Action mechanism of leukocytes by special reagents for automated leukocyte differentiation (DIFF channel)

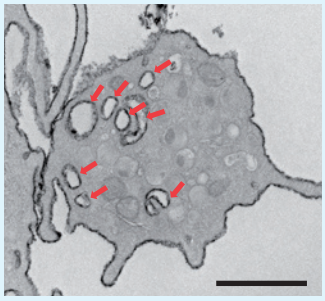
The Cell Analysis Center – Scientific Bulletin Part 5

Stromatolyser-4DL (surfactant), a special reagent used for automated leukocyte differentiation on the XE Series, forms ultramicroscopic pores[1] on the leukocyte surface. Another special reagent, Stromatolyser-4DS (fluorescent dye), rapidly permeates inside the leukocyte through ultramicroscopic pores and efficiently stains the organelles[2] and nucleic acids [1]. In Scientific Bulletin Part 4, [8] scanning an electron microscope image of ultramicroscopic pores formed on a leukocyte surface by the action of Stromatolyser-4DL and a confocal laser scanning microscope image of a leukocyte fluorescently stained by Stromatolyser-4DS were shown [1]. This time, we could confirm that the ultramicroscopic pores observed through scanning on an electron microscope are not merely 'pits', but rather they are 'holes' connected to the outer part of the cell by using a special staining method and observing through a transmission electron microscope. Also, by performing time-lapse staining[3] of cells with Stromatolyser-4DS, the relationship between staining and scattergram[4] was investigated in more detail.

Electron microscope image of a cell that uses ruthenium red[5] (e.g. platelet)

Ruthenium red does not have cell membrane permeability[6]. If the chemical fixation of a cell is done using a reagent which includes ruthenium red, the substance gets attached to the cell surface. Then, during the process of preparing the sample for an electron microscope, it binds with osmium tetroxide[7]. In the cellular specimen prepared during this process, an image of a cell membrane stained with thick black borders can be obtained [1] (Refer to the figure in the left below).

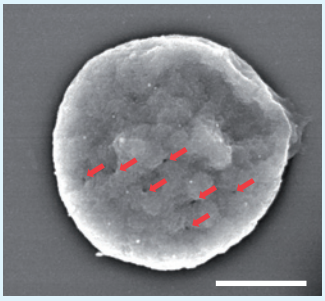
Platelet stained with ruthenium red (Transmission electron microscope image)



In the platelet treated by the fixative solution that includes ruthenium red, it can be observed that the cellular surface was covered with black borders. Inside the platelet, there is a structure known as an open canalicular system[8] connected with the outer part of the cell [3]. By observing through a transmission electron microscope, we found that the inner side of the open canalicular system was also stained with ruthenium red ( ).

Bar=2μm

Surface structure of a platelet (Scanning electron microscope image)



If the surface structure of a platelet is observed by scanning electron microscope, the apertural area of the open canalicular system can be conformed ( ).

Bar=2μm

memo

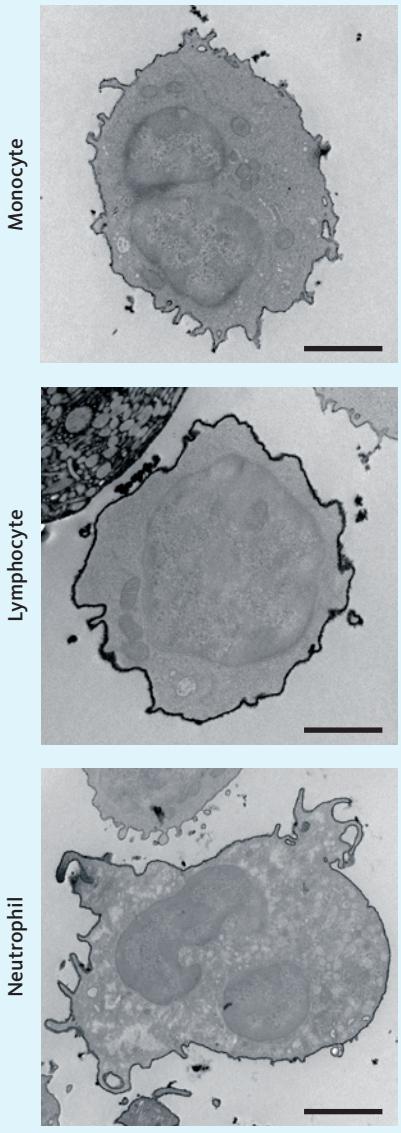
Minute particles appearing as white dots on the platelet surface are colloidal gold[9] of diameter around 10 nm. This platelet is immunostained with colloidal gold labeled with CD41[10] antibody. White dots show the localisation[11] of CD41.

