Cancer Immunol Immunother (1999) 48:483–492

ORIGINAL ARTICLE

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Immunization of mice with fucosyl-GM1 conjugated with keyhole limpet hemocyanin results in antibodies against human small-cell lung cancer cells

Received: 25 March 1999 / Accepted: 5 August 1999

Abstract Fucosyl-GM1 (Fuc-GM1) [Fucα1 → 2Galβ1 → 3GalNAcβ1 → 4(NeuAcα2-3)Galβ1 → 4Glcβ1 → *O*-Cer] is a small-cell-lung-cancer (SCLC)-associated ganglioside initially defined by the murine monoclonal antibody F12. On the basis of its known distribution, Fuc-GM1 is a potential target for active immunotherapy in SCLC patients. Fuc-GM1 has been extracted and purified from bovine thyroid. The immunogenicity of Fuc-GM1 was tested in mice either alone, mixed with carrier protein keyhole limpet hemocyanin (KLH) or covalently linked with KLH, plus immunological adjuvant QS-21. The Fuc-GM1-KLH conjugate plus QS-21 adjuvant was found to be optimal. It induced consistent IgM and IgG enzyme-linked immunosorbent assay (ELISA) titers against Fuc-GM1. These antibodies were strongly reactive with the strongly Fuc-GM1-positive rat hepatoma cell line H4-II-E, and they were moderately reactive with the moderately positive human SCLC cell line H146 by flow cytometry and complement-mediated lysis. Both ELISA and fluorescence-activated cell sorting (FACS) reactions were inhibited with Fuc-GM1or H4-II-E but not with the structurally related ganglioside GM1 or Fuc-GM1-negative colon cancer cell line LS-C. On the basis of these results, a vaccine comprising Fuc-GM1-KLH plus QS-21 is being prepared for testing in patients with SCLC.

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Key words Cancer vaccine · Fucosyl-GM1 · Gangliosides · Immunogen · Immunotherapy · SCLC

Introduction

Fucosyl-GM1 [Fuc-GM1; Fucα1 → 2Galβ1 → 3GalNAcβ1 → 4(NeuAcα2-3)Galβ1 → 4Glcβ1 → O-Cer] is a ganglioside largely restricted to small-cell lung cancer (SCLC). Gangliosides are neuraminic-acid-containing glycosphingolipids that are anchored into the lipid bilayer of the plasma membrane by their lipophilic ceramide moiety. During malignant transformation, gangliosides such as GM2, GD2, GD3, 9-*O*-acetyl GD3 and fucosyl-GM1 are up-regulated [33]. Fucosyl-GM1 was first isolated from bovine thyroid gland [15, 30] and was subsequently identified on small-cell lung cancers by using the murine monoclonal antibody F12 [19]. With the use of F12, Fuc-GM1 was identified in tissue samples of 19 of 21 cases of SCLC and in serum of a few patients with advanced disease [3, 31]. Fuc-GM1 was not identified in the normal lung and bronchus; however, sparsely distributed clusters of small round cells were stained in the thymus, spleen, pancreatic islet cells, and the lamina propia and intramural ganglionic cells of the small intestine [3]. We used F12 to detect the presence of Fuc-GM1 on a variety of tumors and normal tissues and found that Fuc-GM1 had the most restricted distribution on normal tissues of all the gangliosides, and was expressed on four of five SCLC but not on any other common cancers tested [33]. It was not detected on any normal tissue except a small subpopulation (10%) of cells in the islets of Langerhans in the pancreas, and a subpopulation of cells in the dorsal root ganglion [33].

There is a need for more effective adjuvant therapy of SCLC. SCLC accounts for approximately 20% of all lung cancer cases and is the fifth leading cause of death from cancer in the United States [11]. Distant metastases are present in more than two-thirds of patients with SCLC at diagnosis and, in the absence of treatment, median survival is only 2–4 months. SCLC is responsive

to chemotherapy with major responses seen in most patients, but relapses are common, and most patients die within 2 years of their diagnosis. Over the past decade, no additional therapy has been shown to improve overall survival, and the standard therapy is observation alone for patients who have achieved a major response after four to six cycles of chemotherapy. Because of these modest results, new approaches to adjuvant therapy are needed. The restricted normal tissue expression, and the over-expression on SCLC suggest that Fuc-GM1 would be an attractive target for both active and passive immunotherapy.

Fuc-GM1 is an auto-antigen in humans (and presumably in the mouse) and so it would be expected to be poorly immunogenic. We have compared the antibody response in four groups of mice after immunization with Fuc-GM1 plus immunological adjuvant QS-21, Fuc-GM1 mixed with keyhole limpet hemocyanin (KLH) plus QS-21, Fuc-GM1 conjugated with KLH plus QS-21 (liquid), and Fuc-GM1 conjugated with KLH plus QS-21 (lyophilized). We have used Fuc-GM1 purified from bovine thyroid glands to prepare these vaccines. In preparation for clinical trials, the questions we ask here are (1) Can antibodies against Fuc-GM1 be induced? (2) Which of the four approaches is optimal? (3) Will the induced antibodies recognize Fuc-GM1 on the tumor cell surface? And (4) Can these antibodies activate complement at the tumor cell surface and produce complement-mediated cytotoxicity?

