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In vitro Anti-tumor Activity of Anti-c-*erb*B-2 × Anti-CD3ε Bifunctional Monoclonal Antibody

Yoshihiro Sugiyama,[1] Miki Aihara,[1] Masafumi Shibamori,[1] Kyohei Deguchi,[1] Kenichi Imagawa,[1,5] Mikio Kikuchi,[1] Hiroshi Momota,[2] Takachika Azuma,[3] Hidechika Okada,[3] Özge Alper,[4] Jiro Hitomi[4] and Ken Yamaguchi[4]

[1]*Viral Diseases Research Division, Tokushima Research Institute, Otsuka Pharmaceutical Co., Ltd., 463-10 Kagasuno, Kawauchi-cho, Tokushima 771-01,* [2]*Cell Engineering Division, Bioscience Laboratory, Earth Chemical Co., Ltd., 1122-73 Kitamachi, Nishihama, Ako-shi, Hyogo 678-02,* [3]*Department of Molecular Biology, Nagoya City University School of Medicine, Mizuhocho, Mizuho-ku, Nagoya 467 and* [4]*Growth Factor Division, National Cancer Center Research Institute, Tsukiji 5-1-1, Chuo-ku, Tokyo 104*

With the aim of developing an effective cancer immunotherapy for common epithelial cancer, a new class of bifunctional antibody (BFA) was developed; one arm of this BFA recognized c-*erb*B-2 gene product, and the other arm recognized CD3ε, a T-cell specific surface antigen. Application of this BFA with human peripheral blood lymphocytes exhibited specific anti-tumor activity *in vitro* on a breast tumor cell line, ZR-75-1, which expressed abundant c-*erb*B-2 gene product on its cell surface. These results indicate that BFA recognizing an oncogene product on cell surface is a potential new agent for cancer immunotherapy.

Key words: Monoclonal antibody — Anti-c-*erb*B-2 — Anti-CD3ε — Bifunctional antibody — Cancer immunotherapy

Clinical relevance of monoclonal antibodies (MoAb)[6] in classical cancer immunotherapy has been investigated extensively. The major application is passive immunization with MoAbs followed by activation of antibody-dependent cellular cytotoxicity[1)] and/or complement-dependent cytolysis[2)]; however, it is an unusual observation that the potent cytotoxic immune response necessary for cancer therapy is induced. These facts limit the clinical relevance of MoAb-guided cancer immunotherapy. Bifunctional antibody (BFA)[3)] is a potential new tool to overcome this difficulty. One arm of BFA recognizes a tumor cell-associated antigen and the other recognizes a T cell antigen of T lymphocytes. Based on this structure, BFA can bind to cancer cells followed by activation of cellular cytotoxic mechanisms. *In vitro* activities of BFA were studied with several tumors[4–7)] and clinical studies have also been undertaken for glioma,[8)] ovarian carcinoma and lung carcinoma.[9)] To develop BFA-guided immunotherapy for more common epithelial cancers, we developed a BFA targeting c-*erb*B-2 gene product,[10)] and evaluated its *in vitro* anti-tumor activity.

The anti-c-*erb*B-2 gene product × anti-CD3ε BFA developed in the present study is schematically demonstrated in Fig. 1. A murine MoAb against c-*erb*B-2 gene product, GFD-OA-p185-1,[11)] belongs to the IgG_1 subclass, and was proved to recognize the extracellular domain of the protein. Another murine MoAb, UCHT1 (Imperial Cancer Research Technology Ltd., London),[12)] was also of IgG_1 subclass and was specific to human CD3ε-antigen expressed on the cell surface of T lymphocytes.

Generally, the BFA have been prepared from quadroma[13)] or formed chemically.[3)] Nitta *et al.*[14)] originally used DTT to reduce each $F(ab')_2$ to Fab′-SH, added DTNB to block the free -SH moieties of one arm of Fab′-SH and then prepared the BFA.

We employed the method of Nitta *et al.* as follows. The nitrobenzoic acid derivatives of anti-c-*erb*B-2 gene product Fab′ fragments (Fab′-S-NB) and anti-CD3ε Fab′ fragments with free SH group (Fab′-SH) were prepared as described in the legend to Fig. 1. They were mixed in equimolar amounts and incubated at 4°C under mild reoxygenation conditions to produce the BFA. The BFA with a molecular weight of approximately 110 kd was separated from unreacted Fab′ fragments and the residual reactants by high-pressure liquid chromatography using a TSK-gel G-3000SW column (Tosoh, Tokyo). SDS-PAGE analysis revealed that the purified BFA possessed the expected molecular weight.

