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Specificity of Glycopeptide-Specific T Cells[1]

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We examined the specificity of glycopeptide-specific CD4 T cells following procedures similar to those previously reported by us. The disaccharide galabiose (Galα1–4Gal) was attached to the middle of the 52–61 peptide of hen egg lysozyme. This peptide is well known to bind to I-A^k molecules. CBA/J mice were immunized and T cell hybridomas were derived from the popliteal lymph node T cells. For this study, we selected hybridomas that recognized galabiose conjugated to 52–61 at residue Ser 56. We demonstrate here that these hybridomas showed specificity for galabiose and not cellobiose (Glcβ1–4Glc). Peptides containing galabiose at residue 53 did not stimulate the T cell hybridomas and neither did galabiose conjugated to the 34–45 peptide of HEL. Acetylation of the hydroxyl groups of the disaccharide resulted in loss of T cell reactivity. These results need to be contrasted with those in which the T cells were directed to galabiose, attached to the amino terminus of 52–61 or to Ser at residue 53. With these results, the fine specificity of recognition of the disaccharide was not apparent. Our results indicate two sets of glycopeptide-specific T cells. One is probably induced by a conformational change induced by the disaccharide on the peptide bound to class II MHC molecules. The second set contains elements of specificity for both the disaccharide and the peptide. *The Journal of Immunology,* 1995, 155: 1074–1078.

Polysaccharides bind poorly to MHC molecules (1–3), which may explain their poor capacity to induce T cell responses. However, glycopeptides that bind to MHC molecules can elicit a T cell response, which is in part directed to the carbohydrate moiety (2–5). To understand the reaction to glycopeptides, it is important to establish the fine anti-carbohydrate specificity of such T cells, their requirements for recognition of the carrier peptide, and their MHC restrictions. Conceivably, these T cells could be a component of the response to microbial glycoproteins processed by APC. If properly induced, such T cells could also be of importance by reacting against repeating carbohydrate epitopes displayed on the surfaces of microbes and neoplastic cells, which could be stimulatory in the absence of MHC molecules. There are precedents for such a case with T cells directed to haptens (6, 7).

In a recent study, we established that the disaccharide galabiose (Galα1–4Gal) attached to the amino terminus of the I-A^k binding peptide from hen egg white lysozyme (HEL)[3] encompassing residues 52–61 (DYGILQINSR), elicited carbohydrate-specific T cells (2). Fine analysis disclosed that these T cells were most likely elicited by conformational epitopes of the (52–61)–I-A^k complex induced by the disaccharide moiety. Here, we report a detailed analysis with T cells now directed to galabiose attached to the middle of the 52–61 peptide. Importantly, these have a completely different set of specificities, being highly specific to the disaccharide moiety itself. These T cells recognize elements of both the carbohydrate and the peptide, in an MHC-restricted way. The possible role of such T cells in an antimicrobial response should be considered.

Materials and Methods

Peptides and glycopeptides

The main peptide selected for this study was that encompassing residues 52–61 of HEL. Table I shows its amino acid composition as well as that of a second peptide from residues 34–45. In the table, the arrows indicate the TCR contact residues.

The glycopeptides were synthesized as follows: peptides glycosylated on serine were prepared from N^{α}-(9-fluorenylmethoxycarbonyl)-3-*O*-[2,3,6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-β-D-galactopyranosyl]-L-serine (8) and N^{α}-(9-fluorenylmethoxycarbonyl) 3-*O*-[2,3,6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-β-D-glucopyranosyl]-L-serine pentafluorophenyl ester by 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase synthesis essentially as described

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[3] Abbreviation used in this paper: HEL, hen egg white lysozyme.

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Table I. *HEL glycopeptides and peptides tested*

(52–61)	D Y G I L Q I N S R
S53-(52–61)	– S————
S56-(52–61)	———— S————
S40-(34–45)	F E S N F N S Q A T N R

Substitutions at *, **, ***, ⊛:
4-*O*-α-D-galactopyranosyl-β-D-galactopyranosyl residue
* Gal_2-(52–61)
** Gal_2-S53-(52–61)
*** Gal_2-S56-(52–61)
⊛ Gal_2-S40-(34–45)
4-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl residue
* Glc_2-(52–61)
*** Glc_2-S56-(52–61)
2,3,6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-β-D-galactopyranosyl residue
* Gal_2-Ac-(52–61)
** Gal_2-Ac-S53-(52–61)
*** Gal_2-Ac-S56-(52–61)

previously (8). After completion of the synthesis, the *O*-acetylated glycopeptides were cleaved from the resin and purified by reversed-phase HPLC. The *O*-acetyl groups were then removed in saturated methanolic ammonia and fully deprotected glycopeptides were obtained after HPLC purification. Chemical structures were confirmed with amino acid analysis, 500 MHz ^{1}H NMR spectroscopy, and fast-atom bombardment mass spectroscopy. In our previous study (2), the carbohydrate moieties were linked to the peptide amino terminus and used mercaptopropionic acid as a spacer (8, 2).

