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Cellular cancer vaccine induces delayed-type hypersensitivity reaction and augments antibody response to tumor-associated carbohydrate antigens (sialyl Le^a, sialyl Le^x, GD_3 and GM_2) better than soluble lysate cancer vaccine

Mepur H Ravindranath, Philip M Bauer, Amir A Amiri, Seyed M Miri, Mark C Kelley, Ralph C Jones[1] and Donald L Morton

Laboratory of Glycolipid Immunotherapy, John Wayne Cancer Institute at Saint John's Hospital and Health Center, 2200 Santa Monica Boulevard, Santa Monica, CA 90404, USA. Tel: (+1) 310 449-5263; Fax: (+1) 310 449-5259. [1]LCDR MC USA, Department of Surgery, National Naval Medical Center, Bethesda, MD, USA.

Allogenic whole cell and lysate cancer vaccines are associated with very different clinical outcome, which could be due to different immune responses to critical tumor-associated antigens. We used a guinea pig model to evaluate the immune responses to melanoma-associated carbohydrate antigens administered in whole cell and soluble lysate vaccines produced from the same cell lines and administered with or without Bacille Calmette-Guerin (BCG). Animals immunized with whole cell vaccine developed a significantly higher delayed-type hypersensitivity (DTH) reaction. The IgG response to all tumor-associated carbohydrate antigens except GD_2 was significantly higher in animals immunized with whole cell vaccine than lysate vaccine. This study indicates that whole cell vaccine is superior to soluble or lysate vaccine because it induces a better immune response against cell-surface antigens. The addition of BCG significantly increased the antibody response, suggesting that an exogenous adjuvant may immunopotentiate antigens better in the presence of an intact cell membrane.

Key words: Bacille Calmette-Guerin, cancer vaccines, carbohydrate antigens, delayed-type hypersensitivity reaction, gangliosides, GD_2, GD_3, GM_2, IgG antibodies, melanoma, sialyl Lewis^a, sialyl Lewis^x.

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Correspondence to MH Ravindranath

Introduction

Recent clinical trials of immunotherapy in human melanoma suggest that whole cell vaccines elicit a stronger clinical response than lysate vaccines. In phase II trials of adjuvant immunotherapy in 283 patients rendered clinically free of disease after surgical resection of American Joint Committee on Cancer (AJCC) stage III melanoma, 52% of patients receiving an allogeneic irradiated whole melanoma cell vaccine (MCV) with Bacille Calmette-Guerin (BCG) survived disease free at 5 years, compared to 36% of 1474 historical control patients.[1,2] Median disease-free survival was 24.3 months for the control group versus greater than 90 months for patients receiving MCV ($p < 0.001$). In contrast, a study of AJCC stage III melanoma patients receiving allogeneic melanoma cell lysate vaccine with vaccinia virus did not show any significant difference in disease-free and overall survival (4 years) between the treatment ($n = 104$) and the control groups ($n = 113$) after a mean follow-up of 30 months.[3] Although the adjuvants and cell lines used in these trials are different, we hypothesized that the apparent differences in clinical outcome are due to a greater immune response to critical melanoma-associated antigens in patients receiving whole cell vaccine. This hypothesis is supported by observations in animal models, which show better cytotoxic T lymphocyte and antibody responses to whole cell than lysate vaccines.[4,5]

We evaluated the relative efficacy of whole cell vaccine and lysate vaccines produced from the same cell lines, by comparing their ability to elicit a

specific antibody response to well defined, purified or synthetic antigens. The whole cell and lysate vaccines were administered with and without BCG to determine the immunostimulatory potential of this adjuvant. The whole cell vaccine with BCG induced stronger antibody responses to a variety of important melanoma-associated antigens than lysate vaccine alone or with BCG.

Materials and methods

Test antigens

The study of functional sLe^x (*Siaα2,3Galβ1,4 (Fucα1,3)GlcNAcβ1,3Gal*-R) and sLe^a (*Siaα2,3Galβ1, 3(Fucα1,4)GlcNAcβ1,3Gal*-R) structures requires that the molecule be complexed with reporter groups to allow detection and presentation in a biologically significant multivalent format. A 30 kDa multivalent polymer is created by incorporating synthetic sialyl Lewis (sLe) probes into a polyacrylamide matrix (GlycoTech, Rockville, MD). Approximately every fifth amide group of the polymer chain is *N*-substituted by the carbohydrate spacer arm. The polymers are also substituted with biotin in a 4:1 ratio. The multivalent biotinylated polymers are used with streptavidin reporter reagents for immobilization to microtiter plates in ELISA.

