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Anti-Tumor Immunity Provided by a Synthetic Multiple Antigenic Glycopeptide Displaying a Tri-Tn Glycotope[1]

Richard Lo-Man,[2]* Sophie Vichier-Guerre,[†] Sylvie Bay,[†] Edith Dériaud,* Danièle Cantacuzène,[†] and Claude Leclerc*

In many cancer cells the alteration of glycosylation processes leads to the expression of cryptic carbohydrate moieties, which make them good targets for immune intervention. Identification of cancer-associated glycotopes as well as progress in chemical synthesis have opened up the way for the development of fully synthetic immunogens that can induce anti-saccharide immune responses. Here, we synthesized a dendrimeric multiple antigenic glycopeptide (MAG) containing the Tn Ag *O*-linked to a CD4^{+} T cell epitope. This MAG is based on three consecutive Tn moieties (tri-Tn) corresponding to the glycotope recognized by an mAb (MLS 128) produced against the LS180 colon carcinoma cell line. The Abs induced by this MAG recognized murine and human tumor cell lines expressing the Tn Ag. Prophylactic vaccination using MAG provided protection of mice against tumor challenge. When used in active specific immunotherapy, the MAG carrying the tri-Tn glycotope was much more efficient than the mono-Tn analogue in promoting the survival of tumor-bearing mice. Furthermore, in active specific immunotherapy, a linear glycopeptide carrying two copies of the tri-Tn glycotope was shown to be poorly efficient compared with the dendrimeric MAG. Therefore, both the clustering of carbohydrate Ags and the way they are displayed seem to be important parameters for stimulating efficient anti-saccharide immune responses.   *The Journal of Immunology,* 2001, 166: 2849–2854.

T he identification of tumor-associated Ags in melanomas and in several other human cancers has opened up a new basis for cancer vaccines (1). A number of tumor-derived peptides have been identified in the last few years and used as therapeutic immunogens in combination with different adjuvants, such as bacillus Calmette-Guérin, IFA, QS-21 (2, 3), or heat shock protein-peptide complexes (4). Other approaches based on DNA vaccines, recombinant virus shuttles, or tumor cells transfected with genes encoding costimulatory molecules or cytokines (5, 6) have also been developed to stimulate anti-tumor immunity. In the last few years, the use of dendritic cells has emerged as a new exciting area for cancer immunotherapy (7), because dendritic cells loaded with tumor peptides can induce immune responses preventing the outgrowth of tumors in mice (8). All these approaches aimed at stimulating peptide-specific T cell immune responses are promising; however, the efficacy of future immunotherapeutic treatments should rely on the stimulation of both Ab and cellular anti-tumor immune responses (9).

Tumor-associated carbohydrate Ags are also potential targets for anti-cancer therapy (10). Among these Ags, T, Tn, sialyl-Tn, and the ganglioside GM2 are the most well known. Covalent attachment of the carbohydrate Ags to protein carriers is traditionally used to induce anti-cancer Ab responses, and the resulting immunogens were shown to increase survival in patients. The most

promising beneficial effects of tumor vaccination using this strategy were found for the GM2/keyhole limpet hemocyanin (KLH)[3] conjugate against melanoma (11) or the sialyl-Tn/KLH conjugate against breast, ovarian, and colorectal cancers (12).

Recently, we developed a fully synthetic immunogen that does not require protein carrier (13, 14). This system, called multiple antigenic glycopeptide (MAG), is based on a dendrimeric lysine core with four arms analogous to the multiple antigenic peptide (MAP) construct of Tam (15). Each arm is linked to a peptide backbone containing a CD4^{+} T cell epitope (PV peptide) with a monomeric saccharide Tn residue at the N-terminal end of the peptide (MAG:Tn-PV). This construction offers several advantages: the carbohydrate content is much higher than in traditional protein conjugates, the core matrix is nonimmunogenic, and the construction has a well-defined chemical structure. A therapeutic immunization performed with this immunogen was shown to increase the survival of tumor-bearing mice (14).

Polysaccharidic cancer-associated Ags (GM2, GD2, Globo H, Le[y], Sialyl-Tn) can display large glycotopic structures available for Ab binding sites. In contrast, Tn, which is a monosaccharidic Ag (α-GalNAc-Ser/Thr) found on mucin-type glycoproteins and expressed on most human adenocarcinomas (16), was shown to be recognized by different mAbs as Tn clusters (17–20). Recent encouraging results obtained with a linear glycopeptide based on a tri-Tn glycotope recognized by the MLS128 mAb showed that anti-Tn Abs can be successfully induced in mice in the absence of a carrier protein (21). To further improve our MAG vaccines, the introduction of a cluster of three Tn was undertaken to mimic native sources of Tn on tumor cells.

We present here the results of both prophylactic and therapeutic vaccinations using a MAG construct with a lysine core carrying four copies of the PV peptide further extended with a tri-Tn glycotope (MAG:Tn3-PV). In both cases, the MAG:Tn3-PV afforded good protection against the development of Tn-expressing tumor cells.

