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Tumor cell reactivity mediated by IgM antibodies in sera from melanoma patients vaccinated with GM2 ganglioside covalently linked to KLH is increased by IgG antibodies

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Abstract Natural IgM antibodies against the melanoma cell-surface ganglioside GM2, and IgM antibodies induced by vaccination with GM2 adherent to bacillus Calmette-Guerin, have been correlated with increased disease-free and overall survival in melanoma patients in previous phase I and II clinical trials. A vaccine containing GM2 covalently attached to keyhole limpet hemocyanin (KLH) plus the immunological adjuvant QS-21 now induces higher-titer, longer-lasting IgM antibodies against GM2 and has recently entered phase III clinical trials. For the first time this new vaccine also induces IgG antibodies against GM2 in the majority of immunized patients. With regard to immunity against bacteria, IgM antibodies have been described to be 1000-fold more effective than IgG antibodies at opsonification, complement-mediated cytotoxicity and protection from bacterial challenge. Though IgG antibodies have the theoretical advantage of being able to mediate antibody-directed cell-mediated cytotoxicity (ADCC), they may inhibit complement mediated IgM effector mechanisms against melanoma cells. Our goal was to confirm the functional characteristics of the anti-GM2 IgM and IgG antibodies induced by vaccination and to determine the impact that IgG antibodies might have on IgM antibody reactivity with GM2-positive tumor cells. Post-immunization sera from seven immunized patients were separated by size-exclusion chromatography into IgM and IgG fractions and a variety of serological assays were performed with the individual fractions and their combinations. Assays identifying specific IgM or IgG reactivity demonstrated partial inhibition by the opposite fraction. However, when the endpoint was complement-mediated lysis or overall antibody binding, which may more faithfully predict in vivo complement-mediated opsonification and lysis, the combinations of IgM and IgG fractions consistently demonstrated higher reactivity than either fraction alone. In addition, ADCC was induced in all seven patients. The results were the same whether the sera were obtained after 2 months or 2 years of immunizations. These findings suggest that IgG antibodies induced by the GM2-KLH plus QS-21 vaccine will not inhibit and should further augment the clinical impact of induced IgM antibodies.

Key words Complement · Vaccine · Conjugate vaccine · GM2 ganglioside · Melanoma · Fractionation

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Introduction

Gangliosides are sialic-acid-containing glycosphingolipids that are overexpressed at the cell surface of cancers of neuroectodermal origin, including melanomas [1–3]. The importance of gangliosides as targets for immunotherapy has been documented by clinical responses seen after treatment with anti-GM2, anti-GD2 and anti-GD3 monoclonal antibodies (mAb) [4–8]; and by the correlation between IgM antibody induction against GM2 after immunization with GM2 vaccines and improved prognosis [9, 10]. Of the GM2-containing vaccines initially studied, purified GM2 adherent to bacillus Calmette-Guérin (BCG) was the most consistently immunogenic vaccine, inducing antibodies in 85% of patients. However, as with all previous GM2 vaccines tested, antibody responses were predominantly IgM, of moderate titer and short-lived, with a median duration of 8–10 weeks after each immunization [9–11]. To produce higher-titered and longer-lasting IgM antibody

responses against gangliosides and to generate IgG antibodies as well, a variety of conjugate vaccines were explored [12]. GM2, covalently attached to keyhole limpet hemocyanin (KLH), plus 100 μg immunological adjuvant QS-21 was selected as the optimal approach [12, 13]. This has induced IgM antibody detected by enzyme-linked immunosorbent assays (ELISA) and immune staining assays in 30 consecutive immunized patients with a median titer eight-fold greater than that seen with the previous GM2/BCG vaccine. IgG antibodies specific for GM2 were induced in 28 of these patients. Antibody titers were also longer-lasting with a median duration of 4–6 months [13, 14].

