細胞とし，マウスの赤血球を標的細胞とした実験において，両者をmillipore filterや透析膜で隔絶することにより細胞障害性が発揮されなくなったとする報告がある．[15,16] また，活性化したマウス腹腔マクロファージ[19,29]やヒト末梢血単球[35]の培養上清には細胞障害性がないことが報告されており，単球やマクロファージによる標的細胞破壊には可溶性細胞障害因子は関与していないとされてきた．[36] しかしこれに対し，我々の実験結果のごとく，単球あるいはマクロファージから可溶性細胞障害因子が放出されるとする報告もある．マウスの腹腔マクロファージをeffector細胞とした実験において，可溶性細胞障害因子がsyngenicなマウスの赤血球に対して放出されることが明らかにされている．[10] また，このような可溶性細胞障害因子はモルモット[20,21]やラット[11,14]の腹腔マクロファージを用いた実験においても証明されている．更に，マクロファージ培養株化細胞の培養上清にも細胞障害能があるとする報告もある．[13] しかし，ヒト末梢血単球からも可溶性細胞障害因子が放出されるとする報告はほとんど見られない．

We studied the possibility that the soluble cytotoxic factor released from human peripheral blood monocytes might be a lysosomal enzyme. As trypan blue is well known to inhibit the activity of lysosomal enzymes,[37] loss of cytotoxic activity of rat peritoneal macrophages collected after intraperitoneal injection of trypan blue in rats[22] and decrease of cytotoxic activity of activated mice peritoneal macrophages by treatment with 4.2×10^{-4} M trypan blue[24] have been demonstrated. The mechanism of target cell destruction by guinea pig peritoneal macrophages has been studied electromicroscopically, showing that macrophages exhibit cytotoxic activity by contacting with target cells and translocating lysosomal organelles into the cytoplasm of target

我々は，ヒト末梢血単球から放出される可溶性細胞障害因子がリソゾーム酵素の一つである可能性について検討した．トリパンブルーはリソゾーム酵素の活性を抑制する物質として知られており，[37] トリパンブルーを腹腔内に注入して集めたラット腹腔マクロファージには細胞障害性がないことや，[22] 活性化したマウス腹腔マクロファージを4.2×10^{-4} M濃度のトリパンブルーで処理することにより細胞障害性が低下することが報告されている．[24] モルモットの腹腔マクロファージによる標的細胞障害の機序を電顕的に調べると，マクロファージが標的細胞と接触し，リソゾームの内容物を標的細胞の細胞質内に注入させる

cells.[28] Trypan blue readily enters the macrophage vacuolar system by pinocytosis, mixes with the content of the secondary lysosomes and inhibits the activity of lysosomal enzymes, which ultimately inhibits macrophage-medicated cytotoxicity.[36,38] We prepared agarose plates containing trypan blue at several concentrations and investigated its effect on hemolytic zone formation. SRBC hemolytic zone formation was completely inhibited by trypan blue at concentrations exceeding 2.5×10^{-5} M, but the migration of PBMC populations was not inhibited by trypan blue at these concentrations, and surprisingly it was enhanced and the migration distance was extended. These results may suggest that the soluble cytotoxic factor spontaneously released from monocytes to hemolyze SRBC is a lysosomal enzyme.

ことによって細胞障害性を発揮することが認められている.[28] この際，トリパンブルーは嚥飲運動によりマクロファージの液胞系に容易に入りこみ，二次リソゾームの内容と混合し，リソゾーム酵素活性を抑制するため細胞障害性が低下するとされている.[36,38] 我々はトリパンブルーをアガロースプレート内に種々の濃度になるよう混入し，その溶血域形成に与える影響を検討した．その結果，2.5×10^{-5} M 濃度以上のトリパンブルーにより，SRBC 溶血域形成は完全に抑制された．しかしこの程度の濃度のトリパンブルーは PBMC 群の遊走能を抑制せず，驚くべきことにむしろ刺激し，遊走距離は長くなった．このような結果から，単球から spontaneous に放出され，SRBC を溶血させた可溶性細胞障害因子はリソゾーム酵素の一つであると考えられる．