[3] Abbreviations used in this paper: KLH, keyhole limpet hemocyanin; MAG, multiple antigenic glycopeptide; ASI, active specific immunotherapy; CY, cyclophosphamide; MAP, multiple antigenic peptide; aOSM, asialo-ovine submaxillary mucin.

Materials and Methods

Synthesis

The Tn Ags (α-GalNAc-Ser/Thr) were synthesized by classical methods (22, 23). Synthesis of the MAG:Tn-PV, MAG:Tn3-PV, MAP:PV, MAP: PV2, and Tn6-PV was performed by solid phase methodology using Fmoc chemistry as described previously (13, 21). After attachment of the β-alanyl spacer on the Wang resin the lysine core was assembled by coupling successively two levels of FmocLys-(Fmoc)-OH, providing four amino groups. The lysine core was further elongated by the amino acids of the T epitope sequence of the poliovirus (KLFAVWKITYKDT) (24) to produce the MAP:PV. The glycosylated amino acid α-GalNAc-Thr-OH was then introduced on the four branches as its pentafluorophenyl ester in the presence of N-hydroxybenzotriazole (21). The incorporation was repeated, and ultimately α-GalNAc-Ser-OH (pfp ester) was coupled to the construction. After deprotection and cleavage from the resin the products were purified by HPLC using a Perkin-Elmer pump system (Foster City, CA) with a UV detector at 230 nm; the column was a Nucleosil C_{18} (5 μm, 300Å, 250 × 10 mm), and the gradient was performed with water (0.1% trifluoroacetic acid)/acetonitrile over 20 min (MAG:Tn3-PV: gradient from 5 to 60%; retention time, 12 min; amino acid analysis: Ala 3.75 (4), Asp 4.56 (4), Ile 4.13 (4), Leu 4.04 (4), Lys 16.6 (15), Phe 4.0 (4), Ser 3.82 (4), Thr 16.07 (16), Tyr 4.6 (4), Val 4.01 (4)).

Mice and immunization

Five- to 8-wk-old female BALB/c mice were obtained from Iffa Credo (St. Germain sur l'Abresle, France). To test immunogenicity of the MAG construct, mice were injected i.p. three times with Ag mixed with alum (Serva, Heidelberg, Germany) at 3-wk intervals. For vaccination experiments, mice received three i.p. injections of Ag mixed with alum or alum alone at 10-day intervals, and 10 days after the last boost, mice were challenged i.p. with 1,000 or 20,000 TA3/Ha cells prepared as described below. Alternatively, mice were challenged with the CT26 cell line (provided by Dr. R. A. Reisfeld, The Scripps Institute, La Jolla, CA) that does not express Tn. In some experiments, mice were treated with GK1.5 (anti-CD4) or H35.17.2 (anti-CD8) mAb (200 μg of mAb on days −1, 0, and 1 at the time of immunization). In all cases sera were collected after each immunization and tested for the presence of anti-Tn Abs by ELISA or FACS.

ELISA

To test the binding of MLS128 (a mouse IgG3 specific for the Tn Ag (25); a gift from Dr. H. Nakada, Kyoto Sangyo University, Kyoto, Japan) to the MAG constructs, an ELISA was performed as previously described (13) by coating Ags at 1 μg/ml in 50 mM carbonate buffer, pH 9.6. Mouse sera were tested for anti-Tn as previously described (21), using the synthetic glycopeptide Tn3-G6K(Biot)G or the nonglycosylated analogue STTG6K(Biot)G as a control. Briefly, the biotinylated peptides at 1 μg/ml were incubated for 1 h at 37°C on streptavidin-coated microtiter plates. Then, serial dilutions of sera were performed, and bound Abs were revealed using goat anti-mouse IgG or IgM peroxidase conjugate (Sigma) and o-phenyldiamine/H_2O_2 substrates as previously described (13). Plates were read photometrically at 492 nm in an ELISA autoreader (Dynatech, Marnes la Coquette, France). The negative control consisted of naive mouse sera diluted 100-fold. ELISA Ab titers were determined by linear regression analysis, plotting dilution vs absorbance at 492 nm. The titers were calculated to be the $\log_{10}$ highest dilution that gave twice the absorbance of normal mouse sera diluted 1/100. Titers were given as the arithmetic mean ± SD of the $\log_{10}$ titers.