All of the glycopeptides were found to bind to I-A^k molecules. This was tested by competition assay, either in T cell bioassays or by direct chemical binding. In the T cell assay, the glycopeptides were added to an assay with the hybridoma 3A9 specific for residues 48–62 of HEL. The amount of HEL peptides added ranged from 0.1 to 3.0 μM. All glycopeptides competed as efficiently as the lysozyme peptide 52–61. In the biochemical assay, the unlabeled peptides were added to the binding reactions using a standard I-A^k binding peptide of lysozyme residues 52–61 but with Tyr and glutamic acid added at their amino terminus (YEDYGILQINSR). This peptide was labeled with ^{125}I. All of the glycopeptides tested competed but required a 10- to 20-fold excess of the unlabeled lysozyme peptide. Peptide at residues 34–45 of HEL or 52–61 also required a 10- to 20-fold excess. These I-A^k binding peptides were used effectively for immunization and to produce T cell hybridomas. The results with these I-A^k binding peptides are presented in *Results and Discussion*. The glycopeptide with Gal_2-S57-(52–61) did not bind in both assays and did not elicit a T cell response.

Immunization and cellular assays

CBA female mice, 12 to 16 wk of age (The Jackson Laboratory, Bar Harbor, ME) were immunized with 50 nmol of the conjugates in CFA. After 7 days, the popliteal lymph nodes were harvested and cultured with the conjugates (2). T cell hybridomas were produced by fusing using standard protocols to BW5147 cells (TCR-negative) (9). Chosen for examination were representative hybridomas showing the different patterns of responses. All hybridomas chosen for examination reacted with the glycopeptides presented by I-A^k molecules of APC. Ag presentation was inhibited by the anti-I-A^k mAb 10.3.62. Ag presentation was done by standard assays using the B cell lymphoma line M12 C3.F6 cells (10). A typical experiment involved culturing 5×10^4 C3.F6 with 10^5 T cell hybridomas and the indicated concentration of peptides for 24 h in 96-well trays in a total volume of 200 μl DMEM containing 10% FCS. Production of IL-2 was estimated by a bioassay using the CTLL-2 cell line (11). All clones were tested with the different peptides at least three times. The criteria for a poor response was usually a difference of 100-fold in peptide concentration.

Results and Discussion

The disaccharide galabiose was conjugated to the peptide encompassing residues 52–61 of the HEL molecule (Table I). The peptide 52–61 is part of the immunodominant peptide from HEL recognized by the MHC class II molecule I-A^k of H-2^k mice (11–13). Galabiose was conjugated to three residues known from previous studies to be TCR contact residues (12). Tyr at residue 53, Leu at 56, or Gln at 57 were changed to Ser, to which the disaccharide was covalently conjugated (8). CBA mice were immunized with each of the conjugates, after which the draining lymph node T cells were harvested, cultured, and hybridomas were derived from them (2).

Table II. *Summary of T cell reactivities to glycopeptides*

	T Cell Specificity				
	Gal_2-S56-(52–61)	Glc_2-S56-(52–61)	Gal_2-S53-(52–61)		Gal_2-(52–61)
Peptide	Clones 56.15.2 and S56.15.16	Clone SJC.4	Clone S53.4.7	Clone 53.2.4	Reference 2
(52–61)	–	–	+	–	–
(46–61)	–	–	–	–	–
Gal_2-S56-(52–61)	+	–	–	–	
Glc_2-S56-(52–61)	–	+			
Gal_2-S40-(34–45)	–				
Gal_2-Ac-S56-(52–61)	–				
Gal_2-S53-(52–61)	–		+	+	
Gal_2-Ac-S53-(52–61)			+	+	
Gal_2-(52–61)	–		–	–	+
Gal_2-(34–45)					–
Glc_2-(52–61)					+
Gal_2-Ac-(52–61)					+
Interpretation:	Gal_2 and 52–61 specific; MHC-restricted epitope	Glc_2 and 52–61 specific, MHC-restricted epitope.		MHC-restricted conformational change induced by adding Gal_2 on 52–61.	

