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Effects of pH and polysaccharides on peptide binding to class II major histocompatibility complex molecules

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ABSTRACT **The binding of immunogenic peptides to class II major histocompatibility molecules was examined at various pH values. We studied binding of peptides containing residues 52–61 from hen egg lysozyme (HEL) to I-A^k on fixed peritoneal macrophages or to solubilized affinity-purified I-A^k. Optimum binding occurred at pH 5.5–6.0 with accelerated kinetics relative to pH 7.4; equilibrium binding was also higher at pH 5.5–6.0 than at 7.4. Similar enhancement at pH 5–6 was observed for the binding of hemoglobin-(64–76) to I-E^k and of ribonuclease-(41–61) to I-A^k. In contrast, the binding of HEL-(34–45) to I-A^k was minimally enhanced at acid pH. Dissociation of cell-associated or purified peptide–I-A^k complexes was minimal between pH 5.5 and 7.4, with increased dissociation only at or below pH 4.0 [HEL-(46–61)] or pH 5.0 [HEL-(34–45)]. Thus, optimum peptide binding occurs at pH values similar to the endosomal environment, where the complexes appear to be formed during antigen processing. In addition, we examined the effect of a number of polysaccharides on the binding of peptide to I-A^k. None of these competed with the HEL peptide ^{125}I-labeled YE52–61 for binding to I-A^k. [^{3}H]Dextran also failed to bind purified I-A^k. Polysaccharides do not appear to bind to class II major histocompatibility complex molecules, which explains the T-cell independence of polysaccharide antigens.**

Antigen processing involves the intracellular catabolism of proteins to produce immunogenic peptides that bind to class II major histocompatibility complex (MHC) molecules. A significant proportion of class II MHC molecules resides in endosomes (1–3), where binding of peptides appears to occur (2). Antigen processing involves late endosomes and lysosomes, since it is blocked at 18°C (4). Efficient processing occurs with liposome-encapsulated antigens that are sequestered until delivery and release into lysosomes (5). These data suggest that immunogenic peptides produced in lysosomes may be recycled to endosomes to meet and bind to class II MHC molecules.

Endosomes maintain an acidic luminal pH (about pH 6 for early endosomes and pH 5–5.5 in later endosomes, whereas lysosomes may attain pH 4.6–5.0). Alkalinization of the endosomal environment (e.g., by lysosomotropic amines or ionophores) disrupts endosomal/lysosomal processes (6), including antigen processing (7). In this context, class II MHC molecules would be expected to bind peptides efficiently at endosomal pH. Previously published data tend to support this hypothesis (8), but the effects of pH on peptide binding remain unclear in many aspects. Peptide dissociation has also been reported to be greater at acidic pH (pH 4.6–5.6) than at neutral pH (9), although other reports indicate that peptides remain stably bound in this pH range (10, 11).

We have studied (12–14) the binding of peptides to the murine class II MHC molecule I-A^k on whole cells and as a solubilized affinity-purified molecule (12–14). We have now investigated the potential role of pH in regulating the binding of peptides to class II MHC molecules. In addition, we have addressed whether certain polysaccharides might affect the binding of peptides to class II MHC molecules. This is an important issue in the context of the immunogenicity of carbohydrate antigens and in their effects on antigen processing (15). Indigestible carbohydrate substances, which accumulate in lysosomes, inhibit antigen processing.