Flow cytometry

Mouse sera were tested at serial dilutions by flow cytometry on two tumor cell lines expressing the Tn Ag, the human Jurkat cell (26), and the murine TA3/Ha cell (27). Cells were first incubated for 30 min with serial dilutions of sera at 4°C in PBS containing 5% FCS and 0.05% sodium azide. Then, cells were incubated 30 min with anti-mouse IgG conjugated to FITC and with an anti-mouse IgM conjugated to PE (Caltag, Burlingame, CA). Cells were analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA), and analysis was performed with CellQuest software (Becton Dickinson). The titers were calculated to be the $\log_{10}$ highest dilution of sera that gave twice the geometric mean of fluorescence obtained with cells incubated with secondary reagents alone. For competition assay, 5 × 10⁵ Jurkat cells were incubated with 1 μg/ml of the MLS128 mAb mixed with serial dilution of MAG, control MAP, or asialo-ovine submaxillary mucin (aOSM; given by Dr. E. Osinaga, Facultad de Medicina, Montevideo, Uruguay) for 30 min at 4°C, then the binding of MLS128 to Jurkat cells was revealed with an anti-IgG-FITC. Results are expressed as the percent inhibition of the signal obtained with MLS128 alone.

Anti-tumor immunotherapy

For tumor implantation, the preparation of the murine mammary adenocarcinoma cell line, TA3/Ha, differs from our previously published work in which freshly isolated TA3/Ha from in vivo passages was directly used (14). Here, the TA3/Ha cells were grown by passage on BALB/c mice and were frozen to obtain a homogeneous batch. Two or 3 days before tumor graft, cells were thawed and cultured in vitro in DMEM containing 5% FCS. Cells were then recovered and centrifuged at 500 rpm for 5 min. In these conditions, cells recovered from the pellet were homogeneous in size and included <50% dead cells. Cells were checked for Tn expression by FACS using the MLS128 mAb before implantation. One thousand TA3/Ha live cells were administered i.p. to 5-wk-old BALB/c mice, then mice were s.c. injected several times, alternatively at the tail base and in the neck, with 100 μg of MAG constructs with 1 mg of alum. Survival of treated and untreated mice was followed for >100 days. Statistical analysis of survival curves was performed with StatView software (Abacus Concepts, Berkeley, CA) using the log-rank test.

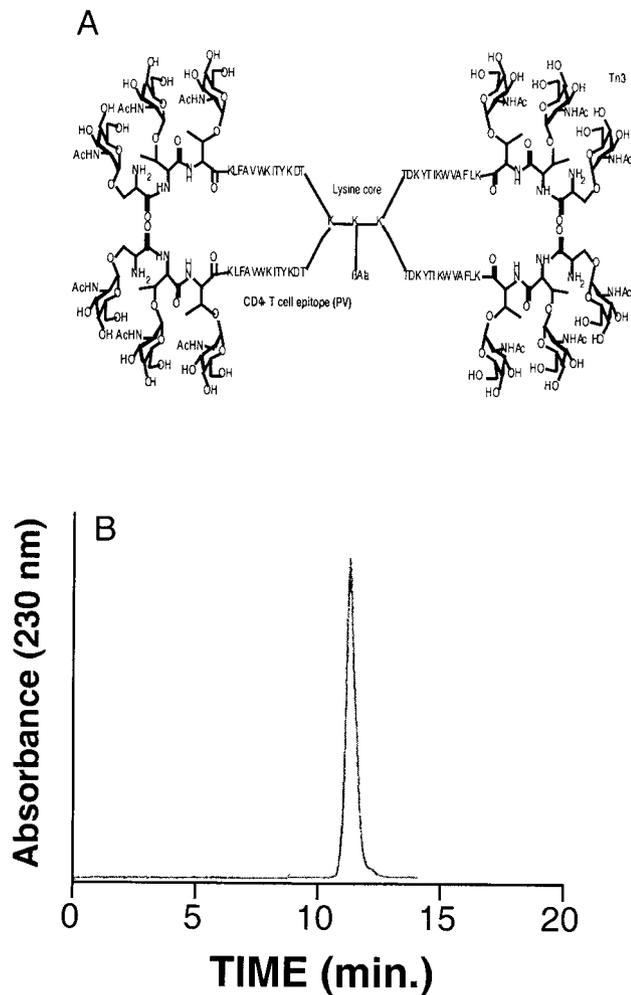## Results and Discussion

Antigenicity of MAG:Tn3-PV

We previously showed that a dendrimeric MAG containing a monosaccharidic Tn motif at the N terminus of its four peptidic arms (MAG:Tn-PV) was able to induce Tn-specific Abs in a T cell-dependent manner (14). As the Tn Ag is expressed as clusters in mucin proteins, it should be advantageous to mimic naturally occurring Tn structures displayed by cancer cells. Moreover, dimeric and trimeric carbohydrate epitopes associated with protein carrier have already been shown to be more effective in generating Ab response than the monomeric analogue (28, 29). Similarly, we showed, using short synthetic glycopeptides, that a Tn cluster was required to induce an efficient immune response (21). To improve our MAG, we have therefore introduced a Tn cluster corresponding to the glycotope recognized by the MLS128 mAb. The MLS128 is a Tn-specific mAb obtained after immunizing mice with the human carcinoma cell line LS180 (25), and this mAb was shown to bind to a tri-Tn cluster ((α-GalNac)-Ser-(α-GalNac)-Thr-(α-GalNac)-Thr) on mucin-like proteins (17, 18). This mAb can also recognize a dimeric Tn within the (α-GalNac)-Ser-(α-GalNac)-Thr-Thr sequence, but with a much lower affinity (30). Although we previously showed that a linear glycopeptide based on three consecutive (α-GalNac)-D-Ser was able to induce anti-Tn Abs (21), the lack of contribution of the aglyconic part of the structure (Ser or Thr residues) to Ab binding is not clearly established. Therefore, based on the initial (α-GalNac)-Ser-(α-GalNac)-Thr-(α-GalNac)-Thr sequence described for MLS128 binding, we synthesized a dendrimeric MAG with four arms containing the PV CD4⁺ T cell epitope (MAG:Tn3-PV; see Table I and Fig. 1). Two dendrimeric MAP controls were also synthesized comprising the