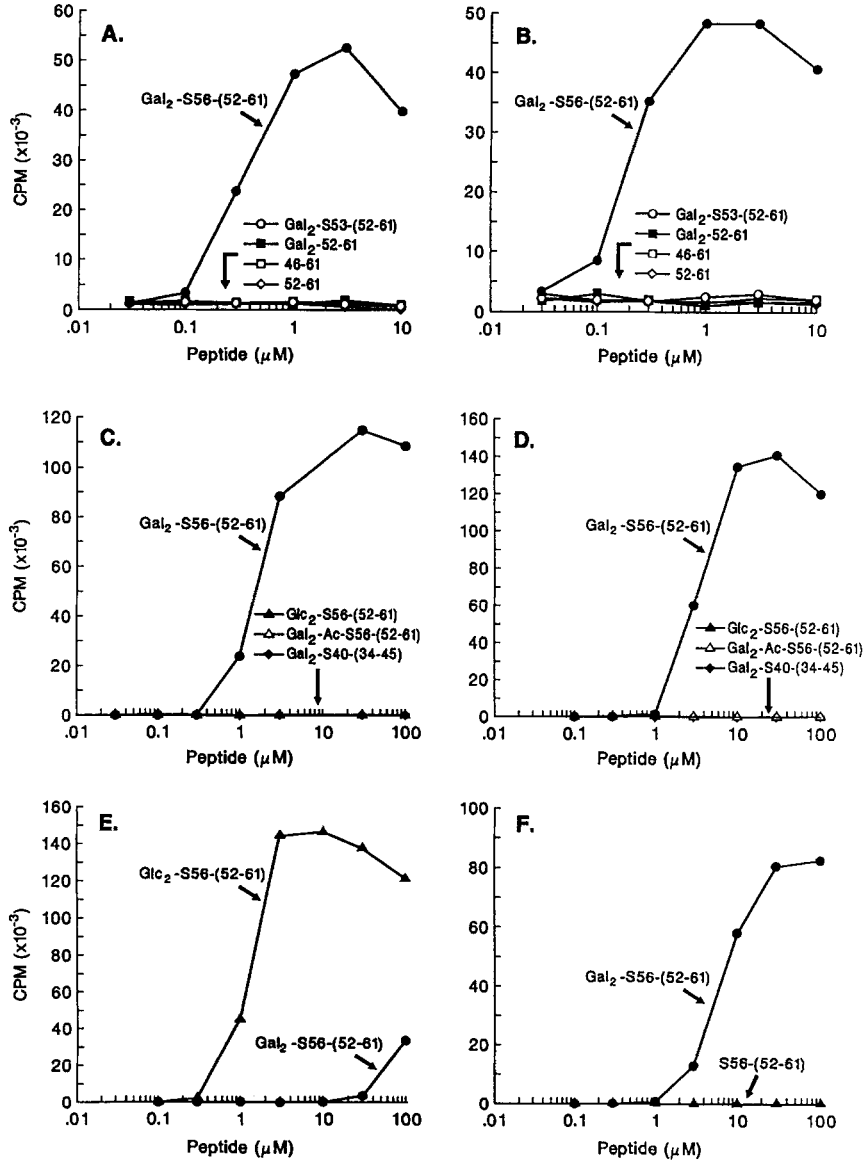

FIGURE 1. Response of T cell hybridomas directed to Gal_2-S56-(52–61) or Glc_2-S56-(52–61). The panels indicate representative experiments showing the different responses of T cell hybridomas induced by immunization with either of the conjugates. *A* and *C* are the results of one hybridoma directed to Gal_2-S56-(52–61) (clone S56.15.2), and *B*, *D*, and *F* are the results of a second one (S56.15.6). *E* shows the reaction of a hybridoma directed to Glc_2-S56-(52–61) (clone SJC.4). The two hybridomas directed to Gal_2-S56-(52–61) respond to it, but not to Gal_2-(52–61), Gal_2-S53-(52–61), 52–61, or 46–61 (*A* and *B*), or to Gal_2-Ac-S56-(52–61), Glc_2-S56-(52–61), or to Gal_2-S40-(34–45) (*C* and *D*). A clone reactive to Glc_2-S56-(52–61) recognizes the conjugate but not Gal_2-S56-(52–61) (*E*). This clone did not react with 52–61 or with 46–61 (not shown). A hybridoma directed to Gal_2-S56-(52–61) (used in *A* and *C*) does not recognize S56-(52–61) (*F*).