High acid phosphatase activity, a marker enzyme of lysosomal enzymes in activated macrophages, has been demonstrated in mice[27] and rats.[22] Increase of neutral protease secretion in activated macrophages has also been disclosed.[39] In our experiment, we found many monocytes and macrophages which showed high acid phosphatase activity in 5-day incubated E-RFC enriched and depleted cells, and acid phosphatase activity was also found outside the cells. Hemolytic zones were more prominent in E-RFC depleted cells than in E-RFC enriched cells, and likewise, cells having a high acid phosphatase activity were also abundant in E-RFC depleted cells. These cells were not found when PBMC without phagocytic cells were allowed to migrate and also hemolytic zones around the wells of agarose plates were not formed. Coincidence of the appearance of cells showing high acid phosphatase activity with the formation of hemolytic zones also suggests that the soluble cytotoxic factor is a lysosomal enzyme.

活性化したマクロファージは，リソゾーム酵素のマーカー酵素である酸性フォスファターゼ活性が高いことがマウス[27]やラット[22]において認められている．また，活性マクロファージ中の中性プロテアーゼの分泌が亢進することも明らかにされている.[39] 我々の実験においても，5日間培養後の E-RFC enriched 細胞群及び E-RFC depleted 細胞群に，極めて酸性フォスファターゼ活性の高い単球又はマクロファージを多数確認し，また酸性フォスファターゼ活性は細胞の外側にも見られた．溶血域は E-RFC enriched 細胞よりも E-RFC depleted 細胞に多く観察された．同様に，酸性フォスファターゼ活性が高い細胞も E-RFC depleted 細胞中に多く見られた．また，PBMC 群から貪食細胞を除いて遊走させると，このような細胞は全く観察されなくなり，アガロースプレート試料孔周囲の溶血域も全く形成されなくなった．酸性フォスファターゼ活性の高い細胞の出現と，溶血域の形成が一致していたことも，可溶性細胞障害因子がリソゾーム酵素の一つであることを示唆する．

One of the reasons why such a cytotoxic factor was difficult to identify is inactivation of the cytotoxic factor by adding serum such as FCS.[10,11] There are some reports which suggest that neutral proteases released from mouse peritoneal macrophages are the cytotoxic factor, but this factor is also readily inactivated by supplemented FCS and cannot be detected at a common FCS concentration in culture fluid.[40,41] Though the culture plates were supplemented with 20% HS of relatively high concentration, the soluble cytotoxic factor was detectable. This might be due to the peculiarity of our assay

このような可溶性細胞障害因子が同定されにくい原因の一つとして，添加する FCS 等の血清による障害因子の非働化が考えられている.[10,11] マウス腹腔マクロファージから分泌される中性プロテアーゼが細胞障害因子である可能性が報告されているが，この細胞障害性は添加する FCS により極めて抑制されやすく，通常の培養液中の FCS 濃度では同定されないとしている.[40,41] 我々は培養プレートに20%とかなり高濃度に HS を添加したが，それにもかかわらず可溶性細胞障害因子を同定することができた．この理由は我々の実験方法の特殊性にあると思われる．

method in which the soluble cytotoxic factor released from monocytes is continuously consumed by SRBC targets suspended in agarose plate, and consequently the total volume of this factor measured is quite large. Another reason is that, because the culture condition of our assay was good, we could preserve monocytes as effector cells in good viability for more than 10 days and long assay time could be employed.

我々の方法では，単球から放出される可溶性細胞障害因子は常にアガロースプレート内 SRBC に作用し続ける訳であり，最終的には非常に大量の細胞障害因子を測定することになる．また，この分析の培養条件が良好であるため，effector細胞である単球を10日以上にわたって生存率のよい状態に保つことができるため，分析時間を長時間とれることにあると思われる．

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