These initial studies included GM2-KLH conjugate prepared at two different centers, two quite different KLH preparations (purchased from Pierce, Rockford, Ill., and Calbiochem, La Jolla, Calif.), and QS-21 doses ranging from 10 μg to 200 μg/vaccination. Significant variability in serological responses to cultured melanoma cells was seen, especially with regard to IgG antibody reactivity to GM2 measured by the mixed hemadsorption assay (MHA) [13, 15]. In a recent analysis only 2 of 22 patients demonstrated high-titer IgG antibodies by MHA, though many had significant IgG titers in ELISA. Some of this variability was shown to be due to immunization with a suboptimal dose of QS-21 [13]. Additional variability may have resulted from the different KLH preparations (neither of which was prepared for human use), different sites and methods of GM2-KLH production, variable quantities of GM2 adherent to the GM2-KLH conjugate but not conjugated, and inhibition of anti-GM2 IgG reactivity by IgM antibodies or of IgM reactivity by IgG antibodies. These variables have been eliminated in recent batches of GM2-KLH vaccine prepared for a multicenter phase III trial of the GM2-KLH plus QS-21 vaccine: (1) all vaccines contain 100 μg QS-21, (2) KLH is now well standardized and prepared for human use by PerImmune Inc., and (3) all detectable nonconjugated GM2 has been eliminated from the vaccines.

We address here, with two additional assays, the apparent low IgG reactivity against GM2 at the cell surface seen with MHA assays in our previous study. Fluorescence-activated cell sorting (FACS) analysis is used as an independent assay of IgG reactivity with cell-surface GM2, and sera have been separated into IgG and IgM fractions to permit evaluation of IgG-induced complement-mediated cytotoxicity. One additonal issue raised by these initial studies with GM2-KLH vaccines is addressed, the possibility that IgM and IgG antibodies raised in the same patient might compete with each other, resulting in lower levels of biological effector functions than might be seen with the induction of either class of antibody alone. This issue is relevant to vaccines against a variety of carbohydrate antigens because conjugate vaccines now make possible the induction of high-titer IgM and IgG antibodies where previous vaccines had resulted predominantly in an IgM antibody response.

For these reasons, sera from 5 patients with a range of antibody responses after 2–3 months of vaccinations and 2 additional patients vaccinated over a 2-year period were fractionated and the IgM and IgG fractions tested individually and in combination by several serological assays.

Materials and methods

Patients and vaccine administration

The 7 patients described here had AJCC stage III or IV melanoma, which had been resected within 8 months prior to treatment. All were free of disease and none had received or were receiving chemotherapy. ELISA results with unfractionated sera from all patients have been described in previous publications. Patients 1 and 5 were part of a phase I trial in 1992 with a GM2-KLH conjugate prepared by Biomira Inc. (Edmonton, Alberta, Canada) [13]. Patients 2, 3, 4, 6 and 7 were immunized in 1993 with GM2-KLH conjugate prepared at Memorial Sloan-Ketterin Cancer Center [14]. These 7 patients were chosen for these studies based on availability of a sufficient quantity of serum for fractionation. All vaccines contained GM2-KLH with 70 μg GM2 and 100 μg QS-21, with the exception of the final three immunizations for patients 6 and 7, which contained GM2-KLH containing 10 μg GM2 and 100 μg QS-21. These 2 patients received this lower dose and were the only patients to receive additional booster immunizations (as indicated in Fig. 1) because of a shortage of GM2-KLH conjugate in early 1994. The vaccine schedule for patients 1, 5, 6, and 7 included four immunizations at 2-week intervals followed by two additional immunizations at 2-month intervals. Patients 2, 3 and 4 received three immunizations at 1-week intervals, followed by a fourth immunization 4 weeks later and a fifth immunization 2 months after the fourth. Sera for fractionation were obtained 6 and 8 weeks after the fifth vaccination and pooled for patient 1, 3 weeks after the fifth vaccination for patient 5, 2 weeks after the fourth vaccination for patients 2, 3 and 4, and 2 weeks after the ninth (final) vaccination for patients 6 and 7.

Size-exclusion chromatography for fractionation of sera

Size-exclusion chromatography using Bio-Gel A-1.5 m was performed as previously described [20]. A 10-ml sample of serum from each patient was precipitated with ammonium sulfate, dialyzed, and applied to the Bio-Gel A-1.5 m column. The IgM and IgG fractions were each concentrated to 6 ml using a diaflow ultrafiltration membrane with a 30-kDa molecular mass cut-off. The concentration from 10 ml serum at the start to 6 ml at the finish was based on a rough estimate of 40% loss during the various steps of this procedure, including the ammonium sulfate precipitation and the several fractionation tubes that were discarded at the overlap points between IgM and IgG fractions. Actual IgM, IgG and IgA concentrations in the fractions were determined subsequently using a SANDF1 QM2000 Immunonethelometer [19].

