

# Peptide Mimotopes as Prototypic Templates of Broad-Spectrum Surrogates of Carbohydrate Antigens for Cancer Vaccination

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**ABSTRACT:** Mechanisms of broad cross-protection, as seen in viral infection and also applied to vaccines, emphasize preexisting antibodies, CD8+ memory T cells, and accelerated B-cell responses reactive with conserved regions in antigens. Another practical application to induce broad-spectrum responses is making use of multispecific antigen recognition by antibodies and T cells. Antibody polyreactivity can be related to the capacity of the antigen-combining site to accommodate a number of different small epitopes or to attain different conformations. A better understanding of the functionality of molecular interactions with graded specificity might help the design of polyreactive immunogens inducing antibody responses to a predefined set of target antigens. We have found this approach useful in targeting tumor-associated carbohydrate antigens in cancer vaccine development. Using combinatorial libraries and pharmacophore design principles, carbohydrate mimetic peptides were created that not only induce antibodies with multiple specificities, but also cellular responses that inhibit tumor growth in vivo.

**KEY WORDS:** peptide mimics, polyspecificity, antigen recognition

## I. INTRODUCTION

The molecular design of vaccines, both for infectious diseases and for cancer, relies on our understanding and combining of relevant B- and T-cell epitopes.<sup>1</sup> An ensuing immune response should target specifically and efficiently the antigen through cross-reactivity with the designed immunogen. The design of vaccines needs to be considered against the backdrop of immunoediting in which the immune response sculpts the tumor into immunologically selected cell populations.<sup>2</sup> Unique antigens are believed to arise from numerous incidental mutations through the development of the tumor, suggesting that immune responses are probably vulnerable to tumor escape through loss of antigen expression. The immunoediting perspective points to a major challenge in cancer vaccinology, namely, how to anticipate and

therefore include individual antigens to make the immune system destroy a tumor cell.<sup>3</sup> Consequently, individualized vaccine therapy, targeting a multiple tumor antigens unique for each patient, may emerge as a major novel principle in cancer immunotherapy.

Several classes of antigens have been suggested as potential broad-spectrum targets and they may actually reflect significant host-tumor interactions as defined by analysis of the reactivity of high-titer circulating tumor-associated antibodies.<sup>4</sup> Several examples are differentiation antigens,<sup>5,6</sup> self-molecules as tumor-associated antigens (TAAs) by virtue of their overexpression,<sup>7</sup> mutated gene products, tumor-specific splice variants, etc. Many of the genes coding for these antigens exist as multimember gene families. The prototypes of this category include the testis antigens<sup>5</sup> and high molecular weight mucins,<sup>8</sup> empha-

sizing the conserved nature of these cytotoxic T-cell targets.<sup>9,10</sup>

The notion of targeting conserved regions parallels an approach in pathogen vaccine development.<sup>11</sup> But sometimes high specificity to an antigenic epitope is not sufficient for a practical application because of the antigenic variability of some pathogens and the antigenic diversity of tumors. Historically, antigenic drift of viral proteins is seen as an obstacle for cross-reactive immune responses against different strains of viruses.<sup>12</sup> In such cases strategies are sought to target a class of similar antigens.

Polyvalent vaccines have been used traditionally for protection against bacterial infections. In terms of rational design of cancer vaccines, a possible approach could be the development of immunogens with polyspecificity encoded in their molecular structure. In its essence, this concept is borrowed from and takes advantage of polyspecific antigen recognition. Polyspecific invariant pattern recognition receptors (PRRs) are characteristic of innate immunity.<sup>13</sup> Many PRRs, such as macrophage scavenger receptor, TLR2,<sup>14</sup> TLR4,<sup>15</sup> DC-SIGN,<sup>16</sup> CD9,<sup>17</sup> NKG2D,<sup>18</sup> etc., recognize multiple, structurally diverse ligands as signals of dangerous changes of the internal environment. Adaptive immune responses are often opposed to innate immunity with respect to the specificity of the antigen receptors. Polyspecificity, however, characterizes major compartments of the antigen receptor repertoires. An individual T-cell receptor (TCR) is capable of recognizing up to 10<sup>6</sup> different peptides.<sup>19</sup> The reason seems to be the imperfect fit of the peptide ligands,<sup>20</sup> stressing the importance of biologically relevant rather than maximal levels of affinity and specificity. A substantial part of the circulating antibodies are also polyspecific.<sup>21</sup> They are characterized, as a rule, by variable regions with few or no somatic mutations indicating that they are either natural antibodies or the products of naïve B cells.

Recent studies underlie the role of paratope flexibility in antibody polyspecificity.<sup>22–26</sup> It seems that in the process of somatic mutation, polyreactive preimmune antibodies evolve a more rigid, specific binding to one selecting antigen. But their specificity is determined by the availability of the initial layer of naïve B-cell clones and the process of maturation, involving T-cell

help and the highly organized microenvironment of the germinal centers. Ultimately, antigen recognition is a function of the system and cannot be reduced to specificity of the antigen receptors.<sup>27,28</sup> The role of polyspecific antigen binding in the immune response is input and classification of biological and structural information ensuring, in a highly efficient way, completeness of the specific antigen receptor repertoire. Optimal use of the knowledge of this initial antigen detection step could greatly facilitate the design of immunogens that can elicit antibodies specific for sets of antigens, thus ensuring the broad specificity necessary, for instance, for efficient HIV vaccines or in tumor immunotherapy.

Tumor-associated carbohydrate antigens (TACAs) are associated with many biological processes (Table 1) and are expressed on multiple proteins and as glycolipids on multiple tumors.<sup>29–33</sup> TACAs are broadly expressed as a product of aberrant glycosylation in a large number of tumors and may represent a unique tool for vaccination.<sup>34</sup> Aberrant glycosylation influences the prognosis and survival of cancer patients, proportionally to the degree of expression.<sup>35</sup> TACAs are present on tumors more frequently than oncogene products (e.g., myc, ras, HER2/neu) and their association with tumor progression is stronger than the deletion or inactivation of tumor-suppressing genes (e.g., p53, p16). The structural degeneracy of carbohydrate (CH) determinants and their broad expression makes them an obvious choice as broad-spectrum targets.

Sustained immunity against TACAs has a beneficial effect on the course of malignant disease and long-term patient survival.<sup>36</sup> Therefore, optimizing sustained immunity against these targets is important. Carbohydrate antigens are, however, a major challenge in vaccine design. To augment responses to TACAs, we have developed carbohydrate mimetic peptides (CMPs). We have made the following striking discoveries in developing CMPs: (1) Immunization with CMPs is effective in eradicating cancer cells and impacting on tumor metastases<sup>37</sup>; (2) CMPs can function as multivalent immunogens, inducing responses to several structurally related TACAs at once and therefore minimizing tumor escape<sup>38</sup>; and (3) CMPs can induce tumor-specific cellular responses,<sup>39,40</sup> suggesting that they can induce

**TABLE 1**  
**Glycomic Profiles Affect Tumor Cell Phenotypes**

Phenotypes	Example of glycosylation change	Initial effect	Upregulates	Downregulates	Result
Apoptosis	Enhance expression of ganglioside-specific sialidase "Neu3" in colorectal cancer	Decrease of GM3 Increase of Lac-cer	Bcl-2	Caspase expression	Promotes tumor invasiveness
Motility	Formation of $\alpha 3$ integrin/CD9/GM3 complex in glycolipid-enriched microdomain	Inhibition of Matrigel- or laminin-S-dependent cell motility		Motility	Inhibits tumor invasiveness
	Gb3 and Gb5 leads to the initiation of monosialo-Gb5 complex with CD9 in breast cancer MCF7 cells	Enhances motility and invasiveness	Motility		Promotes tumor invasiveness
Epidermal growth factor receptor	Inhibition of epidermal growth factor receptor (EGFR) tyrosine kinase by GM3	Inhibition of EGFR		EGFR	Inhibits tumor invasiveness
Angiogenesis	Production of vascular endothelial growth factor (VEGF)	GD3 production	VEGF		Promotes tumor invasiveness
	Secretion of Gn T-V	Promotes metastasis			Promotes angiogenesis
$\beta 6$ GlcNac branching	Upregulation of GnT-V gene leads to enhance expression for branching	Increase branching	Matriptase (matrix-destroying enzyme)		Promotes tumor invasiveness
	Upregulation of GnT-V gene reduces branching of $\beta 6$ GlcNac	Decrease branching		E-cadherin	Metastasis suppression
E-selectins	Expression of SLe <sub>x</sub> and SLe <sub>a</sub> tumor-associated antigens	Promotes adhesion of tumor cells to epithelial cells			Promotes tumor invasiveness
	Lcs leads to Lc4, nLc4 which produce SLe <sub>a</sub> and SLe <sub>x</sub>				
	Lcs to nLc4 also leads to production of myeloglycans				
Cadherin	Enhanced $\beta 6$ GlcNAc reduces $\beta 6$ GlcNac branching	Enhanced cadherin-dependent cell-cell adhesion		Cadherin	Inhibits tumor invasiveness
P-, L-selectins	Transfection of P-selectin glycoprotein ligand-1 (PSGL-1); presence of sialyl-6-sulfo- Lex (L-selectin ligand)	P- and L-selectin-dependent adhesion			Promotes tumor invasiveness
Siglec	Multiple disialogangliosides and sialylated O- or N-linked glycans targeted by siglecs  Ex. Gb3 and Gb5 lead to DSGb5 Ex. Tn leads to STn	Unclear			Variable effects

Source: Adapted from Ref. 31.

degenerate T cells reactive with tumor-associated glycopeptides presented in MHC class I. CMPs can elicit polyspecific CH-reactive antibodies, thereby being defined as templates for inducing broad-spectrum responses. In this context, CMPs can be defined as multiple antigen mimotopes (MAMs). Here, we summarize our experience with developing these novel immunogens and the theoretical basis for their translation into the clinic.