Validation of leukocyte cell membrane permeation by Stromatolyser-4DL using ruthenium red

To clarify the cell membrane permeation mechanism of Stromatolyser-4DL (surfactant), morphological analysis of leukocytes by using ruthenium red was undertaken.

Inner parts of leukocytes were strongly stained with ruthenium red after treatment with a special reagent. This shows that the ultramicroscopic pores formed by the reagent are not merely 'pits', but rather they are 'holes' connected with the outer parts of a cell.

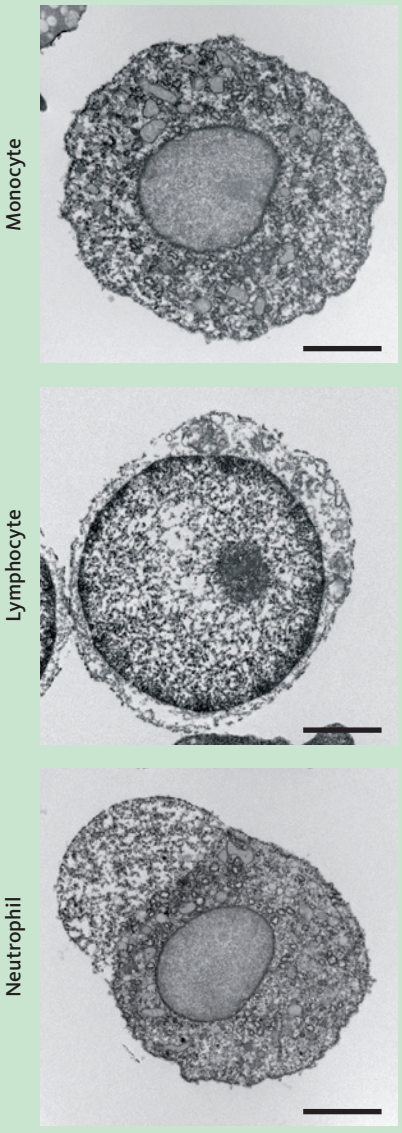
Leukocytes stained with ruthenium red before treatment with Stromatolyser-4DL
(transmission electron microscope image)



Ruthenium red does not have cell membrane permeability, therefore, prior to treatment with Stromatolyser-4DS, the leukocyte's cell surface was stained with thick black borders, but the inside of the cell was not stained.

Bar=2μm

Leukocytes stained with ruthenium red after treatment with Stromatolyser-4DL
(transmission electron microscope image)



Once the leukocyte was treated with Stromatolyser-4DL, ruthenium red entered into the cell and strongly stained the internal part of the cell. Apparently, Stromatolyser-4DL did not alter the cellular morphology significantly, but formed ultramicroscopic pores on the cellular surface.

Bar=2μm

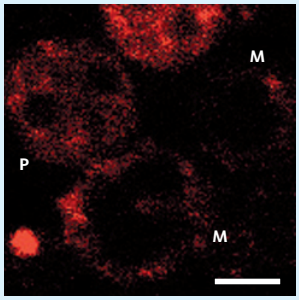
Time-lapse staining of cells by Stromatolyser-4DS

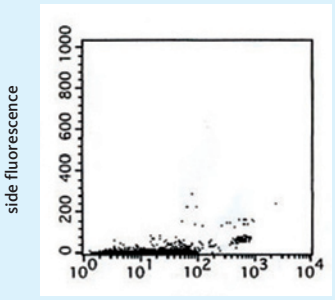
Stromatolyser-4DS (fluorescence dye) permeates through the ultramicroscopic pores formed on the leukocyte surface by Stromatolyser-4DL (Surfactant), and rapidly spreads inside the cell and stains the organelles, nucleic acid and nucleolus.

As a result of analysing the time-lapse staining of a leukocyte, the morphology of the cell corresponded with the side fluorescence on the scattergram.