MATERIALS AND METHODS

The effects of pH and polysaccharides on the binding of peptides to class II MHC molecules were tested with two binding assays: (*i*) on antigen-presenting cells, with binding indicated by the response of peptide–MHC-specific T-cell hybridomas and (*ii*) direct binding of radiolabeled peptides to purified class II molecules. We used the following antigen-presenting cells: the TA3 B-lymphoma cells (H-2^{kxd}) (16) or the activated peritoneal macrophages. The latter were elicited from CBA/J (H-2^k) mice by i.p. injection of *Listeria monocytogenes* and peptone (17) and cultured in plastic plates at 2×10^5 cells per well for 2 hr at 37°C in normal medium [Dulbecco's modified Eagle's medium with 10% (vol/vol) fetal calf serum, 50 μM 2-mercaptoethanol, and antibiotics] (18). The cells were then fixed with 1% paraformaldehyde (18), washed, and placed back in normal medium, 0.15 M citrate, or Hepes buffer at various pH values. Immunogenic peptides were added in unbuffered normal saline and incubated at 37°C for 2 hr (peptides were synthesized and purified as described) (19). The cells were then washed and T-hybridoma cells were added in normal medium. T-cell responses (interleukin 2 secretion) were measured using a CTLL proliferation assay as described (18). We used the following T-cell hybridomas: 3A9, specific for the HEL peptide HEL-(52–61) bound to I-A^k (18); 18.2 (subclone of 18N.30) or A6.A2, both specific for HEL-(34–45)–I-A^k (19); TS1.2, specific for bovine RNase-(43–56)–I-A^k (20); and Y01.6, specific for murine hemoglobin β^{dmin} (Hb)-(67–76)–I-E^k (21). For peptide dissociation experiments, the peptides were allowed to bind to fixed macrophages in normal medium. The cells were then washed, incubated at various pH values in the above buffers, and washed in regular medium prior to addition of T cells. For studies of the carbohydrates, fixed adherent macrophages or TA3 B-lymphoma cells were incubated with peptides in the presence of the carbohydrates, washed, and then incubated with T-hybridoma cells.

I-A^k was purified from cell membranes of CH27 lymphoma cells by affinity chromatography using the anti-I-A^k monoclonal antibody 10.3.6.2. (13, 22). The I-A^k was solubilized in binding buffer (phosphate-buffered saline containing 20 mM MEGA-8 and 20 mM MEGA-9) (Calbiochem). I-A^k–peptide binding was assayed as described (13) with some modifications. Briefly, purified I-A^k (about 45 pmol) was incubated for 2–48 hr at room temperature in binding buffer with the

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Abbreviations: MHC, major histocompatibility complex; HEL, hen egg lysozyme; Hb, murine hemoglobin β^{dmin}.

radioiodinated peptide HEL-(YE52–61) (about 1 pmol; 6 × 10^{18} cpm/mol), with or without lysophosphatidylcholine or lysophosphatidylserine (13). (At saturation, about 30% of the I-A^k molecules bind YE52–61.) Alternatively, binding was performed in 0.3 M Mes/20 mM MEGA-8/20 mM MEGA-9 at various pH values. Binding was assessed by Sephadex G-50 gel filtration (I-A^k-bound peptide appeared in the void volume) (13). Specific binding was 5–20% of the input cpm, and nonspecific binding (with excess unlabeled peptide) was generally 1% of input cpm. Alternatively, binding was assessed by Triton X-114 phase separation (23). The samples were added to 1 ml of 1% Triton X-114 at 4°C and warmed to 37°C for 10 min. The detergent phase was pelleted and washed with saline at 4°C. The samples were then rewarmed, pelleted, and washed as before. The final pellet contained I-A^k and associated molecules and the aqueous phase contained the unbound peptide. The nonspecific background (i.e., the amount of peptide in the detergent phase in the absence of I-A^k) was similar to that obtained with the gel-filtration assay.

Polysaccharides used included dextran T-500 (M_r, 500,000; Pharmacia), dextran T-2000 (M_r, 2,000,000; Pharmacia), sodium dextran sulfate (M_r, 500,000; Pharmacia); lipopolysaccharide from *Salmonella typhi* (Ribi Immunochem), capsular polysaccharide of *Neisseria meningitidis* (MCPS) (courtesy of K. Stein, Food and Drug Administration, Bethesda, MD), pneumococcal capsular polysaccharide (from J. H. Humphrey, Royal Postgraduate Medical School, London), heparin (Sigma; M_r, 5000), Ficoll 400 (Pharmacia), phenolic glycolipid from *Mycobacterium leprae* (from P. Brennan, Colorado State University, Fort Collins, CO), and lipoarabinomannan B from *M. leprae* and *Mycobacterium tuberculosis* (from P. Brennan). Also included were a number of monosaccharides and oligosaccharides, all obtained from Sigma: maltose, lactose, maltose 1-phosphate, thiodigalactose, maltotriose, stachyose, sucrose, *N*-acetylneuraminosyllactose, and uridine 5′-diphosphogalactose. Finally, we also evaluated dextran oligomers (of 5, 10, or 15 saccharide units) produced by J. Baenziger (Washington University). All carbohydrates were tested for inhibition of antigen presentation [ovalbumin-(323–339) presented by I-A^d to the T-cell hybridoma 3DO54.8 or Hb-(64–76) presented by I-A^k to Y01.6] and for competition with YE52–61 for binding to purified I-A^k. [^{3}H]Dextran (M_r, 70,000; 214 mCi/g; 1 Ci = 37 GBq) was from Amersham. Its binding to I-A^k was examined using the Triton X-114 assay.