Table I. *List of compounds used in this study*

Compound[a]	Peptidic Backbone		Glycosidic Moiety No. of Tn Copies	
	PV peptide (no. of copy)	Additional amino acid[b]	Per branch	Per compound
STT-G6K(Biot)G	–	–	–	–
Tn3-G6K(Biot)G	–	–	3	3
MAP:PV	4	–	–	–
MAP:PV2	4	STT	–	–
MAG:Tn-PV	4	S*	1	4
MAG:Tn3-PV	4	S*T*T*	3	12
Tn6-PV	1	(S*T*T*G)₂	3	6

[a] MAP and MAG refer to dendrimeric compounds based on the (Lys)₂-Lys-βAla core. The PV peptide corresponds to the KLFAVWKITYKDT poliovirus sequence.
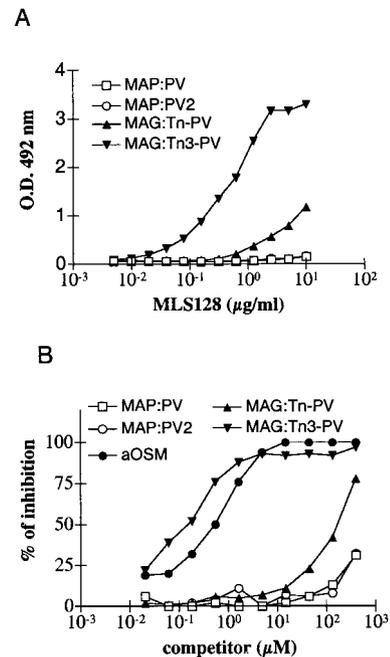[b] Asterisks refer to glycosylated amino acids.

FIGURE 1. *A*, Schematic representation of MAG:Tn3-PV. *B*, HPLC elution profile of MAG:Tn3-PV.

FIGURE 2. Compared antigenicity of mono-Tn- vs tri-Tn-based multiple antigenic glycopeptide. *A*, MLS128 mAb specific for Tn was tested by ELISA for reactivity to the MAG:Tn-PV, MAG:Tn3-PV, or control MAP:PV or MAP:PV2 coated at 1 μg/ml. *B*, Inhibition of MLS128 mAb binding to the Tn-positive Jurkat cell by MAG carrying the Tn Ag. Jurkat cells were incubated with MLS128 at 1 μg/ml together with various concentrations of MAG:Tn-PV, MAG:Tn3-PV, MAP:PV, MAP:PV2, or aOSM. MLS128 binding was detected by FACS using an anti-mouse IgG labeled with FITC. Results are expressed as the percent inhibition calculated from the Jurkat-associated fluorescence obtained with MLS128 alone.

PV sequence alone (MAP:PV) or the same construct with an additional STT peptide (MAP:PV2; Table I).

We first evaluated the recognition of Tn in the MAG:Tn3-PV by the MLS128 mAb. Using direct ELISA, the MLS128 Ab can very efficiently bind to the MAG:Tn3-PV compared with the former MAG:Tn-PV carrying a monomeric Tn (Fig. 2*A*). No MLS128 binding was observed using the control dendrimeric MAP compounds devoid of Tn (MAP:PV and MAP:PV2). To precisely compare the two MAGs, we tested by FACS the ability of the two constructs to inhibit the MLS128 binding to native Tn structures at the surface of the human Jurkat cell line. In these conditions, MAG:Tn3-PV strongly inhibited MLS128 binding to Jurkat cells, whereas MAG:Tn-PV did so only weakly (Fig. 2*B*). The MAG:Tn3-PV inhibition was as efficient as that obtained with aOSM.