Elapse Time

At the first stage of reacting with reagent

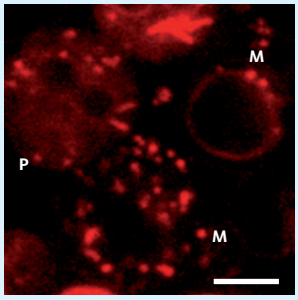


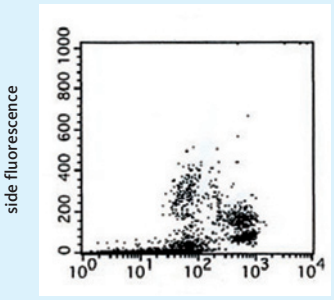


The leukocytes were almost unstained at the primary stage of reacting with the reagent (upper figure). Side flourescence (vertical axis) (lower figure) of cells could not be observed.

M: Mononuclear cells
P: Polynuclear cells
Diagram above: Confocal laser scanning microscope image
Diagram below: Scattergram from a general purpose flow cytometer

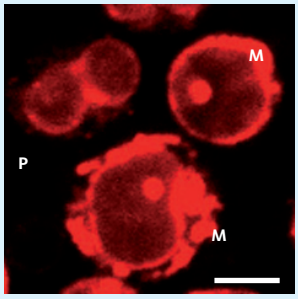
During the intermediate stage of reacting with reagent

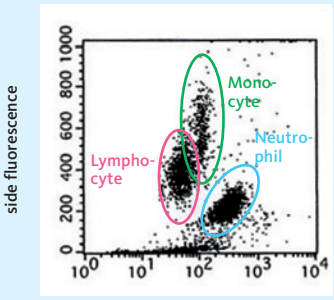




In the leukocyte image taken during the intermediate stage of reaction with reagent, it was observed that organelles were stained in mononuclear cells (M). In polynuclear cells (P), the entire cell was diffusely stained and organelles were stained as well (upper figure). In the scattergram, it could be evaluated that the side fluorescense of the individual cell had strengthened gradually (lower figure).

At the time of measurement in the analyser





In the cell image of mononuclear cells (M), the organelles, nuclei and nucleoli are strongly stained. In polynuclear cells, cell cytoplasm is diffusely stained, while nuclei are relatively strongly stained. (upper figure) In this phased-staining scattergram (DIFF scattergram[12]), distinctive cellular populations[13] are formed based on the strength of side fluorescence of leukocytes and the inner structure of cells.

Bar=2μm

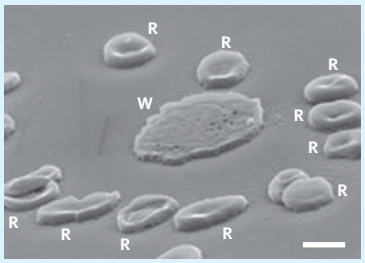
Action mechanism of special reagent on leukocytes based on morphological evidence [1]

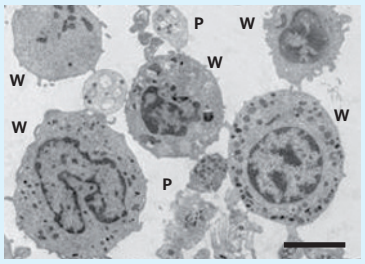
Inside automated haematology analysers, the reaction with special reagent and whole blood[14] is carried out quickly and accurately. Automated leukocyte differentiation is then performed.

Based on the morphological evidence, the action mechanism of automated leukocyte differentiation reagent is represented graphically.

Before the reaction with special reagent

Electron microscopy image of peripheral blood





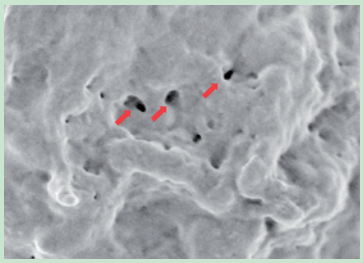
The figure above is the scanning electron microscope image and the figure below is the transmission electron microscope image of peripheral blood. This peripheral blood is treated with special reagent.