## II. CARBOHYDRATE ANTIGENS AS TARGETS FOR SPECIFIC IMMUNIZATION

Many CH antigens are found to be clinically relevant for immunoprotection in infectious disease. Carbohydrate-based vaccines against *Haemophilus influenzae* type b, *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Salmonella enterica* serotype Typhi (S. Typhi) are already licensed,<sup>41</sup> and many similar products are in various stages of development. The success or failure of these trials would also be relevant to the development of cancer vaccines.<sup>42,43</sup> From a different perspective, cancer immunotherapy is akin to the induction of autoimmunity-caused tissue damage.<sup>44,45</sup> Carbohydrate antigen targeting tissue damage is best typified by the natural antibody response directed against the  $\alpha$ -Gal antigen, a major barrier in porcine-to-human xenotransplantation.<sup>46</sup> This tissue rejection supports a mechanistic rationale for targeting TACA because tumor-induced antibody responses resemble the autoimmune response.<sup>47</sup>

The potential impact of vaccines that induce responses to TACA is demonstrated in clinical trials where patient survival significantly correlates with carbohydrate-reactive IgM levels.<sup>36</sup> Some TACAs are glycosphingolipids (GSLs).<sup>32</sup> Antibodies that recognize GSLs such as GD2, GM2, and LeY are demonstrated to mediate complement-dependent cytotoxicity (CDC) and have been suggested to be more cytotoxic to tumor cells than antibodies that recognize protein antigens or TACA linked to protein antigens<sup>48</sup> that kill tumor cells by antibody-dependent cell-mediated cytotoxicity (ADCC). Antibodies to GD2 (Refs. 49, 50) and GM2 (Refs. 51, 52) are also able to mediate apoptosis of tumor cells. GSL-induced responses could augment naturally

occurring carbohydrate-reactive IgM antibodies that trigger apoptosis of tumor cells.<sup>53</sup> As clinical correlates have highlighted IgM responses to cancer cells in humans,<sup>36,53</sup> attention to antibody isotypes is warranted to further understand and develop strategies to augment these responses.

Because TACAs are T-cell-independent antigens and self-antigens, their conjugation to immunologic carrier protein is perceived essential to recruit T-cell help for antibody generation much like they do in bacterial systems. Representative examples of TACA-based conjugate vaccines in clinical development for cancer include those directed toward gangliosides,<sup>54–56</sup> polysialic acid,<sup>57</sup> Globo-H,<sup>58</sup> LeY,<sup>59</sup> and the STn antigen.<sup>60</sup> Pre-clinical assessment of gangliosides and LeY vaccines are purported to induce antibodies in mice of varying titers.

Covalent coupling to an immunogenic protein carrier alters the response to CH in several important ways.<sup>61–63</sup> Conjugation of TACA does not, however, ensure an increase in immunogenicity because conjugation strategies do not uniformly enhance CH immunogenicity.<sup>64,65</sup> Furthermore, even with conjugation, the lack of induction of cellular immune responses that would amplify TACA-reactive humoral responses necessitates constant boosting with vaccine. The relative lack of memory for IgG CH responses is believed to be secondary to the inability of CH to associate with MHC class II molecules and thus a failure to recruit cognate CD4+ T-cell help.<sup>66</sup> Furthermore, in cases where a large number of different CH antigens are required to afford protection, much like that representative of the large number of pneumococcal CH serotypes, carbohydrate-conjugate vaccines will be far more complicated to produce.<sup>63</sup>

## III. MOLECULAR MIMICRY OF TACA AND BROAD-SPECTRUM RESPONSES

Molecular mimicry has become a common approach in vaccine strategies to induce cross-reactive immune responses to pathogens.<sup>67</sup> Peptides selected with antibodies from random peptide libraries have raised considerable expectations as low molecular weight substitutes of natural antigens, at least with respect to a particular epitope.<sup>67–69</sup> CMPs, which are structurally simpler than carbo-

hydrates and in consequence easier to synthesize and manipulate, are particularly compelling to augment carbohydrate-directed immune responses.<sup>70–74</sup> CMPs function as xenoantigens and, consequently, can overcome tolerance to CH self-antigens. More importantly, CMP conjugates can induce carbohydrate-reactive serum antibodies in strains of mice with otherwise impaired antibody production to CH antigens.<sup>72,74</sup>

We have defined many CMPs that antigenically mimic the Lewis Y (LeY) antigen<sup>75</sup> and a lipid-associated, structurally related sialylated Lex containing difucoganglioside (6B ganglioside), expressed on murine Meth A fibrosarcoma cells<sup>37</sup> and on human tumor cells.<sup>76</sup> In particular, peptides 106 (GGIYWRYDIYWRYDIYWRYD) and peptide 107 (GGIYYRYDIYYRYDIYYRYD) cross-react with the LeY reactive antibody BR55-2 and the anti-6B antibody FH6, whereas the peptide 105 (GGIYYPYDIYYPYDIYYPYD) displays diminished reactivity with the BR55-2 and FH-6 monoclonals.<sup>37</sup> The substitution of Trp for Tyr appears not to significantly alter the reactivity of BR55-2 and FH-6, but substitution of Pro for Arg reduces the reactivity of peptide 105 to these antibodies.<sup>77</sup> Nevertheless, all three peptides induce antibodies reactive with fibrosarcoma cells and human cancer cells and are effective *in vivo* in a prophylactic tumor model,<sup>37</sup> suggesting that the three peptides reflect the ability to induce antibodies that recognize CH substituents, for example, peptide 106 induced serum IgM reactive with constituents of neolactoseries antigens (Fig. 1).

