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Anti-cancer Activity of a Human Monoclonal Antibody Secreted from a Human × Human Hybridoma

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Abstract Human × Human hybridoma, CLNH11, derived from a lymph node lymphocyte of a cervical cancer patient, produced human monoclonal IgG_1 antibody (CLN–IgG). CLN–IgG recognized an antigen expressed preferentially on the cell surface of various human tumor cells including cervical carcinomas. In this report, we demonstrated that simultaneous and systemic administration of CLN–IgG into nude mice resulted in the inhibition of subcutaneous growth of human cervical cell line ME–180. Furthermore, *in vitro* analysis showed that the anti-cancer effects of CLN–IgG *in vivo* seemed to be mediated by the mechanism of antibody-dependent cell-mediated cytotoxicity, rather than direct or complement-dependent manner.

Introduction

It is thought that tumor cells express unique antigens which are not detected on normal cells and that host immune systems eliminate the cells with such antigens. In human, it was reported that the serums of melanoma patients contained antibodies directed against auto-cancer cells (Carey *et al.*, 1976; Shiku *et al.*, 1976). Therefore, it seemed possible that such antibodies could be used for therapeutic and diagnostic purposes. But limitation of constant supply of the serum interfered with such attempts.

Monoclonal antibody (MoAb) technique developed by Kohler and Milstein (1975) resolved this problem. After then, murine MoAbs to human cancer cells were generated (Mitchell *et al.*, 1980; Brown *et al.*, 1982; Soule *et al.*, 1983) and some of them were applied to clinical trials (Miller *et al.*, 1982; Dillman *et al.*, 1984; Goodman *et al.*, 1985; Houhton *et al*, 1985; Meeker *et al.*, 1985; Sears *et al.*, 1985). From the results of such experiments, it has become apparent that anti-mouse immunoglobulin responses were induced in many cancer patients treated with mouse MoAbs and that unexpected side effects associated with the responses have limited the usefulness of xenogeneic MoAb in human. Therefore, human MoAbs to human cancer have been required.

Recently, Hagiwara and Sato (1983) have succeeded in establishing a human×human hybridoma, CLNH11, by fusion of cervical cancer patient's lymphocytes with human B-lymphoblastoid cell line UC729–6 by polyethylene glycol. CLNH11 produces human monoclonal IgG_1 antibody (CLN–IgG) which reacted with various tumor cell lines but not with normal cells including fibroblasts, perpheral blood lympho-

cytes and erythrocytes. It was also found that the tumor-associated antigen recognized by CLN–IgG was a molecule exposed on the tumor cell surface. The affinity-purified antigen (TA60/53) consisted of α (molecular weight 60,000) and β subunit (molecular weight 53,000) which were linked by intermolecular disulfide bond(s) (Aotsuka and Hagiwara, 1987). In peroxidase immunostaining of astrocytoma tissue sections, the expression of TA60/53 correlated with their malignancies, suggesting that TA60/53 could be involved in metastasis or tissure invasion of malignant tumor cells.

In this report, we described the inhibitory effects of CLN–IgG on the growth of human cervical carcinoma xenograft in nude mice and mechanism of CLN–IgG-mediated cytotoxicity.

Materials and Methods

Mice. Six-week old female Balb/c athymic nude (nu/nu) mice were used.

Tumor cells. Human cervical carcinoma cell lines, ME–180 were purchased from American Type Culture Collection (ATCC; Rockville, MD, USA). The cell line was maintained in DF medium (1:1 mixture of Dulbecco's modified MEM and Ham's F12) containing 10% fetal bovine serum (FBS).

Antibodies. Human monoclonal antibody CLN–IgG were purified from serum-free culture fluid of human$\times$human hybridoma CLNH11 by using protein A-coupled Sepharose 4B as described elsewhere (Aotsuka and Hagiwara, 1987; Hagiwara *et al.*, 1985). Purity of the antibody was assessed by SDS-polyacrylamide gel electrophoresis (SDS–PAGE) and high performance liquid chromatography. Antibodies of above 95% purity were used.