Serological assays

Enzyme-linked immunosorbent assays (ELISA) were performed against purified GM2 ganglioside using anti-IgM and anti-IgG second antibodies as previously described [9]. To control for non-specific binding, immune sera were also tested on plates that were processed identically, but to which no ganglioside had been added, and the reading was subtracted from the value obtained in the presence of the ganglioside. The titer was defined as the highest dilution yielding a corrected absorbance equal to or greater than 0.1.

Complement-dependent cytotoxicity (CDC) was assayed on the human sarcoma cell line A2394 using a 4-h europium-release assay as previously described [16] with human complement. Spontaneous release (the amount released by target cells incubated with complement alone) was subtracted from both experimental and maximal release values. Maximum release was the amount released by target cells after a 4-h incubation with 1% Nonidet P-40. Specific release was equal to corrected experimental release divided by corrected maximal release.

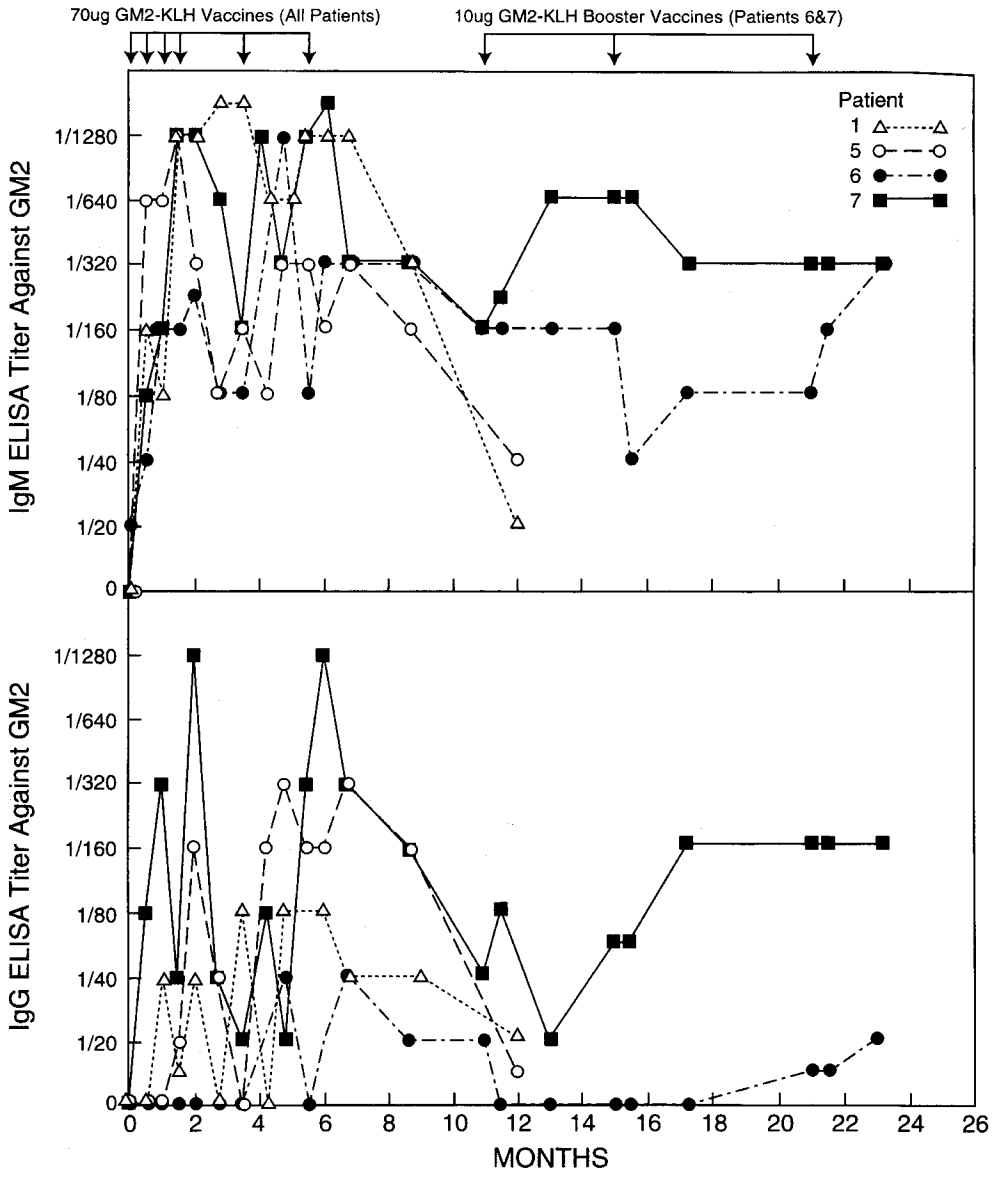


Fig. 1 IgM and IgG antibody responses in unfractionated sera from melanoma patients immunized with GM2-KLH plus QS-21 vaccines. Sequential results for patients 1 and 5 who received six immunizations and patients 6 and 7 who received the same six immunizations plus three additional booster immunizations are presented. *Arrows* vaccination dates. Some of these data have been presented previously [13, 14].

