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Cellular and Tissue Distribution of MRK20 Murine Monoclonal Antibody-defined 85-kDa Protein in Adriamycin-resistant Cancer Cell Lines*1

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A murine monoclonal antibody (MAb) specific to adriamycin-resistant K-562 (K-562/ADM) cells, MRK20, was found to react strongly with an 85-kDa protein present in K-562/ADM and adriamycin-resistant ovarian cancer (2780^{AD}) cells. This protein was present at only very low levels in parental cells (K-562 and A2780), methotrexate-resistant K-562 cells (K-562/MTX3, K-562/MTX4 and K-562/MTX5) and cisplatin-resistant ovarian cells (KFr). Immunoelectron microscopically, the protein was found to be located on the cell membrane of K-562/ADM and 2780^{AD} cells. Furthermore, the presence of the protein in various cell lines, normal tissues and surgical materials from patients given no anti-cancer agents was examined by immunocytochemistry and flow cytometry. MRK20 reacted with granulocytes, monocytes and endothelial cells in various tissues, but did not react with tissue macrophages. This 85-kDa protein recognized by MRK20 seems to be the second multidrug-resistance gene-encoded product appearing in adriamycin-resistant cancer cells, following the characterization of 170–180-kDa glycoprotein, and may be important for elucidating the multidrug-resistance mechanism relevant to adriamycin and *Vinca* alkaloids.

Key words: Distribution — Adriamycin — Resistance — MRK20 monoclonal antibody

Adriamycin (ADM)*5 has been widely used for the treatment of solid tumors such as lung cancer, breast cancer and ovarian cancer, as well as acute leukemia and malignant lymphoma. It has been recently reported that adriamycin-resistant leukemia,[1] daunomycin-resistant Chinese hamster ovary cell mutants and *Vinca* alkaloid-resistant leukemia carry a 170–180-kDa glycoprotein (P-glycoprotein) which can hardly be recognized in their parental cells.[2–6]

In order to clarify the mechanism of multidrug resistance, we obtained an adriamycin-resistant leukemia cell line (K-562/ADM) and subsequently prepared murine monoclonal antibodies (MAbs) specific to K-562/ADM, and MRK16, -17 and -20.[7, 8] MRK16 and -17 recognized 170–180-kDa glycoprotein (P-glycoprotein) in various human multidrug-resistant tumor cell lines,[9] while MRK 20 was found to recognize an 85-kDa protein in adriamycin-resistant cancer cells.[8] As a further step, the present experiments were designed to evaluate the level of the protein recognized by MRK20, employing both normal and cancerous tissues from patients untreated with anti-cancer agents.

This paper is the first to report that MRK20 recognizes granulocytes, monocytes and en-

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*5 Abbreviations used in this paper: ADM, adriamycin; K-562/ADM or K/A, adriamycin-resistant K-562 cells; K-562/MTX, methotrexate-resistant K-562 cells; KFr, cisplatin-resistant KF-1 cells; MAb, monoclonal antibody; FCS, fetal calf serum; ABC-PO, avidin-biotin-peroxidase; ABC-GO, avidin-biotin-glucose oxidase; PLP, periodate-lysine-paraformaldehyde; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MNC, mononuclear cells; PBS, phosphate-buffered saline.

dothelial cells as well as adriamycin-resistant leukemic and ovarian cells, and that the MAb does not react with methotrexate-resistant leukemic cells (K-562/MTX3, K-562/MTX4 and K-562/MTX5) and cisplatin-resistant ovarian cells (KFr).

