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Augmentation by Bispecific F(ab′)$_2$ Reactive with P-Glycoprotein and CD3 of Cytotoxicity of Human Effector Cells on P-Glycoprotein Positive Human Renal Cancer Cells

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A bispecific F(ba′)$_2$ was constructed that was composed of two Fab fragments, one derived from anti-CD3 monoclonal antibody (mAb) (OKT3) and the other from anti P-glycoprotein mAb (MRK 16). This bispecific F(ab′)$_2$ enhanced the binding and cytotoxicity of human peripheral blood mononuclear cells (PBMCs) on P-glycoprotein-positive human kidney cancer cells (ADMHK/E). It had no effect on the cytotoxicity of PBMCs on P-glycoprotein-negative HK/E cells [long-term cultured HK/E (LCHK/E)]. Control F(ab′)$_2$ composed of OKT3 or MRK16 alone did not influence the cytotoxicity of PBMCs on ADMHK/E cells. These findings suggest that the MRK16-OKT3 bispecific F(ab′)$_2$ may be therapeutically beneficial in treatment of human multidrug-resistant cancers.

Key words: Multidrug resistance — P-glycoprotein — CD3 — Bispecific F(ab′)$_2$ — Cytotoxicity

Multiple drug resistance (MDR)[5] has been studied extensively because it is one of major problems in cancer chemotherapy. Recently, P-glycoprotein on the surface of MDR cells was found to play an important role in the mechanism of MDR.[1-3] P-Glycoprotein is now believed to be an energy-dependent drug efflux pump, that reduces the accumulation of drugs in MDR cells.[4, 5] As the P-glycoprotein is known to be expressed by both acquired and intrinsic MDR cells in human cancers,[1-3] therapeutic approaches that target it should be useful in overcoming multidrug resistance. Various chemical compounds (including many dihydropyridine-type calcium channel blockers such as verapamil, R-verapamil, diltiazem, quinidine and trifluoperazine) have been found to reverse multidrug resistance *in vitro,*[6, 7] and in a few instances *in vivo.*[8-10] But unfortunately, most of these agents have undesirable side-effects *in vivo,* such as to cause cardiovascular disorders at the concentrations needed to reverse multidrug resistance *in vitro.*[11, 12]

P-Glycoprotein is located on the membranes of MDR cancer cells, and so could be a suitable antigen for immunotherapy. For this purpose, we constructed two anti-P-glycoprotein monoclonal antibodies (mAbs), MRK16 and MRK17, which recognized different extracellular domains of the P-glycoprotein of MDR human cancer cells.[13] Pastan *et al.* reported the construction of an immunotoxin composed of MRK16 and pseudomonas exotoxin which showed cytotoxic effects against MDR cancers.[14] MRK16 alone effectively prevented the growth of a P-glycoprotein-positive MDR human ovarian cancer line (2780AD) inoculated subcutaneously in athymic mice.[15] In the presence of these mAbs, human effector cells showed enhanced cytotoxicity via an antibody-dependent cell-mediated cytotoxicity (ADCC) mechanism.[16]

Recently, it was reported that CD3 of T cell receptor-CD3 complex played a key role in signal transduction by antigen binding. Anti-CD3 mAb behaved similarly to the antigen. The stimulation of CD3 by anti-CD3 mAb transduced important signals followed by proliferation of the cells and cytokine production of the cells. Thus much attention has been paid to the use of bispecific antibodies, composed of anti-CD3 and anti-tumor specific antigens, to increase the direct interaction between effector cells and target tumor cells, leading to tumor cell killing. Such an antibody was found to link cloned cytotoxic T lymphocytes or peripheral blood mononuclear cells (PBMCs) with tumor cells, and rendered the latter more sensitive to killing by effector cells.[17-21] Nitta *et al.*[22] reported that a bispecific F(ab′)$_2$ composed of anti-glioma × anti-CD3 antibodies rendered PBMCs capable of lyzing the human glioma cell line, U251MG, which is resistant to natural killer cell-mediated cytotoxicity *in*

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[5] The abbreviations used are: MDR, multidrug resistance; mAb, monoclonal antibody; PBMCs, peripheral blood mononuclear cells; ADM, adriamycin; PBS, phosphate-buffered saline; IL, interleukin.

vitro.[22] They used this bispecific $F(ab')_2$ with lymphokine-activated killer cells in treatment of glioma a patients, and obtained promising results.[23]

In this study, we constructed a bispecific $F(ab')_2$ composed of two Fab fragments, one derived from anti-CD3 mAb (OKT3) and the other from anti-P-glycoprotein mAb (MRK16). This bispecific $F(ab')_2$ augmented the binding and killing activities of human PBMCs against P-glycoprotein-positive human renal cancer cells.

