Identification of Sodium Alginate-induced Reddish Purple-stained Cells (ARPC) in Mice

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Abstract: *Cell surface marker analyses revealed that sodium alginate-induced reddish purple-stained cells (ARPC) expressed high levels of CD45 (common leukocyte antigen), F4/80, MHC class II antigen and c-kit but little Gr-1 and FceR. This expression pattern is the same as those of resident peritoneal macrophages and thioglycollate-induced macrophages (TG-macrophages), indicating that ARPC are macrophages. Mac-1 and Mac-2 expression patterns of ARPC were similar to TG-macrophages, but F4/80 expression was higher than that of TG-macrophages. In addition, the average size of ARPC is about 1.3 times as large as that of TG-macrophages. These results indicate that ARPC may be a distinct macrophage subset.*

Key words: *Mice, Cell surface, Reddish purple cells (ARPC), Macrophages.*

It has been reported that the mouse peritoneal cavity contains a set of hematopoietic cells such as lymphocytes, neurotrophils, eosinohilis, macrophages and mast cells. These cells always reside in the cavity, and are referred to as resident peritoneal cells. In previous experiments, intraperitoneal injection of sodium alginate in ddY mice resulted in a great accumulation of cells stained reddish purple with Wright/Giemsa, together with leukocytes including neutrophils, eosinophils, macrophages and lymphocytes. These cells were named alginate-induced reddish purple-stained cells (ARPC) (He et al., 2000).

Hematopoietic cells were identified by various methods including cytochemical staining, ultrastructure, function analysis and cell surface antigen analysis [6]. Cell surface antigen analysis is the most reliable and convenient way, if monoclonal antibodies to the antigens are available. In addition, as it is now possible to stain a cell with multiple monoclonal antibodies that are labeled with different fluorescent colors, cells that have no specific antigens can also be identified.

In previous experiments, it was shown that ARPC accumulated most 3 days after a 10 mg/mouse injection of sodium alginate irrespective of mouse strains (He et al., 2000). In this experiment, therefore, ARPC were identified by analyzing the cell surface antigens with flow cytometry. C57BL/6 mice were used in the present work because this strain is mostly used as a source of cell surface antigens to raise monoclonal antibodies.

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Materials and Methods

Reagents and media

Histopaque 1119 and thioglycollate broth were purchased from Sigma Chemical Co. (St. Louis, MO) and Difco Laboratories (Detroit, MI), respectively. Fetal bovine serum (FBS) was purchased from Bio-Whittaker Inc. (Walkersville, MD) and used after heat inactivation at 56°C for 30 min. Bovine serum albumin Fraction V (BSA) was a product of Boehringer-Mannheim (Mannheim, Germany). RPMI-1640 and phosphate buffered saline (PBS(–)) were obtained from Nissui Pharmaceutical Co. (Tokyo, Japan). Culture media used in this study and their abbreviations are as follows: RPMI-1640-H, RPMI-1640 buffered with 25 mM HEPES (pH 7.4); RPMI-1640-HG, RPMI-1640-H containing 0.1% gelatin (Bio-Rad); BSA-DAB, PBS(–) containing 0.1% BSA and 0.1% NaN_3.

Anti-mouse CD45, anti-mouse F4/80 and anti-mouse Mac-1 rat monoclonal antibodies were purchased from Serotec (Oxford, UK). Fluorescein isothiocyanate (FITC)-conjugated anti-mouse Gr-1 and anti-mouse Mac-2 rat monoclonal antibodies were obtained from Cedarlane Laboratories Ltd. (Ontario, Canada). FITC-conjugated

anti-mouse c-kit rat monoclonal antibody and FITC-conjugated anti-mouse major histocompatibility complex (MHC) class II antigen (I-Ab) mouse monoclonal antibody were purchased from PharMingen (San Diego, CA). Anti-dinitrophenol (DNP) mouse IgE was purchased from Seikagaku Corporation (Tokyo). Phycoerythrin (PE)-conjugated anti-mouse IgE rat monoclonal antibody was a product of Southern Biotechnology Associates, Inc. (Birmingham, AL). FITC-conjugated anti-rat IgG(H+L) goat polyclonal antibody (F(ab')2) which was previously absorbed by mouse serum by the manufacturer was purchased from Protos Immuno Research (San Francisco, CA) and used as the second antibody. Other basic processes for histological examinations were conducted according to routine methods [1].