Materials and Methods

Cell Lines and Tissues K-562 (human myelogenous leukemia) was established by Lozzio and Lozzio,[6] and K-562/ADM, an *in vitro*-induced adriamycin-resistant subline of K-562, was established in the laboratory of one of the authors.[10] An ovarian cancer cell line, A2780 and its adriamycin-resistant strain, 2780^{AD}, were kindly supplied by Drs. R. F. Ozols and T. C. Hamilton, National Cancer Institute, USA.[11,12] A cisplatin-resistant ovarian cancer cell line, KFr, and its parental line, KF-1, were kindly provided by Prof. I. Nishiya, Department of Gynecology and Obstetrics, Iwate Medical College.[13] The three methotrexate (MTX)-resistant K-562 cell lines, K-562/MTX3, K-562/MTX4 and K-562/MTX5, were kindly provided by Dr. S. Koizumi, Department of Pediatrics, Kanazawa University School of Medicine.[14] BALL-1[15] and NALM-6[16] were supplied by Dr. J. Minowada, Fujisaki Cell Bank, Hayashibara Co., Okayama. HL-60,[17] SW-13,[18] Y-1,[19] THP-1,[20] U-937,[21] Raji,[22] Daudi,[23] CEM-CR3,[24] HeLa,[25] MIA PaCa-2,[26] PANC-1,[27] RPMI 8866,[28] Li-7,[29] COLO205[30] and PC-12[31] were supplied by Japan Cell Resources Bank, Tokyo. All of the cell lines were grown in MEM or RPMI 1640 containing 10% fetal calf serum (FCS) and gentamicin sulfate (20 μg/ml). The cell lines were routinely passaged twice weekly.

For immunohistochemical study, human tissues were obtained from biopsies performed on patients for diagnostic purposes at Saitama Medical College Medical Center and the University of Tokyo Hospital, and from autopsies performed within one hour of death on patients with various heart diseases and malignancies. Tissues for frozen-section studies were immediately snap-frozen in liquid nitrogen and stored at -70° until used.

Preparation and Characterization of Monoclonal Antibody, MRK20 The methods used for antigen preparation, immunization, cell fusion, cloning and serological characterization of monoclonal antibody (MRK20) isotype have been previously described.[7]

Immunocytochemistry Immunocytochemistry (ABC-PO and ABC-GO methods) was used for the cultured cell lines and frozen sections (according to an instruction sheet issued by Vector Labs., Burlingame, CA). Briefly, the preparations were reacted with MRK20 (10 μg/ml) at room temper-

Table I. Reactivity of MRK20 with MNC and Cell Lines

Cell line[a]	Reactivity of MRK20 as revealed by immunocyto-chemistry[b]	flow cyto-metry[c] (%)
A2780	−	13.1, 14.3
2780^{AD}	+	90.6, 13.9
COLO 205	−	NT
HeLa	−	NT
KF-1	−	6.7, 5.9
KFr	−	10.1, 8.7
Li-7	−	NT
MIA PaCa-2	−	NT
PC-12	−	26.7, 22.9
SW-13	+	92.8, 11.0
Y-1	−	12.9, 7.5
Daudi	−	19.8, 20.9
Raji	−	33.3, 27.8
RPMI 8866	−	13.1, 14.0
U-937	+	49.1, 5.6
BALL-1	−	4.8, 1.0
HL-60	−	47.5, 51.3
K-562	−	14.5, 10.6
K-562/ADM	+	92.3, 14.7
K-562/MTX3	−	25.4, 29.3
K-562/MTX4	−	24.8, 23.4
K-562/MTX5	−	23.3, 23.0
NALM-6	−	0.4, 0.0
THP-1	+	94.3, 3.5
CEM-CR3	−	6.4, 1.5
Granulocytes	+	4.5, 0.1
Lymphocytes	−	1.7, 0.8
Monocytes	+	61.7, 1.1

a) The tissue types of cell lines used are as follows: A2780 (ovarian cancer), 2780^{AD} (adriamycin-resistant A2780), COLO 205 (colonic cancer), HeLa (uterine cervix cancer), KF-1 (ovarian cancer), KFr (cisplatin-resistant KF-1), Li-7 (hepatoma), MIA PaCa-2 (pancreatic cancer), PC-12 (rat pheochromocytoma), SW-13 (human adrenocortical cancer), Y-1 (mouse adrenal tumor), Daudi (Burkitt lymphoma), Raji (Burkitt lymphoma), RPMI 8866 (lymphoelastoid cell line), U-937 (histiocytic lymphoma), BALL-1 (acute lymphoid leukemia), HL-60 (acute promonocytic leukemia), K-562 (chronic myelogenous leukemia), K-562/ADM (adriamycin-resistant K-562), K-562/MTX3 (methotrexate-resistant K-562), K-562/MTX4 (methotrexate-resistant K-562), K-562/MTX5 (methotrexate-resistant K-562), NALM-6 (acute lymphoid leukemia), THP-1 (acute monocytic leukemia), CEM-CR3 (T-cell hybridoma).
b) Intensity of immunocytochemical staining was classified as follows: −, negative; +, positive.
c) Percentages indicate MRK20-positive cells (%) vs. non-immune mouse serum-treated cells (%). NT: not tested.