Materials and methods

Antigens, adjuvants and reagents

Fuc-GM1 was isolated from bovine thyroid as previously described [15, 30]. QS-21 [9] was obtained from Aquila Biopharmaceutical Inc. (Framingham, Mass.). Keyhole limpet hemocyanin (KLH) was obtained from PerImmune Inc. (Rockville, Mo.). Bovine serum albumin, ganglioside GM1 (GM1) and sodium cyanoborohydride were obtained from Sigma Chemical Co. (St. Louis, Mo.). Purified anti-(Fuc-GM1) monoclonal antibody F12 (mAb F12) was used as a positive control [19]. Goat anti-(mouse IgG) and anti-(mouse IgM) conjugated with alkaline phosphatase and goat anti-(mouse IgM) and anti-(mouse IgG) conjugated with fluorescein isothiocyanate (FITC) were obtained from Southern Biotechnology Associates Inc. (Birmingham, Ala.). Female CB6F1 mice were obtained from the Jackson Laboratory (Bar Harbor, Me.).

Fucosyl-GM1 extraction

Bovine thyroid glands obtained from domestic cows were used to extract Fuc-GM1 according to the method described by Van Dessel et al. [30] and Macher et al. [15], a method very similar to the one used in the preparation of GD2 and GM2 from bovine brain for our many previous clinical trials. In brief, thyroid tissue was lyophilized and extracted by the Folch system with varying concentrations of chloroform and methanol. Non-lipid contaminants were removed by Sephadex G-25 chromatography and Fuc-GM1 was separated by preparative thin-layer chromatography (TLC). Fuc-GM1 purification was performed by Matreya Inc. (Pleasant Gap, Pa.). The purity was checked by TLC with resorcinol and by immune thin-layer chromatography with mAb F12 [19]. A single Fuc-GM1 band with more than 95% purity was obtained by both TLC and immune TLC.

Vaccine preparation

Fuc-GM1-KLH vaccines were prepared as described previously for the GD3-KLH conjugate vaccine [5] with slight modification. The principle involved in the conjugation procedure is cleavage of the double bond of Fuc-GM1 ceramide by ozone, generation of an aldehyde group and conjugation to ε-amino groups of lysine on carrier proteins by reductive amination. In brief, Fuc-GM1 (5 mg) was dissolved in methanol (2 ml) and cooled in an ethanol/solid CO_2 bath. Ozone was generated by an ozone generator (Del Industries, San Luis Obispo, Calif.) and passed through the sample for 5 min. The reaction mixture was stirred for 2 min and excess ozone removed by bubbling N_2. Methyl sulfide (500 μl) was added, and the cleaved Fuc-GM1 sample was stirred at room temperature for 30 min. The sample was dried under a stream of N_2 and treated with *n*-hexane to remove free fatty aldehydes. A 10-mg sample of KLH (5 mg/ml in phosphate-buffered saline, PBS) and sodium cyanoborohydride (2 mg) were added to cleaved Fuc-GM1 and the mixture was stirred for 5 min. The sample was filtered through a 0.22-μm filter under sterile conditions, placed in a sterile vial, capped and incubated at 37 °C for 48 h. Unreacted Fuc-GM1 was removed by a molecular-mass-cut-off filter (M_r 30 000; Centriprep, Amicon Inc., Beverly, Mass.). The protein content was determined by the BioRad dye-binding method according to the manufacturer's instructions and the ganglioside content by estimating sialic acid as described by Svennerholm [29]. The epitope ratio of Fuc-GM1-KLH was 696/1. A portion of conjugate was stored with QS-21 as a liquid at 4 °C and the rest was lyophilized with QS-21.

Vaccination

Groups of five female CB6F1J mice were vaccinated five times with Fuc-GM1 (10 μg) plus QS-21 (10 μg), or Fuc-GM1 (10 μg) and KLH (20 μg) plus QS-21 (10 μg), or Fuc-GM1-KLH conjugate (3 μg Fuc-GM1) as a liquid plus QS-21 (10 μg) or Fuc-GM1-KLH conjugate (3 μg Fuc-GM1) plus QS-21 (10 μg) lyophilized and dissolved in PBS immediately prior to injection. Vaccines in 100 μl PBS were administered subcutaneously to each mouse at weeks 1, 2, 3, 7, and 19.

Serological analysis

Enzyme-linked immunosorbent assay (ELISA)

ELISA were performed to determine the IgM and IgG response against Fuc-GM1, as described previously [12]. Serially diluted sera were added to wells coated with 0.1 μg Fuc-GM1, and incubated for 1 h at room temperature. Goat anti-(mouse IgM) or anti-(mouse IgG) conjugated with alkaline phosphatase served as secondary antibodies. The absorbance was measured at 415 nm. The antibody titer was defined as the highest serum dilution showing an absorbance at least 0.1 unit above that of prevaccination mouse sera.