Animals

Female C57BL/6 mice, 7 weeks old, were purchased from Seak Yoshitomi Ltd. (Fukuoka, Japan) and acclimated for a week to laboratory conditions at 22°C in a polycarbonate cage with wood shaving. During acclimation and the experiment, mice were fed with commercial pellets.

Collection of cells

Sodium alginate was dissolved in saline (0.85% NaCl) at 10 mg/ml and autoclaved. Thioglycollate broth (TG) was dissolved in saline at 3% and autoclaved. Mice were anaesthetized with ether and injected intraperitoneally with 1 ml of sodium alginate solution or with 2 ml of TG solution. Three days after the injection, mice were sacrificed under ether anaesthesia and 5 ml ice-cold RPMI-1640 containing 25 mM HEPES (RPMI-1640-H) was intraperitoneally injected. The abdomen was massaged for 30 sec and the peritoneal fluid was collected using a polypropylene syringe with a 21 gauge needle. The PEC was washed and suspended in 1 ml RPMI-1640-H. Resident peritoneal cells were obtained from non-treated mice in the same manner. For collecting connective tissue type mast cells (CTMC), resident peritoneal cells were layered over 2 ml Histopaque 1119 in a 15 ml centrifuge tube and centrifuged at 1,600 rpm for 20 min at room temperature. The pellet was washed, suspended in 1 ml of RPMI-1640-HG and used as CTMC suspension.

Flow cytometry analysis

The freshly prepared cell suspension was adjusted to 5 x 10^6 cells/ml. An aliquot (200 μl) was centrifuged at 1,000 rpm for 3 min at 4°C in a microcentrifuge tube using a swing rotor. The pellet was suspended in 200 μl of BSA-DAB containing monoclonal antibody at a concentration described in the manufacturer's protocol. The suspension was incubated on ice for 1 h. After incubation, the suspension was added with 1 ml of BSA-DAB, centrifuged at 1,000 rpm for 3 min at 4°C, and the pellet was washed twice with BSA-DAB. The pellet was then suspended in 200 μl of the second antibody and incubated on ice for 1 h. After incubation, the cells were washed as above, suspended in 1 ml of BSA-DAB and filtered through a 50 mesh nylon filter. For detection of Gr-1, c-kit and MHC class II, the second antibody was not used. IgE receptor (FceR) was detected with anti-DNP mouse IgE (10 mg/ml) followed by PE-conjugated anti-IgE. Flow cytometry was performed with EPICS ELITE ESP (Coulter Corporation, Miami, FL). The gating region of ARPC in forward scatter (FS) vs. sideward scatter (SS) profile was previously determined by Wright/Giemsa staining of the sorted cells. The gating regions of TG-macrophages and resident macrophages in FS vs. SS scattergram were F4/80-positive regions.

Results

Cell surface marker analysis of ARPC

Figure 1 summarizes results of the cell surface marker analyses. ARPC showed high forward scatter (FS) and sideward scatter (SS). ARPC expressed a high level of CD45 (leukocyte common antigen), F4/80 and MHC class II but little Gr-1 and FceR. F4/80 and MHC class II are markers of macrophages. ARPC expressed a significant level of c-kit which is one of the mast cell markers, though the level was lower than that of CTMC. However, both TG-macrophage and resident macrophages also expressed similar levels of c-kit to that of ARPC, indicating that c-kit expression is not specific to mast cells but macrophages also express c-kit. It was concluded from these results that ARPC are macrophages.