Human anti-AB blood type IgG antibody was purified from anti-AB serum (BCA; West Chester, PA, USA) by the same method as described above.

Cytotoxicity assay. Complement-dependent cytotoxicity (CDC) was assayed by trypan blue dye exclusion. 4×10^4 human cervical carcinoma ME–180 cells (50 μl) was mixed with 50 μl of antibody. And then, 50 μl of two-fold diluted rabbit serum (Hoechst, West Germany) used as complement sources was added and incubated at 37°C for 3 hr. After adding 150 μl of 0.1% trypan blue dye solution, viable cell number was counted. Percent cytotoxicity was calculated as follows: (viable cell number of control−viable cell number of experiment)/(viable cell number of control)$\times$100, where "control" means the condition in the absence of antibody and complement.

Antibody-dependent cell-mediated cytotoxicity (ADCC) was assayed by ^{51}Cr-release method. ME–180 cells (2×10^6) were labelled with 25 μCi sodium [^{51}Cr] chromate for 1.5 hr at 37°C and washed 2 times with RDF medium (2:1:1 mixture of RPMI1640, Dulbecco's MEM and Ham's F12). Labelled target cells (10^4 in 50 μl) and 1.6–100 μg/ml of CLN–IgG were added to each well of 96-well microtiter plate (Becton Dickinson Labware, Oxnard, CA, USA).

1–8×10^5 human peripheral blood lymphocytes (PBL) were then added to give an effector to target ratio of 10:1 to 80:1. After 18 hr incubation at 37°C, supernatant was removed for counting. The percent specific ^{51}Cr release was calculated as: (experimental−spontaneous release)/(total−spontaneous release)$\times$100. Total count

was measured by lysis of cells using 0.08% Triton X. Spontaneous release in the absence of effector cells and CLN–IgG was 16% of total count.

Nude mice test. 5×10^6 ME–180 cells were mixed with 1–5 mg CLN–IgG and then injected subcutaneously into nude mice. In other experiment, after subcutaneous inoculation of 5×10^6 ME–180 cells, 5 mg of CLN–IgG was administrated intravenously. The size of growing tumor was measured and expressed as follows: Tumor size= (longest length)$\times$(shortest length)$^2\times1/2$.

Results

It is known that there are three ways for antibody to show anti-cancer activity: (1) direct inhibition, (2) complement-dependent cytotoxicity (CDC), and (3) antibody-dependent cell-mediated cytotoxicity (ADCC). CDC and ADCC mechanism require cell surface localization of antigen recognized by antibody.

We have shown that tumor-associated antigen TA60/53 recognized by human monoclonal antibody CLN–IgG was expressed on the cell surface and that its expression seemed to be limited in tumor cells (Aotsuka and Hagiwara, 1987). These results promted us to examine whether CLN–IgG has anti-tumor activity or not.

Effect of human monoclonal antibody CLN–IgG on the tumor growth in vivo

First of all, we investigated the effect of CLN–IgG on the growth of tumor xenograft in athymic nude mice. We chose human cervical tumor cell line ME–180 as target cell, because this cell line expressed TA60/53 antigen, as examined by immunofluorescence staining and ELISA, and grew well in nude mice. When 5×10^6 ME–180 cells were inoculated subcutaneously, tumors of about 1 cm diameter were formed on day 30 in all nude mice. We used normal human IgG (Beriglobin; Hoechst) as irrelevant negative control. When analyzed by ELISA, this antibody has no reactivity with ME–180. We also used anti-blood type AB antibody (BCA) as positive control antibody, because ME–180 cells possess blood type A antigen.