Selected for these studies were T cell hybridomas that preferentially recognized the disaccharide–peptide complexes. Hybridomas were obtained against Gal_2-S53-(52–61) (i.e., galabiose conjugated to 52–61 at Ser 53; Table I) and Gal_2-S56-(52–61) but not against Gal_2-S57-(52–61). The latter peptide was found to bind weakly to purified I-A^k. The major results of representative T cell hybridomas are summarized in Table II and are shown in Figures 1 and 2.

The majority of our studies examined hybridomas that are reactive with Gal_2-S56-(52–61). Two representative clones are shown in detail in Figure 1. These reacted with Gal_2-S56-(52–61), presented by the APC line C3.F6. C3.F6 is a cell from the M12.C3 line expressing I-A^k (10); as expected, these clones did not react when APC bearing I-A^d were used because I-A^d does not bind to Gal_2-S56-(52–61). These two clones are likely to be specific for the sugar moiety as well as for the carrier 52–61 peptide and

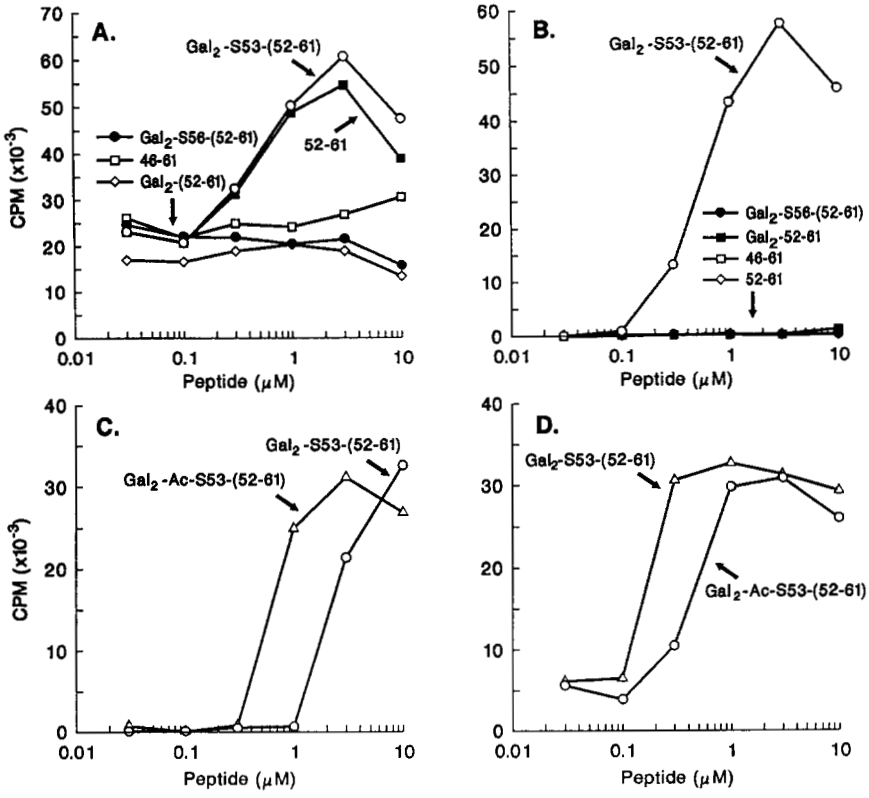

FIGURE 2. Response of T cell hybridomas directed to Gal_2-S53-(52–61). The panels show the response of the two hybridomas induced by immunization with Gal_2-S53-(52–61). *A* and *C* are clone 53.4.7, which responds equally well to Gal_2-S53-(52–61) and (52–61), but does not respond to Gal_2-S56-(52–61) or to Gal_2-(52–61) or 46–61. The other clone (53.2.4); *B* and *D*, only responds to Gal_2-S53-(52–61) and does not respond to the other peptides or to HEL 52–61. *C* and *D* show that both clones respond to the acetylated Gal_2 peptide.

I-A^k. Thus, both did not react with unconjugated short or long peptide (52–61, 46–61, or 52–61 containing a Ser at 56) or to the Gal_2 moiety placed either on S53-(52–61) or on the amino terminus of 52–61 (Fig. 1, *A* and *B*), or with Gal_2-S40-(34–45) (Fig. 1, *C* and *D*). The latter is a peptide that also binds to I-A^k (14). These two clones also did not recognize Gal_2-S56-(52–61), which had an acetylated disaccharide moiety, or 52–61, to which cellobiose (Glc β1–4Glc) was conjugated at S56(Glc_2-S56-(52–61)) (Fig. 1, *C* and *D*). To validate these latter results, clones were then generated to Glc_2-S56-(52–61). These clones reacted with it, but not with Gal_2-S56-(52–61) or 52–61 (Fig. 1*E*).

