Reduction of Disulfide Bonds during Antigen Processing: Evidence from a Thiol-dependent Insulin Determinant

By Peter E. Jensen

From the Department of Pathology, Emory University School of Medicine, Atlanta, Georgia 30322

Summary

Previous studies have demonstrated that insulin, like other protein antigens, requires processing in metabolically active antigen-presenting cells (APC) before it can be recognized by class II–restricted T lymphocytes. Unlike many other proteins, insulin peptides of minimal size retain the requirement for antigen processing. We demonstrate that this requirement can be bypassed by incubation of insulin with reducing agents in the presence of aldehyde-fixed APC. Fixed APC treated in this way were able to stimulate I-A^b- and I-A^d-restricted T cell hybridomas. Data are presented that demonstrate that cloned and polyclonal T cells recognize a determinant within the NH$_2$-terminal 14 residues of the beef insulin A chain with no requirement for B chain residues. The common feature among peptides capable of stimulating these cells in the presence of live APC is the chemical form of the cysteine thiol groups. Those forms that produce free thiols upon reduction are active, whereas those with irreversibly protected sulfhydryls are not. Functional experiments with fixed APC and competition binding experiments with purified I-A^d indicate that only A chain peptides with free thiols are able to stably associate with the peptide-binding site on class II in a form that is recognized by specific T cells. Our findings indicate that reduction of disulfide bonds is both necessary and sufficient for presentation of insulin to a major population of class II–restricted T cells. The results provide strong support for the hypothesis that protein disulfides can be reduced during physiologic antigen processing.

One important function of the endocytic pathway in APC is to alter the structure of protein antigens so that they may interact with class II histocompatibility glycoproteins to form stable complexes that can be recognized by helper T cells (1, 2). It is generally observed that native proteins interact poorly with class II molecules. The requirement for antigen processing is operationally defined by an inability to stimulate appropriate T cells in the presence of aldehyde-fixed APC or by sensitivity of antigen presentation to agents that raise the pH of acidic intracellular organelles. In most instances, peptides derived from antigen can directly interact with class II and therefore do not require further antigen processing (3–7).

Insulin has been an exception to this observation. Disulfide bond–containing peptides of insulin of minimal length to maintain antigenicity still require processing by the operational definition. Naquet et al. (8) have described a minimal determinant comprised of residues A(1–14) disulfide linked to B(7–15) that was recognized by I-A^d- and I-A^b-restricted, loop-reactive T cell hybridomas. Further truncation or cleavage of disulfide bonds in this peptide destroyed its antigenicity. The authors concluded that the T cells recognized a confor-

mational determinant containing essential residues from both the A and B chains of insulin. Others have described experiments in which mouse and human A chain loop-reactive T cells were stimulated by A chain peptides with no associated B chain residues (9–13). These results indicate that at least some loop-reactive T cells recognize determinants within the A chain. To date, no fragment of insulin has been identified that efficiently stimulates T cells in the presence of fixed APC or inhibitors of antigen processing. The case of insulin provides an apparent paradox, where antigenic determinants of minimal size require further processing. Therefore, alterations other than proteolytic cleavage must occur during immunologic processing of insulin.

In the present communication, we demonstrate that the minimal immunodominant T cell determinant recognized by beef insulin–immune H-2^d and H-2^b mice is contained within the A(1-14) segment of beef insulin and that the chemical status of one or more of the cysteine thiols is critical for maintaining peptide antigenicity. A chain peptides with free thiol groups are presented to T cells by fixed APC and therefore do not require further immunological processing. Results from experiments using purified I-A^d suggest that cysteine

sulfhydryls are involved both in the interaction of insulin peptides with the peptide-binding site on class II and in recognition by TCR. Our results support the hypothesis that the minimal processing event required for recognition of insulin by class II–restricted T cells is reduction of disulfide bonds, rather than cleavage by endopeptidases.