MATERIALS AND METHODS

Cancer cells The human renal cancer cell line (Parent HK/E) was provided by Dr. T. Utakoji (Department of Cell Biology, Cancer Institute, Tokyo). This cell line was established from a clear cell renal carcinoma of a patient who had received no chemotherapy. The cell line was maintained in RPMI 1640 medium with L-glutamine and 1% sodium pyruvate, supplemented with 10% FBS, designated as CRPMI 1640 medium.

Freshly recovered Parent HK/E cells that had been stored in liquid nitrogen were composed of a mixed population of P-glycoprotein-positive and negative cells (see Fig. 1). The Parent HK/E cells were used within 2 months after recovery from the frozen stock because the expression of P-glycoprotein declined gradually. We isolated a P-glycoprotein-positive HK/E line (ADMHK/E) by short-term treatment of these Parent HK/E with ADM, which might mimic the clinical situation of emergence of drug resistance. For this, the Parent HK/E cells were treated with 100 ng/ml of ADM, which corresponds to the IC_{50} value of ADM against the cells. On day 3, the culture medium was changed to drug-free medium, and on day 8 the cells were transferred to new dishes without the drug and incubated for 2 weeks with routine medium change. The resulting, growing HK/E cells, named "ADMHK/E" cells, were used for experiments within 1 week, because their expression of P-glycoprotein gradually decreased during culture. A P-glycoprotein-negative HK/E line (LCHK/E) was established by long-term (more than 3 months) culture of the parent cells without ADM.

The human ovarian tumor line A2780 and its ADM-resistant variant 2780AD, used as control cell lines, were kindly supplied by Drs. R. F. Ozols and T. C. Hamilton, National Cancer Institute. The expression of P-glycoprotein by these cell lines has been reported.[24]

Antibodies The anti-P-glycoprotein mAb MRK16 (IgG-2a) was obtained as described previously.[13] The anti-CD3 mAb OKT3 was obtained from an OKT3-producing hybridoma (American Type Culture Collection). These mAbs were purified from ascites by ammonium sulfate precipitation followed by chromatography on DEAE-Sephacel (Pharmacia, Uppsala, Sweden) as described.[25]

Chemicals Recombinant human interleukin (IL)-2 (TGP-3; specific activity, $\times 10$ U/ml) was kindly provided by Takeda Pharmaceuticals, Osaka. Dithiothreitol and 5,5′-dithiobis(2-nitrobenzoic acid) were purchased from Sigma Chemicals (St. Louis, MO). $F(ab')_2$ of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG, and $F(ab')_2$ of peroxidase-conjugated goat anti-mouse IgG were purchased from Cappel (West Chester, PA).

Effector cells Human PBMCs were prepared as described previously.[16] Non-activated PBMCs were plated in flat-bottomed 96-well microtiter plates at a density of 1×10^5 cells/well in 100 μl of CRPMI 1640 medium for use in cytolytic assay.

Flow cytometric analysis HK/E cells were harvested and suspended in CRPMI 1640 medium at 1×10^6/tube. After centrifugation for 5 min, the cells were resuspended in 400 μl of PBS containing MRK16 (10 μg/ml) for 30 min at 4°C. Then they were washed twice with PBS, and resuspended in 400 μl of PBS containing the second-stage antibody, $F(ab')_2$ of FITC-conjugated goat anti-mouse IgG, at a dilution of 1:50 for 30 min at 4°C. They were then washed twice, resuspended in PBS and stored at 4°C in the dark until analysis. Cells incubated with only the second-stage antibody were used for measurement of the background. Flow cytometry was performed in an FCS-1M instrument (Nihon Bunko, Tokyo).