Flow cytometry

SCLC rat hepatoma cell line H4-II-E and human SCLC H146 [2] served as targets. Single-cell suspensions of 2×10^5 cells/tube were washed in PBS with 3% fetal calf serum and incubated with 20 μl 1:20 diluted antiserum or mAb F12 for 30 min on ice. After the cells had been washed twice with 3% FCS in PBS, 20 μl 1:15 goat anti-(mouse IgM) labeled with FITC was added, mixed and incubated for 30 min. The positive population and mean fluorescence intensity of stained cells were analyzed by flow cytometry (FACScan, Becton-Dickinson, Calif.) as described [32].

Complement-dependent cytotoxicity (CDC)

Complement-dependent cytotoxicity was assayed at a serum dilution of 1:40 with H4-II-E cells and human complement by a

chromium-release assay as previously described [32]. In brief, approximately 10^7 cells were labeled with 100 μCi $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, Mass.) in 3% human serum albumin for 2 h at 37 °C. After washing, the concentration of live cells was adjusted to 10^6 cells/ml. Samples consisting of 50 μl labeled cells were added to appropriately diluted pre- or post-immunization serum or mAb F12 (5 μg) and incubated at 4 °C for 45 min. The mixture was incubated at 37 °C for 2 h after addition of 100 μl of a 1:5 dilution of human complement. The amount of ^{51}Cr released was read by a gamma counter (Packard Minaxi Gamma Counter 5000 Series, Downers Grove, Ill.). All assays were performed in duplicate. Controls included cells incubated only with culture medium or complement. Spontaneous release was the chromium released by target cells incubated with complement alone and maximum release was the amount released by target cells after a 2-h incubation in the presence of complement and 1% Triton X-100. The percentage cytolysis was calculated according to the formula:

$$\text{Specific release}(\%) = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

Inhibition assay

Antisera at 1:150 dilution or mAb F12 at 5 μg/ml were mixed with 50 μg Fuc-GM1 or 50 μg monosialoganglioside GM1. The mixture was incubated at room temperature for 30 min and then transferred to an ELISA plate coated with Fuc-GM1-ceramide and assays were performed as described above with alkaline-phosphatase-linked anti-(mouse IgM) or anti-(mouse IgG) as a secondary antibody. FACS were performed on the H4-II-E cell line as described above with FITC-labeled anti-(mouse IgM) or anti-(mouse IgG) as a secondary antibody. Percentage inhibition was calculated as the difference in absorbance (ELISA) or percentage positive cells (fluorescence-activated cell sorting, FACS) between the uninhibited and inhibited serum.

ELISA were also performed with sera that had been inhibited (absorbed) by incubation with H4-II-E or with Fuc-GM1-negative colon cancer cell line LS-C [7]. For this assay 5×10^5 cells were incubated with 1:10 diluted sera for 1 h. After incubation, cells were spun at 1000 rpm for 5 min. The supernatant was removed and ELISA was performed using the supernatant as described above with anti-(mouse IgM)-FITC as a secondary antibody.

Results

Antibody response by ELISA to vaccination with Fuc-GM1 and Fuc-GM1-KLH conjugate

No detectable anti-Fuc-GM1 IgM or IgG antibodies were present before vaccination. After vaccination with Fuc-GM1 alone plus QS-21 or Fuc-GM1 mixed with KLH plus QS-21, antibody responses were delayed and of lower titer than in groups 3 and 4. The IgM and IgG ELISA titers against Fuc-GM1-ceramide in sera from the four groups of mice immunized with Fuc-GM1 vaccines are shown in Table 1. Fuc-GM1 conjugated with KLH induced high IgM and IgG titers. The lyophilized Fuc-GM1-KLH vaccine induced the highest titers. IgM antibody titers remained higher than IgG titers at most assay times, including those after the two booster immunizations and, in general, titers were no higher after the booster immunizations than after the initial immunizations.

Reactivity of antisera with tumor cells

The cell-surface reactivity of anti-(Fuc-GM1) antibodies was tested on Fuc-GM1-positive H4-II-E cells and H146 cells by flow cytometry, and CDC assays, as summarized in Table 2. The median percentages of positive cells, determined by flow cytometry with sera from mice vaccinated with Fuc-GM1 plus QS-21 and Fuc-GM1 mixed with KLH plus QS-21, were respectively 2.95% and 4.79% (IgM), and 13.73% and 15.25% (IgG). Sera from mice vaccinated with Fuc-GM1 conjugated with KLH, either liquid or lyophilized with QS-21, showed significantly stronger IgM and IgG reactivities with H4-II-E cells by flow cytometry ($P < 0.01$ by Dunnet's multiple-comparison test).