Materials and Methods

Cell Lines. The Ia-positive B cell hybridoma, TH2.2 (14), was generously provided by Dr. Richard Asofsky (Bethesda, MD). TH2.2 expresses I-A^b, I-A^d, and I-E^d. Insulin-specific T cell hybridomas were produced in this laboratory (15) from beef insulin–immune BALB/cBy and C57BL/10 SnJ mice. Bb-6.BC2 (Bb-6) is restricted by I-A^b and recognizes beef, but not pork, insulin. Bd-1.4 is restricted by I-A^d and is partially crossreactive with pork insulin. Cell lines were maintained in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, and 5 × 10^{-5} M 2-ME (R10).

Culture Conditions and Lymphokine Assay. Cultures were performed in flat-bottomed 96-well tissue culture plates in a final volume of 0.2 ml R10. T cell hybridomas (10^5/well) were cultured for 24 h in the presence of various antigens and treated or untreated TH2.2 APC. Fixed TH2.2 were prepared by incubation in PBS containing 1% paraformaldehyde for 15 min at 24°C followed by extensive washing in serum-containing media. Lymphokine production, reflecting T cell activation, was quantitated by using the IL-2-responsive cell line HT-2 (16). Culture supernatants (100 μl) were subjected to one freeze/thaw cycle and cultured with 10^4 HT-2 for 40 h. Each well was pulsed with 1 μCi of [^{3}H]thymidine during the final 16 h of culture. The results represent the mean ± SD cell-associated cpm from triplicate cultures.

Lymph Node Proliferation Assays. Mice were immunized subcutaneously with 50 μg beef insulin or 25 μg peptide in CFA. After 9–11 d, draining lymph nodes were removed and single cell suspensions were prepared. Assays were set up in 96-well tissue culture plates using RPMI 1640 supplemented with 0.5% normal mouse serum, 2 mM L-glutamine, 5 × 10^{-5} M 2-ME, 100 U/ml penicillin, and 100 U/ml streptomycin. Plates were incubated for 4 d at 37°C in a 5% CO$_2$ atmosphere, and cultures were pulsed with 1 μCi [^{3}H]thymidine for the final 18 h. Results represent the mean ± SD [^{3}H]thymidine incorporation of triplicate cultures.

Peptide Antigens. All insulin peptides and derivatives used in this study were from the beef insulin sequence. Beef insulin (INS) was purchased from Elanco Products Co. (Indianapolis, IN). Performic acid–oxidized (oxINS) and reduced and carboxyamidomethylated (rcamINS) beef insulins were produced as previously described (17). The sulfonated A chain of beef insulin (A[SSO$_3$]$_4$) (18) was purified from sulfonated insulin by reverse-phase HPLC using a C4 column and a CH$_3$CN gradient in 0.1% TFA. Purity was confirmed by microsequencing. The peptides A(1-14), GIVEQCCASVCSLY; B(1-30), FVNQHLCGSHLVEALYLVCG-ERGFFYTPKA; B(17-30), LVCGERGFFYTPKA; I(1-18), MDD-QRDLISNHEQLPILG; HEL(104-120), GMNAWVAWRNRC-KGTDVY; HEL(105-120), MNAWVAWRNRCKGTDV; and Myo(106-118), FISEAIIHVLHSR were synthesized in the Emory University Microchemical Facility as previously described (19). Cysteine was incorporated into peptides using temporary S-p-methoxybenzyl protection to produce free thiol groups after cleavage in liquid hydrogen fluoride. A(1-14)cm7 was synthesized with a permanent S-acetamidomethyl protecting group in the A7 cysteine. A(1-14)cm6,7,11 was produced by reaction of A(1-14)cm7 with

excess iodoacetamide. Air-oxidized forms of A(1-14) and A(1-14)cm7 were produced by stirring 0.5 mg/ml peptide in 50 mM Tris/HCl, pH 8.5, for 24 h at 24°C.