*MAG:Tn3-PV induces Abs that recognize tumor cells*

We next evaluated the immunogenicity of the MAG:Tn3-PV compound by immunizing mice with MAG:Tn3-PV, MAG:Tn-PV or control MAP:PV2. Mice were immunized three times (days 0, 21, and 42) and were bled 1 wk after each immunization, and then regularly over a 6-mo period. Sera were tested for IgG and IgM Tn-specific Abs by ELISA using a biotinylated glycopeptide containing a tri-Tn cluster (Tn3-G6K(Biot)G). Fig. 3 shows that Tn-specific Abs (both IgG and IgM) were induced by MAG:Tn3-PV and MAG:Tn-PV, but not by the control MAP:PV2. However, the

Tn-specific response induced by the MAG:Tn-PV slowly increased after immunization, whereas that induced by the MAG:Tn3-PV rapidly increased after two injections and then remained stable for 5 mo. The isotypic analysis of MAG:Tn3-PV-induced Abs shows that IgG1 Abs were predominant, but sera also contained a large amount of IgG3 Abs, which traditionally characterize anti-carbohydrate Ab responses in mice (data not shown). The increase in Tn density in the MAG did not modify the Th dependency of the anti-Tn Ab response, because this response was abolished when mice were depleted of CD4 T cells in vivo, but remained unchanged when CD8 T cells were depleted (data not shown). These results show that the introduction of a tri-Tn glycotope, instead of a monomeric Tn, into the MAG strongly increases the immunogenicity of the Tn Ag and makes the response more persistent.

Mucins and mucin-like proteins are characterized by a high degree of *O*-glycosylation through Ser or Thr residues; however, apomucin sequences and glycosylation frequency of acceptor sites remain heterogeneous. Recently, Reis et al. (20) showed that Tn-specific Ab responses induced in mice and rabbits by a glycosylated MUC2 peptide did not recognize Tn on asialo ovine submaxillary mucin, which is commonly used to test Tn reactivity. Therefore, the choice of a given Tn glycotope recognized by a single mAb, here three adjacent Tn on a STT backbone, could lead to the induction of a skewed anti-Tn immune response. To ensure that Tn-specific Abs induced by the MAG:Tn3-PV can recognize native Tn structures, we analyzed by flow cytometry the binding of sera from immune mice to tumor cells. As shown in Fig. 4, titration of sera from MAG:Tn3-PV-immunized mice on murine adenocarcinoma TA3/Ha cells demonstrates positive binding of both IgM
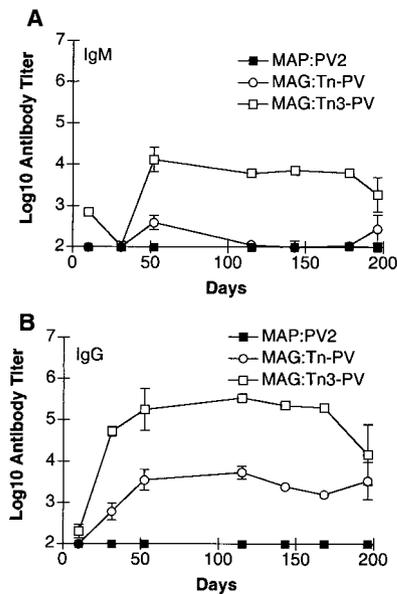
FIGURE 3. MAG:Tn3-PV induces a long-lasting Tn-specific Ab response. BALB/c mice (five per group) were immunized on days 0, 21, and 42 with 20 μg of MAP:PV2, MAG:Tn-PV, or MAG:Tn3-PV mixed with alum. Sera were collected 1 wk after each immunization or at various times until day 200 and were tested for Tn-specific IgM (*A*) and IgG (*B*) Abs by ELISA using Tn3-G6K(Biot)G.

and IgG Abs, as opposed to the lack of binding of sera from mice immunized with MAP:PV2. Likewise, the human Jurkat cell was well recognized by Abs induced after immunization with MAG:Tn3-PV, but not with MAP:PV2. These results show that native Tn structures on tumor cells are recognized by Abs induced by a MAG based on a tri-Tn glycotope, indicating that these Abs can efficiently target Tn-expressing tumor cells.

Preventive vaccination with MAG affords protection against tumor challenge

We next verified the capacity of MAG:Tn3-PV-induced Abs to target and reject the highly tumorigenic TA3/Ha adenocarcinoma in vivo. Mice were left untreated or were vaccinated three times with 10 μg of
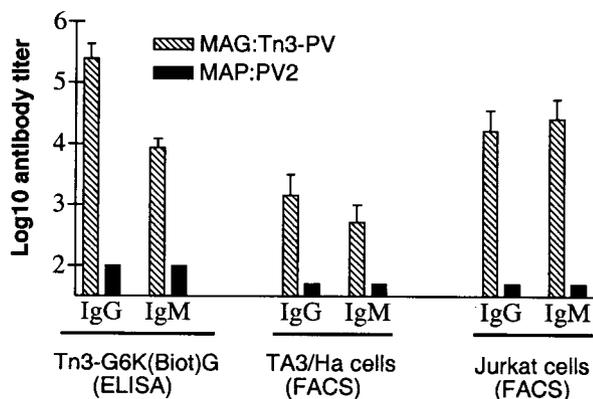
FIGURE 4. MAG:Tn3-PV-induced Abs bind to tumor cells expressing Tn. Serial dilutions of sera from mice (10/group) immunized on days 0, 21, and 42 with MAG:Tn3-PV or control MAP:PV2, as described in Fig. 2, were tested comparatively for binding to the Tn3-G6K(Biot)G by ELISA or to Tn-expressing tumor cell lines, the murine TA3/Ha cell or the human Jurkat cell, by FACS. Ig binding to tumor cells was revealed by double labeling with IgG-FITC and IgM-PE. Results are expressed as the mean of $\log_{10}$ individual Ab titers $\pm$ SD.

