Recimbinant Human Hexamer-Dominant IgM Monoclonal Antibody to Ganglioside GM3 for Treatment of Melanoma

Yumiko Azuma, Yuji Ishikawa, Shigeto Kawai, Toshiaki Tsunenari, Hiroyuki Tsunoda, Tomoyuki Igawa, Shin-ichiro Iida, Masahiko Nanami, Masami Suzuki, Reiko F. Irie, Masayuki Tsuchiya, and Hisafumi Yamada-Okabe

Abstract

Purpose: L612, a human IgM monoclonal antibody produced by an EBV-transformed human B-cell line, binds to ganglioside GM3 and kills GM3-positive human melanoma cells in the presence of complement. It has been shown to be effective in some patients with late-stage melanoma. L612 consists of hexameric IgM (about 20%), pentameric IgM (about 74%), and other minor IgM molecules. Because hexameric IgM activates complement more effectively than pentameric IgM, we developed and evaluated a hexamer-dominant recombinant IgM for clinical applications.

Experimental Design: Chinese hamster ovary (CHO) cells were transfected with heavy- and light-chain genes of L612, with or without the joining-chain gene. Antitumor effects of the recombinant IgM secreted from CHO cells were evaluated in vitro and in vivo.

Results: Recombinant IgM secreted from CHO cells without the joining chain (designated CA19) was ~80% hexameric, whereas recombinant IgM from CHO cells transfected with heavy-, light-, and joining-chain genes (designated CJ45) was about 90% pentameric. Both CA19 and CJ45 recombinant IgMs caused complement-dependent cytotoxicity against human and mouse melanoma cell lines, but the amount of CA19 required for 50% specific cytotoxicity was 5 to 10 times smaller. I.v. injection of CA19 compared with CJ45 or native L612 elicited more profound antitumor activity in nude rats bearing a GM3-positive mouse melanoma xenograft.

Conclusions: A hexamer-dominant human IgM against GM3 may provide a more potent treatment option for patients with GM3-positive melanoma.

In the United States the lifetime risk of developing malignant melanoma is ~1 in 75 (1). The 5-year survival of patients with American Joint Committee on Cancer stage III or IV melanoma is 27% to 70% and 5% to 10%, respectively (2). Although three drugs (dacarbazine, IFN-α, and interleukin 2) have been approved by the Food and Drug Administration for the treatment of advanced metastatic melanoma, none of the three have either as a single agent or in combination improved survival significantly (3–5). Because complete surgical resection of advanced disease is often not possible, there is urgent need for an effective systemic therapy.

Monoclonal antibodies have been one of the most anticipated drugs for melanoma treatment because melanoma cells express tumor-associated ganglioside antigens on their cell surface (6), and monoclonal antibodies can mediate tumor cell lysis via complement-dependent cytotoxicity (CD8; ref. 7) and/or antibody-dependent cell-mediated cytotoxicity (8). Under clinical evaluation is the chimeric monoclonal antibody against ganglioside GD3 (9). Tumor-associated gangliosides are highly immunogenic in humans (10). In studies of active immunotherapy, patients who received a melanoma cell vaccine for treatment of metastatic melanoma had survival rates that correlated with serum concentrations of IgM but not IgG anti-ganglioside antibody (11, 12). In studies of passive immunotherapy, i.t. injection of human IgM antibodies resulted in the regression of cutaneous melanoma metastases (13). These results suggest that IgM class antibodies against gangliosides have therapeutic efficacy against melanoma.

GM3 is the most common ganglioside found on human melanoma cells, where it is expressed at much higher densities and frequencies than on normal cells (14). Previously, several human IgM class antibodies were obtained from EBV-transformed human B cells (15, 16). One of these antibodies, designated L612, binds to GM3 and kills human melanoma cells expressing GM3 in the presence of complement (17). I.v. administration of L612 into nine patients with American Joint Committee on Cancer stage IV melanoma led to complete responses in two patients, a partial response in one patient, a...
mixed response in one patient, and a stabilized disease in one patient (18). Although the expected survival of these patients had been less than 6 months, four patients survived more than 1 year after receiving the first dose of L612; two of the four patients remained tumor-free, exceeding 5 years of follow-up. Despite a very high total dose of IgM (up to three consecutive doses of 1,920 mg), there was no significant toxicity in these patients. The mechanism of action of L612 is not well understood; this monoclonal antibody contained ~20% hexameric IgM, which may have activated complement more efficiently than pentameric IgM in the induction of CDC (19–21).