Class II Purification and Peptide-binding Assay. I-A^d and I-E^d were purified from detergent-solubilized A20 B lymphoma membrane preparations as previously described (20). Solubilized membranes were passed sequentially through Sepharose-4b, mouse IgG-Sepharose, MKD6-Sepharose (16), and 14-4-4-Sepharose (21). Immunoaffinity columns (2 ml) were prepared as described by Gorga et al. (22). After loading, the columns were individually washed with 100-column volumes of 0.5% NP-40, 0.5 M NaCl, 50 mM Tris, pH 8.0, followed by 50 vol of 50 mM Tris, pH 8.0, and 10 vol of 50 mM Tris, 0.15 NaCl, pH 8.0, containing 1% N-octylglucoside. Purified class II was eluted in 50 mM glycine, pH 11.0, containing 1% N-octylglucoside. The eluate was neutralized with 1 M Tris, pH 7.0, dialyzed, concentrated, and stored at 4°C in 1% N-octylglucoside.

HEL(104-120) and Myo(106-118) were biotinylated by reaction with a 2.5 mol excess of biotin-amidocaproate N-hydroxysuccinimide ester in N,N-dimethyl formamide, followed by acetone precipitation and HPLC purification (20). Biotinylated and unlabeled peptides were incubated with 40–80 nM I-A^d or I-E^d at 37°C in a final volume of 30 μl in the presence of 0.2% NP-40, 30–100 mM citrate/phosphate, 1 mM PMSF, and 2 mM EDTA. Sodium azide (0.01%) was included in some experiments. A sixfold molar excess of N-ethyl maleimide was added 60 min before immunoassay in experiments that included dithiothreitol (DTT)[1] during incubation with peptide. Samples were diluted to 200 μl in 200 mM Tris, pH 7.5, containing 5% skim milk, 0.1% Tween 20, and 0.5% NP-40. Class II/peptide complexes were separated from free peptide by incubation on microtiter assay plates coated with MKD6 (I-A^d) or 14-4-4 (I-E^d) mAb. Assay plates were prepared by incubation with 50 μg/ml affinity-purified goat anti–mouse Ig followed by incubation with 50–200 μg/ml purified mAb. Plates were blocked with 50 mM Tris, pH 7.5, containing 5% skim milk, 0.1% BSA, and 0.1% Tween 20, and thoroughly washed before sample addition. Bound biotinylated peptide was detected by incubation with 5 μg/ml avidin-alkaline phosphatase for 60 min at 24°C followed by incubation with 1 mg/ml p-nitrophenyl phosphate in 4 mM MgCl$_2$, 0.5 M Tris, pH 10.0, and measurement of absorbance at 405 nm. Nonspecific absorbance, measured in the presence of excess unlabeled peptide, was generally <5% of the maximum value in the assay and was equal to that observed in the absence of biotinylated peptide and independent of biotin-peptide concentration. Nonspecific absorbance was subtracted from the results. Binding, as measured by this assay, reflects the known specificity of peptide/class II interaction defined by functional assays and is saturable and dose dependent (20).

Results

Bypassing Insulin Processing Requirements by Use of Reducing Agents. We have used the beef insulin–reactive T cell hybridomas, Bd-1.4 and Bb-6, to define antigenic determinants recognized in association with I-A^d and I-A^b, respectively. These hybridomas appear to be representative of the major polyclonal populations induced by beef insulin in H-2^d and H-2^b mice. Despite its relatively small size, insulin must be processed in metabolically active APC before it can be recog-

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nized by class II–restricted T cells (8, 15). Processing is inhibited by chloroquine or prior fixation of APC. Disruption of the disulfide bonds in insulin by performic acid oxidation or reduction and alkylation destroys its capacity to stimulate cloned and polyclonal T cells (8, 12, 13, 17). This can be interpreted to indicate that an intact A chain loop structure or disulfide-linked B chain residues are essential components of the major determinant. We have performed experiments with peptides derived from chymotrypsin and protease V8 digests of insulin that indicate that while such peptides may retain the capacity to stimulate T cells in the presence of live APC, they do not stimulate in the presence of fixed APC (data not shown). We therefore considered the possibility that the insulin determinant may be generated by cellular reduction of disulfide bonds.