To begin with, we tested the effect of simultaneous administration of antibodies and ME–180 cells on *in vivo* tumor growth; 5×10^6 cells were mixed with antibody and inoculated subcutaneously in nude mice. Tumor size of each mice was measured as a function of days after tumor injection. Assuming that growing tumor is spheroid, tumor volume was calculated by the following approximate equation: (longest length)$\times$(shortest length)$^2\times1/2$.

As shown in Fig. 1, when 5 mg of control human IgG together with ME–180 cells was injected, no growth inhibition was observed, compared with the tumor growth of saline-control group. In contrast, when ME–180 cells were inoculated together with anti-AB antibody (5 mg), the tumor growth was almost completely suppressed. In Fig. 2, simultaneous administration of 5 mg of CLN–IgG resulted in the suppression up to 30 days after tumor inoculation, although injection of CLN–IgG seemed less effective than that of same amount of anti-AB antibody. Administration of 1 or 2.5 mg of CLN–IgG induced growth inhibitory effect comparable to that of 5 mg CLN–IgG.

Next we examined the effect of systemic administration of CLN–IgG on the growth

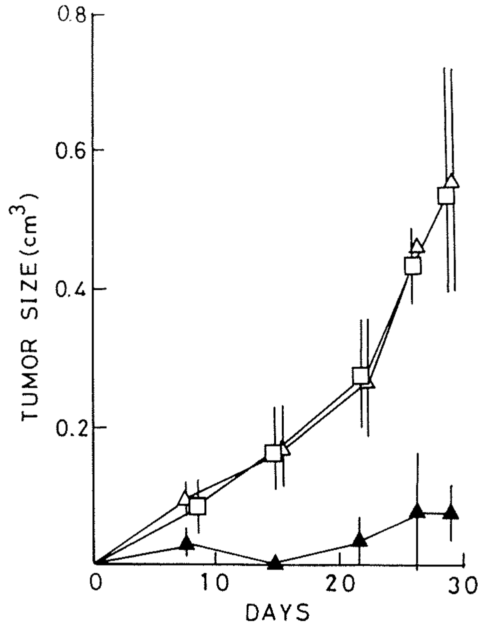

Fig. 1. The effect of simultaneous injection of control antibodies on the growth of human cervical carcinoma ME–180 grown in nude mice. ME–180 cells (5×10^6) were mixed with saline (□), normal human IgG (△) or human anti-blood type AB IgG antibody (▲) and injected subcutaneously into athymic nude mice. Tumor size of each mouse was measured by the following equation: (longest length)×(shortest length)$^2\times1/2$. Each symbol and vertical bar represent mean value and S. D. of 6 mice, respectively.

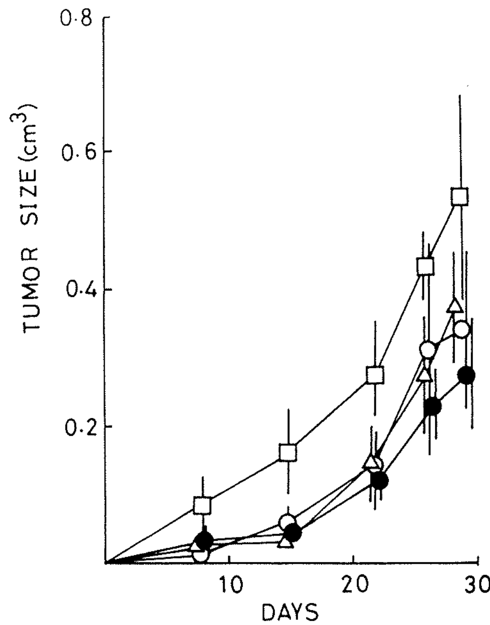

Fig. 2. The effect of simultaneous injection of CLN–IgG on the growth of human cervical carcinoma ME–180 grown in nude mice. ME–180 cells (5×10^6) were mixed with saline (□), 1 mg (△), 2.5 mg (○), 5 mg (●) of CLN–IgG and inoculated subcutaneously into nude mice. Other indications are the same as the legend of Fig. 1.

of ME–180. CLN–IgG (5 mg) was injected intravenously (i.v.) soon after 5×10^6 ME–180 cells were inoculated subcutaneously. As shown in Fig. 3, i.v. injection of CLN–IgG induced significant suppression of the tumor growth, although this treatment seemed less effective than simultaneous injection of same amount of CLN–IgG (Fig. 2). This result suggests that injected CLN–IgG or CLN–IgG-bound effector cells, such as macrophage and K-cell, gathered around the inoculated tumor to show cytotoxic effect.

