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CHEMICAL AND PHYSICAL FACTORS OF FIBERS ON ENZYME LEAKAGE AND MORPHOLOGICAL CHANGES OF GRANULOCYTES ADHERING TO FIBERS^{*1}

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We have evaluated the compatibility of various fibers as the leukocytes separators by the quantification of the amounts of enzymes which were leaked from adhering granulocytes to fibers. When granulocytes adhered to synthetic and natural fibers, changes in the shape of adhering cells and leakage of lysosomal enzymes were observed, depending on the chemical and physical structure of fibers. However, morphological changes and enzyme leakage were not always correlative. Physical factors such as the diameter and the shape of fibers were important. These changes increased as the diameter of fibers decreased. They could be suppressed by coating the surface of fiber with albumin.

INTRODUCTION

In most transfusion, patients need a specific component of blood instead of whole blood. Recently, the blood component therapy is widely used in transfusion. As the result, the establishment of a cell separation system for such purpose is desired at blood centers¹⁾.

The authors have been developing a cell separator of human leukocytes for the preparations of (1) granulocytes and leukocyte-poor erythrocytes for transfusion and of (2) purified lymphocytes for the production of interferon and transfer factor. A new system, using various synthetic fibers, is applied to the development of cell separation on the basis of the so-called adherence chromatography. This methodology has already been applied to filtration leukapheresis for granulocyte transfusion²⁾. It has been, however, pointed out³⁾⁻⁷⁾ that this is inferior to the centrifugation method in regard to the functional damage of the collected cells, although there are many merits in this method, such as the high recovery and high purity of granulocytes. Therefore, the authors have been trying to overcome this defect by coat-

ing the fibers with albumin or gelatin. It was reported in the previous paper¹⁾ that by using protein-coated fibers, improvements in the recovery and selectivity of separation of granulocytes from human leukocytes have been achieved. In this paper, the compatibility of various synthetic fibers as cell separator was evaluated by the quantification of the amounts of leaked lysosomal enzymes which were contained in azurophil or specific granules in cytoplasm. In addition, the relationship between enzyme leakages and the morphological changes of the adhering cells was discussed. Furthermore, natural fibers like silk and wool were also evaluated in comparison with the protein-coated fibers.

MATERIALS AND METHODS

Quantification of The Leakage of Lysosomal Enzymes. Various fibers were packed into a plastic syringe (5 ml, Terumo Co., Ltd.) so as to keep a total surface area of approximately 120 cm². This shows enough capacity for adhering of charged granulocytes to the fibers. Hank's medium (NaCl 137 mM, KCl 5.4 mM, Na₂HPO₄ 0.4 mM, KH₂PO₄ 0.4 mM, NaHCO₃ 0.4 mM, glucose 5.6 mM, CaCl₂ 1.0 mM, MgCl₂ 1.0 mM, pH 7.4) was passed through the column in order to eliminate completely air bubbles in the column. Granulocytes, isolated from human blood of healthy donors by Ficoll-Conray method⁸⁾, had the purity

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and the viability of granulocytes in the collected cells, 95% and 98%, respectively. The granulocytes were suspended in Hank's medium at a cell density of 10^7 cells/ml. This cell suspension (2 ml) was charged into the fiber column and then incubated 30 min at 37°C. After the incubation, the amounts of lysosomal enzymes such as β -glucuronidase, lysozyme and lactic dehydrogenase (LDH) which had leaked out into the medium were quantified by the usual methods: β -glucuronidase was colorimetrically measured with p-nitrophenyl- β -glucuronide as substrate⁹⁾, lysozyme was measured by the turbidimetric rate of lysis of *Micrococcus lysodeikticus*¹⁰⁾, and LDH was measured by the rate of the decrease of NADH in the reduction of pyruvate¹¹⁾. The enzyme leakage is indicated by percentage to the total enzyme activity leaked by 0.2% of Triton X-100.

Scanning Electron Micrographs (SEM) of Granulocytes Adhering to Fibers. After the removal of the medium from the fibers as described in the preceding section, the granulocytes adhering to the fibers were treated with 1.5 wt% glutaraldehyde in 0.1 M cacodylate buffer containing 1% sucrose, pH 7.4, at room temperature for 2 h and further fixed with 1 wt% osmium tetroxide in 0.1 M phosphate buffer containing 4% sucrose, pH 7.4, at 4°C for 1 h. Then, the fixed cells were dried by gradual dehydration with a mixture of ethanol and water and additionally by critical-point drying for SEM observation¹²⁾.