ature for 30 min after a 30-min blocking with 100-fold-diluted horse serum in phosphate-buffered saline (PBS). After three washings with PBS, 50 μl aliquots of 1:100-diluted biotinylated horse anti-mouse IgGs were applied for 30 min at room temperature. Thereafter, 100-fold-diluted avidin-biotin-peroxidase (ABC-PO) or avidin-biotin-glucose oxidase (ABC-GO) solution was applied. After coloration with H_2O_2 and diaminobenzidine (DAB), or with β-D-glucose, nitroblue tetrazolium (NBT) and phenazine methosulfate (PMS), respectively, the preparations were examined with a light microscope (Olympus Optical Co., Tokyo).

Ultrastructural Localization of 85-kDa Protein in K-562/ADM Cells To examine the localization of the 85-kDa protein recognized by MRK20, an immunoelectron microscopic study was carried out. K-562 and K-562/ADM cells (1×10^7/ml) in suspension were first treated with periodate-lysine-paraformaldehyde (PLP) for 45 min and then with 0.2% saponin (Sigma Chemical Co., St. Louis, MO) in PBS for 5 hr. Thereafter, they were treated with MRK20 (20 μg/ml) at 4° for 12 hr. After being washed carefully with PBS three times, 50 μl aliquots of peroxidase-conjugated rabbit anti-mouse IgGs (DAKO PATTS, Copenhagen) were applied. The cells were fixed with 2.5% glutaraldehyde-PBS for 20 min after frequent washing with PBS. Thereafter, 500 μl of DAB-H_2O_2 solution (2.5 mg of DAB and 10 μl of H_2O_2 in PBS) was added for 10 min for coloration. After three washings with PBS, the cells were post-fixed with 1% osmic acid solution for 30 min and embedded in Epon 812. Ultrathin sections were prepared using an ultramicrotome (Ultracut E, Reichert-Jung Co.). These were stained with uranyl acetate and examined with an electron microscope (Model 100C, JEOL, Tokyo).

Flow Cytometry Whole peripheral blood was reacted with either MRK20 (5 μg/ml) of non-immune mouse serum (Sigma, 5 μg/ml) at 4° for 20 min. The various cell lines were also treated with either MRK20 or non-immune serum at 4° for 20 min. After two washings with PBS, they were reacted with FITC-labeled goat anti-mouse IgGs ($F(ab')^2$ fragments) (1:40-diluted, Tago, USA) at 4° for 30 min. After two washings, erythrocytes in the whole peripheral blood were hemolyzed with a lysing reagent (Coulter). Peripheral mononuclear cells (MNC) thus obtained and the cell lines were examined for MRK20 positivity with a Spectrum III (Ortho Diagnostics Systems Inc., Raritan, NJ). A proportion of the cells were saved for further immunocytochemical examination.

The criteria of positivity with MRK20 MAb were as follows: +, percentage of MRK20-positive cells much higher than that of cells treated with non-immune mouse IgGs used as a negative control; −, no statistically significant difference in percentage between MRK20-treated cells and non-immune serum-treated cells.

Immunoblotting To determine whether MRK20-positive cells actually possess 85-kDa protein specifically expressed in adriamycin-resistant K-562 cells and MNC, the immunoblotting technique was employed. MNC, K-562 and K-562/ADM cells (1×10^7/ml) were solubilized according to the method of Laemmli.[32] Briefly, the cells were solubilized with 500 μl of cell lysis buffer containing 1% Triton X100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.15*M* NaCl, 50m*M* Tris-HCl, pH 7.4, and 2m*M* phenylmethylsulfonylfluoride (PMSF). After initial development of the solubilized proteins by SDS-PAGE, they were electrophoretically transferred onto a nitrocellulose membrane filter at a constant voltage of 50 V for 3 hr. Thereafter, immunochemistry (ABC-PO method) was used for the detection of 85-kDa protein.[33]