Anti-(Fuc-GM1) antibodies induced by vaccination were also tested for the ability to mediate CDC. As shown in Table 2, sera from mice vaccinated with Fuc-GM1 alone plus QS-21 or Fuc-GM1 mixed with KLH plus QS-21 induced little or no CDC. Sera from mice vaccinated with Fuc-GM1-KLH plus QS-21 induced CDC of H4-II-E and H146 cells at a serum dilution of 1:10 in the presence of human complement. CDC in sera from the group receiving lyophilized Fuc-GM1-KLH plus QS-21 induced significantly higher CDC than those from groups 1 and 2 ($P \leq 0.05$). Under similar conditions, the specific release with purified mAb F12 was 40%, indicating that H4-II-E, though a high expressor of Fuc-GM1, was quite resistant to CDC. Postvaccination sera in the absence of complement, and complement without sera were not cytotoxic.

Antibody specificity determined by inhibition assays

Inhibition assays were carried out with purified fucosyl-GM1 and GM1 to determine the specificity of the anti-(Fuc-GM1) mouse sera and especially to rule out strong cross-reactivity of these antibodies from the immunized mice with GM1. Inhibition of ELISA reactivity against Fuc-GM1-ceramide and inhibition of FACS reactivity against the strongly Fuc-GM1-positive cell line H4-II-E were both performed. The results of sample experiments, demonstrating the inhibition, by Fuc-GM1 but not GM1, of IgM and IgG antibody ELISA reactivity in the sera of the ten mice vaccinated with Fuc-GM1-KLH conjugate vaccines, are shown in Fig. 1a, b (ELISA inhibition) and Fig. 2a, b (FACS inhibition). The results of ELISA inhibitions indicated that Fuc-GM1 efficiently inhibited anti-(Fuc-GM1) reactivity but that the closely related GM1 ganglioside did not. Fuc-GM1 inhibited more than 90% of the anti-(Fuc-GM1) antibody activity while GM1 did not inhibit the reactivity at all. As shown in Fig. 2, FACS reactivity against cell line H4-II-E was completely inhibited by Fuc-GM1 in seven of ten mice while GM1 showed little or no inhibition in any mouse.

The absorption results (415 nm), indicating anti-(Fuc-GM1) IgM antibody reactivity in the sera of the 10

Table 1 Enzyme-linked immunosorbent assay antibody titers against fucosyl-GM1 before and after immunization with fucosyl-GM1 vaccines. Each mouse number refers to an individual mouse. Vaccines in 100 µl phosphate-buffered saline were administered subcutaneously to each mouse at weeks 1, 2, 3, 7 and 19. *KLH* keyhole limpet hemocyanin. The antibody titer is the highest serum dilution showing an absorbance at least 0.1 unit above that of prevaccination sera

Group no. and vaccine	Mouse number	Antibody titer											
		Before 1st vaccination		10 days after 3rd vaccination		Before 4th vaccination		10 days after 4th vaccination		Before 5th vaccination		10 days after 5th vaccination	
		IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
1. 10 µg Fuc-GM1 + 10 µg QS-21	1.1	0	0	0	0	0	0	100	0	100	0	0	0
	1.2	0	0	0	0	0	0	0	0	0	0	0	0
	1.3	0	0	0	0	0	0	0	0	0	0	0	0
	1.4	0	0	0	0	0	0	100	0	0	0	0	0
	1.5	0	0	0	0	0	0	100	0	0	0	0	0
	Median	0	0	0	0	0	0	100	0	0	0	0	0
2. 10 µg Fuc-GM1 + 20 µg KLH + 10 µg QS-21	2.1	0	0	200	0	0	0	200	0	0	0	400	3200
	2.2	0	0	400	0	400	0	100	0	100	0	800	200
	2.3	0	0	400	0	100	0	400	0	0	0	1600	0
	2.4	0	0	800	0	0	0	100	0	0	0	400	800
	2.5	0	0	800	0	100	0	200	0	0	0	400	800
	Median	0	0	400	0	100	0	200	0	0	0	400	800
3. 10 µg Fuc-GM1-KLH (liquid) + 10 µg QS-21	3.1	0	0	200	0	800	100	3200	1600	200	3200	1600	400
	3.2	0	0	200	50	800	200	1600	400	200	800	400	1600
	3.3	0	0	200	0	400	200	4300	800	100	400	800	12,800
	3.4	0	0	100	0	400	400	12,800	1600	800	0	800	12,800
	3.5	0	0	200	0	800	0	3200	200	200	800	800	1600
	Median	0	0	200	0	800	200	3200	800	200	800	800	1600
4. 10 µg Fuc-GM1-KLH (lyophilized) + 10 µg QS-21	4.1	0	0	800	800	50	3200	100	800	3200	200	1600	800
	4.2	0	0	800	800	1600	400	100	800	3200	800	200	400
	4.3	0	0	200	200	6400	1600	200	1600	3200	200	1600	1600
	4.4	0	0	800	800	200	400	200	800	3200	400	6400	1600
	4.5	0	0	400	400	6400	400	200	800	3200	800	800	12,800
	Median	0	0	800	800	1600	400	200	800	3200	400	1600	1600

