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Carbohydrate-dependent, HLA class II-restricted, human T cell response to the bee venom allergen phospholipase A2 in allergic patients*

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The T cell-independent antibody response to polysaccharide antigen (Ag) is believed to result from their inability to bind major histocompatibility complex (MHC) restriction elements. However, recent studies using glycosylated analogues of known immunogenic peptides revealed that glycopeptides can interact with MHC molecules and are able to elicit specific T cell responses in experimental animals. This raises questions about the possible role which carbohydrates can play in T cell responses following natural exposure to glycoprotein antigens. Analyzing the fine specificity of the human T cell response against the major bee venom allergen phospholipase A2 (PLA), a 16–20-kDa protein glycosylated at a single site (Asn¹³), we have identified several T cell clones which proliferate in response to the glycoprotein but not to its non-glycosylated variants. Neither the carbohydrate moiety alone nor the combination of carbohydrate and non-glycosylated protein could substitute for the intact glycoprotein. Antibody directed against the carbohydrate moiety inhibited Ag-induced proliferation of these clones whereas control clones with known peptide specificity were not affected, providing additional evidence for the involvement of carbohydrates in T cell recognition. Moreover, peripheral blood mononuclear cells of two individuals from whom glycosylation-dependent T cell clones have been isolated showed significantly higher proliferation in response to glycosylated compared to non-glycosylated Ag, suggesting that glycosylation can contribute in some cases extensively to the immunogenicity of a glycoprotein Ag. Thus, this report shows that glycosylation-dependent Ag recognition by T cells can also occur following natural exposure to a glycoprotein.

1 Introduction

Ag recognition by T cells requires the association of an antigenic peptide fragment with MHC molecules of APC, and the subsequent interaction of this complex with the TCR [1–3]. The current concept holds that T cell recognition is confined to Ag-derived linear peptides whereas carbohydrates or lipids cannot induce T cell responses. This assumption is substantiated by the T cell-independent antibody response to some polysaccharide Ag [4, 5] which has recently been proposed to result from the inability of polysaccharides to bind to class II MHC molecules [6]. The question whether glycopeptides can be recognized by T cells has recently been addressed in murine systems using glycopeptide analogues of a known OVA-derived immunogenic glycopeptide [7], glycopeptides from HEL determinants [8] as well as from Sendai virus nucleus protein [9]. Moreover, T cell recognition of carbohydrate on type II collagen in rats has been demonstrated [10]. These studies provided evidence that the carbohydrate moiety can be part of the determinant recognized by T cells. Although these

experimental systems underscore the potential immunogenicity of glycopeptides, their relevance for human T cell recognition following natural exposure to a glycoprotein antigen has not yet been demonstrated.

We have analyzed the specificity of human T cell responses to the major bee venom allergen phospholipase A2 (PLA) [11], a glycoprotein of 134 amino acids with a single carbohydrate unit attached to Asn¹³ [12]. In a previous study, we have identified three immunogenic domains along the primary sequence of PLA. Synthetic peptides of 12 to 18 residues spanning these areas induced proliferative responses in cloned T cells as well as in unfractionated PBMC of sensitized individuals [13]. However, although a large panel of overlapping peptides spanning the entire molecule was tested, several PLA-specific T cell clones failed to recognize any of these peptides. Here we report T cell clones which selectively respond to glycosylated PLA but not to its non-glycosylated variants. Moreover, T cells exhibiting glycosylation-dependent Ag recognition seem to predominate over those with peptide specificity in some individuals, suggesting that glycosylation may contribute significantly to the overall immunogenicity of a glycoprotein in the context of exposure to a natural Ag.