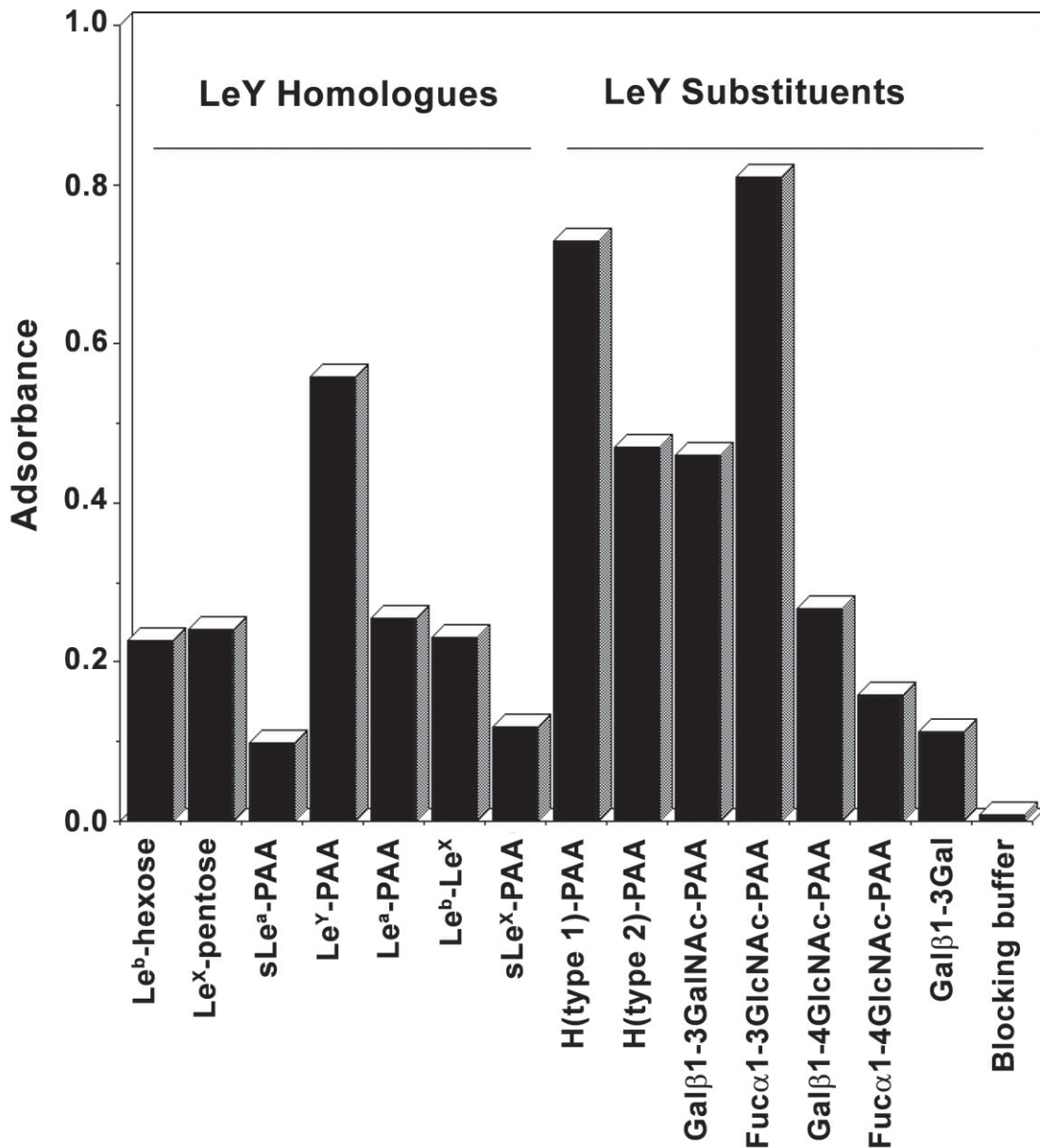
CMPs with a high prevalence of tryptophan and tyrosine occur often.<sup>78</sup> In particular, the YPY motif has been associated with mannose as identified from peptide phage screening with Concanavalin A,<sup>79,80</sup> WRY has been found to mimic 1–4 glucose as identified from analysis of protein binding to  $\alpha$ -amylase,<sup>81</sup> WLY has been found to mimic LeY as identified from peptide phage screening with the anti-LeY monoclonal antibody B3,<sup>82</sup> and the YRY motif derived from an anti-idiotypic antibody has been found to mimic the major C polysaccharide  $\alpha(2-9)$  sialic acid (MCP) of *Neisseria meningitidis*.<sup>71</sup> The sequence similarities that define these peptides suggest that antibodies to homologous peptides might cross-react with similar subunits expressed on what are otherwise dissimilar CH structures. Molecular modeling sug-

gests that the LeY tetrasaccharide structure is similar to the core structure of MCP (Figs. 2A and 2B), highlighting similarities in the Fuc $\alpha$ 1-3GlcNAc structure with MCP. Competition assays further validate reactivity with Fuc $\alpha$ 1-3GlcNAc (Fig. 2E). Furthermore, simple epitope comparison with LeY and  $\alpha(1-4)$ glucose defines a possible common epitope between these sugar moieties (Figs. 2C and 2D).

We have also suggested the possibility to build the mimicry of diverse CH antigens using a small set of amino acids. Most probably, these residues interact with CH-binding sites on antibodies and lectins (Fig. 3) through a degenerate alphabet of simple bonding patterns, mostly of hydrogen bonds and pi stacking, that are common with CH antigens. Many of the bonds established between a mimotope and a template antibody are outside the target CH footprint and increasing the affinity of the interaction with the template may lead to a peptide highly specific for the template but not a better CMP. Therefore, refining the specificity of the mimotopes with respect to the target structures (but not for the templates) may be impossible<sup>83</sup> and it may be advisable rather to use a controlled level of degeneracy of recognition to provide structures that elicit a repertoire of antibodies of predetermined polyspecificity (multiple antigen mimotopes, MAM).<sup>84,85</sup> It is possible that such CH mimics elicit a broader range of anticarbohydrate antibodies than predicted from the initial characterization of these motifs.

#### IV. MOLECULAR MIMICRY AS A TOOL FOR TUNING THE IMMUNE RESPONSE TO TACA

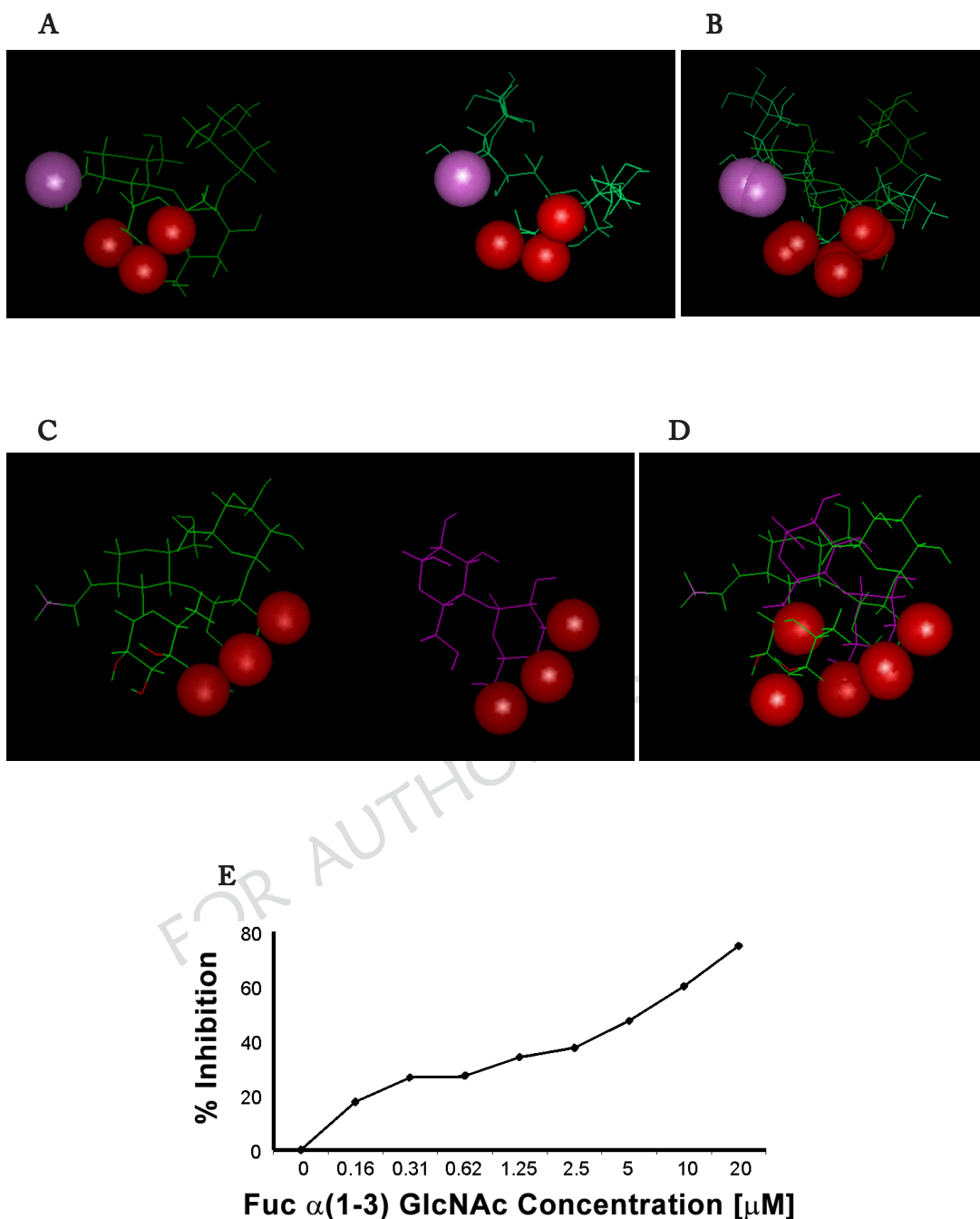
As MAP mimotopes, CMPs demonstrate an ability to induce primarily cross-reactive IgM CH responses on immunization.<sup>37,77,86–90</sup> DNA encoded CMPs also induce carbohydrate-reactive IgM (Ref. 91) and IgG2a isotypes.<sup>73</sup> In contrast to immunization with unconjugated MAP, MAP or monovalent peptide conjugated to immunologic carrier protein induces serum with a high IgG/IgM ratio.<sup>90</sup> Unlike CH antigens and carbohydrate-conjugate vaccines, we have shown that CMPs prime B and T cells for subsequent memory of CH antigens, facilitating long-term