RESULTS

Reactivity of MRK20 with MNC and Cell Lines As shown in Table I, MRK20 reacted with K-562/ADM and 2780^{AD} cells. Figure 1 shows that adriamycin-resistant cancer cells (K-562/ADM and 2780^{AD}) were stained positively and strongly with MRK20. Interestingly, MRK20 also reacted with human monocytes, granulocytes, SW-13 (human adrenocortical tumor line), THP-1 (monocyte-like cell line), and U-937 (human histiocytic lymphoma cell line). However, MRK20 did not react with K-562/MTX3, K-562/MTX4, K-562/MTX5 (methotrexate-resistant K-562 cell lines) or KFr (cisplatin-resistant ovarian cell line). Figure 2 shows that both monocytes and granulocytes were stained positively with MRK20 by immunocytochemistry (ABC-GO method). These observations were further confirmed by flow cytometry. Figure 3 reveals that granulocytes (b) and monocytes (c) were stained positively with MRK20 as observed by flow cytometry.

Staining Reactivity of MRK20 with Normal Tissues and Surgical Specimens from Untreated Patients We carried out an extensive survey of the reactivity of MRK20 with normal tissues and surgical materials from untreated patients. As shown in Table II, no MRK20-positive cells were found in these normal tissues and surgical specimens except that endothelial cells, granulocytes and mono-

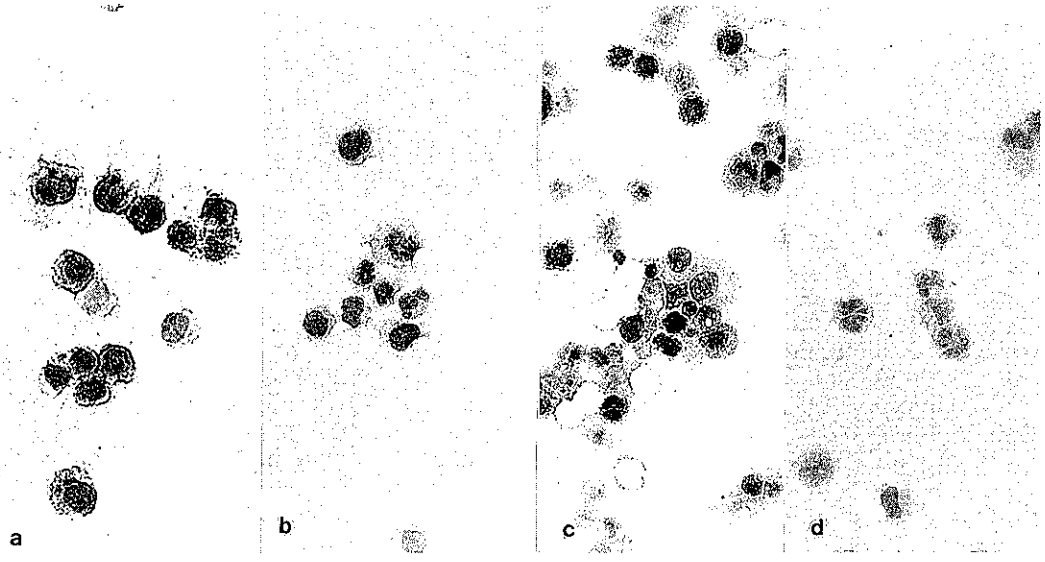

Fig. 1. Immunostaining of 85-kDa protein on K-562/ADM (a), K-562 (b), 2780^{AD} (c), and A2780 (d) by MRK20 (×1,140). ABC-PO method.

Fig. 2. Immunostaining of mononuclear cells (MNC) by MRK20 (×1,600). ABC-GO method. Monocyte (single arrow) and granulocyte (double arrows) are stained positively (a). Negative control (non-immune mouse serum) (b).

cytes in bone marrow were stained positively with MRK20. Tissue macrophages showed no positive staining with MRK20.