To investigate whether the hexameric L612 may have played a dominant role in the observed antitumor activity, we generated both hexameric and pentameric L612 by transfecting Chinese hamster ovary (CHO) cells with L612 heavy (H)- and light (L)-chain genes, with or without the joining (J)-chain gene (20, 21), and compared their antitumor activity in vitro and in an animal model. The study has shown that antitumor activity was greater with hexamer-dominant recombinant IgM than with pentamer-dominant recombinant IgM or native L612. This is the first report to describe generation of a hexamer recombinant IgM against human cancer and show its antitumor potential surpasses that of its pentameric counterpart.

Materials and Methods

Tumor cell lines. Tumor cell lines (GM3-positive M1 human melanoma, GM3-positive B16F10 mouse melanoma, and GM3-negative A549 human lung carcinoma) were maintained in RPMI 1640 supplemented with 5% fetal bovine serum, RPMI 1640 supplemented with 10% fetal bovine serum, and DMEM supplemented with 10% fetal bovine serum, respectively. The M1 cell line, which expresses no gangliosides other than GM3, was obtained from the University of California, Los Angeles, where it had been previously established by one of the authors (R.F.I.). The A549 cell line was obtained from the American Type Culture Collection, and the B16F10 cell line was obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Miyagi, Japan).

Human IgM monoclonal antibodies. L612 and L55 monoclonal antibodies are specific for GM3 and GM2, respectively. These human IgM antibodies are secreted by corresponding EBV-transformed human B-cell lines grown in a serum-free medium (17, 22). The development of recombinant IgM anti-GM3 and anti-GM2 antibodies from L612 and L55 mRNA, respectively, is described elsewhere (17, 23).

CJ45 recombinant IgM, which binds to GM3, was obtained from the spent culture media of CHO DG44 cells that had been transfected with genes for the H, L, and J chains of L612 cells. CA19 recombinant IgM, which also binds to GM3, was obtained from the spent culture media of DG44-CHO cells that had been transfected with genes for the H and L chains of L612 cells. LA74 recombinant IgM, which binds to GM2, was obtained from the spent culture media of DG44-CHO cells that had been transfected with genes for the H and L chains of L55 cells.

Native L612 and the three recombinant IgM antibodies were purified from the spent tissue culture media by sequential column chromatography in three steps: Q-FF anion-exchange column chromatography, hydroxyapatite column chromatography, and Sephacryl S-400 gel filtration column chromatography. The purity of these antibodies was nearly 100%; no proteins other than IgM were detected by SDS-PAGE and silver staining. The antibody binding specificity for ganglioside antigens was analyzed by ELISA (22). Purified IgM fractions were separated on a 3.7% polyacrylamide gel, and the ratio of hexameric to pentameric forms was determined by densitometry analysis of gels that had been stained with SYPRO-Ruby (Bio-Rad Laboratories; ref. 24).

To assess the specific binding of the IgM antibodies to cell-surface GM3 or GM2, tumor cells from the three lines were incubated with 100 μg/mL L612, CA19, CJ45, LA74, or control human IgM (Cappel, MP Biomedical) and then with FITC-labeled anti-human IgM antibody (BD Biosciences PharmMingen). FITC intensity and number of FITC-positive cells were assessed by flow cytometry (EPICS XL, Beckman Coulter).