MAP:PV or MAG:Tn3-PV in alum or with alum alone. Then, 10 days after the last boost, mice were challenged with 1,000 TA3/Ha cells, and survival of mice to the TA3/Ha graft was followed for 100 days. As shown in Fig. 5*A*, all untreated mice died within 30 days following tumor challenge. In contrast, 80% of mice vaccinated three times with 10 μg of MAG:Tn3-PV mixed with alum survived to the tumor challenge, whereas 10% of mice survived in control groups receiving MAP:PV mixed with alum or alum alone (Fig. 5*A*). The protection afforded by MAG:Tn3-PV against the TA3/Ha challenge ranged from 70–90% depending on the dose of MAG used for vaccination (Fig.
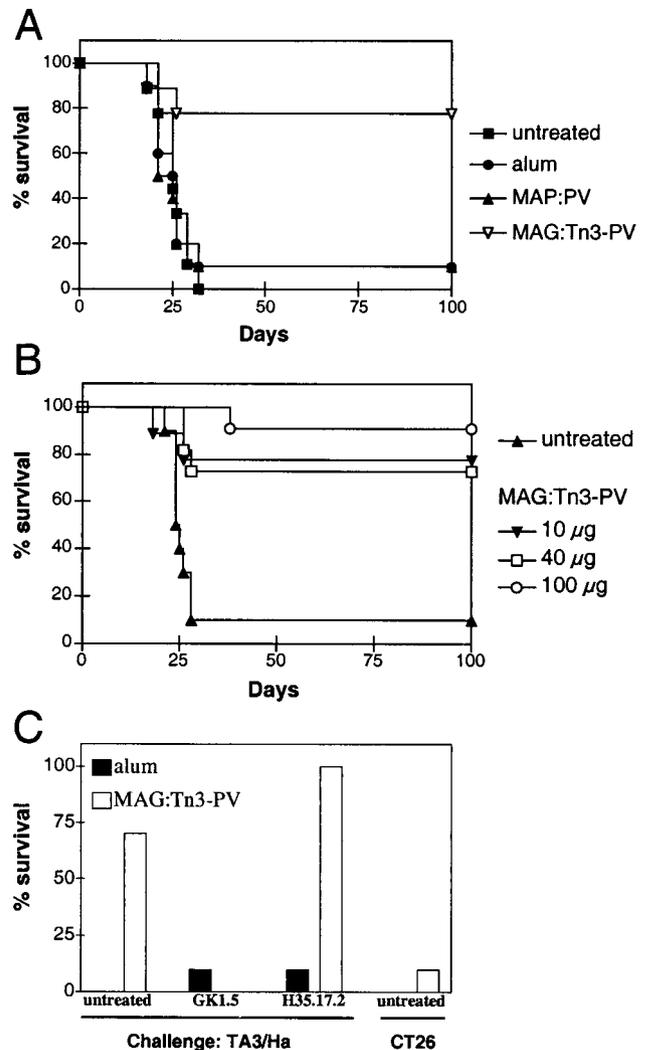
FIGURE 5. MAG:Tn3-PV vaccination protects mice against tumor challenge. *A*, Mice (10/group) were immunized on days −30, −20, and −10 with 10 μg of MAP:PV or MAG:Tn3-PV mixed with alum or with alum alone, or were left untreated. On day 0, mice received 1000 TA3/Ha tumor cells, and survival was followed for 100 days. Statistical analysis of the MAG:Tn3-PV-vaccinated group gave $p < 0.005$ vs untreated group, $p < 0.01$ vs alum-treated group, and $p < 0.01$ vs MAP:PV-treated group. *B*, Following the same schedule as that in *A*, mice (10/group) received three injections of 10, 40, or 100 μg of MAG:Tn3-PV with alum or were left untreated before being challenged with 1,000 TA3/Ha tumor cells. Mice were monitored for survival for 100 days after the tumor challenge. All MAG:Tn3-PV-vaccinated groups are statistically different from the control group ($p < 0.01$). *C*, Untreated, GK1.5 (anti-CD4)-treated, or H35.17.2 (anti-CD8)-treated mice were vaccinated with alum or with 10 μg of MAG:Tn3-PV mixed with alum and then challenged with 20,000 TA3/Ha cells or CT26 cells. Ten mice were used for each group, and survival was followed for 100 days.