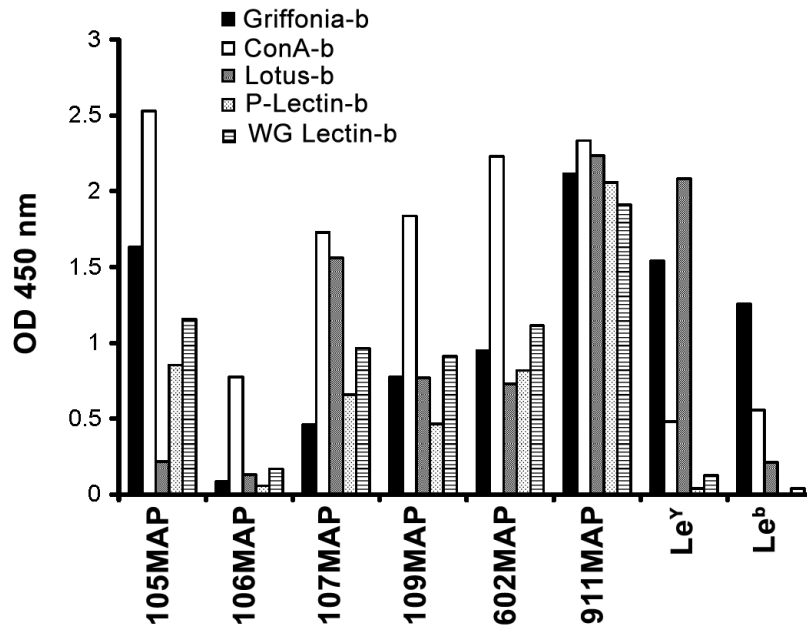
**FIGURE 1.** CMPs induce cross-reactive antibodies. Pattern of the reactivity with neolactoseries constituents of IgM in serum from mice immunized with peptide 106 MAP [(GGIYWRYDIYWRYDIYWRYD)<sub>8</sub>K<sub>7</sub>A] in the presence of QS21 as adjuvant.

surveillance through recall of CH immune responses.<sup>92</sup> This effect is a major advantage that would minimize the need for constant boosting. In contrast to carbohydrate conjugates, CMP conjugates can facilitate cognate interactions between B and T cells after immunization of immunoincompetent *Xid* mice that have a point mutation in Bruton's tyrosine kinase (*btk*).<sup>72</sup> Mice bearing the *Btkxid* mutation lack peritoneal B1 lymphocytes, but show only a 30%–50% reduction in

other B lymphocyte populations. Functionally, *Xid* mice have reduced levels of natural IgM and fail to respond to type 2 T-independent antigens (TI-2s), such as TACA. Unlike the multivalent form of the CMP, the CMP conjugate induced LeY reactive IgG1 responses in *Xid* mice, overcoming the inability of these mice to mount a carbohydrate-reactive response. Although these results indicate that CMPs have the potential to overcome immune deficiencies that suppress vac-



**FIGURE 2.** Examples of minimal common epitopes. **(A)** Low-energy conformers of LeY (left side of panel) and MCP (right side of panel) are compared highlighting the conserved spatial positions of the methyl group on GlcNAc (magenta-colored sphere) and hydroxyl oxygens on the Fuc residue of Fuc $\alpha$ 1-3GlcNAc (red-colored spheres) of the LeY structure and the methyl group of  $\alpha$ 2 sialic acid (magenta colored) and hydroxyl oxygens (red colored) of  $\alpha$ 9 sialic moiety. **(B)** Superposition of the LeY and MCP structures. In this orientation, a common epitope is defined on these two dissimilar carbohydrate antigens. **(C)** Low energy conformers of LeY (left side of panel) and  $\alpha$ (1-4)glucose (right side of panel). **(D)** Superposition of the LeY and  $\alpha$ (1-4)glucose structures—two additional superimposed hydroxyl oxygens are shown to the left and behind the triad initially identified. **(E)** Fuc $\alpha$ 1-3GlcNAc inhibits serum antibody binding to Fuc $\alpha$ 1-3GlcNAc. Fuc $\alpha$ 1-3GlcNAc was used to compete with serum antibodies induced by peptide 106 for binding to Fuc $\alpha$ 1-3GlcNAc-coated ELISA plate.



105 - GGIYYPYDIYYPYDIYYPYD  
 106 - GGIWRYDIYWRYDIYWRYD  
 107 - GGIYRYDGGIYRYDGGIYRYD

109 - GGARVSFWRYSSFAPTY  
 602 - RNVPPIFNDVYWIAF  
 911 - RYRYGRYRSGSRYRYGRYRSGS

**FIGURE 3.** Polyreactivity of CMP based on a limited set of amino acids. The reactivity of a panel of biotinylated lectins was tested against a number of CMPs in MAP format. Some repetitive motifs based on tyrosine, arginine, tryptophan, and proline show broad reactivity.

cine-induced CH-directed responses, multivalent forms of CMPs alone may still behave more like CH antigens as they engage B cells.

There may be an optimal level of TACA mimicry with respect to the clinical relevance of the TACA cross-reactive antibody response elicited by CMPs. Fundamental to this hypothesis is the diversity of the B-cell subpopulations that can be stimulated by TACA versus those stimulated by a CMP. The functional importance of TI-2 responses to CH antigens has been studied mostly in bacterial infections where this type of response is protective and individuals with missing, defective, or immature TI-2 responsive compartments are at higher risk.<sup>93</sup> The protective role of the IgM antitumor response and the structure of TACA point to a possible role of the same immune mechanisms in antitumor immunity.<sup>36,53</sup> We have observed that immunization with a multivalent antigenic peptide (MAP) form of a CMP that emulates the perceived clustering and multivalency of some TACAs induces a much longer lasting IgM re-

sponse than the CH antigen.<sup>92</sup> If TI-2 antigens induce long lasting memory through cells that lose the ability to receive cognate T-cell help, this would explain the inability to induce TD response after encounter with a TI form of the antigen.<sup>94,95</sup>

It is possible that this mechanism is responsible for the split responses to the carrier (TD) and to the conjugated CH epitope (TI).<sup>64,66,96</sup> One way to circumvent the problem is to use CMPs that stimulate a wider repertoire of B cells, similar to DNP to which the “TI sin” does not apply.<sup>94</sup> We hypothesize that for many CMPs a level of mimicry exists at which an optimal number of TACA cross-reactive clones are recruited, sufficiently different to have escaped the TI priming by the CH.

Using nude and *Xid* mice, we have demonstrated that CMPs behave like a typical TI-2 antigen in MAP format.<sup>72</sup> Isolated reports about the possibility for establishment of long term IgM memory demonstrate the participation of MZ cells<sup>97</sup> and at least one finds B1b cells responsible