Ultrastructural Localization of 85-kDa Protein Recognized by MRK20 in K-562/ADM Cells and Endothelial Cells As shown in Fig. 4, 85-kDa protein was distributed abundantly and evenly on the surface membranes of K-562/ADM cells, but not on K-562 cells. As we found light microscopically that MRK20 also recognized endothelial cells (Table II), we examined the distribution of antigens recognized by MRK20. Figures 5 and 6 show that the antigens recognized by MRK20 were located evenly on the cell membranes of endothelial cells in the liver and tonsils. No MRK20-positive macrophages were seen in these tissues.

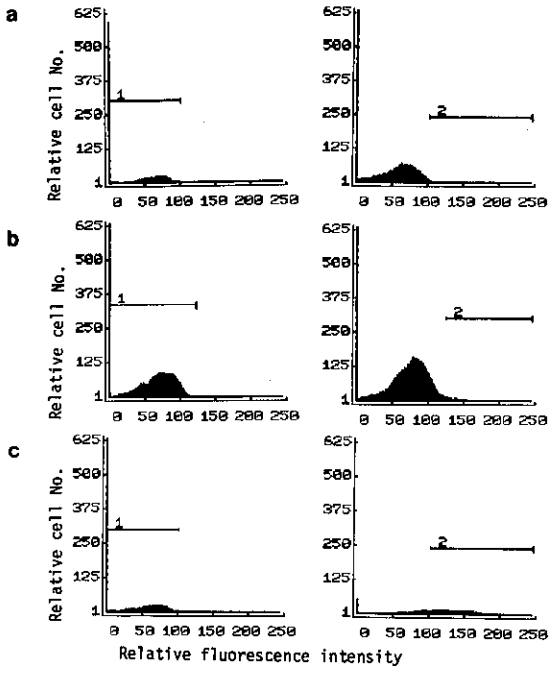

Fig. 3. Flow cytometric analysis of mononuclear cells (MNC) with MRK20. The whole peripheral blood was reacted with MRK20 MAb (5 μg/ml) or non-immune mouse IgGs (5 μg/ml) at 4° for 20 min. Thereafter, they were reacted with FITC-labeled goat anti-mouse IgGs ($F(ab')^2$) (1:40-diluted at 4° for 30 min after two washings. Then, the blood was treated with a hemolyzing reagent. A horizontal line marked 1 shows a negative gated area consisting of MRK20-negative cells. A horizontal line marked 2 shows a positive gated area consisting of MRK20-positive cells. (a) Immuno-reactivity of lymphocytes with MRK20. Non-immune mouse IgG, 0.8%; MRK20, 1.7%. (b) Immuno-reactivity of granulocytes with MRK20. Non-immune mouse IgG, 0.1%; MRK20, 4.5%. (c) Immuno-reactivity of monocytes with MRK 20. Non-immune mouse IgG, 1.1%; MRK20, 61.7%.

Immunoblotting In order to confirm that the antigens on MNC and endothelial cells recognized by MRK20 were compatible with the 85-kDa protein present on K-562/ADM cells, immunoblotting was performed. The lanes in Fig. 7 represent the solubilized proteins from K-562 cells, K-562/ADM, MNC, and liver, respectively, after SDS-PAGE and blotting with MRK20. A band with a molecu-

Table II. Reactivity of MRK20 with Normal Tissues and Surgical Specimens from Untreated Patients

Tissue	Reactivity of MRK20[a]
Fetal adrenal	− (3)[b]
Fetal pancreas	− (2)
Fetal kidney	− (2)
Fetal spleen	− (3)
Fetal lung	− (2)
Fetal liver	− (3)
Fetal thymus	− (2)
Fetal heart	− (2)
Neonatal adrenal	− (1)
Adult adrenal	− (10)
Placenta	− (2)
Kidney	− (5)
Pancreas	− (5)
Rectum	− (2)
Gallbladder	− (1)
Thyroid	− (5)
Lymph node	− (5)
Liver	− (5)
Prostate	− (2)
Stomach	− (3)
Lung	− (3)
Submandibular gland	− (2)
Pituitary	− (2)
Mammary gland	− (2)
Large intestine	− (2)
Small intestine	− (2)
Spleen	− (2)
Heart	− (2)
Skeletal muscle	− (1)
Cerebrum	− (2)
Cerebellum	− (2)
Esophagus	− (3)
Skin	− (1)
Femoral bone marrow	+ (1)
Colon cancer	− (4)
Pancreatic cancer	− (2)
Renal cancer	− (5)
Malignant lymphoma	− (10)
Gastric cancer	− (2)
Neuroblastoma	− (1)
Malignant fibrous histiocytoma	− (1)
Osteogenic sarcoma	− (1)
Lung cancer	− (12)
Ovarian cancer	− (2)
Uterine cervix cancer	− (1)
Breast cancer	− (9)
Thymoma	− (2)
Retinoblastoma	− (2)
Hepatoma	− (2)
Carcinoid of the bronchus	− (1)
Myeloma	− (2)
Endothelial cells	+