[5] To whom requests for reprints should be addressed.

[6] The abbreviations used are: MoAb, monoclonal antibody; PBL, peripheral blood lymphocytes; BFA, bifunctional antibody; DTT, dithiothreitol; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

The immunological reactivities of the two $F(ab')_2$ fragments generated from the original MoAbs, anti-c-*erb*B-2 gene product MoAb and anti-CD3ε MoAb, and the developed BFA were tested using 7 human tumor cell lines and peripheral blood lymphocytes (PBL) prepared from a healthy volunteer (male, 38 years old). These cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland), and were ZR-75-1 (human breast carcinoma), SK-BR-3 (human breast adenocarcinoma), MCF-7 (human breast adenocarcinoma), PANC-1 (human pancreas, epitheloid adenocarcinoma), MIAPaCa-2 (human pancreatic carcinoma), AsPC-1 (human metastatic pancreas adenocarcinoma), and BxPC-3 (human pancreas, primary adenocarcinoma). The tumor cells or PBL were washed with PBS containing 0.1% BSA and 0.1% NaN_3, and were incubated with the same buffer containing 10 μg of antibodies for 30 min on ice. The cells were washed twice, incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG (Tago, Inc., Burlingame, California) for 30 min on ice, and again washed twice. The reactivities of the antibodies were measured by flow cytometry using an Ortho Spectrum III (Ortho Diagnostic Systems, Westwood, Massachusetts). The analysis yielded a log scale fluorescence histogram. Positive cells were identified on the basis of their stronger fluorescence intensity than non-stained cells.

Anti c-*erb*B-2 gene product $F(ab')_2$ fragments reacted strongly with two breast tumor cell lines, ZR-75-1 and SK-BR-3, both of which express abundant c-*erb*B-2 mRNA.[16] This $F(ab')_2$ reacted weakly to MCF-7, MIAPaCa-2, PANC-1 and AsPC-1 compared to the former cell lines, reacted very weakly to BxPC-3, and did not react with PBL. Anti-CD3ε $F(ab')_2$ fragments reacted with PBL alone. The BFA reacted strongly with two human tumor cell lines, ZR-75-1 and SK-BR-3, and