Fibers. Synthetic and natural fibers which were offered by Mitsubishi Rayon Co., Ltd., were washed with ethanol and sufficiently with distilled water before use. The diameters of the fibers used were measured by SEM. A surface area of fibers was calculated on the assumption that the fibers are cylindrical.

Coating of Fibers with Albumin. The fibers packed in a syringe were dipped in Hank's medium containing 1 wt% of human serum albumin (HSA) for 3 h at room temperature. Then, a large amount of Hank's medium was passed through the syringe. HSA was isolated from pooled plasma by the Cohn method¹³⁾ at this center.

RESULTS AND DISCUSSION

The Enzyme Leakage and The Morphological Changes of Granulocytes Adhered to Fibers.

Granulocytes contain two kinds of granules in cytoplasm, azurophil and specific granules, in which many enzymes participating in bactericidal function are involved¹⁴⁾. In this work, β -glucuronidase and lysozyme, contained in azurophil and specific granules, respectively, were determined as a marker which indicates the functional damage of granulocytes¹⁾, when they adhere to fibers. In addition, LDH was also measured as a marker of the damage of plasma membranes¹⁾. The order of the degree of enzyme leakage for three kinds of lysosomal enzymes by adhering to the fibers was as follows: lysozyme > LDH $\gg$ β -glucuronidase. β -glucuronidase showed a very low leakage (max. 4%) at 37°C up to 120 min. The phenomenon that the amount of lysosomal enzyme which is leaked from granulocytes adhered to fibers is different between azurophil and specific granules, has already been reported by Wright et al.¹⁵⁾ and it is also found in phagocytosis of microorganisms¹⁶⁾. When a granulocyte is allowed to adhere for 5 min to nylon 6 at 37°C, pseudopod radiate from the spreading cell, as the cell flattens itself on the fiber surface. A cell after 30 min contact with the fibers fully spread out to a flattened hemisphere. In a granulocyte plated onto nylon 6 for 60 min, the cytoplasm is already withdrawn from the periphery. However, although the morphological changes of granulocytes adhered to the fibers were thus marked, the enzyme leakage, especially the leakage of LDH, was not so extensive.

Effect of Chemical Structure of Fibers. Fig. 1 and Fig. 2 show the degrees of leakage of lysozyme and LDH, and the morphologies of granulocytes adhering to various fibers for 30 min at 37°C. These observations show: (1) In the case of hydrophilic fibers such as cellulose (rayon and acetate) and vinylon, the enzyme leakage and the morphological change are relatively low. (2) In the case of polypropylene, the leakage of lysozyme and the morphological change are relatively low in spite of the highest leakage of LDH among the fibers used here. It is thus suggested that the degree of the leakage of lysosomal enzymes does not always correspond to the extent of the morphological change of adhering granulocytes. (3) In the case of nylon fibers, the degrees of both the leakage of lysozyme and morphological change

Fig. 1 Effects of chemical structure of fibers on enzyme leakage. The open and closed columns indicate the degrees of leakage of lysozyme and LDH, respectively. Enzyme leakage was determined at 30 min after adhesion to fiber at 37°C. The diameters of fibers (μm) used here were polyester (12.4), nylon 6,6 (13.7), nylon 6 (13.7), rayon (11.9), acetate (12.7), vinylon (12.9), polypropylene (15.3), and polyacrylonitrile (13.0).

Fig. 2 Shapes of granulocytes adhering to various fibers 30 min after adhesion to fibers at 37°C. The fibers used here were the same as in Fig. 1.

are the highest. (4) In the case of acryl fibers, the degrees of the leakage of both LDH and lysozyme are relatively low, and in addition, the shape of adhering cells is mostly round.

In addition, more than 90% of granulocytes which were charged into the adherent column adhered to all fibers used in this experiment under the conditions described in the preceding section so that the difference in the degree of enzyme leakage was not due to the difference in the degree of adhesion of granulocytes to fibers. Moreover, there was no apparent loss of activities of the enzymes during the incubation period with fibers.