These results need to be contrasted with those reported before, when galabiose was placed on the amino terminus of 52–61 (2) (Table II). The set of clones directed to Gal_2-S53-(52–61) behaved very similarly to the previous results (Fig. 2). For example, note that clone 53.4.7 was not stimulated by Gal_2 linked to the amino terminus of 52–61, nor by 46–61 (while reacting to 52–61) (Fig. 2*A*). Clone 53.2.4 appears to be specific for the carbohydrate molecule (Fig. 2*B*), yet, as shown in Figure 2, *C* and *D*, neither it nor 53.4.7 was affected by acetylation of the OH groups, a most likely indication that they are not reacting to the saccharide moiety itself. In the more detailed study using hybridomas directed to Gal_2-(52–61), there was equal recognition of Glc_2-(52–61) (2).

Two additional issues were examined that are relevant for interpreting the results presented in the figures. First, all of the glycopeptides, except Gal_2-S57–52–61, bound to I-A^k (Table I). Second, repeated attempts failed to demonstrate directly interaction of the T cells solely with the disaccharide moiety. We attempted to inhibit Ag presentation with free galabiose (i.e., by the standard assays shown in Figure 1, pulsing with 0.1 to 1.0 μM peptide in the presence of up to 5 mM galabiose or other disaccharides: there was a variable inhibition at 2 mM which was entirely nonspecific in that the response to the 52–61 peptide by the hybridoma 3A9 was likewise inhibited). We also attempted, unsuccessfully, to stimulate the T cell hybridomas to galabiose bound to culture plates. (Different amounts, 16 mM to 0.01 mM, of galabiose with a mercaptopropionic acid spacer were attached to Covalink plates from Nunc; these contained reactive amino groups attached to their surface. We followed exactly the procedure detailed in Elofsson et al. (15). Hybridomas were incubated on the plates and the release of IL-2 was measured after 24 h.) Finally, prolonged incubation (up to 3 days) of the Gal_2-S56-(52–61)-reactive hybridomas with

galabiose in soluble form or bound to plates never inhibited their subsequent reaction to the glycopeptide presented by APC. We interpret these results to mean that the binding energy of the TCR interaction with the Gal_2 moiety is low.

In summary, the evidence from this and our other studies (2), as well as from the work of others (3–5), does indicate the presence of carbohydrate-specific T cells. Our own studies show two strikingly different sets of specificity. In one, the direct recognition of the disaccharide moiety is in question in that other disaccharides were recognized (2) and acetylation of saccharidic hydroxyl groups did not impair recognition. This took place when the disaccharide was placed on the amino terminus of the peptide or on the second amino acid from the amino terminus (i.e., the T cells directed to Gal_2-(52–61) or, like clone S53.2.4, to Gal_2-S53-(52–61); see Table II). Conceivably, the bulky sugar moiety may distort the MHC class II combining site, favoring its opening in ways that now produce a modified display of the 52–61 peptide. For the other set, the evidence indicates that there is fine discrimination of the disaccharide by T cells when the sugar moiety is displayed on residues in the middle of the peptide at known TCR contact residues (summarized in Table II). This is analogous to the hapten-specific T cells studied by others, in which there is indeed a fine recognition of the haptenic group (6, 7, 16–18). Ongoing studies are in progress to define further the specificity of the glycopeptide-specific T cells, i.e., vs Gal_2-S56-(52–61). Recently, class I MHC-restricted T cells were elicited against glycopeptides, and here the positioning of the carbohydrate was likewise of importance (4).

Carbohydrate-specific T cells could be involved in the immune response to glycoproteins that form part of the structure of relevant foreign Ags, such as bacteria, viruses, or tumor cells. Knowing that the carbohydrate moiety can indeed be part of the epitope recognized by CD4 T cells and CD8 T cells (4) necessitates the search for glycopeptides in class I or II MHC molecules following processing of foreign or autologous Ags.