Drug sensitivity assay Drug sensitivity was measured as described previously.[26] Briefly, cells (1×10^3/well) were cultured at 37°C for 5 h in 50 μl of CRPMI 1640 medium in flat-bottomed 96-well microtiter plates. Then the cells were incubated with or without graded concentrations of ADM for 72 h. Drug sensitivity was measured by MTT assay.[26]

Preparation of bispecific $F(ab')_2$ Bispecific $F(ab')_2$ was prepared using dithiothreitol and 5,5′-dithiobis(2-nitrobenzoic acid) according to a published procedure.[23] The molecular weight of the $F(ab')_2$ constructed was determined by 7.5% polyacrylamide gel electrophoresis in the presence of 0.1% SDS under non-reducing conditions.[27]

Measurement of affinity of bispecific $F(ab')_2$ The affinity of MRK16-OKT3 bispecific $F(ab')_2$ was measured by enzyme-linked immunosorbent assay (ELISA) as described previously.[28] Human PBMCs were prepared from normal volunteers as described.[16] IL-2-activated PBMCs were prepared by overnight treatment of the cells with 1 U/ml of TGP-3. Monolayers of LCHK/E, ADMHK/E, A2780, 2780AD, non-activated PBMCs and IL-2 -activated PBMCs in 96-well flat-bottomed plates were treated with 10 μg/μl of bispecific $F(ab')_2$, or $F(ab')_2$ of MRK16 or OKT3. Data are presented as differences in optical densities at 495 nm in the presence and absence of the $F(ab')_2$. Backgrounds for each cell

line were determined by incubations without the first stage antibodies.

Binding assay Samples of 1×10^5 HK/E cells in 2 ml of CRPMI 1640 medium in 3 cm dishes were cultured overnight at 37°C in a CO_2 incubator. The supernatants were then removed and 400 μl of ^{51}Cr-labeled PBMCs (5×10^5/ml) was added to each well with or without MRK16-OKT3 bispecific F(ab′)$_2$ (0–10 μg/ml). In blocking experiments, 10 μg/ml of F(ab′)$_2$ of MRK16 or OKT3 was added with 10 μg/ml of bispecific F(ab′)$_2$. After incubation for 30 min at room temperature, the supernatant was removed and the wells were washed twice with pre-warmed medium. The PBMCs bound to HK/E cells were lysed by addition of 500 μl of 0.1% NaOH, the radioactivity of the cell lysate was counted in a gamma counter, and the number of bound PBMCs was calculated. Data are presented as differences in the numbers of bound cells in the presence and absence of the F(ab′)$_2$.

Cytolysis assay Cytolytic activity was determined by ^{51}Cr release assay as described previously.[16] The effector: target ratio was 10, and percentage cytolysis was calculated as follows:

$$\%\ \text{Cytolysis}=\frac{\text{experimental cpm}-\text{spontaneous cpm}}{\text{total cpm}-\text{spontaneous cpm}}\times 100.$$

The total radioactivity was determined by addition of 1% Triton-X to 100 μl of target cells. Spontaneous release was determined by incubating the target cells in CRPMI 1640 medium without the antibody. The spontaneous release was always less than 15% of the total release.

Other methods Protein was measured by the method of Smith *et al.*[29] Statistical significance was analyzed by using Student's 2-tailed *t* test.

RESULTS

Characterization of HK/E cell lines Flow cytometric analysis showed that the parent HK/E cells were composed of a mixed population of P-glycoprotein-positive and negative cells (Fig. 1A). LCHK/E cells, which had been incubated for three months in drug-free conditions had no P-glycoprotein antigen (Fig. 1 C), whereas ADMHK/E cells, obtained after short-term treatment of HK/E cells with ADM, expressed P-glycoprotein strongly (Fig. 1D). The LCHK/E and ADMHK/E cell lines were morphologically similar to the Parent HK/E cells (data not shown).

The IC_{50} values of ADM for the Parent HK/E cells and the P-glycoprotein-negative LCHK/E cells were 0.185 μg/ml and 0.045 μg/ml, respectively (Fig. 2). More than 50% of the ADMHK/E cells survived treatment with ADM at 1 μg/ml, and the IC_{50} for these cells was not determined.