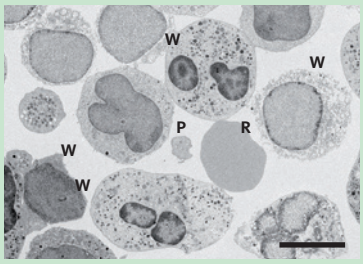
W: Leukocyte
R: Erythrocyte
P: Platelet

Bar=2µm

After the reaction with special reagent

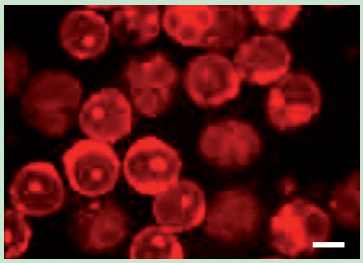
Change in ultramicro morphology by Stromatolyser-4DL (surfactant)

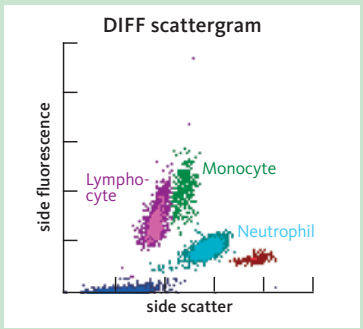




Stromatolyser-4DL acts on the leukocyte cell membrane and forms ultramicroscopic pores on the cellular surface (↗). Leukocyte treatment with special reagent does not destroy this outer formation significantly and the organelles in the cells remain they are.

DIFF scattergram and fluorescent staining image of a cell by Stromatolyser-4DS (fluorescent dye)





From the ultramicroscopic pores formed on the surface of leukocytes, Stromatolyser-4DS permeates inside the cell and stains the organelle, nucleic acid and nucleous. In the DIFF scattergram, the cellular population reflects the characteristics of a leukocyte after treatment with special reagent, and the automated differentiation is performed.

Bar=2µm

Terminology

1 Ultramicroscopic pore

Holes of the diameter 10–50 nm, formed on a leukocyte cell membrane by the action of Stromatolyser-4DL including a surfactant.

2 Organelle

Includes mitochondria, endoplasmic reticulum, Golgi apparatus etc.

3 Time-lapse staining

Refers to the process of continuously gathering the data of the ingestion of fluorescent dye in a cell using flow cytometer and confocal laser scanning microscope over time.

4 Scattergram

Two parameters graphical representation of optical information of cells collected with a flow cytometer. Physical and chemical characteristics of cells are presented [4].

5 Ruthenium red

Ruthenium red is a hexavalent cation. If a cell is processed with a fixative solution which includes this reagent, ruthenium red binds with the acid mucopolysaccharide on the cell surface. Ruthenium red attached with the cell surface binds with osmium tetroxide during the process of sample preparation for electron microscope. If this high electron density substance attached to blood cell surface is observed with a transmission electron microscope, you can see that the cellular surface is stained with black borders.

6 Ruthenium red does not have cell membrane permeability

Because ruthenium red can not pass through the cell membrane, cellular surface connected to the outside of the cell is stained with black borders. However, the inside of the cell is not stained.

7 Osmium tetroxide

It performs a chemical fixation of phospholipids (e.g. in a cell membrane) and some portions of unsaturated fatty acids. This reaction also results in a staining effect in electron microscopy.

8 Open canalicular system

An open canalicular system usually has 15–20 opening pores on the surface of a platelet and inside the cell. It forms a vascular system by changing the inner diameter and direction. This functionality is thought to be for quick granule release inside the platelet and quick fetching in of stimulating substances in deep parts inside the cell [3].

9 Colloidal gold

Gold particles (1–40 nm) used for immunoelectron microscopy. Gold with a high electron density is visible as black particles under a transmission electron microscope and as white particles under a scanning electron microscope.

memo

By observing the magnetic beads attached to a cell by MACS method, we showed that
- **magnetic beads can be conformed by electron microscope and**
- **it is possible to quickly locate an antigen [5], [6].**

10 CD41

CD41 appears in the platelet and megacaryocyte. It binds with CD61 and forms a receptor for fibrogen, fibronectrin, von Willebrand factor and vitronectin. CD41 is a molecule that works as a contact of cellular substratum of platelet and agglomeration of platelets [3].

11 Localisation

The location at which a substance is present.

12 DIFF scattergram

Cell poplulation analysis graph used in automated leukocyte differentiation functionality of XE Series automated haematology analysers [7].

13 Cellular population

Cells with similar characteristics are summarily described as populations. In a scattergram, they are depicted as signal clusters [4].

14 Reaction with special reagent and whole blood

In the automated leukocyte differentiation unit inside heamatology analysers, two types of reagents (Stromatolyser-4DL and 4DS) are mixed with the whole blood sample and incubated for 22 seconds at 35°C.

Reference

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