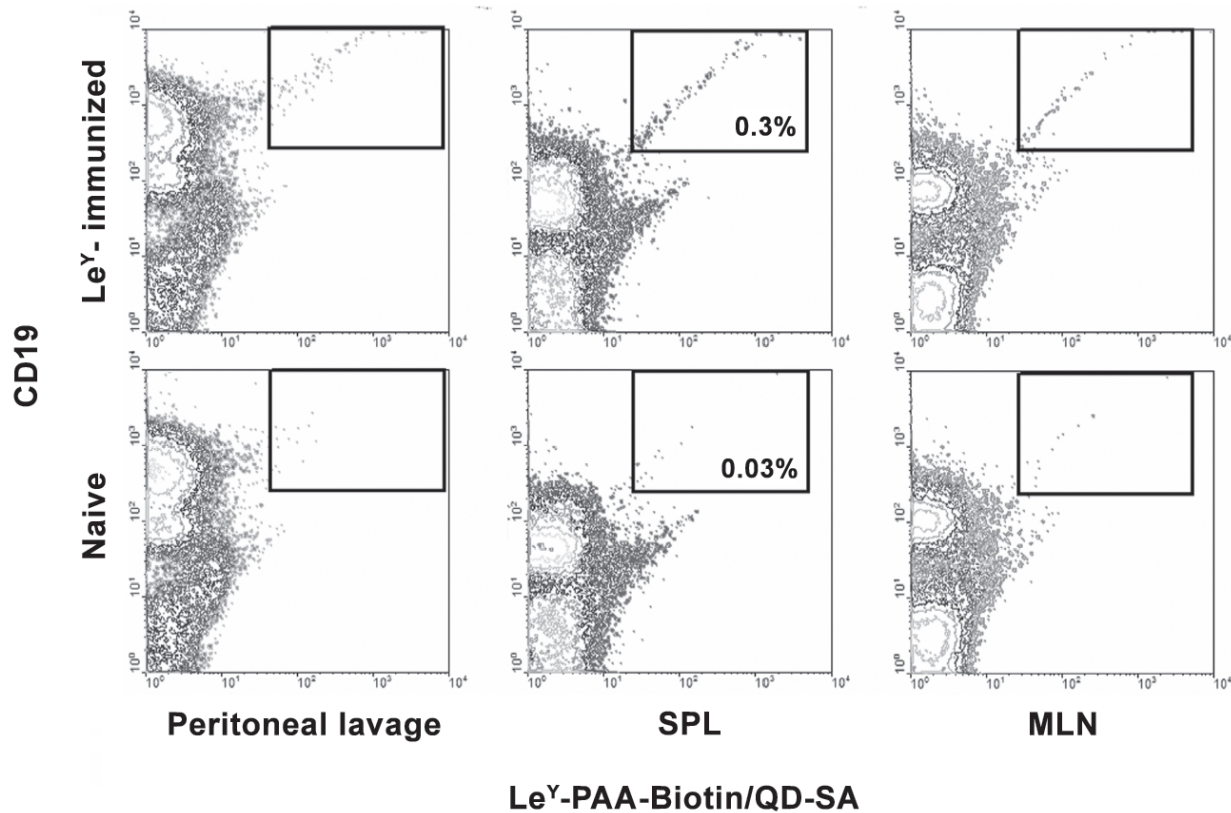


for TI-2 specific IgM memory.<sup>98</sup> The induction of long lived MZ memory cells may be related to forming GC with or without T-cell participation,<sup>97</sup> although there are indications that these GCs do not yield PC and memory responses.<sup>99</sup> It would be interesting to study comparatively the ability of CH and CMP immunogens to stimulate the different B-cell compartments. Thus, correlating structural, antigenic, and immunogenic properties will help elucidate both the optimal strategy for designing CMPs as well as increase our understanding of TI-2 responses to TACAs and their mimotopes.

Although the number of antigen-specific B cells in a naïve host is on the order of  $10^{-5}$ , it may be possible to stain detectable numbers of antigen-specific cells in immunized animals and analyze their phenotype.<sup>100</sup> Quantum dots (QDs) are stable, bright fluorophores that can have high quantum yields, narrow fluorescence emission bands, high absorbency, very long effective Stokes

shifts, and high resistance to photobleaching, and can provide excitation of several different emission colors using a single wavelength for excitation.<sup>101</sup> To test the feasibility of application of QDs for detection of antigen CH antigen-specific B cells, CH complexes were prepared by incubating ZnS/CdSe QD (Quantum Dot Corporation, Hayward, CA) and emitting at 565 nm coupled to streptavidin with biotinylated Lewis Y polyacrylamide polymers at 5:1 or 10:1 molar ratio (excess of CH polymer). Different cells were then stained with preformed complexes and analyzed by flow cytometry (Fig. 4). Splenocytes, mesenteric lymph node cells, and peritoneal lavage cells of a mouse, sacrificed one week after a single immunization with LeY-PAA, were compared to those of a naïve mouse. A 10-fold increase of the QD/LeY-PAA stained CD19 positive cells was observed after immunization in all three compartments.

One working hypothesis in our development of CMPs is that sequential immunization with a

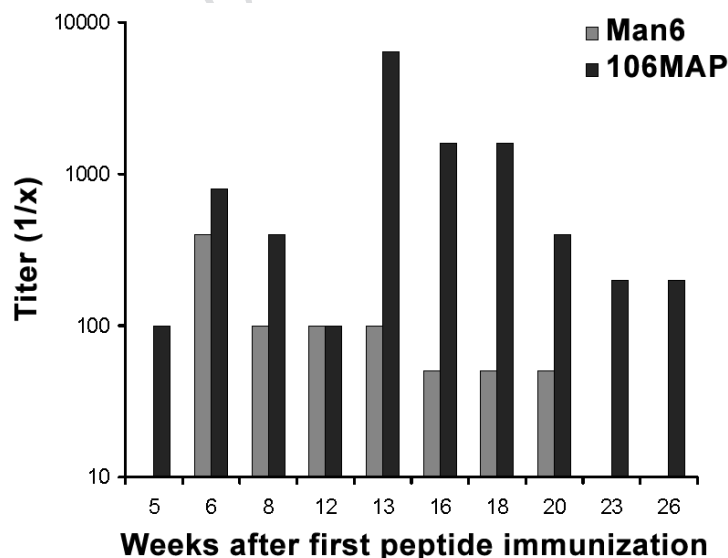


**FIGURE 4.** Flow cytometric analysis of CH-specific B cells. Peritoneal lavage, spleen, and mesenteric lymph node cells from LeY immunized and naïve mice were stained with CD19 and Quantum Dot-Streptavidin-LeYPAA-biotin complexes. The frame indicates LeY-binding B cells.

mimetic and with an original antigen can expand the B-cell population, generating a strong immune response to the self-antigen. A self-TAA does not induce an immune response to itself in the autologous host, since the corresponding high affinity B-cell clones have been eliminated during the establishment of self-identity.<sup>102</sup> In contrast, TAA mimics may stimulate B-cell clones, which have not been eliminated because of low affinity for the self-TAA. Somatic hypermutations in the course of the immune response elicited by a TAA mimic may enhance the association constant of antibodies for the original TAA. Boosting with the original TAA may expand the B-cell population producing mutated antibodies, thus generating a strong immune response to the self-TAA. Since selected CMPs also stimulate Th1 responses with IFN- $\gamma$  production, priming with CMP may induce a unique type of memory for the generation of longer lasting serum IgM on subsequent CH boost. To illustrate this possibility, we primed mice with 106 MAP and then boosted the mice with Man6 ( $\alpha$ -D-Man-6-Phosphate-PAA). Reactivity of collected serum was checked against the corresponding sugars. Priming with CMP 106

followed by Man6 boost mediated a longer lasting IgM CH-reactive response (Fig. 5). End-point titer of anti-Man-6 cross-reactive serum antibodies was 1:800 one week after peptide boost and dropped 5 weeks later. Man-6 immunization of peptide-primed animals boosted the end-point titer to 6400 a week after boost and showed a stable performance for several weeks. In either case, priming with CH induced a degree of reactivity, which died off soon and was not boostable on the second immunization, a characteristic of T-cell-independent antigens.

Conjugation of CMPs to carrier protein can affect IgG responses.<sup>72</sup> Likewise, it has been shown that carbohydrate-conjugate priming followed by nonconjugated boost may enhance particular IgG isotype profiles.<sup>103</sup> In this regard, we studied the effects of immunization with a MAP conjugate on induction of cross-reactive antibodies. 105 MAP, a peptide mimetic of pneumococcal capsular polysaccharide type 14 (Pn14), was conjugated to BSA and used in a set of prime-boost experiments.<sup>104</sup> Mice that were primed with 105-MAP conjugate displayed high IgG1 activity (Fig. 6). As expected for CH antigens, Pn14, while priming



**FIGURE 5.** Peptide immunization primes for an anticarbohydrate cross-reactive memory IgM response. Mice were prebled and groups of mice were immunized with 106 MAP twice at weeks 1 and 4. Other groups were immunized with Man-6 ( $\alpha$ -D-Man-6-Phosphate-PAA) once at week 4. Serum was collected at week 0 (prebleed), after peptide boost or after first sugar immunization for every 2 to 3 succeeding weeks. All groups received a sugar immunization at week 12. Mice were bled individually, the collected sera pooled, and the reactivity against indicated carbohydrates was detected by standard ELISA. The highest serum dilution with a 2SD higher OD than preimmune serum was determined as the end-point titer. Data are representative of three independent experiments.

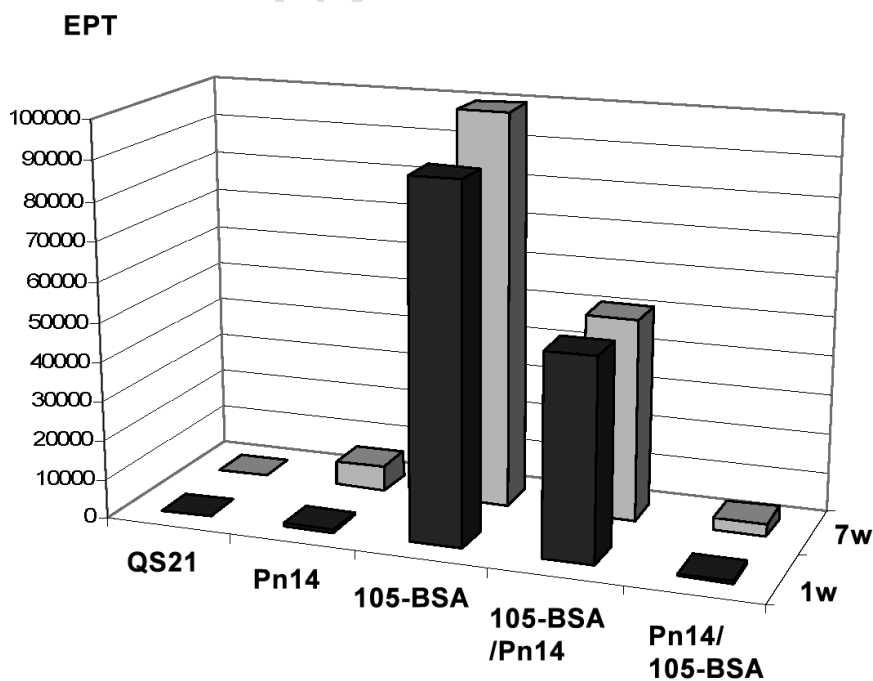
and boosting agents induced the production of specific IgG3, two injections of 105-BSA did not substantially increase IgG3 production.