a) Intensity of immunohistological staining was classified as follows: −, negative; +, positive if less than 50% of the tissue was stained.
b) Numbers in parentheses indicate numbers of case(s).

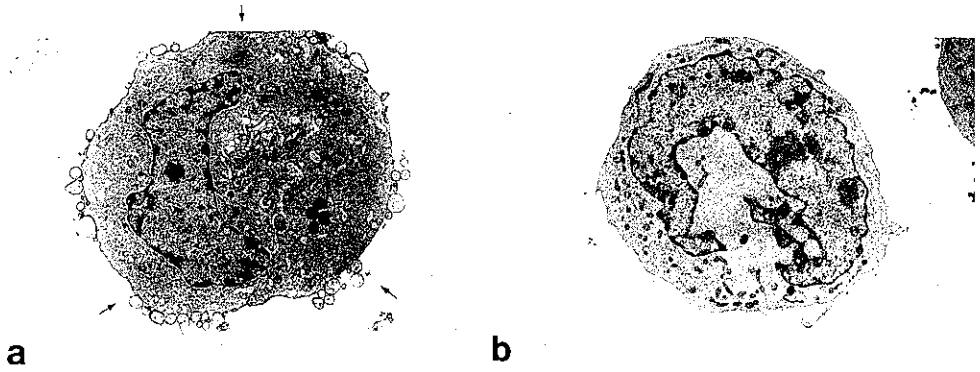

Fig. 4. Ultrastructural localization of 85-kDa protein on K-562/ADM (a) and K-562 (b) by MRK 20 (×8,300). The protein is localized on the cell membranes of K-562/ADM (arrow), but not on the cell membrane of K-562. ABC-PO method.