Antibody-dependent cell-mediated cytotoxicity and CDC. IgM-induced cytotoxicity was measured by 51Cr-release assay. For CDC, target cells that had been labeled with 51Cr-sodium chromate were seeded on 96-well plates (104 per well). IgM antibodies were added at the indicated final concentrations, and the cells were incubated at 4 °C for 60 min. Complement derived from rabbit rabbit serum (BRC, Cedarlane Laboratories), F344/N Jcl-rnu nude rat serum, BALB/c nude mouse serum, or human serum from healthy volunteers was added, and cells were incubated at 37°C for 90 min. After centrifugation at 1,000 rpm for 3 min at 4°C, radioactivity in the supernatant was counted in a gamma counter. Radioactivity in the supernatant of cells incubated without complement or antibody was considered spontaneous 51Cr release; radioactivity after incubation in 1% NP40 solution was considered maximum 51Cr release. Cytotoxicity (%) was calculated as follows: (A - C) / (B - C) × 100, where A, B, and C represent 51Cr release in each experiment, maximum 51Cr release, and spontaneous 51Cr release, respectively. All experiments were done in triplicate.

For antibody-dependent cell-mediated cytotoxicity, the procedure was identical except for the use of peripheral blood mononuclear cells from healthy volunteers as the source of effector cells instead of serum. Peripheral blood mononuclear cells were added to target cells at an effector to target (E/T) ratio of 100.

In vivo antitumor activity. Male F344/N Jcl-rnu nude rats between 6 and 8 weeks of age were i.p. injected with 5 × 106 B16F10 mouse melanoma cells. On the same day (day 0), five groups of six to nine rats each received i.v. injections of L612, CA19, CJ45, LA74, or control human IgM (vehicle), and survival was monitored. The serum level of IgM antibody was 10 mg/kg, injected thrice at 3-h intervals.

All rats were obtained from Clea Japan, kept in sterilized cages, given water and CE2 (Clea Japan), and treated in accordance with the ethical guidelines of animal care, handling, and termination promulgated by Chugai Pharmaceutical Co., Ltd.

Results were analyzed with an SAS statistical package (version 6.12), and differences with P < 0.05 were considered significant.

Immunohistochemistry. Antibody binding to metastatic tumor resected from patients with American Joint Committee on Cancer stage IV melanoma was determined by direct immunohistochemical staining. Tissue sections were frozen but were not permanently fixed by paraformaldehyde because of the effect of formalin and heat on ganglioside antigens. Control human IgM was purchased from Rockland Immun Diagnostics. CA19 was biotinylated according to the instruction manual provided by the manufacturer (Pierce). After the tissue sections were fixed with cold acetone, 2 μg/mL of biotinylated CA19 or biotinylated control IgM was added. Antibody binding was detected by immunoperoxidase staining with alkaline phosphatase (ABC-AP AK-5000; Vector Laboratories) and red substrate (SK-5100, Vector Laboratories).

Results

Analysis by SDS-PAGE (Fig. 1) revealed that native L612 comprised hexameric (19.8%), pentameric (73.6%), tetrameric (3.7%), and aggregate (2.9%) forms of IgM. Of its two recombinant IgMs, CA19 was 79.2% hexameric IgM, 9.3% pentameric IgM, 3.5% tetrameric IgM, and 6.5% aggregate IgM, whereas CJ45 was 4.5% hexameric IgM, 91.5% pentameric IgM, and 4% aggregate IgM. Both recombinant forms had strong
binding specificity to GM3. A similar ratio of hexameric and pentameric forms of IgM was observed with the recombinant form of L55 (LA74), which bound strongly and specifically to GM2 in ELISA (data not shown).

Antibody binding to ganglioside antigens of the murine melanoma (B16F10), the human melanoma (M1), and the human lung cancer (A549) cell lines was assessed by fluorescence-activated cell sorting analysis. M1 and B16F10 cells expressed no gangliosides other than GM3 on their surface, whereas A549 cells expressed GM2 but not GM3. L612 bound strongly to these cells but did not bind to A549 cells, whereas L55 did not bind to B16F10 or M1 cells but bound very strongly to A549 cells. At an antibody concentration of 100 μg/mL, binding to the two melanoma cell lines was similar for CA19 and L612 and weaker for CJ45 (Fig. 2A and B). LA74, which was used as a control recombinant antibody, did not bind to either melanoma cell line but was strongly positive for lung cancer cells, against which CA19, CJ45, and L612 were negative (Fig. 2C). Fluorescence-activated cell sorting analysis confirmed antigen-specific binding of the recombinant antibodies to cell-surface GM3 or GM2 antigens.