Unexpectedly, 105-conjugate immunization did not induce a significant amount of anti-Pn14 IgG, despite the observation that this peptide competes in a concentration-dependent manner with Pn14 for binding to a Pn14 reactive monoclonal antibody. Nevertheless, priming with the conjugate followed by boosting with Pn14 led to both high IgG2a and IgG3 end-point titers. The concentration of the anti-Pn14 IgG increased from  $0.16 \pm 0.007$  to  $4.9 \pm 0.5 \mu\text{g/mL}$  after boost. As importantly, the Pn14 reactive IgM portion also was boosted from  $1.3 \pm 1$  to  $15 \pm 0.5 \mu\text{g/mL}$ .

## V. STRUCTURAL BASIS FOR POLYREACTIVITY AND ANTIGEN MIMICRY

The structural basis for functional peptide mimicry of CH antigens is only partially understood. It is clear, however, that mimicry is an instance of

polyspecificity and the mechanisms of the latter are to a great extent also mechanisms of mimicry. At the level of molecular interaction, polyspecificity involves binding of multiple ligands to the same binding site. Theoretically, there are several mechanisms for a specific binding site to accommodate different ligands.<sup>13,105</sup> The current view favors paratope conformational plasticity as the main mechanism of antibody polyreactivity.<sup>22-26</sup> Earlier on, the finding that antigenicity correlated better with temperature factors than with hydrophilicity<sup>106</sup> led to the hypothesis that the mobility of an antigenic determinant facilitates the binding to an antibody site not fashioned to fit the exact geometry of a protein. This illustrates another mechanism of polyreactivity,<sup>107</sup> exemplified by the binding to the same antibody of apparently distinct flexible antigens that can attain very similar conformations or at least engage with a similar bonding footprint. Flexibility is typical of CH and peptide ligands and this probably facilitates cross-reactivity contributing both to mimicry between peptides and CH and cross-reactivity of one peptide with multiple CH antigens.<sup>85</sup>



**FIGURE 6.** Anti-Pn14 IgG1 titer assessed by ELISA. Mice were primed and boosted as indicated. End-point titers (EPTs) for serum anti-Pn14 IgG subisotypes of primed and boosted mice were determined as the last dilution at which the immune sera had statistically significantly higher OD than naïve serum.

At least two modes of flexible binding are considered, namely, induced fit and conformational selection.<sup>108–110</sup> The notion of the simple “lock and key” comes from enzyme-substrate binding<sup>111</sup> and implies predetermined complementarity. The induced fit concept evolved from this, postulating that the complementarity is not necessarily preexisting and may be a result of the interaction,<sup>112</sup> implying flexibility of at least one of the ligands. Recently, it has been proposed that this mode of binding, although thermodynamically reasonable, may often be too slow for biologically meaningful reactions because the rate determining step is the formation of an unstable initial complex.<sup>109</sup> Instead, a number of studies point out the possibility of selection of preexisting conformations in the molecular ensemble leading to a much higher overall reaction rate.<sup>109,110,113,114</sup> The induced fit mode may still be preferred when the ligands have considerable complementarity in the initial complex and the bonding is necessary to lower the energy barrier, making the stable complex conformation accessible.

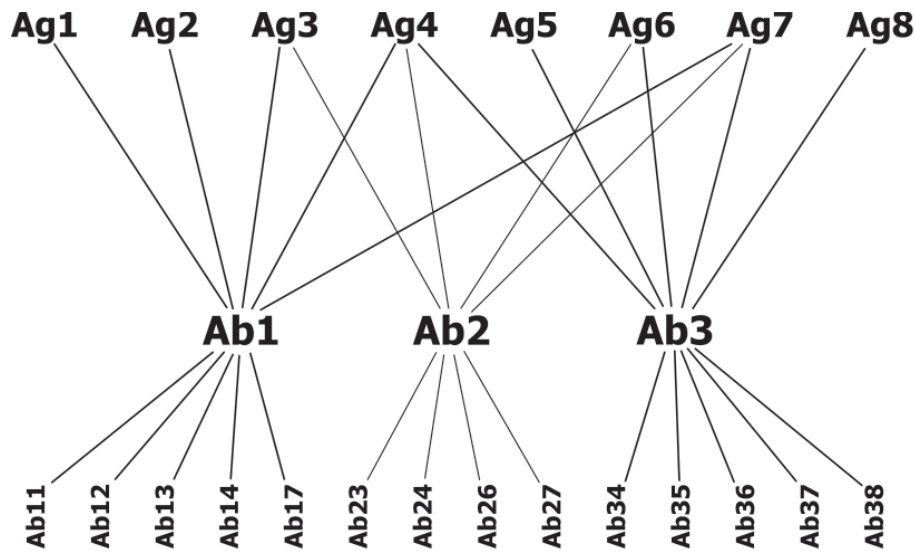
Flexibility of the interacting interfaces may not be the only path to polyreactivity. Possibly the simplest form of polyreactivity is the recognition of the same epitope on different molecules. For instance, a protective monoclonal antibody E5 to *Schistosoma mansoni* recognizing the CH antigen LeX, a leukocyte marker (CD15) known also as stage specific embryonic antigen 1, binds and neutralizes HIV-1 virus.<sup>115,116</sup> Although most of the antibodies binding to CH epitopes should fall into this category, there is an insufficiently understood specificity for a very limited set of glycoprotein expressing the sugar epitope. An interesting version of this mode is the interaction of the same or very similar footprint consisting mostly of a set of hydrogen bonds with defined directionality. This common footprint may, in general, belong to completely different molecules and represents best the material substrate of molecular mimicry.<sup>117</sup>

Epitope mapping techniques and crystallographic analysis have repeatedly demonstrated that antibodies bind to epitopes of three to seven amino acids within a peptide sequence.<sup>86,118–120</sup> This binding usually does not fully engage the binding site. Neither does binding of a CH epitope. Antibody binding sites are larger than many

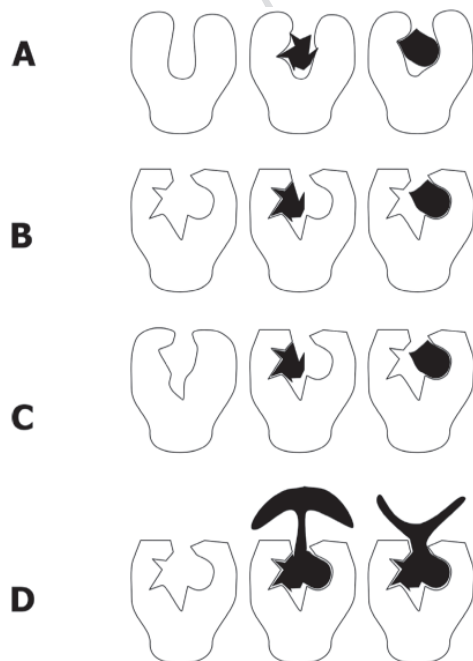
epitopes and haptens, providing room to accommodate different structures in different paratopes. This mode of polyspecificity is compatible with rigid binding, but the most intriguing example of it came recently in the context of flexible binding of preimmune antibodies. Sethi et al. found that a typical flexible paratope on binding attains the same rigid conformation when it combines with different ligands in different binding regions,<sup>26</sup> reminiscent of the rigid adaptation described for NKG2D.<sup>107</sup> Thus, another function of the plasticity may be restricted to the nonbound state, which channels different plastic epitopes to binding by the single conformation of the antibody in the bound state. The different modes of polyspecific interactions are illustrated in Figure 7.

Polyspecificity is sometimes confused with nonspecific stickiness, implying mostly hydrophobic interactions with little complementarity. James and Tawfik addressed this issue using a polyreactive IgE antibody and showed that its interactions depend on specific hydrogen bonds.<sup>121</sup> This finding is most consistent with the enumerated possible mechanisms of polyspecificity as molecular interaction, specific for a set of ligands rather than a single ligand.