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2 Materials and methods

2.1 Antigens

Purified natural PLA was purchased from Boehringer (Mannheim, FRG) and used without prior chemical inactivation. Alternatively, PLA was purified from whole bee

venom (obtained from Nectarcop, Sofia, Bulgaria) by size fractionation and ion exchange chromatography [14]. Recombinant PLA (rPLA) was produced in *Escherichia coli* and prepared as described [15]. None of these PLA preparations showed cytotoxic effects up to 100 µg/ml when assayed on EBV-transformed human B cells [11]. Natural Ag devoid of the carbohydrate moiety was produced by enzymatic deglycosylation (PLA-deg) or by biochemical purification of the naturally occurring non-glycosylated PLA variant (PLA-0). Enzymatically deglycosylated PLA was obtained by incubating PLA for 24 h at 37°C with 0.9 units endoglycosidase-F/N-glycosidase-F (Boehringer) per mg PLA in 25 mM Tris-HCl, 10 mM EDTA, pH 8.0 in the absence of denaturing agents. To monitor deglycosylation, protein samples were resolved by SDS-PAGE [16] and stained with Coomassie brilliant blue R-250 (Bio-Rad, Richmond, CA). PLA-0 was isolated by lectin affinity chromatography on immobilized concanavalin A (Sigma Chemical Co., St. Louis, MO) carried out as described [14]. The samples were desalted by reverse phase HPLC on a Bio-Rad RP304 column (4 × 250 mm) using an acetonitrile gradient from 0 to 20% in 0.1% trifluoroacetic acid for 25 min at a flow rate of 1 ml/min. This procedure also removed trace amounts of lectin leaking from the column and yielded a protein preparation which was free of mitogenic activity when assayed on nonspecific T cells. The carbohydrate-free nature of PLA-deg and PLA-0 was tested by ELISA using rabbit anti-horseradish peroxidase antibody (Sigma) which shares extensive cross-reactivity with the carbohydrate epitope of PLA [17]. Concentrations were determined according to the Bradford method using a commercial kit (Bio-Rad) or were calculated from the absorbance using an ϵ_{280} 1% of 13.0 [15]. Asparagine-linked oligosaccharide was prepared by pronase digestion of PLA followed by size exclusion chromatography as described [18].

2.2 Cell lines and culture conditions

A detailed description of the isolation and maintenance of PLA-specific $\alpha\beta^+$ CD4⁺ T cell clones and EBV-transformed autologous B cell lines is given elsewhere [11]. In addition to the clones MeT1.1A9, MeT3.2E5, MeT5.3A7 and BuT1.2A2 described previously [11], PLA-specific clones ReT16 and ReT41 isolated by the same method were analyzed in this study. Cells were cultured in RPMI 1640 medium supplemented with 1 mM sodium pyruvate, 1% MEM nonessential amino acids, 1% MEM vitamins, 2 mM glutamine, 100 µg/ml kanamycin, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.05 mM 2-ME (all from Gibco, Basel, Switzerland) containing 10% heat-inactivated FCS (Seralab, Crawley Down, GB) and were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

2.3 Proliferation and HLA-D restriction of T cell clones

Triplicate samples of serially-diluted Ag were incubated for 48 h with 2×10^4 PLA-specific T cells and 4×10^4 irradiated (6000 rad) autologous EBV-B cells in 150 µl medium in 96-well flat-bottom microtiter plates (Costar). After an additional 12-h pulse with 1 µCi/well of [³H] dThd (Amer-

sham Int., Amersham, GB), proliferation was assessed by liquid scintillation in an LKB β -plate counter. HLA restriction was determined by inhibition of T cell proliferation using suboptimal Ag concentration and mAb against monomorphic epitopes on DR, DQ and DP molecules (mAb L243, SK10, Biotest, Dreieich, Germany and B7/21, Becton Dickinson, Basel, Switzerland, respectively). Identical assays were set up to assess the influence on T cell proliferation of antibody specific to the carbohydrate moiety of PLA.