RESULTS

Effects of pH on HEL-(46–61) or HEL-(YE52–61) Binding to I-A^k. The binding of peptides to I-A^k was examined at various pH values using either the antigen-presentation assay or the direct-binding assay with purified I-A^k. Fixed peritoneal macrophages were incubated at 37°C with HEL-(46–61) in buffers of various pH values and then washed thoroughly. 3A9 T-hybridoma cells (specific for I-A^k and the 52–61 epitope within this peptide) were then added, their response serving as a measure of binding during the peptide incubation (Fig. 1). Binding of HEL-(46–61) to I-A^k occurred at pH 7.4 but was enhanced at more acidic pH, with optimum binding at pH 5.5. The kinetics of peptide binding were markedly accelerated at pH 5.5 (Fig. 1*C*).

The effect of various pH values on the binding of peptide to purified detergent-solubilized I-A^k was examined using a HEL peptide, ^{125}I-labeled YE52–61 (12, 13), that binds I-A^k with affinity similar to HEL-(46–61). HEL-(52–61) represents the minimal-size peptide that binds and is recognized by 3A9 cells. After the binding incubation at room temperature, Sephadex G-50 gel filtration was used in most experiments to separate free peptide (included volume) and peptide bound to

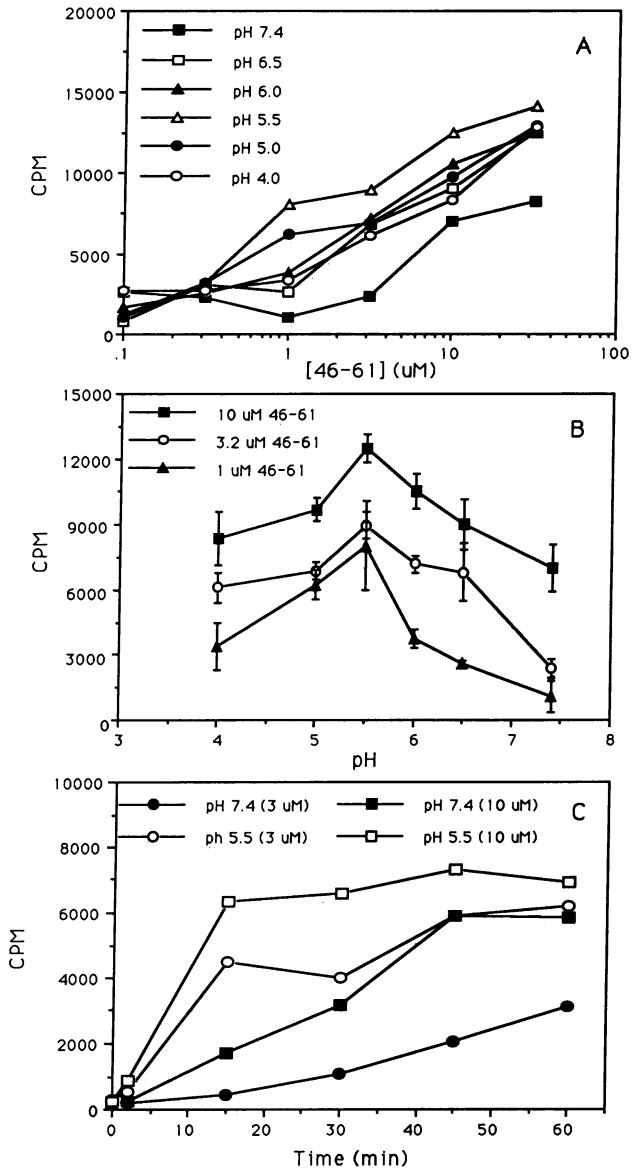

FIG. 1. Effects of pH on the binding of HEL-(46–61) to cell-associated I-A^k. (*A* and *B*) Fixed peritoneal macrophages were incubated with peptide for 2 hr at various pH values and then washed. 3A9 T-hybridoma cells, specific for this peptide bound to I-A^k, were then added. Secretion of interleukin 2 by the T cells, assessed by a CTLL proliferation bioassay, served as a measure of HEL-(46–61)–I-A^k complexes formed during the peptide incubation. *A* and *B* show data from the same experiment. (*C*) The kinetics of peptide binding were assessed as above except that the time of incubation with peptide was varied as indicated.