The purified gangliosides GD_3, GD_2 and GM_2 were obtained from Fluka Chemical (Ronkonkoma, NY) or Sigma (St Louis, MO). The purity of the ganglioside antigens was assessed by thin-layer chromatography and nuclear magnetic resonance (NMR) spectroscopy. Fluka provides upon request the NMR profiles for their purified gangliosides. Immunological reactivity of the gangliosides and sLe antigens was assessed with a variety of monospecific monoclonal antibodies (Table 1).[6–9] KM 93 and KM 231 have been extensively characterized for epitope specificity and used as a cocktail for immunodiagnostic purposes.[6,7] The monoclonal antibodies were diluted with PBS (pH 7.2) containing 4% HSA. The antibodies were used at a dilution of 1/100 with the exception of 14.G2a which was diluted 1/4 or specified otherwise. Mouse IgG3, IgG1, IgG2a and IgM (Southern Biotechnology Associates, Birmingham, AL) were used as negative controls that did not react with the antigens in ELISA plates. The control antibodies were always used after adjusting their protein concentration to that of their respective primary antibodies.

MCV

MCV and lysate of MCV cells were formulated from the human melanoma cell lines M10-v, M24 and M101. These cells are cryopreserved and thawed as described earlier.[2] All melanoma cell lines were cultured in RPMI 1640 medium (JRH Biosciences, Lenexa, KS) supplemented with 5% FBS (Gemini Bioproducts, Calabassas, CA) and an antibiotic–antimycotic (Pen-Strep-Fungizone, Gibco/BRL 15240-013). Cells were harvested using quality controlled manufacturing practice and stored at −80°C.[2] The human melanoma cell lines, FBS and media were tested for mycoplasma (Mycotrim Assay), HIV (PCR) and HBV (PCR), and were negative. To prepare MCV or MCV lysate, a known volume of RPMI containing a known number of cells was separated into equal aliquots. One aliquot was used as intact cells and the other was used as MCV lysate by repeated freezing, thawing and vortexing the cells. The lysate was examined for the presence of viable cells by Trypan blue exclusion.

Immunization in guinea pigs

Thirteen guinea pigs (strain: HsD, Poc:DH, females, weight 300–350 kg) were immunized on weeks 0, 2, 4 and 6. The vaccine formulations were administered intradermally in the right posterior dorsolateral regions of the body. Each dose contained $1 \times 10^{-}$ cells consisting of an equal number of cells

Table 1. Monoclonal antibodies used in this investigation

Antigen	Mab	Isotype	Purity (P)	Concentration	Source	Ref.
Sialyl Le^x	KM93	IgM	affinity-P	100 μg/500 μl	Kamiya	6
Sialyl Le^a	KM231	IgG2a	affinity-P	100 μg/500 μl	Kamiya	7
GD_3	Mel-1	IgG3	affinity-P	225 μg/ml	Signet	8
GD_2	14.G2a	IgG2a	cult.sup.	–	Dr R Reisfeld	9

Source: Kamiya Biomedical (Seattle, WA); Signet (Dedham, MA); Dr Ralph Reisfeld (Scripps Institute, La Jolla, CA).

from each cell line. The animals were divided into four immunization groups: group 1: MCV with BCG ($n = 4$); group 2, MCV without BCG ($n = 3$); group 3, MCV lysate with BCG ($n = 3$) and group 4, MCV lysate without BCG ($n = 3$). BCG (1×10^6 bacterial cells) was admixed with MCV (4×10^7) or MCV lysate (volume and the number of cells immunized as lysate were same as that used for MCV) just before injection. Animals were bled on week −1, 0, 3, 5, 7 and 9.

Measurement of DTH reaction

The DTH reaction to the vaccine cells was studied 1 day after intradermal injection of 1×10^6 viable cells intradermally on weeks 0, 2, 4 and 6. Erythema and induration were measured with vernier calipers in two dimensions. DTH is expressed as the mean diameter of induration.