Antibody-dependent cell-mediated cytotoxicity (ADCC) assays were performed using a 6-h europium-release assay [16] and normal donor mononuclear cell/tumor cell ratios between 50/1 and 150/1.

Flow-cytometry (FACS) assays were performed with single-cell suspensions of A2394 sarcoma cells as previously described using 1:15-diluted fluorescein-isothiocyanate-labelled goat anti-(human IgM) or anti-(human-IgG) [16]. Two rosetting assays were performed as previously described: immune adherence for detection of IgM antibody and goat anti-(human IgG) mixed hemadsorption (MHA) for detection of IgG antibody [17, 18].

Sera were considered positive by ELISA if the titer of reactivity was at least 1/40, by FACS if at least 20% of target cells were positive, by CDC and ADCC if there was 10% or more specific release, by immune adherence and MHA if 10% or more of target cells were at least 50% covered with red cell rosettes. For each assay, a plus or a minus sign was assigned to a patient if reactivity with a serum fraction was more or less than with the mixed fractions. The two-sided sign test was applied for statistical significance.

Results

Antibody response in unseparated sera against GM2 after vaccinations

Sequential IgM and IgG anti-GM2 antibody titers before and after vaccination with GM2-KLH + QS-21 are shown in Fig. 1 for patients 1, 5, 6, and 7. No patient had GM2 antibodies prior to immunization and, as has been our previous experience, all patients produced high-titer IgM antibodies (equal to or greater than 1/160), and most patients produced IgG antibodies as well.

Patients 6 and 7 received three additional booster immunizations and maintained their GM2 antibody titers for a period greater than 2 years. Serological results for patients 2, 3 and 4 are not included in Fig. 1 because these patients were vaccinated according to a separate schedule. Peak post-vaccination IgM antibody titers were 1/1280, 1/1280,

and 1/640 for patients 2, 3, and 4, and their peak IgG antibody titers were 1/640, 1/80, and 1/160.

Analysis of fractionated sera

Unseparated sera and the IgM and IgG fractions were analyzed for IgG, IgM and IgA content. Fractionation by size-exclusion chromatography resulted in a more than 99% decrease of IgG in the IgM fractions in 6 of 7 patients. Patient 7 had a 96% decrease. An 88%–96% decrease of IgM in the IgG fractions was seen (median 94%). Overall, IgA was twice as concentrated in the IgG fraction than in the IgM fraction.

In the studies listed below, sera were tested at the indicated titers. When IgM plus IgG fractions were mixed, equal quantities of each at a twofold concentration were used, resulting in a final dilution (titer) of IgM or IgG as indicated. Results with fractionated sera are in some assays generally higher than with unfractionated sera (ELISA) or lower than with unfractionated sera (ADCC, IgM FACS). Meaningful comparisons are between IgM, IgG and combined IgM + IgG fractions.

Study of fractionated sera by ELISA (Fig. 2a, b)

Inhibition of IgM antibody reactivity against GM2 by the IgG fraction was present in patients 1–5 (Fig. 2a) and inhibition of IgG reactivity against GM2 by the IgM fraction was present in patients 2, 4 and 6 (Fig. 2b). The inhibition generally resulted in a two- to four-fold decrease in titer.

Effect of serum fractionation on immune adherence and MHA rosetting assays (Fig. 2c, d)

For the immune adherence assay, which measures predominantly IgM antibody reactivity, addition of the IgG fraction to the IgM fraction resulted in decreased antibody titers (compared to results with the IgM fraction) in all 6 patients with immune adherence reactivity (all but patient 7). MHA, which measures predominantly IgG antibody reactivity, showed no reactivity for patients 3–7. Patients 1 and 2 had a titer of 1/40 but only a titer of 1/10 in the IgG fraction was found for patient 1 and no reactivity was seen in the IgG fraction for patient 2 (Fig. 2d). Since MHA titers were low in the IgG fractions, clearly the low MHA titers in the unseparated sera were not a consequence of inhibition by IgM antibodies. No conclusions are possible concerning the impact on MHA reactivity of addition of the IgM fraction to the IgG fraction in this setting.