I-A^k (void volume) (Fig. 2). Relative to pH 7.4, binding at pH 5.5 occurred with both enhanced rate and increased equilibrium binding (binding at 48 hr approached equilibrium) (13) (Fig. 3). The optimum pH for binding was pH 5.5–6.0.

We also examined the effect of various pH values on the dissociation rate of peptide previously bound to I-A^k molecules, both purified and cell-associated. As reported (12), dissociation of isolated HEL-(YE52–61)–I-A^k complexes was exceedingly slow. Over 48 hr we observed minimal dissociation above pH 4. Only at or below pH 4 did we observe dissociation of the complexes (Fig. 4*A*). Similar results were obtained with cell-associated I-A^k. Fixed macrophages were incubated with HEL-(46–61) at pH 7.4 for 24 hr, washed, and incubated at various pH values for 24 hr. The cells were then washed and incubated with 3A9 cells. HEL-

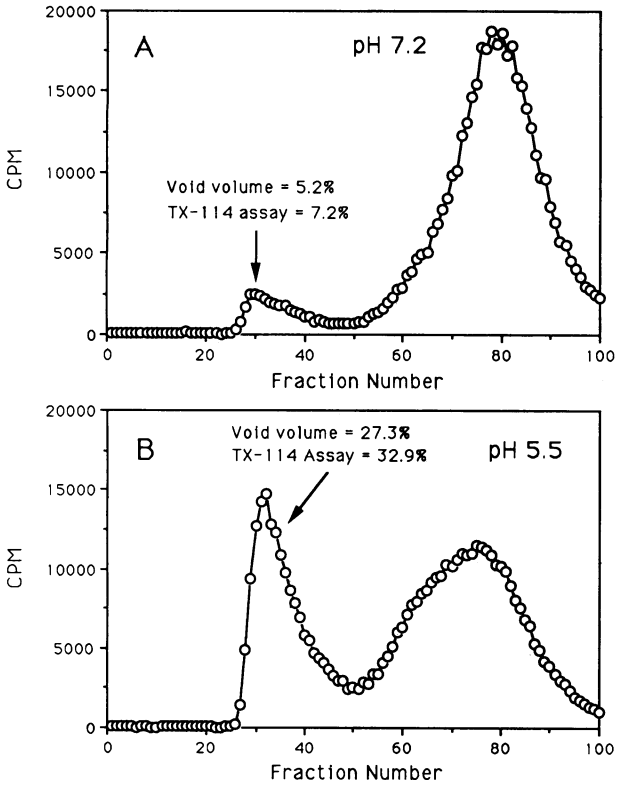

FIG. 2. Binding of ^{125}I-labeled HEL-(YE52–61) to purified I-A^k. Detergent-solubilized I-A^k was incubated with ^{125}I-labeled YE52–61 for 48 hr at pH 7.2 (*A*) or pH 5.5 (*B*), and binding was assessed by Sephadex G-50 gel filtration (bound peptide was eluted with I-A^k in the void volume, fractions 28–42). Binding was also measured by the Triton X-114 assay after 72 hr. The nonspecific background in the absence of I-A^k was 0.8% (in the void volume) or 1.5% (Triton X-114 precipitate). Other experiments showed similar levels of nonspecific binding in the presence of I-A^k plus excess unlabeled YE52–61.