References

1. Harding, C. V., R. W. Roof, P. M. Allen, and E. R. Unanue. 1991. Effects of pH and polysaccharides on peptide binding to class II major histocompatibility complex molecules. *Proc. Natl. Acad. Sci. USA 88:2740.*
2. Harding, C. V., J. Kihlberg, M. Elofsson, G. Magnusson, and E. R. Unanue. 1993. Glycopeptides bind MHC molecules and elicit specific T cell responses. *J. Immunol. 151:2419.*
3. Ishioka, G. Y., A. G. Lamont, D. Thompson, N. Bulbow, F. C. A. Gaeta, A. Sette, and H. M. Grey. 1992. MHC interaction and T cell recognition of carbohydrates and glycopeptides. *J. Immunol. 148: 2446.*
4. Haurum, J. S., G. Arsequell, A. C. Lellouch, S. Y. C. Wong, R. A. Dwek, A. J. McMichael, and T. Elliott. 1994. Recognition of carbohydrate by major histocompatibility complex class I-restricted, glycopeptide-specific cytotoxic T lymphocytes. *J. Exp. Med. 180: 739.*
5. Michaëlsson, E., V. Malmström, S. Reis, A. Engström, H. Burkhardt, and R. Holmdahl. 1994. T cell recognition of carbohydrates on type II collagen. *J. Exp. Med. 180:745.*
6. Ganju, R. K., S. T. Smiley, J. Bajorath, J. Novotny, and E. L. Reinherz. 1992. Similarity between fluorescein-specific T cell receptor and antibody in chemical details of antigen recognition. *Proc. Natl. Acad. Sci. USA 89:11552.*
7. Siliciano, R. F., T. J. Hemasath, J. C. Pratt, R. Z. Dintzis, H. M. Dintzis, O. Acuto, H. S. Shin, and E. L. Reinherz. 1986. Direct evidence for the existence of nominal antigen binding sites on T cell surface Ti α/β heterodimers of MHC-restricted T cell clones. *Cell 47:161.*
8. Elofsson, M., S. Roy, B. Walse, and J. Kihlberg. 1993. Solid-phase synthesis and conformational studies of glycosylated derivatives of helper T cell immunogenic peptides from hen-egg lysozyme. *Carbohydr. Res. 246:89.*
9. White, J., M. Blackman, J. Bill, J. Kappler, P. Marrack, D. P. Gold, and W. Born. 1989. Two better cell lines for making hybridomas expressing specific T cell receptors. *J. Immunol. 143:1822.*
10. Wade, W. F., Z. Z. Chen, R. Maki, S. McKercher, E. Palmer, J. C. Cambier, and J. H. Freed. 1989. Altered I-A protein-mediated transmembrane signaling in B cells that express truncated $I\text{-}A^k$ protein. *Proc. Natl. Acad. Sci. USA 86:6297.*
11. Allen, P. M., and E. R. Unanue. 1984. Differential requirements for antigen processing by macrophages for lysozyme-specific T cell hybridoma. *J. Immunol. 132:1077.*
12. Allen, P. M., G. R. Matsueda, R. J. Evans, J. B. Dunbar, Jr., G. Marshall, and E. R. Unanue. 1987. Identification of the T cell and Ia contact residues of a T cell antigenic epitope. *Nature 327:713.*
13. Nelson, C. A., R. W. Roof, D. W. McCourt, and E. R. Unanue. 1992. Identification of the naturally processed form of hen egg white lysozyme bound to the murine major histocompatibility complex class II molecule $I\text{-}A^k$. *Proc. Natl. Acad. Sci. USA 89:7380.*
14. Lambert, L. E., and E. R. Unanue. 1989. Analysis of the interaction of the lysozyme peptide HEL(34–45) with the $I\text{-}A^k$ molecule. *J. Immunol. 143:802.*
15. Elofsson, M., J. Broddefalk, T. Ekberg, and J. Kihlberg. 1994. Synthesis of a water-soluble serine-based neoglycolipid which can be covalently linked to solid phases. *Carbohydr. Res. 258:123.*
16. Hanna, N., and S. Leskowitz. 1973. Structural requirements for in vivo and in vitro immunogenicity in hapten-specific delayed hypersensitivity. *Cell. Immunol. 7:189.*
17. Hertel-Wulff, B., J. W. Goodman, C. G. Fathman, and G. K. Lewis. 1983. Arsonate-specific murine T cell clones. I. Genetic control and antigen specificity. *J. Exp. Med. 157:987.*
18. Baskar, S., G. H. Sunshine, and S. Leskowitz. 1990. The presentation of L-tyrosine-azobenzenearsonate by different mouse Ia molecules uses a common agretope. *Mol. Immunol. 27:79.*