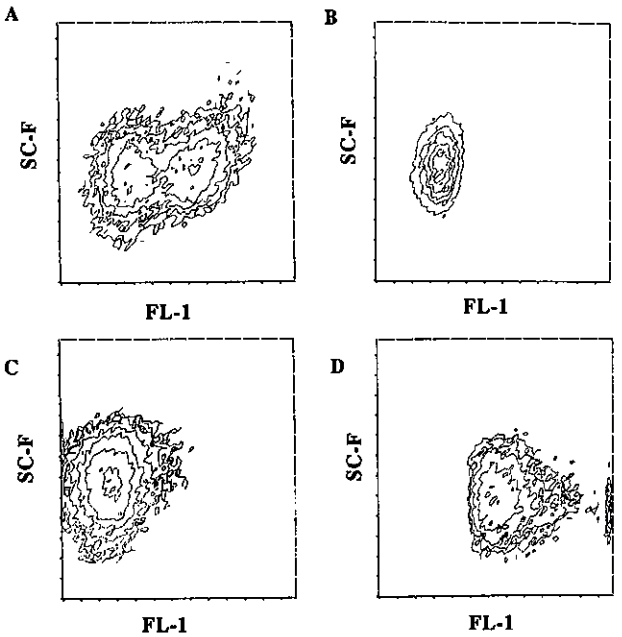

Fig. 1. Expression of P-glycoprotein on HK/E cells. Samples of 1×10^6 HK/E cells were incubated with or without MRK16 at 10 μg/ml. The expression of P-glycoprotein was examined in an FCS-1M apparatus (Nihon Bunko, Tokyo) using the second-stage FITC-conjugated goat anti-mouse IgG F(ab′)$_2$. FL (fluorescence)-1 indicates the expression of P-glycoprotein. SC-F (forward scatter) indicates the size of the cells. A, Parent HK/E; B, Parent HK/E without MRK16; C, LCHK/E; D, ADMHK/E.

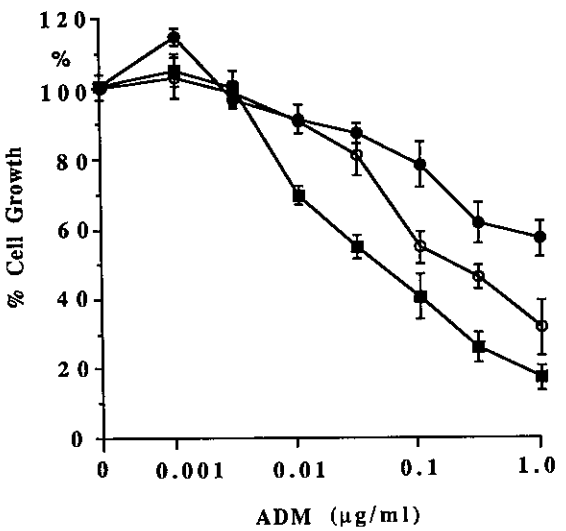

Fig. 2. ADM sensitivities of Parent HK/E, LCHK/E and ADMHK/E cells. Sensitivities were examined by MTT assay as described in "Materials and Methods." ○, Parent HK/E cells; ■, LCHK/E cells; ●, ADMHK/E cells.

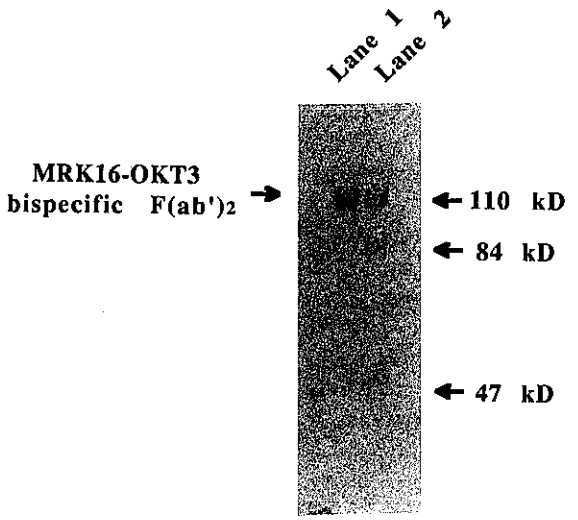

Fig. 3. Molecular weight of the MRK16-OKT3 bispecific F(ab′)$_2$ purified by Sephacryl S300 column chromatography. Lane 1, MRK16-OKT3 bispecific F(ab′)$_2$ without mercaptoethanol treatment; lane 2, molecular weight markers.