(46–61)–I-A^k complexes remained stably expressed after incubations above pH 4.0. Only at pH 2.5–3.5 did we see loss of presentation of these complexes to the 3A9 cells (Fig. 4*B*).

Effect of pH on Binding of Other Peptides. There was also increased binding of both RNase-(41–61) and Hb-(64–76) at pH 5.0 using the antigen-presentation assay. With both of these peptides, the binding during a 1- to 2-hr incubation at pH 7.4 was minimal (Fig. 5). The same effect was found in the single experiment done using the direct binding assay with

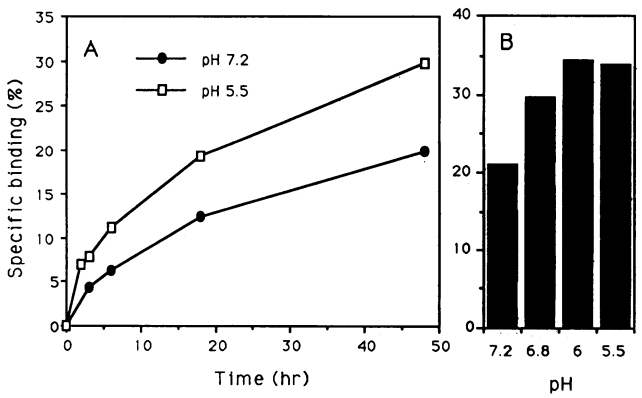

FIG. 3. Effects of pH on peptide binding to I-A^k. (*A*) Binding of ^{125}I-labeled HEL-(YE52–61) to I-A^k was determined (Fig. 2) after incubation at pH 5.5 or 7.2 for various periods. (*B*) Another experiment showing equilibrium binding (48 hr) of ^{125}I-labeled YE52–61 to I-A^k at various pH values. This preparation of I-A^k was from a different batch than that shown in Fig. 2.