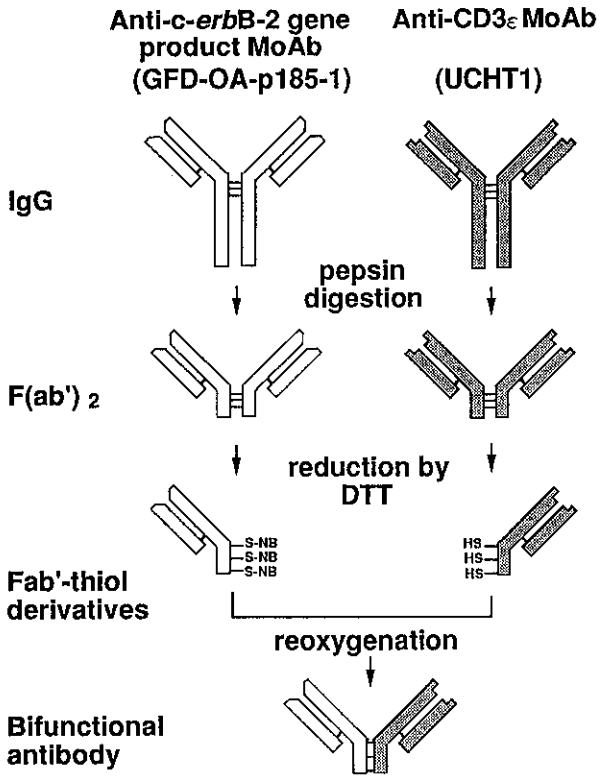

Fig. 1. Preparation of the BFA. Both MoAbs were digested with pepsin to generate $F(ab')_2$ fragments.[15] After purification of pepsin digests on a TSK-gel G-3000 SW column (Tosoh, Tokyo), GFD-OA-p185-1 $F(ab')_2$ fragments were reduced with DTT at a final concentration of 2 m*M* for 30 min at room temperature, and then the reaction was stopped by the addition of DTNB at a final concentration of 5 m*M*. The nitrobenzoic acid derivatives of the GFD-OA-p185-1 Fab′ fragments (Fab′-S-NB) were separated from the excess reagents and the other reaction products by means of a Sephadex G-25 column. UCHT1 $F(ab')_2$ fragments were also reduced with DTT at a final concentration of 2 m*M* for 30 min at room temperature, and thus-generated Fab′ fragments with free SH groups (Fab′-SH) were separated from the excess reagents by using a Sephadex G-25 column. Fab′-SH and Fab′-S-NB fragments were then mixed at a ratio of 1:1, incubated for 4 h at room temperature and stored at 4°C. After a few days, the resulting preparation was applied to a TSK-gel G-3000 SW column to remove the remaining Fab′ fragments (data not shown). Generation of BFA was checked by comparison of the molecular weight with those of the parental $F(ab')_2$ and reduced Fab′ by SDS-PAGE on a 7.5% gel under non-reducing conditions (data not shown).

Table I. The Reactivities of Anti-c-*erb*B-2 Gene Product $F(ab')_2$ Fragments, Anti-CD3ε $F(ab')_2$ Fragments and Their BFA to Various Tumor Cell Lines and PBL

Cell	Control[a]	$F(ab')_2$ fragment		BFA
		anti-c-*erb*B-2 gene product	anti-CD3ε	
PBL	2.7[b]	4.9	82.7	82.8
ZR-75-1	8.2	85.5	2.6	92.9
SK-BR-3	2.0	99.5	2.9	99.9
MCF7	4.8	40.3	1.9	47.4
PANC-1	4.6	24.4	3.6	25.9
MIAPaCa-2	2.2	61.4	5.2	61.9
AsPC-1	9.6	33.5	NT[c]	22.1
BxPC-3	2.8	8.8	2.4	8.6

a) Second antibody alone.
b) Percent of cells reacted with respective antibodies.
c) NT; not tested.
The reactivities were accessed by flow cytometry, representing percent positivity of tumor cell lines and PBL reacted with the respective preparations.