As described above, fibers having hydrophilic surface like cellulose or vinylon do not strongly damage granulocytes but have a low effect on the recovery and purity of granulocytes¹⁾. There appears a contrary correlation in the separation of granulocytes by synthetic fibers between the capability of selective separation and functional integrity as indicated by lysosomal enzyme leakage. Moreover, these morphological changes indicate the degree of detachment of adhered cells as reported in our previous paper¹⁾.

Effect of Protein Coating of Fibers. Fig. 3 shows a comparison of enzyme leakage and the shape of adhering cells between HSA-coated fibers (nylon 6) and natural fibers (wool and silk). The HSA coating inhibited the leakage of lysozyme and LDH to a significant extent and also offered a suppression effect on morphological changes comparatively. Wool has a scale-like surface structure as shown in the SEM pictures of Fig. 3, and there appeared a tendency that granulocytes preferentially adhered to the border areas between the scales by SEM observations of about 500 cells. There were observed both round and spread cells on the different surface areas. The degrees of the enzyme leakage of wool were almost the same as those of non-coated nylon 6. In the case of silk which has an elliptical cross section of fiber, pseudopod radiated from adhering granulocytes along the axis of the fibers, and the degree of enzyme leakage was significantly lower than those of wool and HSA-coated nylon 6. In addition, the degree of adhesion of granulocytes to silk or wool was as high as that to nylon 6 or to HSA-coated nylon 6. The differences in the morphology of adhering cells and enzyme leakage between wool

Fig. 3 Effects of protein coating of fibers on enzyme leakage and the morphological change of granulocytes 30 min after adhesion to fibers at 37°C. The notations are the same as in Fig. 1.

and silk may be due to (1) the difference in such chemical factors as the structure of the proteins (keratin and fibroin) and/or due to (2) the difference in such physical factors as the shape of the fibers (diameter, shape of cross section, surface structure and others).

Effect of Diameter of Fibers. It was found in Fig. 4 that the degrees of enzyme leakage and the morphological changes increased as the diameter of acrylic fibers decreased. As shown in the electron micrograph of granulocytes adhered to the fiber of 3.8 μm in diameter, it seems as if granulocytes phagocytosed the fibers. An observation similar to these results has been reported¹⁷⁾: when granulocytes phagocytose, the rate of phagocytosis is greatly dependent on the size of the particles which are taken into the cytoplasm of granulocytes. It is thus shown that both morphological change and enzyme leakage are not only dependent on the chemical factors of fibers but also on their physical factors, such as the diameter and the shape of the fibers. Latter factors are very important in the cell interaction.

CONCLUSION

The following points for the evaluation of fibers as a leukocyte separator should be checked: (1) recovery (degrees of adhesion and detachment), (2) selectivity of separation of granulocytes and lymphocytes from leukocytes, (3) morphology of adhering cell, (4) leakage of lysosomal enzymes and (5) functional integrity.

In the markets, 1.5 or 3.0 denier nylon (Leukopak®, Fenwal, Morton, Grove, III.) or polyester (Leukalum®, Terumo Co., Ltd.) fiber columns are commercially available for granulocytes separation. However, functions and viability of collected granulocytes are not so good as mentioned in this paper. Finally, we recommend

Fig. 4 Effects of diameter of acrylic fibers on enzyme leakage and morphological change of granulocytes 30 min after adhesion to fiber at 37°C. The notations are the same as in Fig. 1.

the HSA-coated fibers which have a diameter over 25 μ m.

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ADDITION

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顆粒球の粘着形態および酵素漏出に及ぼす繊維の物理・化学的要因 細胞粘着法による血球分離(II)

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著者らは、繊維に粘着した顆粒球からの漏出酵素を定量することにより、白血球分離用としての繊維の適合性を評価した。顆粒球は種々の合成繊維や天然繊維への粘着に伴い、形態変化が起こり、細胞内から種々の酵素が漏出した。これらの変化は、繊維の化学構造や物理的形状により異なり、また、粘着形態変化と酵素漏出量は、

必ずしも対応しなかった。繊維の形状は細胞との相互作用には、重要であり、繊維の太さが、顆粒球より小さい程、形態変化と酵素漏出量は共に増加した。ヒト血清アルブミンをコートした繊維は、細胞分離を高く維持しながら、細胞の機能保持にも効果が認められた。