Antibody-induced CDC against the three tumor cell lines was assessed by a 51Cr release assay (Fig. 3). In the presence of human serum complement and with an antibody concentration of 5 μg/mL, maximum CDC activity against B16F10 was approximately the same for CA19, CJ45, and L612. However, at lower antibody concentrations, CDC was stronger for CA19 than for CJ45 or L612; the concentration of antibody required for 50% specific cytotoxicity was 5 to 10 times smaller for CA19 compared with CJ45 (Fig. 3A). Although M1 was less sensitive than B16F10 as a target for CDC, differential CDC activities were observed with CA19 and CJ45 against M1, even at the antibody concentration of 5 μg/mL (Fig. 3A). CJ45 and L612 showed equivalent CDC against both melanoma lines at all antibody concentrations tested. Similar results were obtained with nude rat serum complement (Fig. 3B) and baby rabbit complement (data not shown).

The fact that neither L612 nor its two recombinant forms (CA19 and CJ45) induced CDC against GM2-positive A549 lung cancer cells, whereas LA74 induced CDC only against A549 cells (Fig. 3B), indicates that target-cell expression of GM3 is necessary for CDC induction by L612, CA19, or CJ45. None of these three antibodies induced antibody-dependent cell-mediated cytotoxicity against the two melanoma cell lines, even at an E/T ratio of 100 and an antibody concentration of 100 μg/mL (data not shown).

In vivo antitumor activity of the recombinant antibodies was assessed in F344/N-rnu nude rats inoculated with B16F10 mouse melanoma cells. We did not use syngeneic nude or severe combined immunodeficient mice because their sera did not induce CDC activity in vitro, whereas F344/N-rnu rat complement was as effective as human complement with respect to induction of CDC against melanoma cells (Fig. 3). The rats received an i.p. injection of 5 × 106 B16F10 cells followed the same day by three i.v. 10 mg/kg injections of native, recombinant, or control human IgM (vehicle). The survival duration of vehicle-treated rats fluctuated, but >78%
died within 25 days. All vehicle-treated or LA74-treated rats died by day 40, compared with only 44% of CA19-treated rats (*P* < 0.01; Fig. 4A). By day 60, the survival rate for CA19-treated rats was 25%, clear evidence of antigen-specific tumor growth inhibition by CA19. Compared with vehicle, CA19, CJ45, and L612 prolonged survival by 238%, 162%, and 155%, respectively (Fig. 4B). CA19 did not affect tumor growth or survival of rats inoculated with GM3-negative A549 tumor cells (data not shown).

Serum levels of 5-S-CD have been shown to be associated with melanoma progression in mice bearing B16 melanoma and in humans (26, 27). In this study, serum levels of 5-S-CD on days 15 and 22 were low in nude rats that received CA19 instead of LA74 or vehicle (*P* < 0.01 on day 15, *P* < 0.05 on day 22), and serum 5-S-CD levels were negatively correlated with survival (Fig. 5).

Immunohistochemistry was also done to confirm that CA19 can bind to human melanoma tissues. CA19 was biotinylated and adjusted to 2 μg/mL for direct immunohistochemical assay. All five melanoma tissue specimens (1 each from skin and soft tissue, 2 from lymph nodes, and 1 of unknown origin) showed moderate staining, and two normal skin specimens showed only background staining. Immunohistochemical results of two representative melanoma specimens and one normal skin specimen are shown in Fig. 6A to C. None of the five melanoma cells stained positive with the control IgM (data not shown). Macrophages were also weakly stained by CA19, but CA19 did not react with fibroblasts or adipocytes (Fig. 6C). The results indicate that GM3 is expressed by human metastatic melanoma tissue and tumors of which a potential target for CA19 treatment.