Effect of fractionation on CDC and ADCC assays (Fig. 2e, f)

Pretreatment sera generally lacked detectable antibodies mediating CDC. After immunization, at a serum dilution of 1/10, the median percentage europium release was 58% for the IgM fraction, and 28% for the IgG fraction. Combination of the IgM plus IgG fractions did not result in an additive response but did result in a median europium release of 66%, higher in all cases than either the IgM or IgG fractions alone ($p = 0.016$).

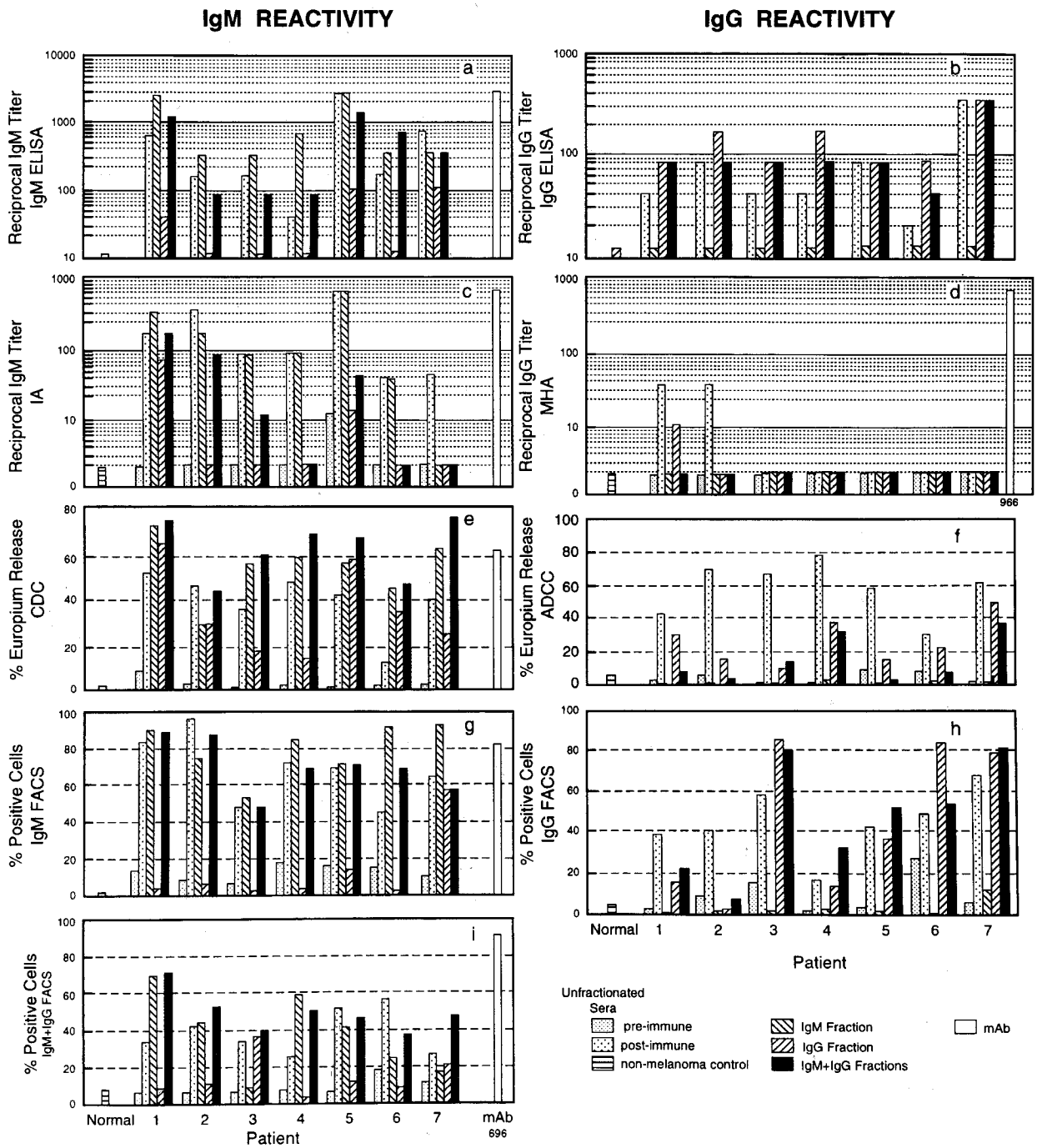
Significant ADCC (>10%) was not seen in sera drawn prior to immunizations. After immunization at a dilution of 1/15, the median percentage ADCC was 61% for the unseparated sera, 22% for the IgG fractions, 0% for the IgM fractions and 8% for the IgM+IgG pool. In all 6 cases with significant ADCC in the IgG fraction, this was decreased by addition of the IgM fraction ($p = 0.031$). The basis for the decreased ADCC in IgG fractions compared to unseparated sera is unclear but it has been a consistent finding. Similar IgG levels, IgG FACS results and IgG ELISA titers in IgG fractions and whole sera suggest no loss of reactive IgG antibodies.