Molecular interaction between the variable region of an antibody and another molecule becomes antigen recognition only as a function of the immune system.<sup>122</sup> Thus, functional mimicry would imply molecular interaction of a surrogate antigen with antigen receptors that elicits immune response, cross-reactive with the target antigen. Antigen recognition involves the process of the maturation of the immune response in which the flexibility of the binding site is lost and the polyreactive preimmune antibodies evolve highly specific rigid binding to one of the different antigen shapes accessible initially<sup>24</sup> (Fig. 8). Therefore, a CMP antigen (binding to antigen receptors) would also be an immunogenic mimotope (inducing immune function) if it triggers an immune response that yields specific antibodies that cross-react with the template CH antigen. For instance, a CMP selected from a random peptide library, with the help of a CH-specific antibody, may or may not bear structural similarity with a CH antigen in the context of binding to the same contact residues in an antibody-combining site.<sup>86,120,123–125</sup> Therefore, when used as an immunogen, this peptide may or



**FIGURE 7.** Polyspecific antibodies have characteristic patterns of recognized epitopes, which may overlap with those of other antibodies. Thus, the size of the preimmune repertoire is multiplied to become complete, and at the same time the size of the population of cells reactive with a particular antigen is increased. In the next stage, adaptation through somatic mutations leads to the production of a potential repertoire of antibodies, which preserves the completeness, but is specific. Although the number and the size of the different clones in the potential specific repertoire are both larger than those of the preimmune repertoire, the overall size of the population does not increase much because the actual specific repertoire comprises only the clones most relevant to the particular immune response. Polyspecificity of the preimmune repertoire acts as a classification mechanism that, when coupled to the subsequent somatic diversification, helps project the space of epitope specificities onto the space of paratopes in a highly efficient way.



**FIGURE 8.** Different types of polyreactive paratopes. **(A)** Preimmune antibodies have flexible paratopes that can adapt to different epitopes. **(B)** Mutated antibodies with rigid paratopes can accommodate different small epitopes in parts of their comparatively larger paratope. **(C)** Recently described<sup>26</sup> paratope, which is flexible in the free state but attains a rigid conformation on binding different epitopes in different binding sites. Somewhat surprisingly, the rigid conformation is the same although the binding mode for each epitope is quite different. **(D)** Highly specific, rigid paratope recognizes the same binding groups found in a different structural context. The recognized domain of the antigen could be identical (e.g., common domains in different proteins or glycans associated with proteins backbone or glycolipid), but it could also be just a common bondage footprint associated with chemically different antigens (e.g., carbohydrates and CMPs).

may not induce antibodies carrying the CH-binding paratope. A disadvantage of the polyspecificity of a CMP is, thus, the potential to induce a whole range of different antibodies of which only a small proportion may be cross-reactive with the CH antigen. Indeed, mimotope immunizations usually induce higher titers of antipeptide than anticarbohydrate responses. An approach that may improve the quality of CH mimicry in this respect is to select and/or model peptides that bind simultaneously and in different ways to different CH-reactive antibodies and lectins with specificity for the same CH antigen.

Another aspect of specificity as an emergent property of the immune system is the filtering of the structural polyspecificity of the receptors when the outcome depends on molecular recognition through cellular cooperation. The polyreactivity of the T-cell receptors is restricted by the accessibility of professional antigen presenting cells, antigen processing, affinity of MHC-peptide binding, competition for MHC binding,<sup>126,127</sup> qualitatively different outcome of the quantitatively different binding relative to certain affinity thresholds (altered peptide ligands),<sup>128,129</sup> etc. On the other hand the importance of the polyspecificity of TCR should also be considered in view of the large number of possible different peptides. The probability of encountering one of  $10^6$  cross-reactive peptides for a given TCR among  $10^{11}$  (for class I) or  $10^{12}$  (for class II) possible peptides is low.

Thus, at the molecular level antigen recognition is often polyspecific and the understanding of its structural basis amounts, to a great extent, to understanding the mechanism of polyspecificity or its restriction by a number of molecular and systemic mechanisms.

## VI. T-CELL RESPONSES TO GLYCANS

The posttranslational modification, particularly glycosylation, plays an important role in the folding and function of proteins of higher organisms. Glycosylation is frequently observed in surface and secreted proteins as well as in most of the molecules involved in innate and adaptive immunity. There are three types of commonly found glycosylations, namely, (1) N-glycosylation where glycan is attached to an Asp side chain via GlcNAc,

(motif Asn-X-Ser/Thr or Asn-X-Cys); (2) O-linked glycans, which bind to an Ser or Thr side chain; and (3) CH components of glycosylphosphatidylinositol (GPI) anchors, which are glycolipids.

T cells recognize peptides that are products of proteolytic processing of protein antigens, which are presented as complexes with MHC molecules class I or II. This seems to define very strictly the necessity for protein structures as T-cell epitopes. Moreover the glycan moieties of glycoproteins are enzymatically removed from the glycopeptides. But in the last decade, a number of researchers report that both CD4+ and CD8+ T cells can recognize glycopeptides carrying mono- and disaccharides in a MHC-restricted manner, provided the glycan group is attached to the peptide at suitable positions.<sup>82,130-148</sup> In such glycopeptides, the primed T cells recognize the glycan structure with high fidelity. These observations are very important to understanding how the immune system responds to tumor cells, as well as to glycoproteins of different pathogens.

Although TACAs are targets for antibody generation in cancer vaccine applications, they may also be targets for CH-reactive T cells. Because of an incomplete formation of glycan side chains resulting from premature glycosylation events, the restrictive distribution of short glycans such as the Thomsen-Friedenreich (TF or T) antigen ( $\text{Gal}\beta 1\text{-3GalNAc}\alpha$ ), Tn ( $\text{GalNAc}\alpha$ ), and sialyl Tn ( $\text{NeuAc}\alpha 2\text{-6GalNAc}\alpha$ ) in normal tissues and their extensive expression in a variety of epithelial cancers make them excellent targets for immunotherapy. The carbohydrate-based vaccine design for T-cell responses is strongly supported by several HLA-peptide complexes resolved by crystallography.<sup>136,146</sup> The structure of different crystals describes the core of the peptide(s) as critical for TcR recognition with a "cavity" corresponding to the CDR3 region that often accommodates aromatic amino acid residues, similar in size and conformation to small CH molecules, such as TF, or the monomer Tn.

We have demonstrated that P106 immunization regresses established murine tumor mediated by T cells.<sup>40</sup> P106 induces Th1 with production of IFN- $\gamma$  and tumor specific CD8+ T cells.<sup>39,40</sup> The ability of TACA-specific T-cell clones to recognize CH antigen in the context of different peptide sequences is very relevant to validate this vaccine

approach (Table 2). Moreover, it is important to determine the fine specificity of the carbohydrate-specific T cells, addressing the recognition of the amino acid linker together with the sugar moiety. Cell-based ELISA assays indicate that the CMP 106 binds biotinylated lectins that recognize GalNAc and GlcNAc moieties and that lectins can differentially recognize these simple sugars expressed on tumor cell surface.<sup>40</sup> The interaction between CMPs and lectins that are reactive with simple monosaccharides and disaccharides associated with tumor-associated glycopeptides suggests that select CMPs may mimic these fundamental moieties associated with TACA.

Molecular modeling studies of CMPs overlaid onto glycopeptide-TCR crystal structures suggest that the spatial positioning of the sugar moiety can be effectively emulated by aromatic and charged residues.<sup>78</sup> The interaction between peptide 106 and wheat germ agglutinin in particular suggests that the peptide may mimic GlcNAc glycans.<sup>40</sup> Although this possibility requires further validation, it may explain why immunization with peptide 106 mimic leads to induction of CD8+ T cells

specific to a glycosylated epitope of tumor-rejection antigens present on Meth A fibrosarcoma cells.<sup>40</sup> The novelty of our approach lies in its attempt to induce tumor-specific cellular responses to CH-associated tumor antigens by CMP vaccines, and to analyze the significance and the relative importance of both humoral and cellular immunity directed toward TACA in the control of cancer.