5B). In contrast, MAG:Tn3-PV vaccination did not protect mice against the Tn-negative carcinoma CT26 cell line, showing the Tn specificity of the protection afforded by MAG. Prior to the tumor challenge, Tn-specific IgG were only detected in MAG:Tn3-PV-vaccinated mice (Ab titers ranging from 5,000 to 100,000 as detected by ELISA; data not shown). When mice were depleted of CD4 T cells in vivo, no anti-Tn Ab was induced, and the protection against TA3/Ha tumor challenge was totally abrogated, whereas CD8 T cell-depleted mice were still protected (Fig. 5C). Together, these results clearly show that the anti-Tn immune response induced by the MAG:Tn3-PV is able to confer a high protection rate against a tumor cell line expressing Tn, and that this protection depends on the induction of anti-Tn Abs requiring T cell help.

MAG-based active specific immunotherapy (ASI)

Preventive vaccination has limited interest when targeting the immune response to cancer-associated Ags; therefore, cancer vaccination should prove efficacious toward pre-existing tumor cells and be used as a therapeutic tool. Using the TA3/Ha tumor model, Fung et al. (31) showed that ASI treatments based on a β-Gal(1–3)α-GalNAc glycotope conjugated to the KLH (TF-KLH) can afford a substantial survival rate (25%) of TA3/Ha-bearing mice. Given the success obtained with the MAG:Tn3-PV vaccination on the survival of mice challenged with TA3/Ha cells, we next tested its efficacy in immunotherapeutic protocols. Mice were given 1000 TA3/Ha cells and were then treated with 100 μg of MAG:Tn3-PV in adjuvant (aluminum hydroxide or Freund's adjuvant) or with adjuvant alone. In these conditions, about 35–40% survival was observed in MAG:Tn3-PV-treated groups, whereas all mice died in groups treated with adjuvant or left untreated (Fig. 6A).

In various experimental models, anti-tumor immunity has been shown to be increased when immunotherapeutic treatments are combined with cyclophosphamide (CY) (32). The potentiation of anti-tumor immune response following CY treatment has been recently reported for both CD8 and CD4 T cell subsets, suggesting that CY treatments may indirectly contribute to the production of T cell growth factors (33, 34). Although the molecular basis of CY activity remains to be clarified, low doses of CY definitely increase delayed-type hypersensitivity and Ab responses. In the TA3/Ha tumor model, when a single dose of CY was given next to the TA3/Ha tumor graft, the efficiency of ASI based on TF-KLH or epiglycanin was highly increased, leading to a 50–90% survival rate of tumor-bearing mice (31). Likewise, a significant protection against TA3/Ha was also reported with a treatment combining CY and desialylated ovine or bovine submaxillary mucin (35). We therefore associated CY administration together with the MAG:Tn3-PV treatment to determine whether this could improve the efficacy of the ASI. Following TA3/Ha tumor implantation, mice received on day 1 a single dose of 10 or 50 mg/kg of CY. As shown in Fig. 6B, CY followed by immunization with alum alone did not modify the mortality of TA3/Ha tumor-bearing mice. When mice were given a 10 mg/kg dose of CY, followed by the MAG:Tn3-PV treatment given with alum on days 2, 5, 11, and 17, only 37.5% of mice survived. However, when MAG:Tn3-PV treatment was given after the administration of a 50 mg/kg dose of CY, the survival of TA3/Ha-bearing mice reached 80% (Fig. 6B). These results indicate that CY can improve the efficacy of the MAG:Tn3-PV treatment.