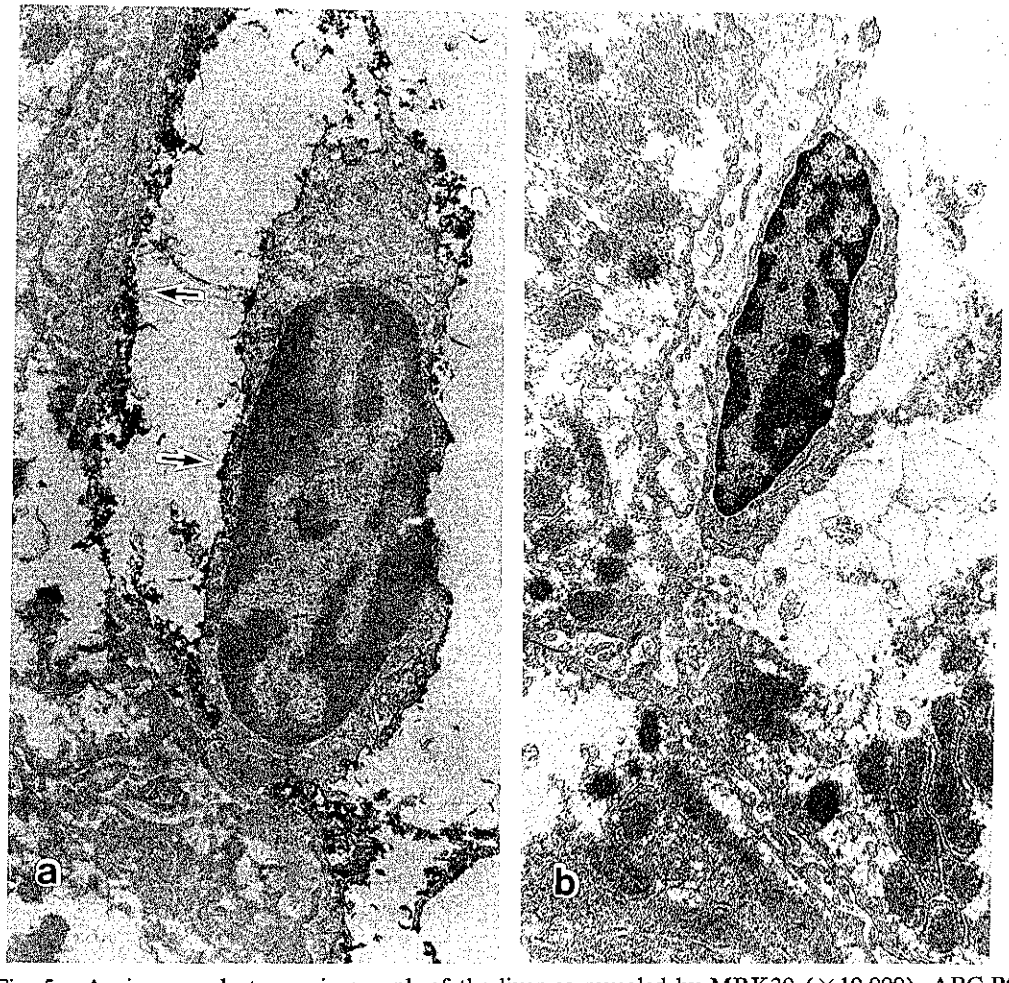

Fig. 5. An immunoelectron micrograph of the liver as revealed by MRK20 (×10,000). ABC-PO method. Sinusoidal endothelial cell showing positive staining (arrow) (a). Negative control (non-immune mouse serum instead of MRK20) (b).

lar weight of ca. 85 kDa was observed in the lanes for K-562/ADM, MNC and liver.

Discussion

Our present study showed that 85-kDa protein recognized by MRK20 was present on the surface membranes of K-562/ADM and 2780^{AD} cells and that the protein was also present on monocytes, granulocytes and endothelial cells in various tissues. These observations raise several points that warrant comment.

First, what is the relationship between P-glycoprotein recognized by MRK16 and the 85-kDa protein recognized by MRK20? As already discussed with regard to the tissue distribution of P-glycoprotein revealed by MRK16, MRK16 reacted with adrenal cortex, renal tubules and placenta as well as K-562/ADM cells and 2780^{AD} cells.[34] In contrast, MRK20 reacted with monocytes, granulocytes and endothelial cells as well as K-562/ADM and 2780^{AD} cells. Thus, the tissue distributions of their antigens are totally different from each other. Furthermore, we performed MRK20-blocking experiments with MRK16, employing liver tissue. MRK16 did not cross-react with MRK20 with regard to the staining reactivity of endothelial cells and Kupffer cells (data not shown). It is intriguing that MRK16 reacts with monocytes but not with mature tissue macrophages.

Although MRK20 MAb seemed to bind to Fc receptors on various cancer cells, MRK 20-positive cells in some cell lines were more numerous than the background non-immune mouse serum-treated cells. This immunopositivity was further confirmed by immuno-