Table 2 Fluorescence-activated cell sorting (*FACS*) and complement-mediated cytotoxicity (*CDC*) assays on H4-II-E and H146 cell lines with peak titer anti-(Fuc-GM1) immune sera. In FACS analysis the pretreatment serum from each mouse was set at 10%. The value is the true increase after vaccination. In CDC the immunized serum value is subtracted from the corresponding CDC value. Monoclonal antibody F12 (IgG3) showed 99.58% positive cells and 40% lysis on H4-II-E cells. Vaccines in 100 µl phosphate-buffered saline were administered subcutaneously to each mouse at weeks 1, 2, 3, 7 and 19

Group no. and vaccine	Mouse number	H4-II-E rat hepatoma cell line					H 146 human SCLC cell line				
		Positive cells by FACS				Lysis by CDC (%)	Positive cells by FACS				Lysis by CDC (%)
		IgM		IgG			IgM		IgG		
		(%)	*n*(mean)	(%)	*n*(mean)		(%)	*n*(mean)	(%)	*n*(mean)	
1. 10 µg Fuc-GM1 + 10 µg QS-21	1.1	13.24	123.71	13.73	111.52	0	16.17	120.71	15.81	44.11	4
	1.2	20.34	130.57	12.15	105.43	6	25.97	196.81	14.72	70.24	2
	1.3	2.06	136.96	15.54	113.83	0	14.58	150.65	10.65	46.42	19
	1.4	2.95	200.48	15.52	113.61	0	29.01	134.40	16.76	66.74	9
	1.5	2.63	154.56	11.99	106.99	0	8.89	169.23	9.65	27.76	3
	Median	2.95	136.96	13.73	111.52	0	16.17	150.65	14.72	46.42	4
2. 10 µg Fuc-GM1 + 20 µg KLH + 10 µg QS-21	2.1	15.46	132.47	23.64	121.02	0	18.31	183.68	36.11	61.82	0
	2.2	24.01	124.70	13.02	106.07	0	13.83	140.83	29.44	55.63	0
	2.3	3.80	161.41	21.86	123.31	0	10.84	127.06	16.73	71.85	0
	2.4	4.79	130.45	15.25	111.01	0	22.63	184.10	20.65	43.03	0
	2.5	3.80	200.96	13.42	108.36	0	51.21	142.13	20.60	63.28	0
	Median	4.79	132.47	15.25	111.01	0	22.63	142.13	20.65	61.82	0
3. 10 µg Fuc-GM1-KLH (liquid) + 10 µg QS-21	3.1	96.46	599.68	52.93	123.46	22	53.26	188.68	28.03	49.42	22
	3.2	88.56	268.89	32.17	119.07	12	55.84	182.69	73.78	55.90	0
	3.3	92.43	399.93	70.27	204.19	0	80.03	141.32	80.80	56.44	0
	3.4	89.32	245.79	65.26	159.65	0	45.45	180.31	37.80	48.75	12
	3.5	96.70	398.83	35.15	88.43	0	38.74	184.78	58.22	46.97	14
	Median	95.91	398.83	52.93	123.46	0	53.26	182.69	58.22	49.42	12
4. 10 µg Fuc-GM1-KLH (lyophilized) + 10 µg QS-21	4.1	56.69	155.57	42.79	111.02	0	9.05	249.38	52.93	123.46	18
	4.2	94.67	290.27	82.28	236.62	54	25.22	201.00	32.17	119.07	16
	4.3	99.03	553.36	90.00	204.96	35	55.36	191.27	70.27	204.19	49
	4.4	97.01	354.76	48.27	107.87	23	5.01	178.97	65.26	159.65	24
	4.5	96.61	325.38	82.27	219.24	14	73.24	219.53	35.15	88.43	40
	Median	96.75	325.38	82.27	204.96	23	25.22	201.00	52.93	123.46	24