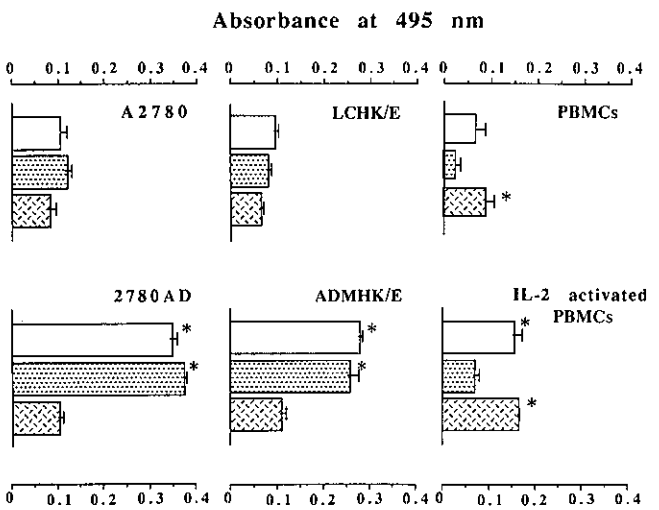

Fig. 4. Affinities of F(ab′)$_{2s}$. Affinities were measured by ELISA as described in "Materials and Methods." Data are presented as increments of $A_{495\,nm}$ by the F(ab′)$_{2s}$ at 10 μg/ml. □, with bispecific F(ab′)$_2$; ▨, with F(ab′)$_2$ of MRK16; ▩, with F(ab′)$_2$ of OKT3. Columns and bars show means+SD for triplicate determinations. * Significant difference from the control value without F(ab′)$_2$.

Preparation and characterization of MRK16-OKT3 bispecific F(ab′)$_2$ Fig. 3 shows the results of SDS-PAGE analysis of the products of the reaction. The bispecific F(ab′)$_2$ showed a molecular weight of 100–110 kD,

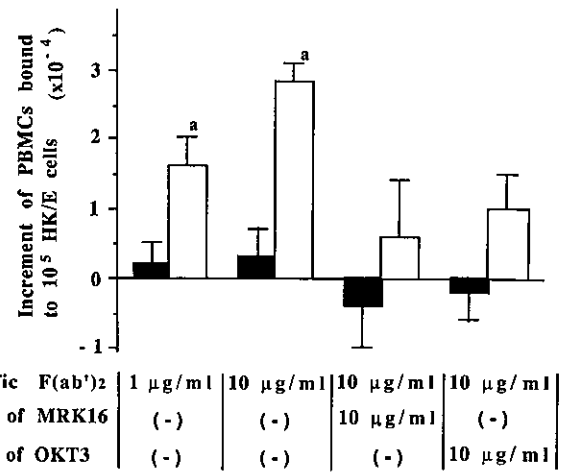

Fig. 5. Effects of F(ab′)$_{2s}$ on binding of PBMCs to HK/E cells. ^{51}Cr-Labeled PBMCs (5×10^5/cells) were incubated with confluent Parent HK/E, LCHK/E or ADMHK/E cells with (1–10 μg/ml) or without MRK16-OKT3 bispecific F(ab′)$_2$ for 30 min at room temperature. Then bound PBMCs were solubilized, their radioactivities were counted and the numbers of bound cells were calculated. Columns and bars show means +SD for triplicate measurements and are corrected for blank values [numbers of cells bound in the absence of F(ab′)$_2$]. □, ADMHK/E cells; ■, LCHK/E cells. a, Significant difference from control value without bispecific F(ab′)$_2$.

which was in good agreement with that estimated from the molecular weights of F(ab′)$_2$ fragments obtained by enzyme digestion. The purity of the bispecific F(ab′)$_2$ was 75–80% as determined by densitometry (data not shown).