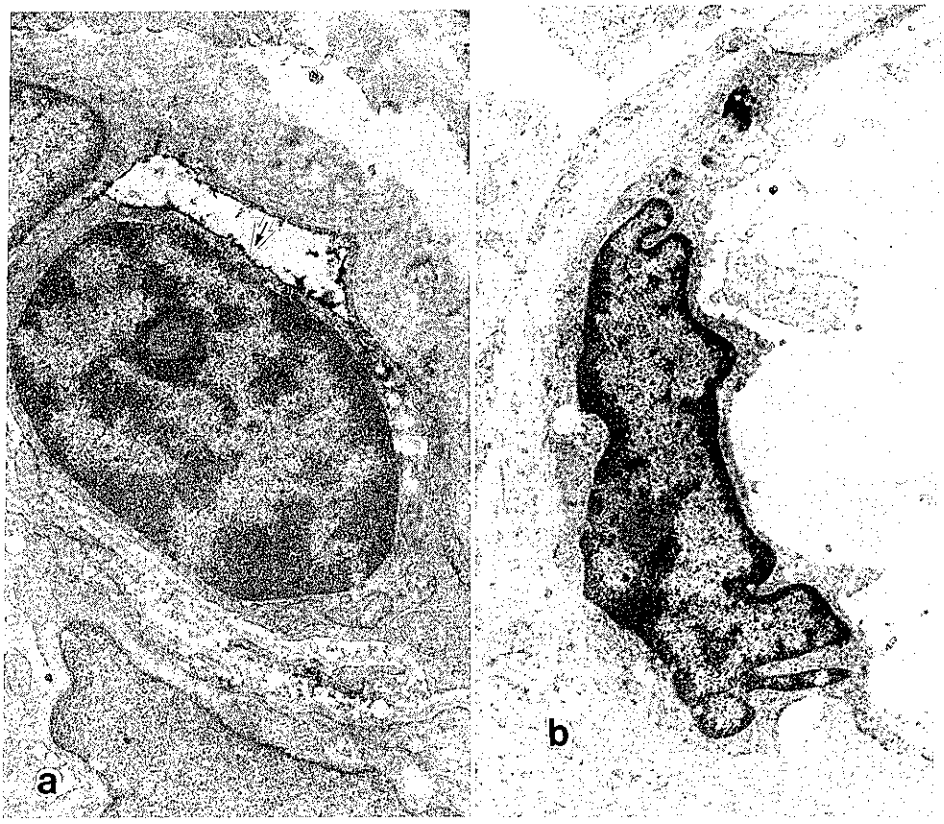

Fig. 6. An immunoelectron micrograph of a human tonsil as revealed by MRK20 (×7,900). ABC-PO method. Endothelial cell showing positive staining with MRK20 (arrow) (a). Negative control (non-immune mouse serum) (b).

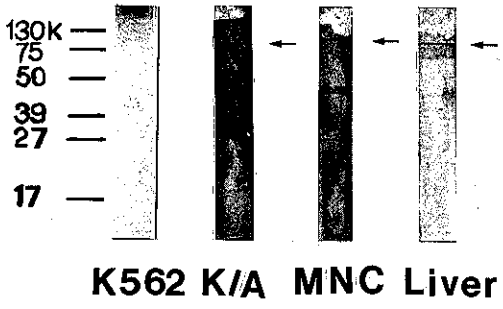

Fig. 7 Western blot analysis of solubilized proteins from K-562, K-562/ADM (K/A), MNC and liver. Approximately 50 μg of each solubilized protein was subjected to SDS-PAGE under reducing conditions prior to blotting, and then reacted with MRK20. Arrows show the band with a molecular size of 80–85 kDa. The MNC contained about 84% granulocytes, 10% lymphocytes and 6% monocytes, as assessed by flow cytometry.

cytochemistry. Therefore, we believe that some cancer cell lines bear 85-kDa protein on their membranes.

Second, what is the role of 85-kDa protein in the multidrug-resistance mechanism? The complete amino acid sequence of this protein is still unknown (Dr. Y. Sugimoto, personal communication). As the 85-kDa protein is not present in methotrexate- and cisplatin-resistant cancer cells, it is possible that it may play a role in adriamycin-induced multidrug resistance. We hope to elucidate the role of the 85-kDa protein in the near future after cloning of its cDNA.

Third, is the protein observed under clinical conditions in cases where adriamycin, *Vinca* alkaloid, and other agents have been administered? Both immunochemistry and flow cytometry have shown that the 85-kDa protein is present on blastic cells in cases of hematological malignancy such as chronic myelogenous leukemia, acute myelomonocytic leukemia, acute promyelocytic leukemia and malignant thymoma (manuscript in preparation). However, as described already (Table II), immature myeloid cells showed no positive staining with MRK20 MAb.

Finally, what is the clinical usefulness of MRK20? Since the antibody does not recognize lymphocytes from normal healthy volunteers (Fig. 4), it may be useful for detecting MRK20-positive blastic cells in lymphoid malignancy after administration of adriamycin and *Vinca* alkaloid. It may also be employed for selective *ex vivo* killing of cancer cells containing high levels of the 85-kDa protein.

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