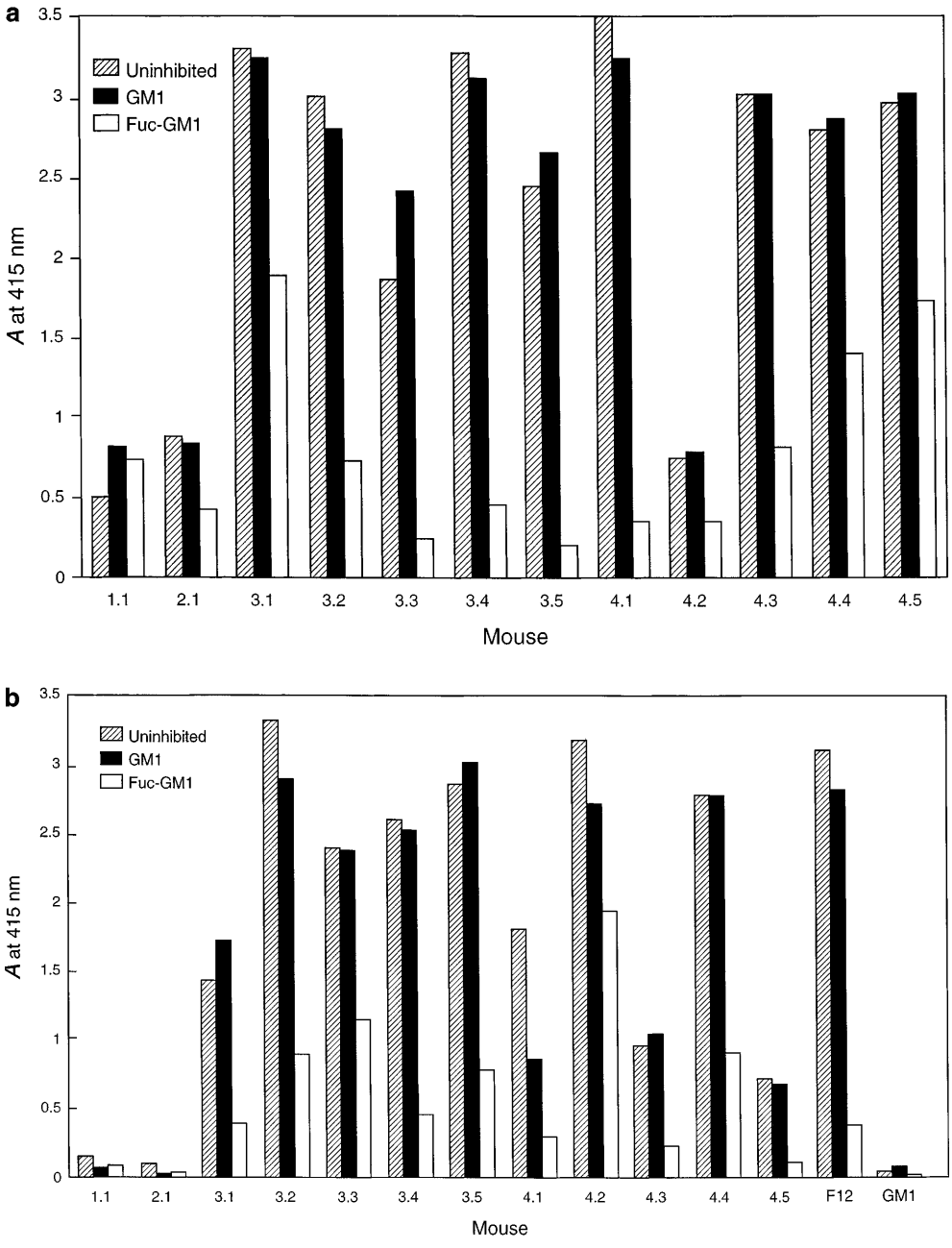


Fig. 1a, b Inhibition of enzyme-linked immunosorbent assay (ELISA) reactivity against Fuc-GM1 from the sera of mice immunized with Fuc-GM1 vaccines. Each mouse serum was preincubated with 50 μg Fuc-GM1 or GM1 or without any antigen at 37 °C for 1 h and then added to the Fuc-GM1-coated plate. ELISA was performed as described in Materials and methods, using either anti-(mouse IgM) or anti-(mouse IgG) labeled with alkaline phosphatase. **a** Anti-(Fuc-GM1) IgM antibody. **b** Anti-(Fuc-GM1) IgG antibody

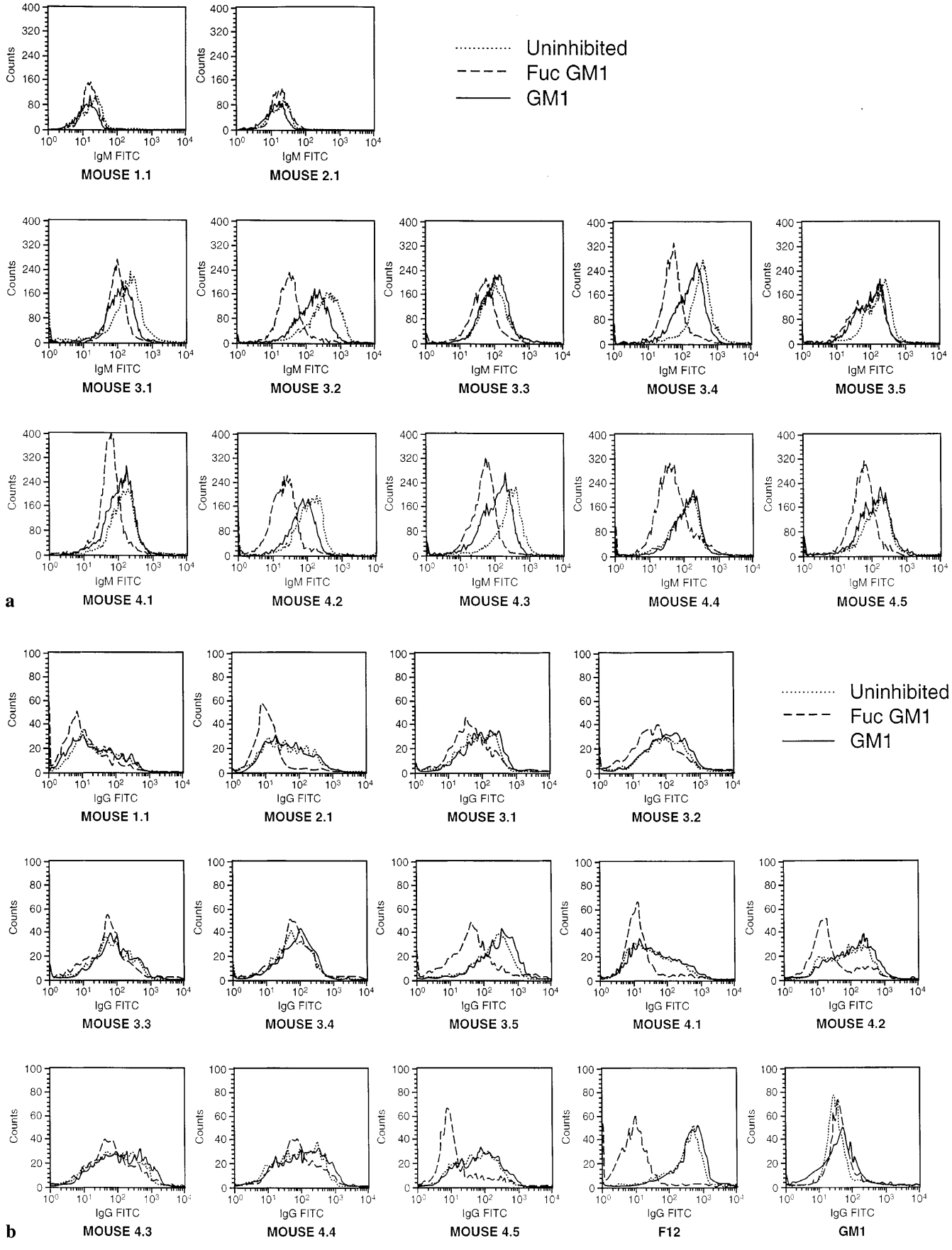
mice receiving the conjugate vaccine with Fuc-GM1-positive (H4-II-E) and Fuc-GM1-negative (LS-C) cell lines, are shown in Fig. 3. More than 75% of the ELISA reactivity against Fuc-GM1 was lost following incubation with H4-II-E cells. No decrease in the activity was observed following incubation with Fuc-GM1-negative LS-C cells. Comparable results were obtained with mAb F12.