Quantitation of cell surface antigens and adjuvants with cell-suspension ELISA (Cs-ELISA)

Quantitation of cell surface antigens and adjuvants was carried out by a Cs-ELISA.[10,11] Use of trypsin was avoided due to its influence on cell surface antigen expression.[11] All cells were suspended in RPMI–4% HSA, collected by centrifugation and examined for viability using Trypan blue exclusion. Cell suspensions (0.2 or 0.5×10^6) in 50 μl of solution per vial (microcentrifuge tubes) were separated as *background* (treated only with the second antibody), *experimental* (treated with first and second antibodies) or negative control (treated with class-matched isotypes of the first antibodies). One hundred microliters of the first antibody, e.g. KM 93 (1/100) or KM 213 (1/100), or Mel-1 (1/100), or 14.2Ga (1/4) in PBS–4% HSA, was added and incubated for 1.5 h in a shaker at below 10°C. After washing the cells three times, 150 μl of a 1/5000 dilution of peroxidase-coupled goat anti-guinea pig IgG antibody was added and incubated for 1 h in a shaker at below 10°C. After washing two times, the contents were transferred into fresh vials containing 1 ml of PBS–4% HSA and centrifuged to recover the pellet. The substrate solution (50 μl) was added to cells in each vial and incubated in the dark at room temperature for 40 min. The supernatant was recovered after centrifugation and transferred to microtiter wells containing 60 μl of 6 N H_2SO_4. The absorbency was measured at 490 and 650 nm. The controls used were: cells treated with substrate only (*blank*), cells treated with peroxidase-coupled second antibody (*background*) and cells treated with non-specific purified isotypes corresponding to first antibodies (*negative*). All values were adjusted to the background and corrected for negative control.

Anti-sLe antibody ELISA

An ELISA was developed to detect anti-sLe antibodies in the sera of guinea pigs. Microtiter plates (Falcon Probind 3915) were coated overnight with 100 μl of streptavidin (0.5 μg/well) (Sigma) suspended in PBS, pH 7.2 at 4°C. After washing excess streptavidin with PBS, pH 7.2, the plates were coated overnight with biotinylated-PAA-sLe polymer (Glycotech) in carbonate buffer, pH 9.6. Box titration showed the optimal concentration to be 3.3 pmol (for both sLe^x and sLe^a). After washing and blocking with PBS–4% HSA PBS, pH 7.2, the plates were incubated at 37°C for 90 min and exposed to sera or first antibodies as described elsewhere.[12] Briefly, 100 μl of diluted sera (diluted in PBS–4% HSA and preincubated at 37°C for 1 h) or monoclonal antibodies was added to wells and incubated at 37°C for 90 min. Wash buffer contained Tween-20 (0.1%) and 0.1% HSA in PBS. Peroxidase-coupled second antibody diluted 1/5000 was used. Only IgG antibodies could be detected in guinea pigs because of non-availability of second antibody specific to IgM. *o*-phenylenediamine dihydrochloride in 25 ml of citrate phosphate buffer, pH 5.0, was used as substrate and the plates were incubated at room temperature in the dark for 45 min. After arresting the enzyme activity with sulfuric acid (60 μl of 6N), absorbency was measured at 490–650 nm. The controls used include: wells without antigen and treated with second antibodies and substrate (*blank*), wells without antigen-treated with first and second antibody and substrate (*background*), and antigen-coated plates treated with class-matched non-specific purified isotypes of first antibody (*negative*). All values were adjusted to the background and corrected for the negative control.

Descriptive statistics

We used Mann–Whitney's non-parametric two-tailed *T*-test to determine the significance of differences in the DTH response. Preimmune and postimmune serum antibody titers were analyzed with an analysis of variation after Bonferoni adjustment for sample

size. The statistical package of GraphPad Instat and Microcal Origin was used with significance ascribed for $p \leq 0.05$.

Results

Glycocalyx of MCV

The three human melanoma cell lines constituting MCV expressed the gangliosides GD_3, GD_2 and oligosaccharide residues of sLe^a and sLe^x (Figure 1A). M24 cells overexpressed sLe^a and expressed small amounts of sLe^x. M10-v cells expressed a large amount of sLe^x, whereas M101 cells expressed equal amounts of sLe^x and sLe^a. The profile presented in Figure 1(B) shows that the predominant constituent of the glycocalyx of MCV is sLe^a, with GD_3 and sLe^x expressed in equal amounts.