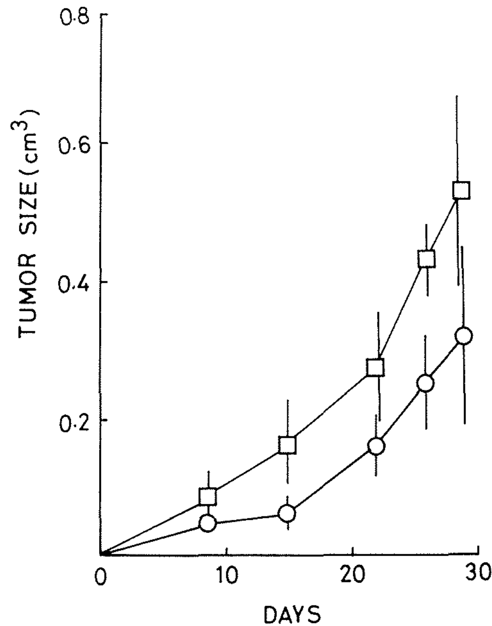

Fig. 3. The effect of intravenous injection of CLN–IgG on the growth of human cervical carcinoma ME–180 grown in nude mice. After 5×10^6 ME–180 cells were inoculated subcutaneously into nude mice, 5 mg of CLN–IgG (○) or saline (□) was injected intravenously. Other indications are the same as in the lengend of Fig. 1.

Table 1. CLN-IgG has no inhibitory effect on the growth of ME–180 cells *in vitro*[a].

CLN-IgG (μg/ml)	Cell number ($\times10^4$)[b]				
	Day				
	1	2	3	4	5
0	2.6±0.3	6.1±0.7	14.7±4.1	31.9±3.1	50.0±2.9
3	2.1±0.3	6.0±0.6	14.1±4.4	31.2±1.1	44.7±4.1
10	2.8±0.3	7.7±2.8	13.4±1.8	31.7±3.0	46.0±1.1
30	1.7±1.4	7.0±0.7	14.3±1.3	30.2±2.8	46.7±2.3
100	2.1±0.8	5.1±1.1	13.0±1.6	28.4±2.2	50.0±1.3

[a] 10^4/ml of ME180 cells were cultured in DF medium containing 10% FBS in the absence of or in the presence of CLN-IgG. Cells were removed from culture dish by using 0.25% trypsin and 0.02% EDTA for 15 min at 37°C and then viable cells were counted by trypan blue dye (0.05%) exclusion method.

[b] mean value±S.D. of triplicate cultures.

Mechanism of growth inhibitory effect of CLN–IgG

In order to determine which mechanism was responsible for anti-cancer activity of CLN–IgG *in vivo*, we assessed the three mechanisms (direct, CDC and ADCC) *in vitro*.

First of all, we examined the direct effect of CLN–IgG on the growth of ME–180.

Table 2. Complement-dependent cytotoxicity of CLN-IgG and anti-AB antibody to ME–180 cells[(a)].