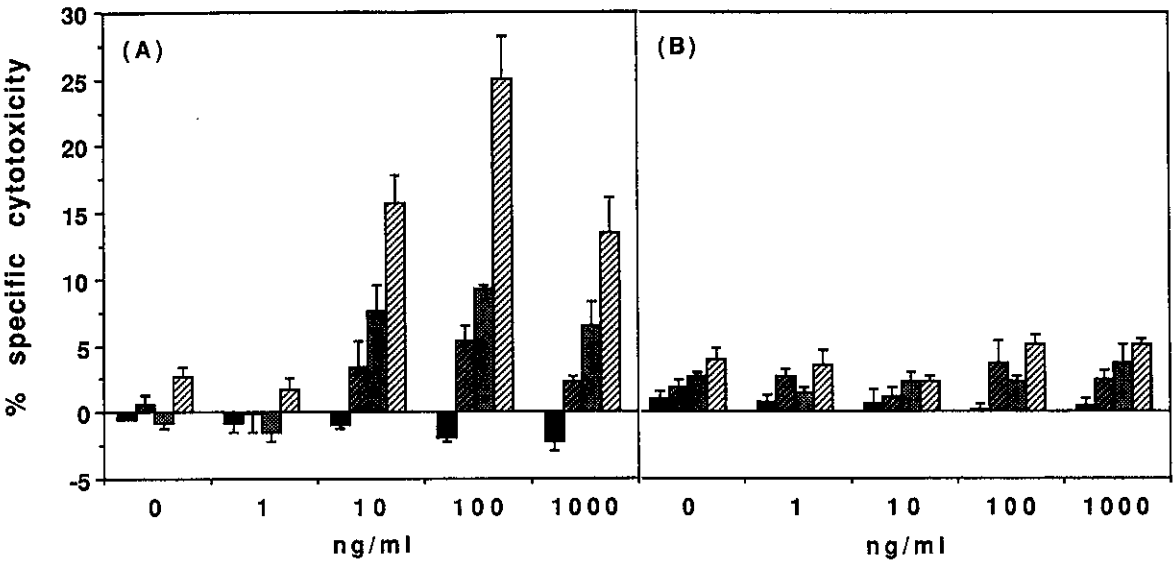

Fig. 2. The cytotoxic activities of BFA. The cytotoxic activities of BFA (A) and the mixture of two $F(ab')_2$ fragments (B) with PBL on ZR-75-1 were measured as follows. Tumor cells (5×10^6 cells) were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ (Amersham Corp., Arlington Heights, Illinois) in 0.5 ml of RPMI 1640 supplemented with 10% FBS for 1 h at 37°C, then washed twice. PBL of healthy volunteer were prepared by using lymphocyte separation medium (Flow laboratories, McLean, Virginia). Then 50 μl of various numbers of effector cells, 100 μl of various concentrations of BFA or the mixture of two $F(ab')_2$ fragments and 50 μl of ^{51}Cr-labeled target cells (10^4 cells) were added in quadruplicate to 96-well round-bottomed microtiter plates (Becton Dickinson Labware, Lincoln Park, New Jersey) and incubated for 4 h at 37°C. The supernatant of each well was collected by using a Supernatant Collection System (Skatron Inc., Sterling, Virginia) and radioactivity was counted with a γ-counter (Packard Instrument Company, Inc., Downers Grove, Illinois). The specific cytotoxicity was calculated according to the following equation; % specific cytotoxicity $=[(a-b)/(c-b)]\times100$; a: the mean cpm of experimental release, b: the mean cpm of target alone (spontaneous release), c: the mean cpm of maximal release (cells were lysed by 1% NP-40). (■), E/T=0; (▨), E/T=5; (▩), E/T=10; (▨), E/T=20.

PBL. For other cell lines, the reactivities of BFA were the same as that of anti c-*erb*B-2 gene product $F(ab')_2$ fragments (Table I). These data indicated that the BFA possessed the expected immunological characteristics.

We tested the cytotoxic activity of BFA with PBL against ZR-75-1 and SK-BR-3, both of which reacted well with this BFA, by means of ^{51}Cr-release assay as described in the legend to Fig. 2. In the case of ZR-75-1, the BFA in conjunction with PBL expressed a significant anti-tumor activity (Fig. 2). This activity was observed at concentrations of BFA greater than 1 ng/ml and at values of the effector/target (E/T) ratio greater than 5. The maximal specific cytotoxicity reached approximately 20% at 100 ng/ml of BFA and at the E/T ratio of 20. On the other hand, the cytotoxicity decreased at the BFA concentration of 1,000 ng/ml (Fig. 2A); it is likely that excess of BFA binds to cells, and interferes with the effective cross-linking between tumor cells and T-lymphocytes. In contrast, at the same E/T ratio, the mixture of the two $F(ab')_2$ fragments (1:1) had no significant anti-tumor activity at concentrations up to 1,000 ng/ml (Fig. 2B), and neither of the parental $F(ab')_2$ fragments showed any significant cytotoxic activity at concentrations up to 1,000 ng/ml (data not shown). Moreover, anti-tumor activity toward ZR-75-1 of another BFA developed in the same way was tested. The other BFA[17] was prepared from the same anti-CD3ε MoAb (UCHT1) and anti-HIV env protein MoAb (0.5β)[18] provided by Dr. Matsushita (University of Kumamoto, Kumamoto). The 0.5β was proved not to bind to ZR-75-1 and PBL by flow cytometry. Under the same experimental conditions, this BFA with PBL had no significant anti-tumor activity (data not shown).

These results indicated that the present BFA with PBL possesses specific anti-tumor activity toward ZR-75-1, and that the effect was dependent on specific immunoreactivities induced by one arm of BFA recognizing c-*erb*B-2 gene product. In the case of SK-BR-3, however, the remarkable cytotoxicity was observed with PBL alone and the specific cytotoxicity induced by PBL with BFA was not observed. We have not yet clarified the cause of this phenomenon, but we presumed that this cell

line was sensitive to natural killer cells in the PBL and so the specific cytotoxicity was not observed. The cytotoxicity of PBL with BFA to other tumor cell lines and primary tumor cells should be further studied to confirm the efficacy of this BFA *in vitro*.

It was established that c-*erb*B-2 gene product is expressed very frequently in various epithelial cancer cells,[19] that its expression in normal adult tissues is very rare,[20,21] and that the expression of this protein is well correlated with worse prognosis of patients with breast[22,23] and ovarian[24] cancer. The present study demonstrates that the BFA of the present form with PBL possessed anti-tumor activity *in vitro*, suggesting that c-*erb*B-2 gene product is useful as a targeting molecule for cancer immunotherapy. Further studies will be required to elucidate the *in vivo* anti-tumor effect of the present BFA using animal models with intact immune systems.

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Addendum: After submission of this manuscript, Nishimura *et al.*[25] reported the anti-tumor effect of anti-c-*erb*B-2 antibody × anti-CD3ε antibody BFA, although the effector cells were different from those in our study.

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