2.4 PBMC proliferation assay

From the same donors (Bu, Me, Re) from whom PLA-specific T cell clones have been established previously [11], PBMC were isolated by Ficoll-Paque density gradient centrifugation and assessed for primary proliferative responses *in vitro*. Proliferation assays were set up in RPMI medium containing 10% heat-inactivated autologous serum instead of FCS. Triplicate samples of serially-diluted Ag were incubated in 150 µl medium in 96-well flat-bottom microtiter plates with 3×10^5 PBMC for 5 days followed by an overnight pulse with 1 µCi/well of [³H] dThd. Proliferation was measured as before, and the stimulation index (SI) was calculated as the ratio of radioactivity incorporated in the presence of Ag to background.

3 Results

3.1 Characterization of T cell clones

To study the human immune response to the bee venom allergen PLA, we have generated a panel of PLA-specific CD4⁺ T cell clones from several bee venom-sensitized subjects [11]. Subsequent attempts to map T cell epitopes with a large array of overlapping synthetic dodeca- and octadecapeptides spanning the entire PLA molecule allowed the identification of three immunogenic peptides, each of them eliciting a proliferative response of both cloned T cells and unfractionated PBMC from sensitized individuals [13]. For some clones, however, the antigenic structure could not be assigned with this approach. The lack of response to overlapping peptides might have been due to insufficient peptide length, peptide degradation or the presence of discontinuous epitopes [13]. In a systematic approach to circumvent the drawbacks inherent to short linear peptides, rPLA produced in *E. coli* was used as an Ag source. When assessing the proliferative response of clone MeT3.2E5, which scored negative with the entire panel of linear peptides, we found that rPLA also failed to induce proliferation (data not shown).

3.2 Carbohydrate-dependent Ag recognition by T cell clones

Since the most obvious structural difference between natural PLA, which induces proliferation and the recombinant form, which does not, is the lack of glycosylation of rPLA, we reasoned that the carbohydrate moiety might be required for Ag recognition by particular T cells. This was

tested with the aid of natural PLA devoid of carbohydrate by two different approaches. Deglycosylated PLA (PLA-deg) was prepared by enzymatic removal of N-linked oligosaccharide from natural PLA through extensive treatment with glycosidases. Alternatively, the non-glycosylated PLA variant PLA-0 occurring as a minor isoform in natural PLA preparations was separated from the glycosylated isoforms by lectin affinity chromatography. Both PLA preparations showed the expected increase in electrophoretic mobility on SDS-PAGE [14] and were essentially free of carbohydrate as shown by the virtual lack of binding of anti-carbohydrate antibody (Fig. 1).

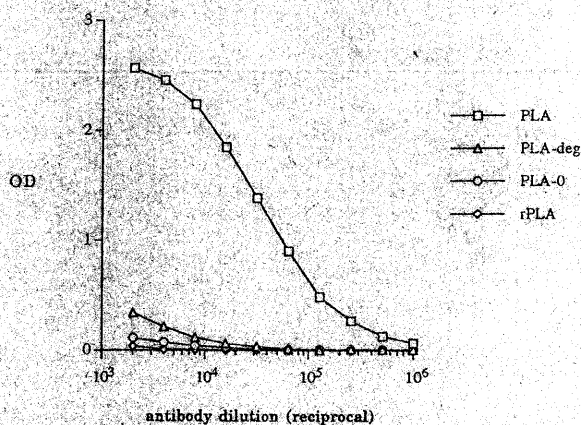


Figure 1. Presence and absence of the carbohydrate epitope in PLA variants. The binding of a rabbit antibody specific for the carbohydrate moiety of PLA to solid-phase glycosylated (□), deglycosylated (Δ), naturally non-glycosylated (○) and recombinant (◇) PLA variants was assessed by ELISA. Binding assays were carried out as described [15] except that phosphatase-labeled rat anti-rabbit antibody (Zymed) was used to detect bound antibodies.