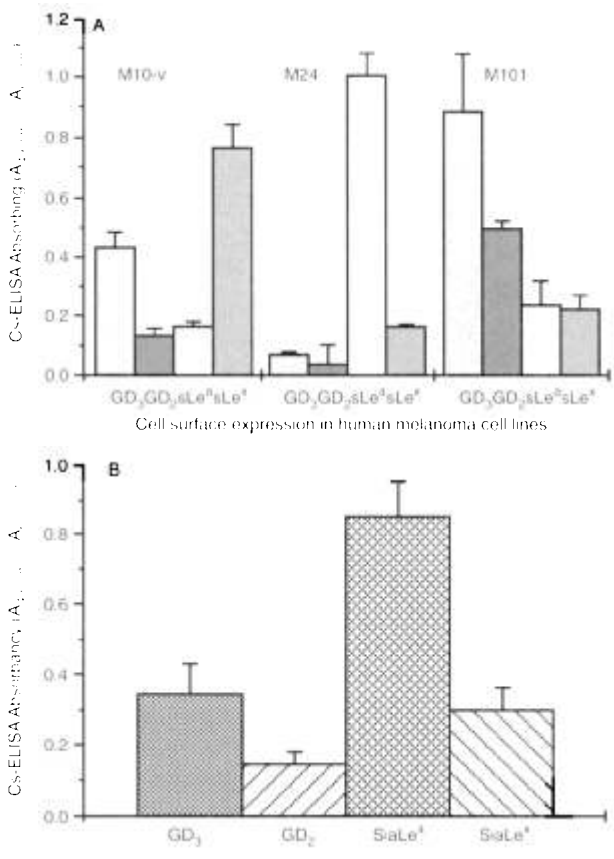

Figure 1. Surface expression of sLe^a, sLe^x, GD_3 and GD_2 in MCV cell lines (M10-v, M101 and M24) (A) and in a batch of MCV (B). Overexpression of sLe^x compared to other glycoconjugates distinguishes the glycocalyx of M10-v. Overexpression of sLe^a and feeble expression of GD_3 are unique features of M24. Cell surface expression of the glycocalyx is monitored by cell suspension ELISA using monospecific murine monoclonal antibodies as described in Materials and methods. The vertical bars represent SD of five analyses.

DTH reaction to MCV

No animal developed a DTH response to MCV prior to immunization. A mild (nine of 13 animals) to prominent (four of 13) erythema with or without induration was seen in most animals after the first immunization. The maximum size of induration was attained after the third immunization. Figure 2 shows that MCV with BCG induced a significantly higher ($p = 0.05$) DTH response than MCV lysate with BCG.

Augmentation of antibodies to melanoma-associated sLe^x, sLe^a, GD_3, GD_2 and GM_2

To test the hypothesis that tumor cell viability and membrane integrity is required for immune recognition and antibody response for major carbohydrate antigens, we compared the IgG antibody response induced by MCV and MCV lysate. The IgG antibody response after four immunizations was highest for GD_3, followed by GD_2, GM_2, and then sLe antigens (Table 2).

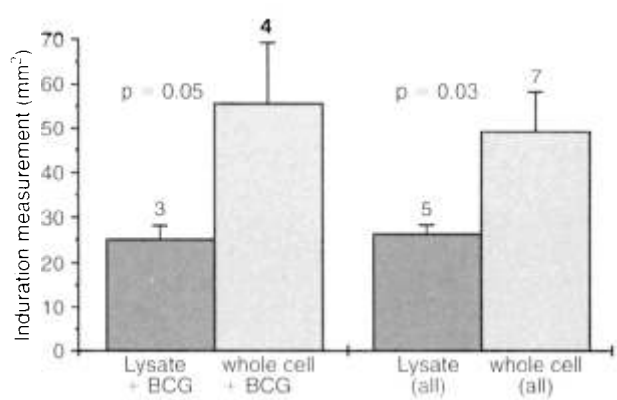

Figure 2. DTH reaction to xenogeneic melanoma cells in the skin of guinea pigs after the fourth immunization with different formulations of MCV. No DTH response was observed before immunization. No distinct induration was seen after the first immunization. The size of induration increased after the third immunization. Viable, irradiated MCV cells (1×10^6) were injected intradermally in guinea pigs. The viability of MCV was greater than 85%. (all) refers to vaccine with or without BCG. The vertical bars represent SD. The sample size for each experiment is indicated above the vertical bars. Non-parametric *t*-test values are indicated.