Effect of serum fractionation on flow-cytometry analysis (Fig.2g, h, i)

When IgM- and IgG-specific second antibodies were used, prefractionation and IgM fraction IgM reactivities against A2394 cells were quite similar. Overall, IgM fraction reactivity was not affected by addition of the IgG fraction, nor was the IgG fraction reactivity altered by addition of the IgM fraction. On the other hand, when the second antibodies measured IgM and IgG, all 7 patients showed the same or higher GM2 reactivity with two fractions together than with either alone. The specificity of this cell-surface reactivity for GM2 was confirmed by pretreating (inhibiting) the combined fractions with GM2-KLH or GD3-KLH before the FACS analysis. Inhibition ranged from 0 to 10% with GD3-KLH and from 60% to 90% with GM2-KLH when 10 μg ganglioside was used in each case.

Discussion

The relative efficacy of IgM and IgG antibodies for adherence to cell-surface antigens and for complement-mediated opsonification and cytotoxicity, as well as in vivo protection, has not been tested in tumor models. With regard to immunity against bacteria, initial studies with polyclonal antisera [20, 21] or monoclonal antibodies [21] resulted in conflicting conclusions concerning the relative merits of IgM and IgG antibodies, perhaps because of significant differences in the antigens detected and antibody affinity. Subsequently, with the use of class-switching technologies, IgM and IgG monoclonal antibodies of constant affinity and titer against defined bacterial



antigens were compared. IgM was found to mediate significantly greater opsonification in vitro and protection in vivo against both *Escherichia coli* and group B streptococci [22]. The potency of IgM was a consequence of increased valancy resulting in increased efficiency for fixing and activating complement, since the efficacy of IgG antibodies could be greatly increased by the construction of IgG dimers [23]. In these studies, it was felt than the conclusions were especially applicable to relatively low-affinity antibodies such as the antibodies induced against most carbohydrate antigens. Although the potency of the IgM antibodies in some of these studies was 1000–4000-fold that of the IgG antibodies, no attempts were made to

Fig. 2a–i Results are demonstrated for enzyme-linked immunosorbent assays (ELISA), immune adherence (IA) and mixed hemadsorption (MHA), complement-dependent (CDC) and antibody-dependent (ADCC) cytotoxicity assays and FACS assays with unseparated pre- and post-immunization sera and separated post-immunization serum fractions from 7 patients immunized with GM2-KLH plus QS-21 vaccines. Each panal (a–i) represents the results of a single experiment with serum and serum fractions from 7 patients as well as a control. All experiments were repeated to confirm consistency. Target cells for rosetting assays, cytotoxicity assays and FACS analysis were human sarcoma cell line A2394. The target for ELISA assays was purified GM2 ganglioside. The anti-GM2 monoclonal antibodies (*mAb*) used as positive controls were the mouse IgM mAb 696 [25] and the chimeric mouse/human IgG mAb 966 [26], both kindly provided by Dr. Nobuo Hanai, Kyowa Hakko Co. Ltd., Tokyo Research Laboratories, Tokyo, Japan

determine whether the IgG antibodies had an inhibitory capacity in this setting. Together, these studies suggest that, in the relatively low-affinity response to most carbohydrate antigens, IgM antibodies are likely to have far greater in vitro and in vivo activity. However, they do not address the issue of whether the presence of IgG antibodies in the reaction mixtures or sera can suppress complement lysis or opsonification mediated by IgM antibodies, or whether IgM antibodies can inhibit the ADCC reactivity mediated by IgG antibodies. These issues were the focus of this analysis, which involved separation of immune sera into IgM and IgG fractions.