**Discussion**

This is the first report of a hexamer-dominant recombinant human IgM antibody against cancer. CA19, produced by transfection of CHO cells with L612 genes for H and L chains without the J chain, had greater antitumor activity than native L612 or its pentamer-dominant recombinant (CJ45). Whereas about three fourths of the L612 was pentameric IgM, CA19 was...
enriched with hexameric IgM that caused stronger CDC than L612 at concentrations lower than 5 \( \mu g/mL \), presumably reflecting increased binding avidity to activate complement. The binding affinities of CA19 and CJ45 to GM3 on M1 cells were not significantly different, ranging from 10 to 14 nmol/L.

Previous reports have shown a greater magnitude of CDC activity between the hexameric form and the pentameric form, and that J-chain protein is associated only with the pentameric form and not with any of the intermediate polymers smaller than a pentamer (20, 28). However, the magnitude of the CDC activities of hexameric and pentameric forms depends on the origin of the complements. Chimeric mouse V/human C\( _4 \) IgM hexamer activated guinea pig complement 100-fold more efficiently than did the chimeric pentamer, but this hexamer was only 4- to 13-fold more active than the pentamer when assayed with human complement (19). Furthermore, whereas mouse hexamer was 100-fold more active than mouse pentamer in activating guinea pig complement, it was only ~2-fold more active than mouse pentamer when assayed with human complement (19).

Administration of CA19 significantly prolonged the survival of nude rats bearing B16F10 mouse melanoma. In addition, serum levels of 5-S-CD remained at low levels in rats that received CA19. Several lines of evidence suggested that 5-S-CD can be used as a surrogate marker of tumor burden (29, 30) and distant metastasis (31). In facts, serum levels of 5-S-CD decreased after treatment with IFN-\( \alpha \) and DTIC (32). Therefore, low levels of serum 5-S-CD in rats that received CA19 suggest inhibition of tumor growth at the sites where tumor cells migrated and survived, and the inverse correlation between survival and serum levels of 5-S-CD indicate that death was primarily due to progression of tumor growth. The mechanism of the in vivo antitumor action of CA19 in nude rats is not fully understood but has been attributed in part to CDC. In a preliminary experiment, we administered CA19 with or without baby rabbit complement to nude or C57BL/6N mice of which complement was not effective in causing CDC in vitro. CA19 elicited antitumor activity only in mice receiving complement; neither CA19 alone nor complement alone prolonged survival (data not shown).

In the aforementioned clinical pilot study, a single i.v. dose of L612 caused complete regression of distant metastatic tumors. This could suggest certain mechanisms other than CDC may have been involved; CDC may have played an important role only in the initial destruction of tumor. We tested if any antibody responses against B16F10 were induced in the tumor-bearing nude rats after CA19 administration; sera from surviving nude rats were collected and transferred to untreated nude rats inoculated with B16F10 melanoma. None of the serum-treated rats survived longer than the rats in the vehicle group. The results suggest that the observed antitumor activity of CA19 may be unrelated to a serologic factor secondarily induced in the animals. We did not test cell-based antitumor immunity because it is unlikely to be the case in

Fig. 5. Correlation between serum 5-S-CD levels and survival of B16F10 melanoma bearing nude rats after administration of CA19 or LA74. After i.p. injection of 5 \( \times \) 10\(^6\) B16F10 melanoma cells, nude rats received 30 mg/kg (three i.v. injections of 10 mg/kg) of CA19 (blue), LA74 (red), or vehicle control (black). Blood concentrations of 5-S-CD on day 15 (A) and day 22 (B) were analyzed as described in Materials and Methods and plotted against survival. Each group consisted of nine animals.

Fig. 6. Immunohistochemistry of human normal skin and melanoma tissues with CA19. Frozen sections of normal human skin (A) and two melanoma tissue specimens from patients with stage IV disease (B and C). Tissues were incubated with 2 \( \mu g/mL \) of biotinylated CA19. Bar, 20 \( \mu m \).
these animals. Therefore, CDC seems to be the primary mechanism of antitumor action in this animal model. Further study is necessary to understand the mechanisms underlying the antitumor effects of CA19 in vivo.