Table 2. Preimmune and postimmune serum IgG titers in guinea pigs

Antigen (per well)	Preimmune titer ($n = 10$)	Serum IgG titers after fourth immunization: number of fold increase from preimmune titer			
		MCV + BCG ($n = 4$)	MCV ($n = 3$)	MCV lysate + BCG ($n = 3$)	Lysate ($n = 3$)
sLe^a (3.3 pmol)	164 ± 22	×3	×1	× < 2	×1
sLe^x (3.3 pmol)	189 ± 41	×3	×2	× < 2	×1
GD_3 (1 pmol)	190 ± 94	×7	×6	×6	×3
GD_2 (1 pmol)	90 ± 45	×5	×3	×3	×3
GM_2 (1 pmol)	71 ± 30	×4	×2	×2	–

Anti-sLe^a IgG antibody response. Figure 3(A and C) shows that the titer of anti-sLe^a IgG differed significantly between prevaccine and postvaccine sera in animals immunized with MCV-BCG. The increase in antibody titer is significantly higher ($p < 0.001$) after the fourth immunization after Bonferoni adjustment for sample size. A similar significant ($p < 0.01$) increase was observed in animals immunized with MCV without BCG. However, no such increase was observed in animals immunized with MCV lysate–BCG or MCV lysate only.

Anti-sLe^x IgG antibody response. Figure 3(B and C) shows that the titer of anti-sLe^x IgG is significantly higher after four immunizations with MCV–BCG. The serum anti-sLe^x IgG titer did not vary between pre- and postimmunization in animals immunized with MCV without BCG or with MCV lysate vaccine with or without BCG.

Anti-ganglioside IgG antibody responses. Figure 4(A–C) shows serum IgG response to GM_2, GD_3 and GD_2, the major gangliosides of MCV. The serum anti-GM_2 IgG antibodies (Fig. 4A) showed a significant increase after the fourth immunization with MCV–BCG. No such increase was observed after immunization with MCV without BCG or with MCV lysate vaccines with or without BCG. Serum anti-GD_3 IgG (Fig 4B) also increased significantly after four immunizations with MCV–BCG ($p < 0.005$), MCV lysate–BCG ($p < 0.02$) and MCV without BCG ($p < 0.05$). The anti-GD_3 IgG antibody response also differed significantly between MCV with and without BCG ($p = 0.05$). Results obtained with anti-GD_2 IgG titers are strikingly different from those observed with other antigens. All vaccine formulations induced a significantly ($p < 0.05$) higher response to this antigen and there was no difference among the different vaccine groups after four immunizations.

Discussion

The gangliosides GD_3, GD_2 and GM_2 are important human melanoma-associated antigens[13,14] which do not require T cell help for immune recognition.[15] These gangliosides are, therefore, important constituents of whole cell[2] or lysate vaccines.[3] A recently developed cell suspension ELISA quantifies the cell-surface expression of these major melanoma-associated gangliosides. Cell viability and antigen density on the cell surface are important factors in determining the magnitude of immune response to the antigens. The results of the present investigation indicate that membrane integrity is important for immune recognition and induction of humoral and cellular immune responses to the glycocalyx of melanoma cells.[4,5]

The glycocalyx of melanoma cells may fall into two or more categories: group 1 expressing high sLe^x, GD_3 (lower, higher or equal to sLe^x) and GD_2 and low sLe^a (exemplified by M10-v cells and M101), and group 2 expressing high sLe^a and GM_2 and low sLe^x GD_3 and GD_2 (exemplified by M24 cells). MCV contains members of both groups to balance the expression of all the major antigens as shown in Figure 1(B).

We have used a xenogenec vaccine in a guinea pig model. Although xenogenic immunization may not be suitable for evaluating immune responses to HLA-dependent antigens, our study may shed light on the humoral responses to carbohydrate antigens, which are T cell independent and therefore not HLA-restricted.[20–22] The antibody responses may not be due to anti-HLA antibodies since we have evaluated the humoral responses against purified antigens.