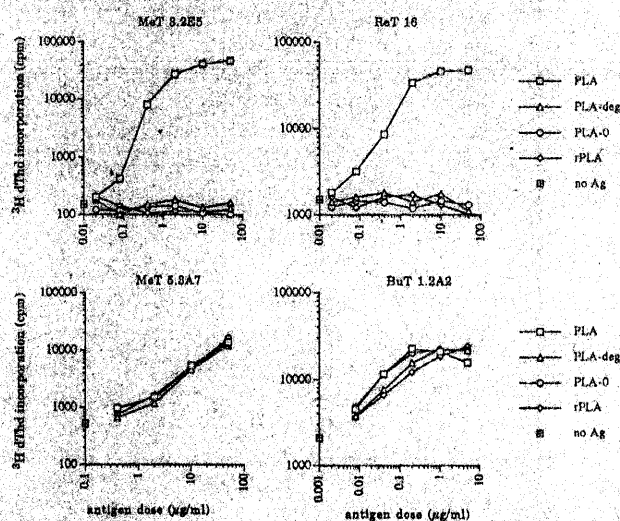


Figure 2. Proliferation of PLA-specific T cell clones in response to glycosylated and non-glycosylated Ag variants. MeT 3.2E5 and ReT 16 scored negative with a large panel of overlapping peptides; control clones MeT 5.3A7 and BuT 1.2A2 map to residues 45–62 and 81–92, respectively [13].

The Ag preparations lacking the carbohydrate moiety were assayed for T cell reactivity using the human PLA-specific T cell clones which were unable to respond to the entire panel of overlapping PLA peptides [11, 13]. As shown for clones MeT3.2E5 and ReT16 (Fig. 2, top panels), all preparations of carbohydrate-free PLA also failed to induce proliferation. This included several truncated recombinant PLA peptides as well as a synthetic 25-residue amino terminal peptide spanning the Asn¹³, which is the residue glycosylated in the natural protein (Table 1). Moreover, clones MeT3.2E5 and ReT16 failed to respond to α -mannosidase-digested PLA, whereas stimulation of the PLA-specific control clones could be achieved. The same observation was made for clone ReT41. Accordingly, the mannose-containing end of the carbohydrate moiety may be required for agretope recognition of processed PLA by the HLA-class II desotope. The structure of the PLA carbohydrate [14] is shown in Fig. 3. The same pattern of reactivity was found with two out of three clones isolated from donor Me and in both clones from donor Re. In a third individual (Bu), only T cell clones specific for the previously described immunogenic PLA-peptides were found [13].

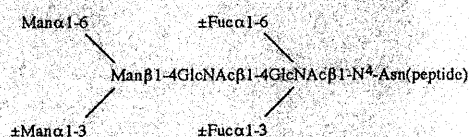


Figure 3. Chemical structure of PLA Asn¹³ carbohydrate [14].

Since neither asparagine-linked oligosaccharide [18] alone nor in combination with non-glycosylated Ag was able to induce proliferation (data not shown), we inferred that Ag recognition by these T cells is exclusively confined to the glycosylated protein. To demonstrate that the carbohydrate-dependent Ag recognition is a property of the particular T cell, the two PLA-specific clones MeT5.3A7 and BuT1.2A2 previously shown to map to PLA peptides 45–62 and 81–92, respectively [13], were tested. As shown in Fig. 2 (bottom panel), peptide-specific T cell clones responded equally well to either natural glycoprotein or its carbohydrate-free variants. This provides firm evidence that immunogenic peptides from both glycosylated and non-glycosylated Ag are equally processed and presented to reactive T cells and further suggests that the carbohydrate-dependent Ag recognition reflects a T cell specificity for a carbohydrate dependent epitope. The reactivity pattern of the PLA-specific T cell clones are summarized in Table 1.