Expression of Mac-1 on the surface of ARPC was almost similar to that of TG-macrophages and a little higher than that of resident peritoneal macrophages. Mac-2 expression of ARPC was also similar to that of TG-macrophages but remarkably higher than that of resident peritoneal macrophages.

Discussion

In mammals, especially in humans and mice, the surface antigens of hematopoietic cells are identified by the use of monoclonal antibodies. Some of the antigens are used as marker proteins independently or in

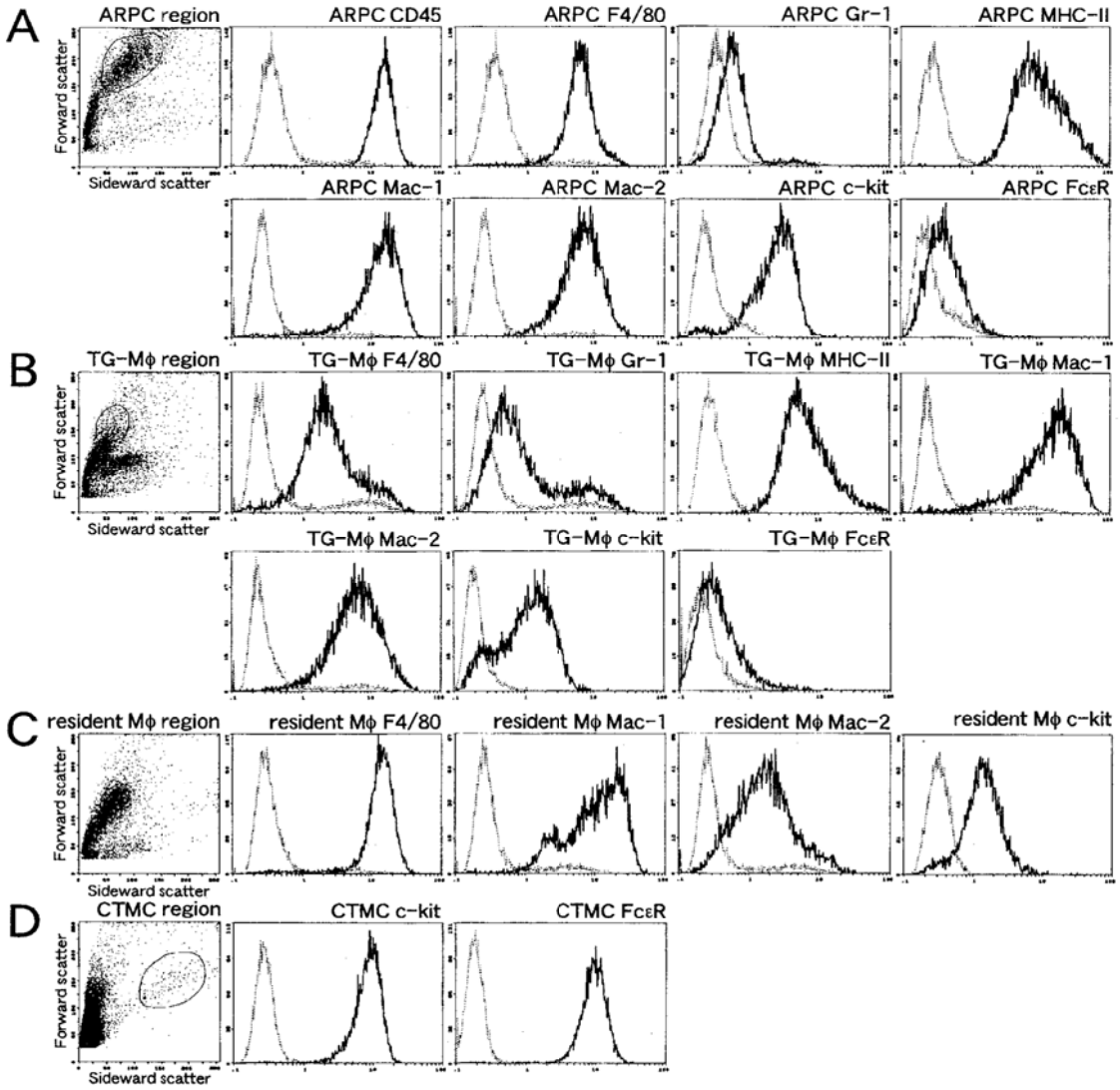

Fig. 1. Flow cytometry analysis of surface marker antigens on C57BL/6 mouse alginate-induced reddish purple-stained cells (ARPC). Peritoneal exudate cells 3 days after sodium alginate injection (10 mg/mouse) were individually stained with monoclonal antibody against one of the indicated markers. Gate for ARPC (A), thioglycollate-induced macrophages (TG-Mφ) (B), resident macrophages (C) or connective tissue type mast cells (CTMC) (D) were set in the forward scatter and sideward scatter histogram (the leftmost), and the frequency vs. fluorescence intensity profiles of the gated cells are shown on the right (solid lines). CTMC were used as the positive control for c-kit and IgE receptor (FcεR) staining. Control cells (dotted lines) for direct and indirect stainings were non-stained cells and cells stained only with FITC- or PE-conjugated second antibody, respectively. Control cells for FcεR staining were treated with IgE alone.

combination with other markers and this enables a cell to be identified. Murine macrophages are identified by the presence of F4/80 and MHC class II antigen [2]. The function of F4/80 is not clear yet. MHC class II antigen was originally identified as a major antigen which causes histocompatible rejection in tissue transplantation, and was later recognized to be a complex which binds to a phagocytosed and digested foreign peptide and presents it to T-lymphocytes on the cell surface [3]. ARPC express MHC class II antigen, indicating they have phagocytic activity and could be associated with the elimination of foreign substances. However ARPC have not been identified in inflammatory responses in mice. Further investigation of ARPC's functions is nec-

essary to clarify their role in the immune system.