Discussion

A variety of approaches have been adopted to increase the antibody response against carbohydrate antigens expressed on the cell surface of malignant tumors. These include chemical modification of the carbohydrate [26, 27], non-covalent complexing with bacteria such as R595 and bacillus Calmeste-Guérin or traditional adjuvants, and covalent attachment to immunogenic protein carriers [5, 6, 22, 24]. We have demonstrated, initially with gangliosides but more recently with other carbohydrates

▶

Fig. 2a, b Inhibition of fluorescence-activated cell sorting (FACS) reactivity against Fuc-GM1-positive H4-11-E cell line from the sera of mice immunized with Fuc-GM1 vaccines. Each mouse serum was preincubated with 50 μg Fuc-GM1 or GM1 or without any antigen at 37 °C for 1 h and then added to the H4-II-E cells. FACS was performed as described in methods using either anti-(mouse IgM) or anti-(mouse IgG) labeled with fluorescence isothiocyanate (*FITC*). **a** Anti-(Fuc-GM1) IgM antibody. **b** Anti-(Fuc-GM1) IgG antibody


Uninhibited
Fuc GM1
GM1
Counts
IgM FITC
IgG FITC
MOUSE 1.1
MOUSE 2.1
MOUSE 3.1
MOUSE 3.2
MOUSE 3.3
MOUSE 3.4
MOUSE 3.5
MOUSE 4.1
MOUSE 4.2
MOUSE 4.3
MOUSE 4.4
MOUSE 4.5
F12
GM1
a
b

and with peptides, that the optimal approach is covalent attachment to the immunogenic carrier protein KLH plus the use of immunological adjuvant QS-21 [5, 6, 8, 21, 23]. QS-21 is a homogeneous saponin fraction purified from the bark of *Quillaja saponaria* Molina. This is the approach applied here to augmenting the immunogenicity of Fuc-GM1.

The differential expression of carbohydrate antigens on tumor cells has been a major determinant in their selection as antigens for anticancer vaccine construction and testing. Gangliosides, such as GM2 and GD2 have been identified as targets for active immunotherapy [4, 6]. The presence of natural or vaccine-induced antibodies against GM2 in melanoma patients correlates with a significantly improved disease-free and overall survival [13, 14]. Disaccharides Thomsen-Friedenreich (TF) antigen, sialyl-Tn (sTn) and globo H have been synthesized and their immunogenicity in conjugate vaccines confirmed [1, 16, 25, 28]. Antibody titers against synthetics Tn have been reported to correlate with improved prognosis in breast cancer patients [17, 18]. Pivotal phase III trials to assess the clinical impact of vaccination with GM2-KLH or sTn-KLH in patients with melanoma or breast cancer (respectively), in the adjuvant setting, are under way. The distribution of Fuc-GM1 on normal tissues is more restricted than any of these antigens and it is abundantly expressed on most small-cell lung cancers. This background suggests that Fuc-GM1 may be an excellent target for active immunization. The availability of Fuc-GM1 has permitted us to compare approaches for augmenting Fuc-GM1 immunogenicity in a series of preclinical studies in the mouse.