As shown by immunohistochemical analysis, binding of CA19 to all five melanoma specimens from resected stage IV metastases occurred only at antibody concentrations of 2 μg/mL. Weak staining of macrophages from CA19 was probably not antigen specific. Although IgM reportedly can bind nonspecifically to tissues of macrophages and certain epithelial cells, in vivo binding of IgM to noncancerous tissues is unlikely to be detrimental because the antibody binding observed with immunohistochemistry is not due to antigen-antibody complexes. In fact, the nine melanoma patients who received high doses of L612 (three injections of up to 1,920 mg per injection) in the pilot study showed no more than grade 1 toxicity (18).

Compared with L612, CA19 has ~5 to 10 times more CDC activity against melanoma, which indicates that it could be administered at a lower dose and for a lower cost. Furthermore, transfecion of CHO cells with the L612 immunoglobulin genes alone avoids EBV genome involvement. Our study suggests that CA19 could be an alternative and more effective anticancer drug candidate than L612.

References
Article on Recombinant Human Hexamer-Dominant IgM Monoclonal Antibody to Ganglioside GM3 for Treatment of Melanoma

In the article on hexamer IgM for melanoma treatment, beginning on page 2745 of the May 1, 2007, issue of Clinical Cancer Research, the figures below should have appeared in color.


**Fig. 2.** Fluorescence-activated cell sorting analysis of the binding of L612, CA19, CJ45, and LA74 to GM3-positive or GM2-positive cancer cell lines. GM3-positive mouse B16F10 melanoma cells (A), GM3-positive human M1 melanoma cells (B), and GM3-negative but GM2-positive human A549 lung cancer cells (C) were incubated with 100 μg/mL CA19, CJ45, L612, LA74, control human IgM (Cont. IgM), or no antibody (No 1st Ab) and analyzed as described in Materials and Methods.
Fig. 3. CDC from L612, CA19, and CJ45 against GM3-positive or GM3-negative culture cells. Antibody-induced CDC against B16F10 mouse melanoma cells, M1 human melanoma cells, and A549 human lung cancer cells was tested using human serum complement (A) or nude rat serum complement (B). B16F10, M1, and A549 cells that had been labeled with $^{51}$Cr-sodium chromate were incubated with 0.04, 0.1, 0.2, 1, 5, and 10 $\mu$g/mL L612, CA19, CJ45, LA74, or control human IgM. Human serum was added to B16F10 cells for a final concentration of 25% and to M1 cells for a final concentration of 25%. Nude rat serum was added to the B16F10, M1, and A549 cells for a final concentration of 25%. Cytotoxicity (%) was determined from the radioactivity of the supernatants. Points, mean of three independent experiments; bars, SD.

Fig. 4. Survival of B16F10 melanoma-bearing nude rats after administration of antibodies. On the same day after i.p. injection of $5 \times 10^6$ B16F10 cells, rats received an i.v. injection of CA19, CJ45, L612, or LA74. A, survival (%) associated with CA19 and LA74, both at a total dose of 30 mg/kg (three injections of 10 mg/kg). B, survival (%) associated with CA19, CJ45, and L612, each at a total dose of 30 mg/kg (three injections of 10 mg/kg). Control animals received commercially available human IgM (vehicle). Each group consisted of six to nine animals. * , significant difference from the vehicle-treated group.
Fig. 5. Correlation between serum 5-S-CD levels and survival of B16F10 melanoma bearing nude rats after administration of CA19 or LA74. After i.p. injection of $5 \times 10^6$ B16F10 melanoma cells, nude rats received 30 mg/kg (three i.v. injections of 10 mg/kg) of CA19 (blue), LA74 (red), or vehicle control (black). Blood concentrations of 5-S-CD on day 15 (A) and day 22 (B) were analyzed as described in Materials and Methods and plotted against survival. Each group consisted of nine animals.

Fig. 6. Immunohistochemistry of human normal skin and melanoma tissues with CA19. Frozen sections of normal human skin (A) and two melanoma tissue specimens from patients with stage IV disease (B and C). Tissues were incubated with 2 µg/mL of biotinylated CA19. Bar, 20 µm.
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