The basis for preparing melanoma vaccines capable of inducing higher-titer and longer-lasting IgM antibodies against GM2 is clear: the correlation of IgM antibody production after vaccination with improved survival [9–11, 24], and the improved disease-free and overall survival following vaccination with GM2/BCG [10], a vaccine that induces only IgM antibodies in the majority of patients. The basis for the induction of IgG antibodies against GM2 is theoretical, the increased body distribution pool of IgG antibodies compared to IgM antibodies, and the potential for induction of ADCC. The studies described here were designed to address a potentially negative aspect of IgG antibody induction against GM2: inhibition by IgG antibodies of complement-mediated cytotoxicity and opsonization, the two major in vivo mechanisms proposed for antitumor efficacy of IgM antibodies. We demonstrate that, with in vitro assays measuring specifically the in vitro reactivity of IgM or of IgG antibodies, addition of the opposite fraction is frequently able partially to inhibit reactivity. However, when these assays measure more clinically relevant effects, such as total complement-dependent cytotoxicity or overall cell-surface Ig binding by flow cytometry, both of which measure the net reactivity of IgM plus IgG, reactivity with the two fractions together was equal to or greater than the reactivity with either fraction alone. With regard to complement-dependent cytotoxicity, this is consistent with our previous finding that IgG antibodies induced by the GM2-KLH plus QS-21 vaccine are primarily of the IgG1 and IgG3 subclasses, both of which are strong binders of complement [9, 13], and with our finding here that the IgG fractions did indeed mediate complement lysis. IgG1 and IgG3 are also known to be effective mediators of ADCC, consistent with our finding that IgG antibody induction in these 7 patients resulted in significant ADCC. Since ADCC reactivity is not mediated by IgM antibodies, it was not surprising that ADCC was partially inhibited by addition of the IgM antibody fraction. Nevertheless, ADCC was present in unfractionated sera from all 7 patients, a second potential benefit from the IgG antibodies induced by this vaccine. These studies also suggest that if a vaccine were to induce IgG antibodies of the IgG2 or IgG4 subclass (which bind complement poorly or not at all), it might be counterproductive, blocking vaccine-induced or natural IgM antibody reactivity.

Another reason for initiating these studies was that, in some of our previous vaccination studies with GM2-KLH plus QS21 vaccines, IgG responses detectable on purified GM2 by ELISA were not detectable on GM2-positive tumor cells by MHA [15]. High-titer IgM antibodies in post-vaccination sera from all 7 patients studied here reacted with purified GM2 assayed by ELISA, and also reacted with GM2 expressed at the tumor cell surface as shown by flow cytometry, complement lysis, and immune adherence assays. This includes sera drawn from 2 patients after receiving GM2-KLH plus QS-21 immunizations over a 2-year period. IgG antibodies in unfractionated sera and the purified IgG fraction were detectable in all patients by ELISA. IgG antibodies were also detected in 6 of 7 patients by complement lysis assays and in 4 of 7 patients by flow cytometry, but reactivity was not demonstrable in any patient by the MHA assay. The lack of MHA reactivity in these sera was consistent with our previous experience [15] but cannot be a consequence of inhibition by IgM antibodies since MHA reactivity was not detectable in the IgG fraction. Other possible explanations are that the MHA assay is less sensitive than the other three assays and that it may be more dependent on IgG antibodies of high affinity.

In conclusion, the GM2-KLH plus QS-21 vaccines used to treat the 7 patients in this study induced IgM and IgG antibodies against GM2 in all patients. These antibodies reacted with both purified GM2 and tumor cells expressing GM2. This was true with sera drawn after 2 months of immunizations and with sera drawn after 2 years of immunizations. Using IgM or IgG fractions with assays detecting specifically IgM or IgG antibodies, partial inhibition of the specifically detected Ig resulted from addition of the other antibody fraction. However, when the more biologically relevant complement-mediated cytotoxicity assay was used, reactivity with and lysis of tumor cells mediated by IgM was only increased by the additional presence of IgG antibody, which also induced complement-mediated cytotoxicity. In addition, sera from all 7 patients were able to mediate ADCC. These findings suggest that IgG antibodies induced by the GM2-KLH plus QS-21 vaccine will not inhibit and should further augment the clinical impact of induced IgM antibodies.

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