The relative efficacy of whole cell vaccine versus lysate vaccine was evaluated by measuring the antibody titers to some of the components of the gycocalyx of MCV. In general, all antigens tested in this study induced IgG antibody response in guinea

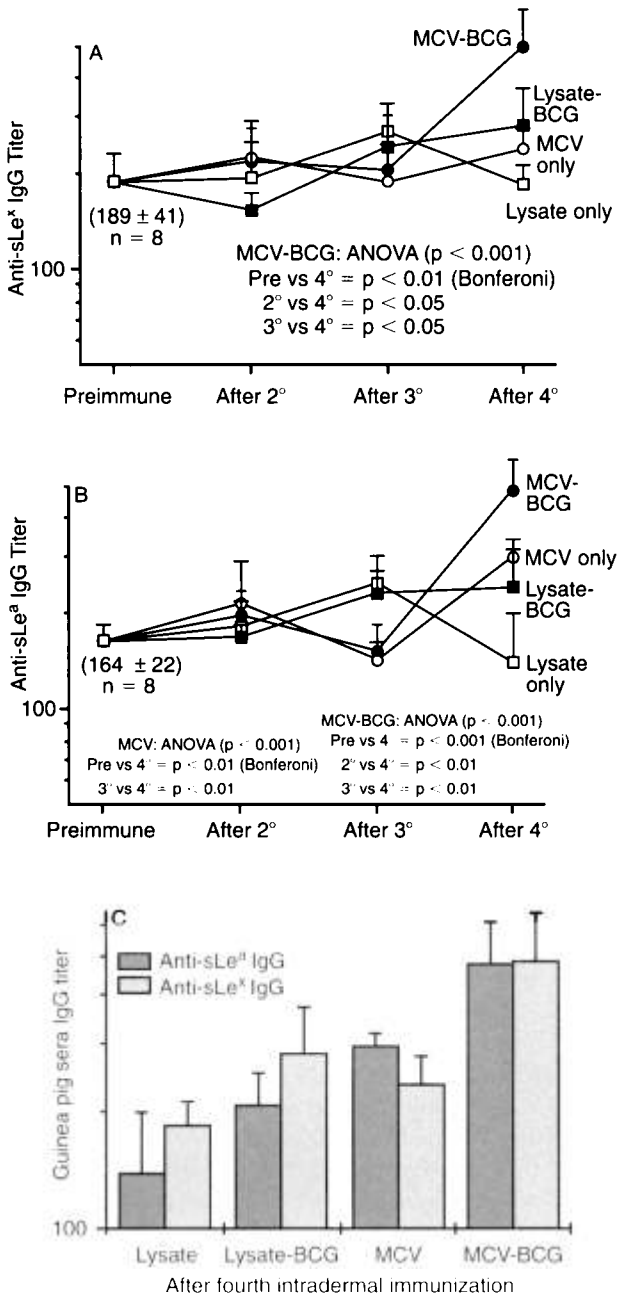

Figure 3. Anti-sLe IgG antibody response induced by MCV and MCV lysate in guinea pigs. Both sLe^a and sLe^x are immunogenec in guinea pigs. Vaccines were administered with or without BCG. Sera were analyzed against purified antigens. After four immunizations, MCV–BCG induced a significantly higher antibody response to sLe^x (A) and sLe^a (B). MCV lysate with or without BCG was poorly immunogenic. Panel (C) summarizes the superiority of whole cell vaccine in inducing antibody response to sLe^a and sLe^x. The preimmune value is the mean of eight analyses. The results of analyses of variance as well as the levels of significance among pre- and post-immune groups are provided after Bonferoni adjustment for the sample size. The vertical bars in immunization groups refer to SD of four analyses.