Additional evidence for the implication of the carbohydrate structure in T cell recognition is provided by inhibition studies using antibody reacting with the carbohydrate moiety of PLA. Fig. 4 shows that the proliferation of MeT3.2E5 is inhibited by antibody to the carbohydrate in a dose-dependent fashion, whereas the control clone MeT5.3A7 specific for PLA peptide 45–62 [13] remained unaffected. Thus, this antibody does not generally decrease the efficiency of Ag uptake, processing and presentation of immunogenic peptides such as PLA 45–62, but rather

Table 1. T cell proliferative response to PLA and PLA derivatives

Compound	T cell clone				
	MeT 3.2E5	ReT 16	ReT 41	MeT 5.3A7	BuT 1.2A2
PLA-nat.	+	+	+	+	+
PLA-Asn ¹³ -CH	+	+	+	+	+
Asn ¹³ -CH (Pronase)	+	+	+	+	+
PLA-O	-	-	-	-	-
r PLA (<i>E. coli</i>)	-	-	-	+	+
r PLA fragments (<i>E. coli</i>)	-	-	-	+	+
PLA deg (Endoglycosidase F/N-Glycosidase F)	-	-	-	+	+
PLA (α -Mannosidase)	-	-	-	+	+
PLA 1-25	-	-	-	+	+
PLA 45-65	-	-	-	+	-
PLA 81-92	-	-	-	+	-
Overlapping 12-18 PLA peptides	-	-	-	-	+
PLA-O + Asn ¹³ -CH	-	-	-	+	+
Mellitin, Hyaluronidase	-	-	-	+	+

CH: Carbohydrate

seems to inhibit the response of clone MeT3.2E5 in an epitope-specific manner. Since both experiments were performed with the same Ag and identical APC, the inhibition by anti-carbohydrate antibody suggests that this moiety might be involved in the TcR/Ag/MHC interaction.

All of the clones showing glycosylation-dependent proliferation recognized their antigenic determinant in the context of the autologous HLA-DR molecules, since antibody to DR, but not to DP or DQ, inhibited Ag-induced proliferation, and since autologous, but not unmatched control APC were able to induce T cell proliferation (data not shown). This shows that these T cells follow the accepted rules of MHC class II-restricted Ag recognition. Since none of the 120 dodecapeptides overlapping at 10 positions and none of additional 18 to 25 mer peptides [13] could activate the cells, we exclude the possibility that different processing of the glycosylated and non-glycosylated forms as the primary reason for the non reaction.

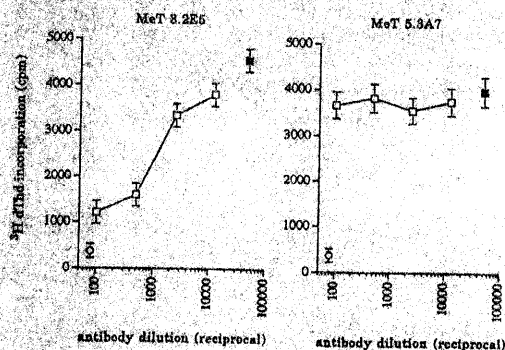


Figure 4. Inhibition of PLA-induced proliferation by antibody reacting with the carbohydrate moiety. Glycosylation-dependent clone MeT 3.2E5 and control clone MeT 5.3A7 were incubated with the same APC and suboptimal doses of glycosylated PLA in the absence (filled symbols) or presence (open symbols) of antibody. Circles show background proliferation in the absence of antigen.

3.3 Influence of glycosylation on primary PBMC proliferation

To test the prevalence of this particular specificity among the PLA-specific circulating T cells, we measured the proliferative response of mononuclear cells from donors Bu, Re and Me. Stimulation indices obtained with optimal concentrations of glycosylated and non-glycosylated natural PLA are depicted in Fig. 5. PBMC from donor Re, from whom two glycosylation-dependent T cell clones have been derived, showed a threefold higher response to the glycoprotein compared to its carbohydrate-free variant. This indicates that a significant portion of peripheral T cells exhibit the same specificity as the particular clones. A significantly increased proliferative response to glycosylated versus non-glycosylated PLA was also observed with donor Me, although responses were generally low. In contrast, PBMC from donor Bu, from which two peptide-reactive but no glycosylation dependent clones had been isolated, proliferated equally to both glycosylated and non-glycosylated Ag. This confirms that glycosylation does not generally affect the efficiency of presentation of immunogenic peptides and points to the possibility that glycosylation-dependent recognition of PLA represents a major T cell specificity in some individuals.