Rabbit complement[(b)]	Antibody concentration (μg/ml)	Cytotoxicity (%)[(c)] CLN-IgG	Cytotoxicity (%)[(c)] Anti-AB[(d)]
+	0	5±2	5±2
−	100	6±4	5±3
+	100	18±8	88±5
+	30	12±10	63±9
+	10	8±2	57±4
+	3	0±7	49±9

a) human cervical carcinoma ME–180 cells (4×10^4 in 50 μl) were mixed with 50 μl of antibody and 50 μl of rabbit complement. After 3 hr incubation at 37°C, 150 μl of 0.1% trypan blue dye solution was added and viable cell number was counted.

b) two-fold diluted rabbit serum (Hoechst) was used as complement source.

c) percent cytotoxicity was calculated as follows: (viable cell No. of control −viable cell No. of experiment)/(viable cell No. of control)×100. "control" means the condition in the absence of antibody and complement.

d) IgG fraction of human anti-AB antiserum (BCA) purified by affinity chromatography using protein A-Sepharose.

Table 3. Dose dependency of CLN–IgG in ADCC activity to ME–180[(a)].

CLN–IgG (μg/ml)	% specific ^{51}Cr release[(b)]
0	4.0±0.5
1.6	4.3±3.1
3.2	11.3±2.6
6.3	10.4±1.5
12.5	15.1±0.6
25.0	12.5±0.4
50.0	6.2±0.6
100.0	3.4±0.9

a) ME–180 cells (2×10^6) were labelled with 25 μCi sodium [^{51}Cr] chromate for 1.5 hr at 37°C and washed 2 times with RDF medium. Labelled target cells (10^4 in 50 μl) and indicated concentration of CLN–IgG were added to wells. 4×10^5 human peripheral blood lymphocytes were then added to give an effector to target ratio of 40:1. After 18 hr incubation at 37°C, supernatant was removed for counting.

b) percent specific ^{51}Cr release was calculated as: (experimental−spontaneous release)/(total−spontaneous release)×100. Spontaneous release in the absence of effector cells and CLN–IgG was 16% of total count. Results are represented by mean±S. D. of triplicate examination.

The cells (10^4/ml) were cultured in DF containing 10% FBS in the presence of 3–100 μg/ml of CLN–IgG and viable cell number was counted daily by trypan blue dye exclusion method. As shown in Table 1, at any concentration of CLN–IgG, no growth inhibition could be observed.

In Table 2, CDC activity of CLN–IgG and anti-AB antibody against ME–180 were shown. ME–180 cells were allowed to react with indicated concentration of antibody and rabbit complement, and viable cell mumber was counted after 3 hr incubation. Anti-AB antibody provided strong cytotoxicity, whereas CLN–IgG, even at 100 μg/ml, could hardly killed ME–180 cells.

Next we investigated ADCC activity of CLN–IgG. ^{51}Cr-labelled ME–180 cells were mixed with peripheral blood lymphocytes (PBL) from healthy individuals together with CLN–IgG and then released ^{51}Cr was counted. Table 3 showed the dose dependency

Table 4. Effect of effector/target ratio in ADCC activity of CLN–IgG to ME–180 cells[a].

E/T ratio[b]	% specific ^{51}Cr release
1:10	4.0±1.8
1:20	11.7±0.5
1:40	15.0±1.4
1:80	15.8±1.7

a) ^{51}Cr-labelled ME–180 cells (10^4) were mixed with 12.5 μg/ml of CLN–IgG. Peripheral blood lymphocytes ($1–8\times10^5$) were then added to give an effector/target ratio of 1:10 to 1:80. After 18 hr incubation, ^{51}Cr in supernatant was counted. Results are presented by mean±S. D. of triplicate examination. Other indications are the same in the legend in Table 3.

b) effector/target ratio.

Table 5. Individual difference in CLN–IgG-mediated ADCC activity to ME–180 cells[a].