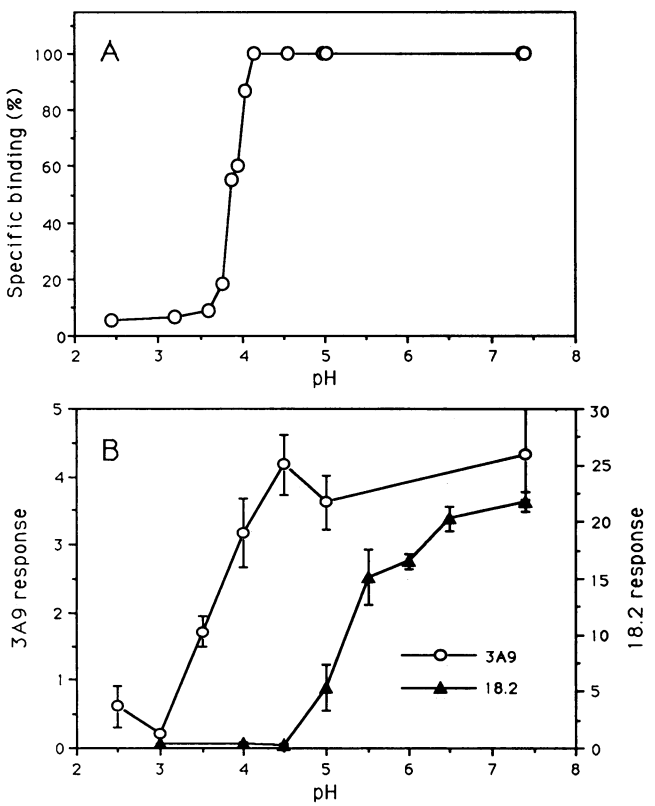

FIG. 4. Effects of pH on the dissociation of peptide–I-A^k complexes. (*A*) Purified ^{125}I-labeled HEL-(YE52–61)–I-A^k complexes were isolated by gel filtration chromatography (Fig. 2), concentrated, and then dialyzed at various pH values in the presence of excess unlabeled YE52–61. The level of binding remaining after dialysis is indicated. (*B*) HEL-(46–61) or HEL-(34–45) were allowed to bind I-A^k on fixed macrophages at pH 7.4. The cells were then incubated at various pH values, and T-hybridoma cells (3A9 or 18.2) were finally used to assess the level of persisting peptide–I-A^k complexes, expressed as cpm per 1000 cells.

RNase-(41–61). In contrast, binding of HEL-(34–45) to I-A^k assayed with two hybridomas, A6.A2 and 18.2, was similar over a wide pH range (pH 5.0–7.4) but declined below pH 5.0 (Fig. 5*C*). Cell-associated HEL-(34–45)–I-A^k complexes showed acid-induced dissociation beginning at pH 5.0 (Fig. 4*B*). Direct binding studies with HEL-(34–45) have been difficult and not reproducible because of aggregation of the peptide that interferes with the Sephadex G-50 and Triton X-114 assays.

Effect of Polysaccharides. All polysaccharides and monosaccharides failed to block presentation of exogenous peptides by fixed macrophages or B cells (including presentation by I-A^k, I-A^d, and I-E^k). None of the polysaccharides tested competed with or inhibited the binding of peptides to purified I-A^k as assessed by gel-filtration chromatography (Fig. 6). These encompassed a number of thymus-independent microbial polysaccharide antigens of biological relevance as well as other monosaccharides, oligosaccharides, and polysaccharides. Furthermore, direct incubation of [^{3}H]dextran with purified I-A^k revealed no significant binding. This was assessed by precipitation of I-A^k and associated molecules by Triton X-114 phase separation; this procedure gave results similar to gel filtration for the binding of HEL-(YE52–61) to I-A^k. In the absence of I-A^k, 0.025 ± 0.007% of the [^{3}H]dextran partitioned into the detergent phase. In the presence of I-A^k, this did not increase (0.015 ± 0.007% of the [^{3}H]dextran partitioned with I-A^k in the detergent phase). Thus these data suggest that polysaccharides generally do not bind to class II MHC molecules.