Our studies here with Fuc-GM1 vaccines were modeled on previous studies with other ganglioside vaccines. The optimal conjugation procedure for GM2 and GD2 involved ozone cleavage of the double bond, introduction of an aldehyde group and coupling to protein aminolysyl groups by reductive amination [5]. In this study the double bond in the Fuc-GM1 ceramide was cleaved with ozone and the product conjugated with KLH. Important characteristics of the antibody response to immunization with Fuc-GM1-KLH conjugate plus QS-21 include the pattern of antibody response and the specificity of the antibodies. IgM and IgG antibody titers against Fuc-GM1 were significantly higher than seen previously after vaccination with other gangliosides in the mouse [5]. As described previously for GM2 and GD3, IgM antibodies against Fuc-GM1 remained higher than IgG antibodies at all assay times and booster immunizations were not able to raise antibody titers above the level after initial immunization. This is consistent with a relatively T-cell-independent antibody response, as expected against carbohydrate antigens. Interestingly, the highest-titer antibodies were induced by the Fuc-GM1-KLH plus QS-21 vaccine, which had been lyophilized for more efficient long-term storage.

Specificity and functional analysis of the antibodies generated by immunization with the Fuc-GM1-KLH plus QS-21 vaccine confirmed the relevance of this antibody response. (1) The antibodies did not cross-react with GM1. This is crucial since GM1 is highly expressed in normal brain, and antibodies against GM1 have been correlated with autoimmune diseases such as multiple sclerosis and amyotrophic lateral sclerosis [10, 20]. (2) No evidence of toxicity was seen in immunized mice (no

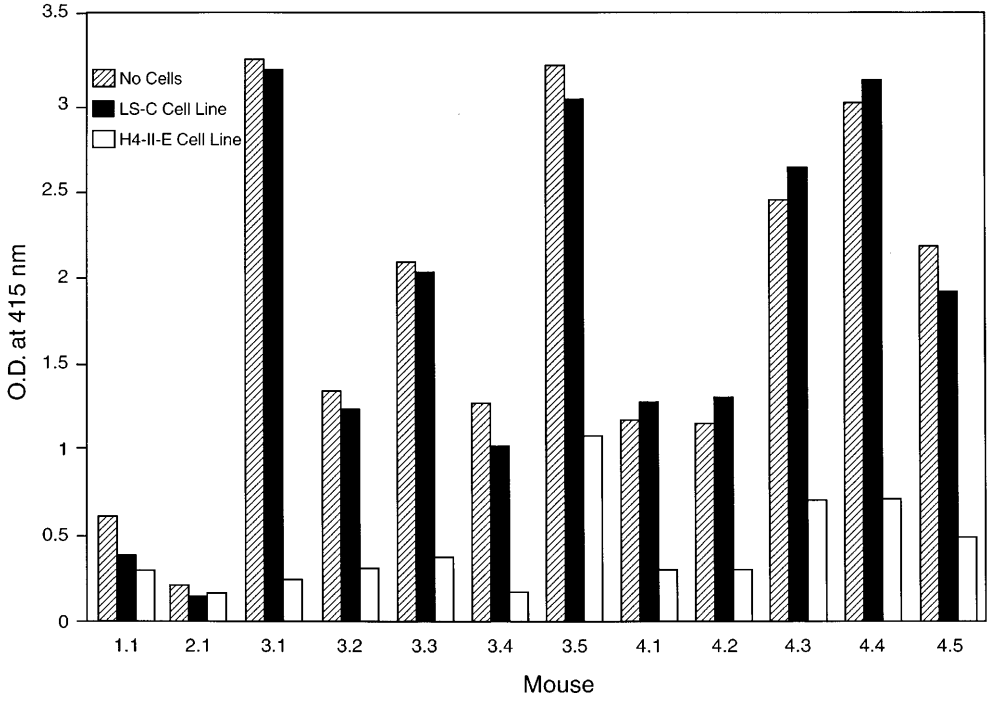


Fig. 3 Absorption values for ELISA reactivity against anti-(Fuc-GM1) IgM by Fuc-GM1-positive H4–11-E and Fuc-GM1-negative LS-C cells from the sera of mice immunized with Fuc-GM1 vaccines. Each mouse serum was preincubated with 5×10^5 cells for 37 °C for 1 h and then added to the Fuc-GM1-coated ELISA plates. ELISA were performed as described in Materials and methods

weight loss was observed, data not shown). (3) Antibodies reacted strongly with Fuc-GM1 expressed at the tumor cell surface, as demonstrated by flow cytometry on cell lines including H146 human SCLC cells. (4) Antibodies were able to mediate complement-mediated cytotoxicity. On the basis of these observations, we plan to initiate clinical trails with the lyophilized Fuc-GM1-KLH plus QS-21 vaccine in patients with small-cell lung cancer.

Acknowledgements This work is supported by grants from the National Institute of Health (CA 33049) and The Milstein Family Foundation.

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