The specificity of the bispecific F(ab′)$_2$ was tested by enzyme-linked immunosorbent assay. As shown in Fig. 4, at a concentration of 10 μg/ml, it showed higher affinities for the P-glycoprotein-positive 2780AD and ADMHK/E cells than for the P-glycoprotein-negative A2780 and LCHK/E cells. The bispecific F(ab′)$_2$ and F(ab′)$_2$ of MRK16 at 10 μg/ml showed similar affinities for 2780-AD and ADMHK/E, respectively, indicating retention of the full reactivity of the bispecific F(ab′)$_2$ against P-glycoprotein. The bispecific F(ab′)$_2$ also showed similar binding affinities to those of the F(ab′)$_2$ of OKT3 for non-activated human PBMCs and IL-2 activated human PBMCs, indicating the retention of the full reactivity of the bispecific F(ab′)$_2$ against CD3. These results clearly indicate that the bispecific F(ab′)$_2$ retained full binding specificities for both P-glycoprotein-positive and CD3-positive cells.

Increment by MRK16-OKT3 bispecific F(ab′)$_2$ of the binding capacity of PBMCs to ADMHK/E cells The MRK16-OKT3 bispecific F(ab′)$_2$ enhanced the binding

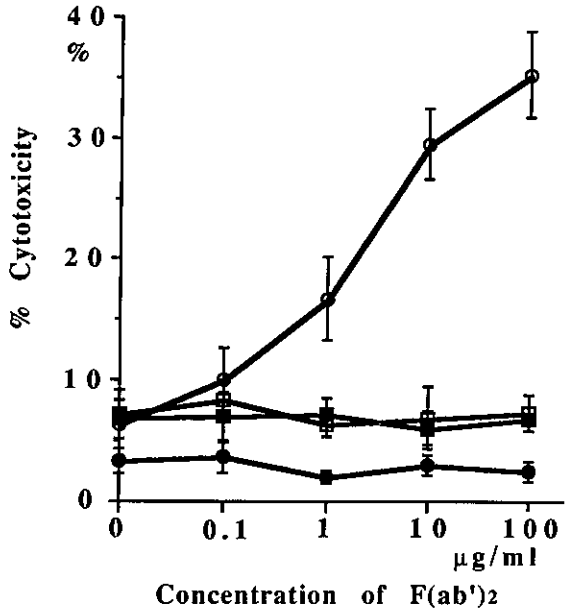

Fig. 6. Effects of bispecific $F(ab')_2$ on the cytolytic activity of PBMCs. Target cells were labeled with $Na^{51}CrO_4$ and pretreated with various concentrations of bispecific $F(ab')_2$ for 30 min at 37°C. Then they were incubated with fresh PBMCs at an E/T ratio of 10. The radioactivity released was determined as described in "Materials and Methods." ○, ADMHK/E cells with bispecific $F(ab')_2$; □, ADMHK/E cells with $F(ab')_2$ of MRK16; ■, ADMHK/E cells with $F(ab')_2$ of OKT3; ●, LCHK/E cells with bispecific $F(ab')_2$. Bars represent SDs for triplicate measurements.

capacity of PBMCs to ADMHK/E cells, but at 1 or 10 μg/ml it had no significant effect on the numbers of PBMCs bound to P-glycoprotein-negative LCHK/E cells (Fig. 5). At 1 and 10 μg/ml it induced increases in the number of PBMCs bound to ADMHK/E cells of $1.6\pm0.4\times10^4$ and $2.8\pm0.3\times10^4$ cells, respectively. Both $F(ab')^2$ of MRK16 and OKT3 inhibited the binding of PBMCs to ADMHK/E cells at 10 μg/ml bispecific $F(ab')_2$. The bispecific $F(ab')_2$, $F(ab')_2$ of MRK16 and $F(ab')_2$ of OKT3 had no effects on the binding of PBMCs to ADMHK/E cells.

Enhancement of the killing activity of PBMCs against HK/E cell lines The bispecific $F(ab')_2$ augmented the killing activity of PBMCs on ADMHK/E cells. The % cytotoxicity of PBMCs on ADMHK/E cells was 6.4 ± 1.2% in the absence of bispecific $F(ab')_2$, but 10.0 ± 2.7, 16.8 ± 3.4, 29.7 ± 2.9 and 35.0 ± 3.6% in the presence of the bispecific $F(ab')_2$ at 0.1, 1, 10 and 100 μg/ml, respectively. $F(ab')_2$ of MRK16 and $F(ab')_2$ of OKT3 did not increase the cytotoxicity of PBMCs on ADMHK/E cells, and the bispecific $F(ab')_2$ had no effects on the cytotoxicity of PBMCs on LCHK/E cells (Fig. 6).