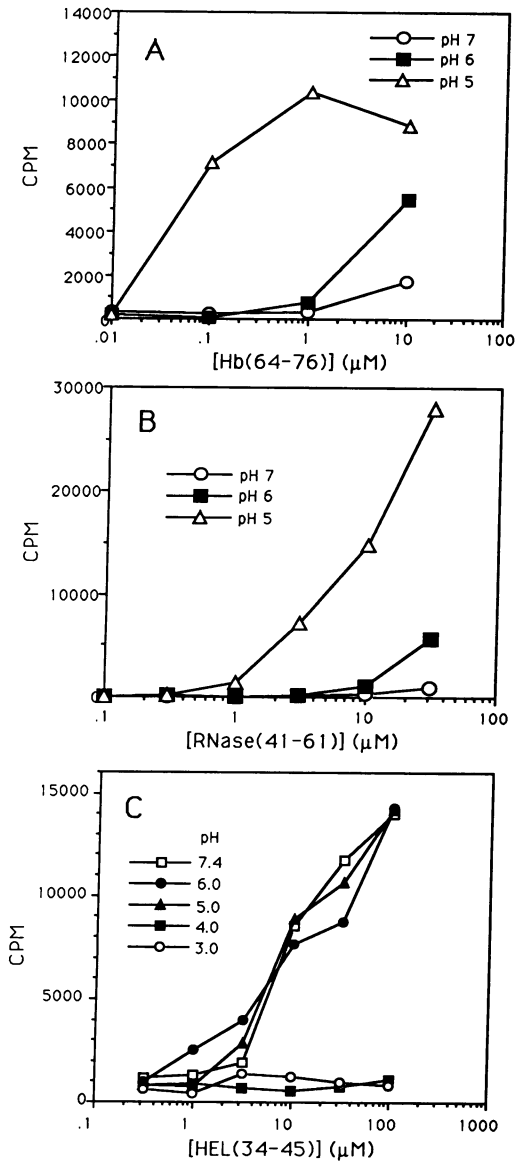

FIG. 5. Effects of pH on the binding of peptides to I-A^k and I-E^k. Binding to cell-associated I-A^k or I-E^k was determined at various pH values (Fig. 1). (*A*) Binding of Hb-(64–76) to I-E^k on fixed macrophages assessed with the YO1.6 T hybridoma. (*B*) RNase-(41–61) binding to I-A^k on unfixed macrophages (subsequently fixed), measured by the response of TS1.2 cells. (*C*) HEL-(34–45)–I-A^k complexes generated on fixed macrophages assayed with 18.2 cells.

DISCUSSION

Our results clearly demonstrate increased binding of HEL-(52–61) (or derivative peptides) to I-A^k, RNase-(41–61) to I-A^k, and Hb-(64–76) to I-E^k at an acidic pH (5.5) that is present within endosomes, in agreement with previous data (8). At pH 5.5, peptide–I-A^k complexes formed more rapidly than at pH 7.4. The equilibrium binding level was also somewhat enhanced at pH 5.5, suggesting a change in the affinity of peptide binding at this pH (Fig. 3). The kinetic enhancement of binding at pH 5.5 may be particularly significant, since binding kinetics limit the level of receptor-ligand binding in many systems (6), and this would be particularly true of peptide–class II MHC complexes, which form very slowly (9, 13).

The binding of various peptides may exhibit different pH dependence, and this may be one important determinant of the immunodominance of particular peptide epitopes. Increased binding of a peptide under endosomal conditions would favor dominance, whereas poor binding at pH 5.5 would limit the immunogenicity of a given peptide. There was a striking difference in the pattern of binding between HEL-(34–45) and the other peptides. HEL-(34–45) bound to I-A^k at acid pH, but this binding was not enhanced beyond the level seen at neutral pH. This difference may in part explain why priming/immunization with intact HEL elicits much more response to HEL-(46–61), which binds optimally at pH 5.5, than to HEL-(34–45).

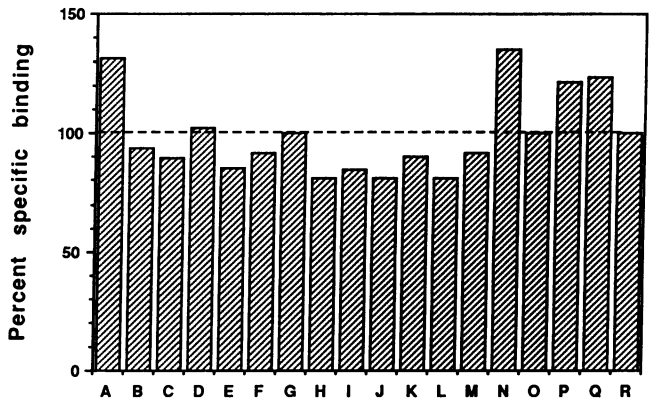

FIG. 6. Polysaccharides do not inhibit YE52–61 binding to I-A^k. The graph includes representative experiments in which the polysaccharides were added, usually at a 5000 M excess over the ^{125}I-labeled HEL-(YE52–61) peptide. The specific binding in the presence of the polysaccharides is expressed as the percentage of the specific binding obtained in their absence (addition of excess unlabeled YE52–61 reduced binding by 90% or more). Bars: A, dextran sulfate (46 mg/ml); B, dextran T-500 (46 mg/mg); C, dextran T-2000 (91 mg/ml); D, lipopolysaccharide (409 μg/ml); E, Ficoll 400 (8 mg/ml); F, pneumococcal capsular polysaccharide (8 mg/ml); G, polyvinylpyrrolidone (1.7 mg/ml); H, polyethylene glycol (3.3 mg/ml); I, phenolic glycolipid of *M. leprae* (12 mg/ml); J, dextran (glc5) (391 μM); K, dextran (glc10) (296 μM); L, dextran (glc15) (261 μM); M, lactose (150 μM); N, *N*-acetylneuraminosyllactose (150 μM); O, UDPgalactose (150 μM); P, stachyose (150 μM); Q, meningococcal polysaccharide (6.4 mg/ml); R, heparin (714 μg/ml).