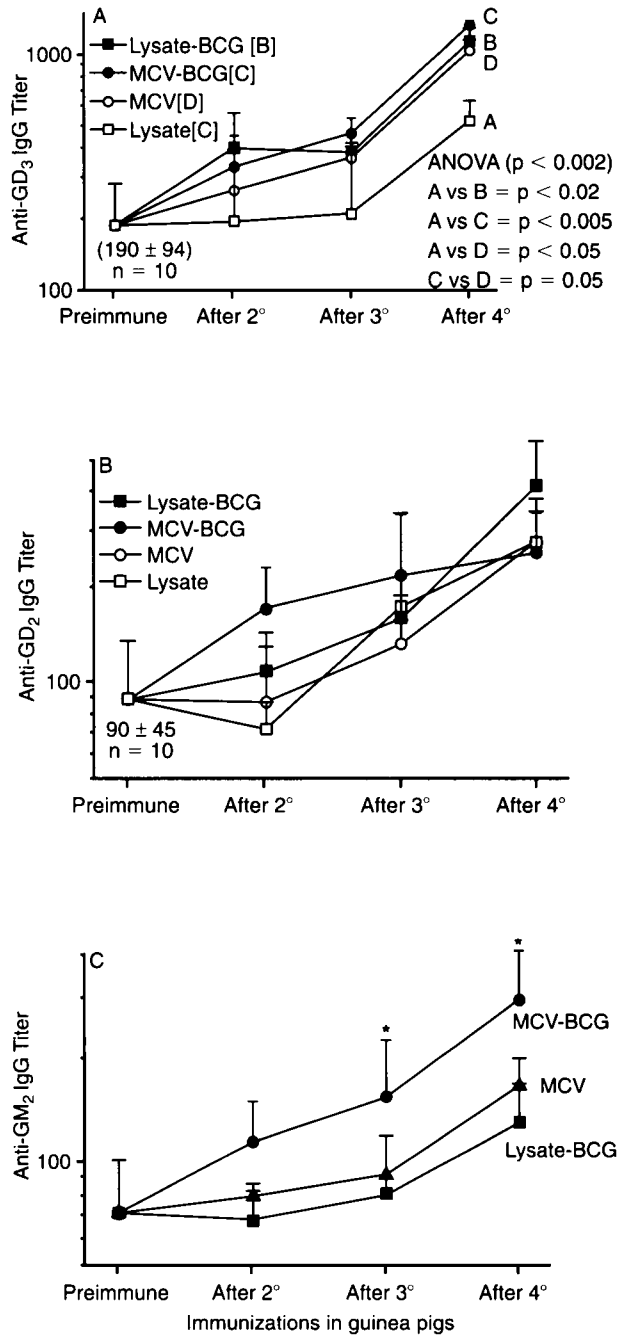

Figure 4. Antiganglioside IgG antibody response in guinea pigs after immunization with different formulations of MCV. Panels (A), (B) and (C) refer to serum anti-GD_3, anti-GD_2 and anti-GM_2 IgG titers respectively, before and after immunization with xenogeneic MCV. Sera were analyzed against purified antigens. The preimmune value is the mean of 10 analyses. The results of analyses of variance as well as the levels of significance among different vaccine groups are provided. The vertical bars in immunization groups refer to SD of four analyses. Asteriks in panel (C) indicate that the antibody titers in animals immunized with MCV–BCG are significantly ($p < 0.05$) higher than other immunization groups.

pigs. The antibody response to GD_2 did not vary significantly among different vaccine formulations; specifically there was no difference between MCV–BCG and MCV lysate–BCG vaccines. However, MCV with BCG produced a significantly greater antibody response to GD_3 and GM_2 than MCV lysate vaccine with or without BCG. BCG also significantly augmented the anti-GD_3 IgG response of MCV.

Whole cell vaccine also induced a greater IgG antibody response to the sialyl Lewis antigens than lysate vaccine. Lysate vaccine was incapable of inducing an anti-sLe^x or anti-sLe^a IgG response even after admixture with BCG. In contrast, MCV with BCG induced a significant antibody response against both the sialyl Lewis antigens, suggesting that the immune recognition of these antigens is possible only in the presence of viable cells with an intact membrane.

Although we could not analyze serum IgM antibody response due to the lack of a sensitive anti-guinea pig IgM antibody, our data based on the IgG response suggest that for the antigens constituting the glycocalyx of human melanoma, lysate or soluble formulations of vaccine may not yield effective antibody response. It is accepted that in contrast to peptide antigens of human melanoma (e.g. MAGE-1 and MAGE-3), carbohydrate antigens do not require HLA restriction. They may be highly immunogenic in the absence of HLA presentation as documented by the strong cellular and humoral responses to these antigens on human melanoma cells in this guinea pig model. It is not clear why these antigens require cell viability or membrane integrity to induce maximum antibody response. It is possible that there may be membrane-bound co-stimulatory molecules involved in B cell recognition of carbohydrate antigens. Alternatively, adjuvants such as BCG may play the role of a co-stimulatory molecule in conjunction with an intact cell-membrane.

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