DISCUSSION

To date, the therapeutic effects of anti-cancer drugs on human renal cancers have been disappointing because of the intrinsic drug-resistance of the cells. Recently, much attention has been focused on methods to overcome renal cancer MDR.[30-32]

The expression of P-glycoprotein on renal cell cancers has been demonstrated by Northern blot analysis and immunostaining with mAbs.[30-32] As is generally the case, the P-glycoprotein-positive renal cancers were MDR in the present chemotherapy. The Parent HK/E cell line was established by *in vitro* culture from an untreated clear cell carcinoma, and was found to consist of a mixed population of P-glycoprotein-positive and negative cells (Fig. 1A). During 3 months' culture in drug-free conditions, these cells lost their ability to express P-glycoprotein (Fig. 1C). But, to our surprise, short-term treatment of the Parent HK/E cells with ADM resulted in a predominance of P-glycoprotein-positive cells. Such emergence of drug resistance may reflect the clinical situation of the present chemotherapy against renal cell carcinoma. It was not due to induction of P-glycoprotein but due to the elimination of P-glycoprotein-negative HK/E cells in Parent HK/E cell populations, because the same treatment of P-glycoprotein-negative LCHK/E cells did not induce a P-glycoprotein-positive cell population (data not shown). As the expression of P-glycoprotein by the renal cell lines used in this study correlated well with their ADM sensitivities (Figs. 1 and 2), we used these cell lines as target cells for studies of the effect of bispecific $F(ab')_2$.

The MRK16-OKT3 bispecific $F(ab')_2$ bound to the target cells expressing P-glycoprotein (Fig. 4). It also augmented the binding of human PBMCs to the P-glycoprotein-positive target cells (Fig. 5), and enhanced their cytotoxicity depending on the expression of the P-glycoprotein (Fig. 6). We thought that the effector cells in this assay might be CD3 T lymphocytes in PBMCs on the basis of previous reports.[33-36] The effect in augmenting the cytotoxicity of the PBMCs seemed to be mediated through enhancement of cell-to-cell binding and activation of the effector cells. Indeed, OKT3 is known to bind to CD3 molecules on lymphocytes and to enhance their lymphokine production, cytotoxicity and synthesis of perforin molecules.[33-35] Moreover a bispecific $F(ab')_2$ containing OKT3 was shown to stimulate lymphocytes.[36] However, in our assay system $F(ab')_2$ of OKT3 alone had no effect (Fig. 6), so the involvement of PBMC activation by OKT3 is unlikely. The enhancement of lymphocyte-mediated cytotoxicity by the present bispecific $F(ab')_2$ thus seems to be mediated through the enhancement of the binding of PBMCs to the cells followed by their cytolysis. The binding of

IL-2 activated PBMCs to P-glycoprotein-positive target cells was enhanced greatly as compared to non-activated PBMCs (Fig. 6), but no enhancing effect of the bispecific $F(ab')_2$ in the cytotoxicity assay could be found (data not shown). Probably, IL-2-activated PBMCs alone had already gained highly enhanced cytotoxicity by IL-2 activation, and the effects of bispecific $F(ab')_2$ did not become evident.

Therapeutically, bispecific $F(ab')_2$ has several advantages over the original murine mAbs. First, as $F(ab')_2$ fragments lack the Fc portion, the $F(ab')_2$ does not bind to FcR^+ cells in the reticuloendothelial system, and thus its rapid clearance is avoided, so it should remain in the circulation for a long time. Second, the murine Fc portion is strongly antigenic in humans, but there is little risk that the $F(ab')_2$ will cause undesirable immune reactions. The P-glycoprotein plays an important role in MDR by reducing the intracellular drug concentration.[1-5] As the protein is a common antigen expressed on MDR cells,[24] targeting therapy against P-glycoprotein seems one of the most promising ways of overcoming drug resistance. Besides targeting therapy with anti-P-glycoprotein mAbs, such as immunotoxin conjugation of the mAb MRK16[14] and ADCC by the mAb MRK-16,[15,16] the augmentation of lymphocyte cytotoxicity by the bispecific $F(ab')_2$ described here could be beneficial because of the unique mechanism of action and low toxicity of therapy.

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