The ability of a given peptide to bind at various pH values is presumably affected by the protonation of residues involved in peptide–class II MHC binding. For example, we observed abrogation of binding of HEL-(52–61) below pH 4.0. The binding of HEL-(52–61) (DYGILQINSR) to I-A^k is mediated primarily by three peptide residues: Asp-52, Ile-58, and Arg-61 (24). Of these three residues, only Asp-52 undergoes hydrogen ion titration in this range (model pK_a = 4.0) (25). Substitution of alanine for Asp-52 blocks binding to I-A^k (24), suggesting a requirement for a negative charge at this residue. Thus, protonation of Asp-52 could prevent binding of HEL-(52–61) to I-A^k below pH 4.0. However, analysis of all titratable residues in HEL-(52–61) does not explain the optimum of peptide binding at pH 5.5 and decline at pH 7.4. Therefore, it is likely that most of the pH effects on peptide binding are due to protonation/deprotonation of I-A^k residues and resulting conformational changes (actual pK_a values may be altered by protein conformation and near neighbor interactions) (25). These changes could differentially alter various specific peptide binding areas or pockets. Since different peptides bind to class II MHC molecules in different ways (26), perhaps utilizing different pockets (27), this would still allow for differential pH effects on the binding of various peptides to the same class II molecule.

We observed acid-induced dissociation of preexisting HEL-(YE52–61)–I-A^k complexes only at or below pH 4, although HEL-(34–45)–I-A^k complexes began to dissociate with pH as high as 5.0. Previous reports (10, 11) established the stability of other peptide–class II MHC complexes at endosomal pH, although Buus *et al.* (9) did report some

increase in dissociation at pH 4.6 and 5.6. The acid pH values found within vesicular compartments are not intrinsically sufficient to induce significant dissociation of most immunogenic peptide–class II MHC complexes. Any dissociation of these complexes within endosomes or on the cell surface would require additional active catalysis by other proteins. Weakly binding peptides, which may not be immunogenic, may dissociate at less acidic pH values.

The inability of polysaccharides to bind to class II MHC molecules explains the independence of the antibody response to pure carbohydrate antigens from the MHC-restricted T-cell regulation that is integral to protein antigen responses. Thymus-independent antigens induce an immune response in the virtual absence of T lymphocytes and predominantly include polysaccharides (e.g., bacterial capsular polysaccharides and lipopolysaccharides from Gram-negative bacteria) (for example, refs. 28–33). The cellular triggering of B cells is believed to take place without the participation of T cells as a result of the engagement of membrane immunoglobulin by the repeating epitopes of the polysaccharides and/or their intrinsic mitogenic property (as with lipopolysaccharide). The biochemical explanation of the lack of T-cell responses to these antigens has not been clear. Our data indicate that typical thymus-independent antigens do not bind to class II MHC molecules, depriving these antigen molecules of the capacity to engage the T-cell receptor of CD4 T cells and elicit T-cell help, delayed hypersensitivity reactions, and T-cell immunity.

A previous study (15) indicated that undigestible polysaccharides inhibited antigen processing by macrophages. Since class II MHC molecules themselves and antigen catabolism were unaffected by the polysaccharide inhibitors, their effect may be in a derangement of intracellular transport or lysosomal recycling mechanisms. Since immunogenic peptides may be generated in lysosomes, where these inhibitors accumulate, the inhibitors could block proposed peptide recycling mechanisms (5) to reduce the efficiency of delivery of peptide to the class II MHC-containing endosomal compartment. Unpublished experiments (C.V.H.) have indicated that polysaccharides do not affect antigen processing by B-lymphoma cells. The relative susceptibility of macrophages to these inhibitors may be due to their greater rate of fluid-phase uptake and a greater accumulation of the inhibitors within lysosomes.

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