PBL[b] donor	% specific ^{51}Cr release
1	8.9 ± 2.6
2	11.1 ± 0.6
3	11.5 ± 3.0
4	13.8 ± 2.1
5	8.9 ± 0.1
6	15.3 ± 0.4
7	19.8 ± 6.8
8	37.9 ± 4.7

a) ^{51}Cr-labelled ME–180 cells (10^4) were mixed with 12.5 μg/ml of CLN–IgG. Peripheral blood lymphocytes (4×10^5) from each healthy individual were then added to give an effector/target ratio of 40:1. After 18 hr incubation, ^{51}Cr in supernatant was counted. Results are expressed by mean±S. D. of triplicate examination. Other indications are the same in Table 3.

b) peripheral blood lymphocytes from healthy persons.

of CLN–IgG in such experiment. At 40:1 of effector/target (E/T) ratio, 12.5. μg/ml of CLN–IgG provided maximum cytotoxicity (16%).

Table 4 demonstrated the effect of E/T ratio on the ADCC activity of CLN–IgG at a concentration of 12.5 μg/ml. At more than 40:1 of E/T ratio, maximum release was observed.

In maximum condition (CLN–IgG, 12.5 μg/ml; E/T ratio, 40:1), we examined the individual differences in ADCC activity to ME–180 cells. It became evident that healthy persons showed 16% cytotoxicity on the average to ME–180 (Table 5). Maximum cytotoxicity of anti-AB antibody was 48% (data not shown).

Together with these results, we concluded that the growth inhibition induced by CLN–IgG *in vivo* seemed to be mediated primarily by the mechanism of antibody-dependent cell-mediated cytotoxicity, rather than direct or complement-dependent manner.

Discussion

We described here that human monoclonal antibody CLN–IgG, secreted from a cervical cancer patient's lymphocyte-derived human×human hybridoma CLNH11, exhibited growth suppressive effect on human cervical carcinoma ME–180 cells grown subcutaneously in nude mice. Both simultaneous and intravenous injection of CLN–IgG were effective. However, activity of CLN–IgG was somewhat weaker than that of anti-AB antibody which suppressed the tumor growth almost completely (Figs. 1, 2).

Anti-AB IgG we used here is polyclonal antibody so that many antibodies of different idiotypes and subclasses were contained in it. It has been demonstrated in several systems that mixture of two kinds of antibody directed against different determinants or different epitopes of identical molecule in the cell surface showed synergistic augmentation of the cytotoxic activity in combination with complement or effector cells (Elliot *et al*, 1978; Hellstrom *et al*, 1981). Thus, it seems likely that monoclonal antibody alone shows slight inhibition, compared with polyclonal antibody.

Actually, in *in vitro* analysis, although anti-AB antibody provided strong cytotoxicity in the presence of rabbit complement, CLN–IgG could not (Table 2). However, our preliminary data showed that combination of CLN–IgG and another human MoAb SLN–IgG, which recognizes tumor cell surface determinant of 37,000 molecular weight, yielded high CDC activity snergistically against human glioblastoma cells, despite failure of each antibody alone in the tumor-killing effect together with complement. It appears, therefore, that CLN–IgG could bind complement, but not activate it effectively on ME–180 cells. This inability may be attributed to low density of CLN–IgG-defined antigen TA60/53 on the cell surface and heterogeneity of the antigen expression of the tumor cells, as observed in immunofluorescence staining (Aotsuka and Hagiwara, 1987).

However, these *in vivo* experiments were carried out in nude mouse system. ADCC analysis by using human peripheral blood lymphocytes as effector cells showed that some individual was found to possess high cytotoxicity activity (38% cytotoxicity). Therefore, it is possible that stronger effect could be achieved if CLN–IgG would be injected in cancer patients.

Katano *et al.* (1984) demonstrated that simultaneous injection of human MoAb directed against ganglioside GD_2, which are expressed on tumor of neuroectodermal

origin (Irie *et al.*, 1982), led to prolongation of tumor-free time in nude mice system. Recently, Irie and Morton (1986) has performed first clinical trial to a melanoma patient using the human MoAb and reported complete remission of the tumor. Therefore, our results suggest the possibility that CLN–IgG would also be useful for cancer therapy in human.

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