We further compared the efficacy of the MAG:Tn3-PV treatment with other immunogenic compounds. In a previous work, we showed
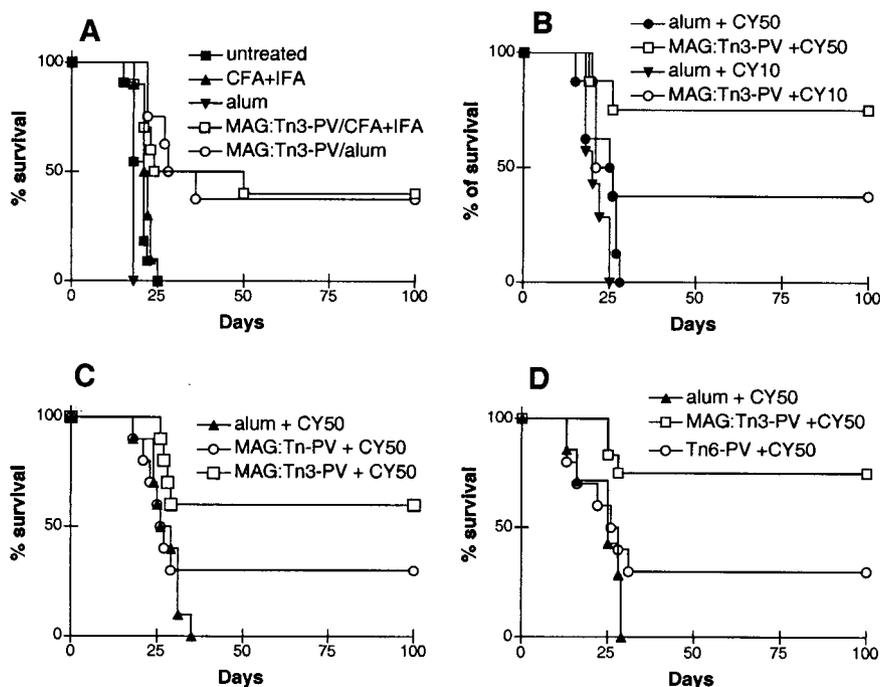
FIGURE 6. MAG-based immunotherapy successfully improved the survival of mice carrying a tumor expressing Tn. Mice received 1,000 TA3/Ha cells on day 0 and were then treated as described below or were left untreated, and survival was followed for 100 days. A, Mice (8–10/group) were treated with 100 μg of MAG:Tn3-PV in CFA/IFA or in alum or with adjuvant alone (CFA/IFA or alum) on days 2, 5, 11, and 17 ($p < 0.02$ between MAG:Tn3-PV/CFA and CFA groups and $p < 0.0001$ between MAG:Tn3-PV/alum and alum groups). B, Mice (8/group) received 10 or 50 mg/kg of CY on day 1 and then were treated with 100 μg of MAG:Tn3-PV in alum or with alum alone on days 2, 5, 11, and 17 ($p < 0.05$ between MAG:Tn3-PV/CY10 and alum/CY10 groups and $p < 0.005$ between MAG:Tn3-PV/CY50 and alum/CY50 groups). C, Mice (10/group) received 50 mg/kg of CY on day 1 and then were treated on days 2, 4, 6, 8, and 12 with alum alone or together with 100 μg of MAG:Tn3-PV or with 100 μg of MAG:Tn-PV ($p = 0.057$ between MAG:Tn-PV/CY50 and MAG:Tn3-PV/CY50 groups). D, Mice (7–12/group) received 50 mg/kg of CY on day 1, and then were treated on days 2, 5, 11, and 17 with alum alone or together with 100 μg of MAG:Tn3-PV or 100 μg of Tn6-PV ($p < 0.01$ between Tn6-PV/CY50 and MAG:Tn3-PV/CY50 groups).

that a MAG compound based on a monomeric Tn (MAG:Tn-PV) was able to improve the survival of TA3/Ha-bearing mice (14). Fig. 6*C* shows that ASI treatment performed with the MAG:Tn-PV together with CY leads to a 30% protection rate of tumor-bearing mice compared with the 60% protection afforded by the MAG:Tn3-PV-based ASI. This result is in agreement with the compared immunogenicity of both compounds (Fig. 3). More recently, we showed that a linear glycopeptide containing a T cell epitope associated with a double tri-Tn glycotope (Tn6-PV) was able to induce anti-Tn Abs that recognize Tn on tumor cell lines (21). The advantage of such a linear glycopeptide over the MAG strategy is that synthesis, yield, and purification are easier to achieve. When Tn6-PV was used in ASI, only 30% of mice survived to the TA3/Ha implantation compared with the 75% survival observed in the MAG:Tn3-PV-treated group (Fig. 6*D*). In summary, we show that the efficacy of ASI afforded by MAG is improved when the Tn Ag is incorporated as a tri-Tn cluster rather than as a mono-Tn moiety, and that the tri-Tn glycotope is much more efficient when displayed on the MAG structure compared with a linear glycopeptide.

It is difficult to ensure that the improved immunogenicity and therapeutic effects of the MAG:Tn3-PV compared with the MAG:Tn-PV result from the use of the tri-Tn glycotope and do not result from a clustering effect due to the higher amount of Tn incorporated into the immunogen. However, the results obtained with the linear glycopeptide (Tn6-PV) indicate that the beneficial effects of Tn clustering may be limited if the number of Tn Ag per branch is considered. Moreover, the injection of the same microgram doses of Tn6-PV and MAG:Tn3-PV corresponds to a 1.5-fold greater amount of Tn or tri-Tn in the case of the Tn6-PV, whereas it shows lower efficacy. Therefore, our results suggest that other parameters should be taken into account, for instance the flexibility of the saccharide moiety. Indeed, there is probably much more rigidity in the double tri-Tn cluster structure of the Tn6-PV, whereas the MAG can display a large variety of Tn glycotopes through its four flexible peptidic arms that can better mimic the diversity of natural Tn clusters. These issues are currently under investigation.

Conclusion

Together, our results show that a synthetic multiple antigenic glycopeptide including a saccharide tumor-associated Ag together with an appropriate CD4$^+$ T cell epitope is highly immunogenic and can efficiently allow rejection of implanted tumor cells when used in therapeutic treatment. Our results also provide evidence that the introduction of well-defined glycotope clusters in carbohydrate-based immunogens is important for the induction of an efficient and long-lasting antitumor response.

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