The crystal structure analysis and lectin profiling data suggest that T cells recognizing CH moieties may do so in a degenerate fashion, further suggesting that such T cells are recognizing a broad-spectrum set of antigens. From a design perspective, this would imply that an emphasis for such designer glycopeptides be placed on defining carbohydrate-peptide conjugates that bind with high affinity class I MHC molecules and that have appropriate structure to induce a T-cell response that is skewed toward the recognition of the haptenic moiety.<sup>149</sup> The critical parameters to be considered in designing peptides (and glycopeptides) with high binding affinity for Class I molecules are (1) optimal length and the presence of critical anchor residues,<sup>150</sup> (2) positioning of the

**TABLE 2**  
**Sequences of Some Glycopeptide T-Cell Epitopes**

Peptide sequence	Glycan moiety	MHC allele	Reference
D PQSGALYISKVQKEDNSTYI	GlcNAc(β)-	HLA-DRB1*0801	156
GALYISKVQKEDNSTYI	GlcNAc(β)-	HLA-DRB1*0801	156
IVIKRSNSTAATN	GlcNAc(β4)-GlcNAc(β)-	HLA-DQA1*0301/ DQB1 *0302	157
SIHRYHSDNSTKAASWD	Man(α6) Fuc(α6) Man(β4)-GlcNAc(β4)- GlcNAc(β)-Man(α3)	HLA-DRB1*0401/DRB4*0101	158
IHRYHSDNSTKAAWD	Man(α6) Fuc(α6) Man(β4)-GlcNAc(β4)- GlcNAc(β)-Man(α3)	HLA-DRB1*0401/DRB4*0101	158
GEPGIAGFKGEQGP	Gal(β)- or Glc(β2)-Gal(β)-	H2-A <sup>a</sup>	159
GIAGFKGEQGPKEPAGP	Gal(β)-	HLA-DRB1*0401	160
AHGVTSAPDTRPAGSTAPPA	Gal(β3)-GalNAc(α)-	H2-A <sup>b</sup>	161
SAPDTRPA	GalNAc-	H2-K <sup>b</sup>	162
NLTISDVSV (nonrepeat region)	GalNAc-	HLA-A*0201	163
SLSYTNPAV (nonrepeat region)	GalNAc-	HLA-A*0201	163
ALASTAPPV (nonrepeat region)	GalNAc-	HLA-A*0201	163
KIFGSLAFL		HLA-A2	164
SIISAVGI		HLA-A2	164
ILHNGAYSL		HLA-A2	164

peptide backbone to allow hydrogen bonding between main chain atoms in the peptide and the atoms in the MHC molecule, and (3) avoidance of detrimental residues at nonanchor positions that interfere with the docking of the peptide in the MHC binding groove.

There is limited data on MHC binding glycopeptides or glycoepitopes. In contrast, a large number of MHC binding peptides or T-cell epitopes is known. It is not known if these peptides are potential glycopeptides. There are large numbers of peptides that have come from glycosylated proteins or have the potential of glycosylation. For example, a number of MHC binders have serine or threonine at nonanchor positions (position 4). It is also known in some cases that T cells cross-react with both glycosylated and nonglycosylated peptides.<sup>151</sup> The ability of T cells to recognize mono- and disaccharides attached to peptides might indicate that such T cells are degenerate in recognizing glycopeptides. Such T cells might then be considered to recognize a set of broad-spectrum antigens. As a simple exercise, we experimentally examined proven 7924 MHC class I binders from the MHCBN database<sup>152</sup> (Table 3). We calculated how many binders have serine at position 4, 5, and 6, and found 393, 409, and 561 binders, respectively. Similarly, we found 398, 367, and 397 binders that have Thr at position 4, 5, and 6. The same trend was observed for 3282 CTL epitopes that have Ser and Thr at position 4, 5, and 6.

It is clear from the above analysis that Ser and Thr are present in experimentally known binders for different alleles. The question is which peptides will be glycosylated and which ones will not. Unfortunately, O-linked glycosylation has no significant motif and the sole condition seems to be that a residue should be accessible. On the basis of crystallographic data of glycopeptides interacting with a TCR, the glycan residue should be pointing toward the TCR in order to simulate a CTL response against glycan or glycosylated peptides. In this context, we analyzed MHC bound peptides of different MHC alleles in order to determine the positions with high surface accessibility (Table 4). MHC bound structures and surface accessibility were assigned using the DSSP software,<sup>153</sup> indicating that positions 4–6 and 8 are the most accessible in the A2 and H2-Db alleles. Using the relative positioning of the Ser residue, Table 4 suggests alternative T-cell epitopes for Her 2 and Muc 1.

On the basis of the outcomes of this study, and on available knowledge on the antigen processing and CTLs activation mechanisms, it seems that glycosylated peptides in most cases would serve as inferior targets for antitumor therapy compared to the nonglycosylated peptides. Despite the fact that most of the proteins are heavily glycosylated and there are distinct patterns of glycosylation in tumor cells versus normal cells, a majority of the presented peptides are derived from the newly synthesized nonglycosylated protein molecules that

**TABLE 3**  
Frequencies of Potential O-glycosylation Sites on Known T-Cell Epitopes

MHC alleles	Total binders	Binders having Ser at position			Binders having Thr at position		
		4	5	6	4	5	6
HLA-A*0201	1154	71	45	58	42	61	51
H2-B	16	1	0	2	0	1	0
H2-D	92	4	5	7	3	2	2
H2-Db	200	21	11	13	8	5	7
H2-Kb	243	7	3	9	9	5	12
H2-Kd	313	18	13	8	11	18	20

Note: In the case of H2-Kb, only a few binders have Ser and Thr in the center of the binder. Except for H2-Kb, most of the alleles have no restriction for Ser and Thr for the potential glycan at the center positions of the binders. Glycosylation of Ser or Thr at position 8 might impact on interactions between MHC and TCR.



**TABLE 4**  
**Surface Accessibility of Potential O-glycosylation Sites on Known T-Cell Epitopes**

MHC alleles	Average surface accessibility								
	1	2	3	4	5	6	7	8	9
HLA-A*0201	21.8	2.5	11.25	69.8	65.5	99.8	27.6	60.24	5.4
H2-Db	22.3	2.0	2.7	72.5	35.1	69.3	42.7	55.8	0
H2-Kb	28.8	3.4	8.0	64.0	55.8	43.2	32.8	27.8	2.8

Note: In all these alleles, anchor positions are in the terminal positions, and center residues are not used in peptide MHC interactions. This means that side chains of center residues of bound peptides (positions 4, 5, and 6) are free to interact with TCR and to bind with glycan (as average surface accessibility -A2).

did not pass through Golgi and endoplasmic reticulum where glycosylation takes place.

An additional source of the presented peptides, that is, degradation of the glycosylated proteins at the end on their life span, is expected to result in a low extent of MHC class I-restricted glycopeptide presentation compared to the corresponding peptide due to (1) partial glycosylation of the amino acid backbone (i.e., only part of the potential glycosylation sites are actually glycosylated) and (2) part of the glycopeptide residues are expected to be cleaved during the glycopeptide processing. In addition, due to a highly variable glycosylation pattern, the presented glycopeptides are expected to bear different glycosylation moieties, and mounting an immune response against specific glycopeptide means that only a small fraction of the presented glycopeptides will serve as potential targets for the cellular immune response. Nevertheless, it is possible that in certain cases a specific glycopeptide may serve as a specific target for cytotoxic immune therapy in the case that the combination of MHC binding and specific cytotoxic immunogenicity for this glycopeptide will outweigh the low efficiency of its presentation and will permit selective cytotoxic responses targeted against the cells presenting such a peptide.

## VII. CONCLUSIONS

Several concepts have emerged from our studies. First, the construction of an effective CMP prob-

ably requires improved fitting between bound mimetic and the antibody to maximize particular interactions within the antibody heavy and light chains. Since there can be a discontinuity between CH and peptide epitopes, induced antibodies can be of different subsets and of unique structures.<sup>154,155</sup> Consequently, selection of peptides reactive with particular anticarbohydrate antibodies can identify peptides that are not faithful mimetics in that they do not make requisite contacts with both the heavy and light chain, as does the nominal CH antigen.<sup>86</sup> This effect has been highlighted in several studies of peptide mimetics.<sup>119,155</sup> Second, it is possible that antibodies see peptides with greater specificity than they do carbohydrates. We have advocated using rational design approaches coupled with reactivity patterns to increase the fidelity of mimicry.<sup>37,86,120</sup> Third, conformational properties of CMPs may influence reactivity to isolating antibodies and the type of antibodies CMPs induce. On the one hand, this aspect may facilitate the design of single CMPs to render effective humoral responses not only to a single CH form, but also to multiple tumor-associated CH antigens at once. Such peptides would simplify currently available vaccine approaches, yet may still require more extensive polymerization to fully emulate a native antigen. A fourth concept that is emerging is the kinetics of interaction and its impact on immunogenicity. Valence plays a key role in protein-carbohydrate interactions. Fifth, CMPs can impact on T-cell responses. Details of how interactions govern immune responses to CH are few. The immunological significance of in-

ducing peptide-specific T cells in helping to promote CH-directed cellular responses awaits further definition.

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