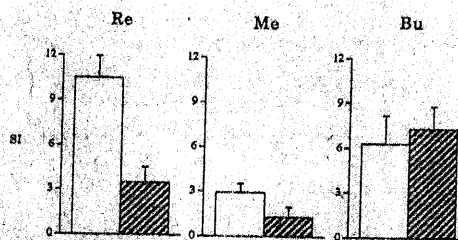


Figure 5. T cell proliferation of unfractionated PBMC from donors Re, Me and Bu. White bars represent the SI $\pm$ SD induced by glycosylated PLA, hatched bars show the same values obtained with carbohydrate-free Ag.

4 Discussion

The principle ability of glycopeptides to bind class II MHC molecules and to elicit specific T cell responses has recently been demonstrated in two murine model systems. Using synthetic glycopeptide analogues of an OVA-derived immunogenic peptide, Ishioka et al. [7] provided evidence that the carbohydrate moiety can be an important part of the antigenic structure recognized by T cells. Similarly, Harding et al. [8] reported MHC binding of a disaccharide linked to the N-terminus of an immunogenic T cell peptide determinant from lysozyme and the isolation of T cell hybridomas specific to the glycopeptide conjugate. In this latter instance, the results suggested no direct involvement of the carbohydrate in the interaction between glycopeptide and TcR, but rather the recognition of a peptide conformation specific to the glycopeptide/MHC complex.

In this report we show that several independently derived PLA-specific T cell clones from different donors, which cannot be stimulated by a large panel of overlapping peptides representing the entire PLA sequence, respond to the glycoprotein, but not to its non-glycosylated variants. Since control clones specific to PLA-derived peptides proliferated equally in response to glycosylated or non-glycosylated Ag, we propose that the glycosylation-dependent proliferative response reflects the recognition of a carbohydrate-dependent epitope. Additional evidence is provided by the selective inhibition by anti-carbohydrate antibody of clone MeT3.2E5, but not of peptide specific control clone MeT5.3A7.

We further show that a preferential response of peripheral T cells to glycosylated PLA can be observed in some allergic patients. In contrast to this observation, there are few reports describing carbohydrate-dependent T cell recognition, suggesting that this specificity is of minor importance in the immune response to glycoproteins. In our case, the occurrence of glycosylation-dependent T cell recognition may be explained by the unusual structure of the PLA-linked oligosaccharide which has not been found in mammals [17, 19]. Interestingly, the carbohydrate structure of PLA has also been shown to represent a major focus of the antibody response in both rabbits [17] and humans [20].

Regarding the molecular basis of the MHC/Ag/TcR interaction, it is not possible to determine whether the carbohydrate moiety of PLA is directly involved in the glycopeptide/TcR interaction or whether it merely introduces conformational constraints for T cell recognition. However, detailed structural analysis revealed the existence of at least 14 isoforms [19] of the N-linked saccharide, raising the possibility either that subsets of these isoforms are recognized by a particular T cell, or that the common structural carbohydrate backbone is involved. The latter may be more plausible since treatment of native PLA with α -mannosi-

dase inhibits recognition. Further investigations will be necessary to determine whether it is indeed the unusual carbohydrate structure of PLA not present in mammalian cells which confers this marked immunogenicity. It will also be of interest to investigate whether this type of T cell recognition might influence the allergenic potential of PLA, since the glycopeptide-dependent T cell clones and the carbohydrate-dependent recognition by PBMC was found coincidentally in allergic individuals.

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