ARPC expressed both Mac-1 and Mac-2 at similar levels to those of TG-macrophages, and these expressions were higher than those of resident macrophages. These results indicate that ARPC are distinct from resident macrophages and similar to cells which are mobilized in response to inflammatory phenomenon like TG-macrophages. Mac-1 antigen was originally identified as a macrophage marker but was later found to be expressed more intensively in neutrophils. Mac-1 is composed of CD11b and CD18 subunits and functions as a complement C3 receptor [4]. Mac-2 is a member of the S-lectin family which binds to galactose-containing ligands, and it has been characterized as being highly expressed on the surface of TG-macrophages [5].

Many similarities are found between ARPC and TG-macrophages, but there are also some differences. First of all, the cell sizes are clearly different. The average size of ARPC is about 1.3 times larger than that of TG-macrophages. The expression patterns of surface antigens on ARPC were almost similar to those of TG-macrophages, but that of F4/80 was different between these two macrophages. From these results it is indicated that ARPC might be a distinct subset of murine macrophage.

It was deduced from the results of sequential sampling of ARPC from the mesentery and peritoneal cavity of alginate-injected mice, that ARPC migrated to the peritoneal cavity via the mesentery. In both cases ARPC existed in the mesentery and in the peritoneal cavity. Vacuole-like organelles (VLO) in the cells became largest 12 h after sodium alginate injection, and thereafter they became smaller but increased in number, finally occupying the greater part of the cytoplasm. Electron microscopy revealed that VLO contained very small amounts of an electron-dense substance. The substance was stained reddish purple with Wright solution. ELISA using anti-alginate monoclonal antibody and uronic acid determination indicated that the substance is not phagocytosed sodium alginate but ARPC-derived cellular materials. It was also shown that VLO were not eosinophilic nor basophilic but stained with azure A and azure B, both of which are main constituents of Wright solution. It is necessary to ascertain whether the VLO are granules or not and to clarify their function and the significance of their formation.

In our previous study, injecting mice with sodium alginate which elicited dynamic migration of leukocytes in carp led to the indentification of a possible novel subset of macrophages [6].

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