Indeed, the presence of reducing agent was found to obviate the need for live APC in the presentation of insulin to class II-restricted T cells (Table 1). Fixed TH2.2 B cells, incubated overnight in buffer containing insulin and 2 mM DTT, were able to activate Bb-6 and Bd-1.4 T cells. The reducing agent was necessary but not sufficient, in the absence of insulin, to mediate this effect. Similar results were obtained with reduced glutathione and 2-ME (data not shown). Two potential mechanisms were considered to explain the observed effect of thiol reagents. DTT might act through reduction of one or more disulfide bonds in insulin, allowing reduced derivatives to interact directly with cell surface class II glycoproteins. Alternatively, reducing agents may activate cell surface proteins, such as thiol proteases, that facilitate the formation of functional insulin/class II complexes. To address the latter possibility, fixed APC were treated with DTT, washed, and then exposed to insulin. Pretreatment with DTT was not sufficient to allow presentation of insulin by fixed APC (Fig. 1 a). This result was consistent with a mechanism involving reduction of disulfides in insulin rather than in cell surface proteins associated with the APC. Optimal T cell responses were observed when APC were exposed to

Table 1. *Bypassing the Insulin Processing Requirement by use of DTT*

Antigen	APC treatment		Bd-1.4	Bb-6
	DTT	APC		
			cpm × 10⁻³	
INS	No	Fixed	3.8 ± 3.7	1.3 ± 0.1
INS	Yes	Fixed	40.1 ± 1.2	40.1 ± 8.7
oxINS	No	Fixed	2.1 ± 2.2	0.7 ± 0.1
rcamINS	No	Fixed	0.8 ± 0.3	0.6 ± 0.1
INS	No	Live*	74.1 ± 4.2	64.6 ± 3.2

Fixed TH2.2 were incubated with 40 μM antigen ± 2 mM DTT in PBS for 18 h at 37°C, washed, and cultured with T cells. Results represent lymphokine production.
* T cells were cultured with live TH2.2 and 40 μM INS.

insulin in the presence of 0.1–1 mM DTT, representing ∼10-fold molar excess of DTT to insulin disulfides (Fig. 1 b). The concentration of 2-ME used in tissue culture media (5 × 10⁻⁵ M) is not sufficient to allow processing-independent presentation of insulin.

The Minimal T Cell Determinant Is Confined to the A Chain of Insulin. Various beef insulin derivatives were tested for their capacity to stimulate Bb-6 and Bd-1.4 T cell hybridomas in the presence of live APC. A chain peptides were capable of stimulating these T cells although their potency was generally reduced as compared to intact insulin. Sulfonated A chain, with reversibly protected sulfhydryls, stimulated both I-A^b- and I-A^d-restricted T cells to a level comparable to that observed with 3–10-fold lower concentrations of intact insulin. By contrast, rcamINS (Fig. 2), oxINS (Table 1), and the purified A chain from oxINS insulin (data not shown) do not stimulate these T cells even at high concentration. The latter preparations have irreversibly protected A chain thiol groups.

The importance of the thiol moiety is further emphasized by a comparison of the potency of air-oxidized forms of the synthetic peptide A(1-14) and A(1-14)cm7. Alkylation of the A7 sulfhydryl destroys the stimulatory activity of this peptide. The air-oxidized preparation of the A(1-14) peptide has three cysteine thiols that form intrachain and interchain disulfides producing a complex pattern on reverse-phase HPLC analysis. Air-oxidized A(1-14)cm7 is homogeneous on HPLC and contains a normal loop disulfide structure. The relative potency of these derivatives indicates that the status of the A7 cysteine sulfhydryl, which participates in an interchain disulfide bond in insulin, is critical, and that an intact loop is not sufficient for antigenicity. Indeed, the activity of sulfonated A chain suggests that an intact loop structure (23) is not an essential component of the T cell determinant. The common feature distinguishing stimulatory from nonstimulatory insulin derivatives is the capacity to regenerate free cysteine thiol groups in the A chain by reduction. The reduced A(1-14) peptide, by contrast to air-oxidized preparations, is essentially inactive when incubated with APC in serum-containing media. It is likely that the availability of this peptide is limited because of its potential reactivity with serum components. Together these results indicate that the minimal determinant recognized by Bb-6 and Bd-1.4 is contained within the first 14 residues of the A chain of insulin with no requirement for loop conformation or B chain residues.
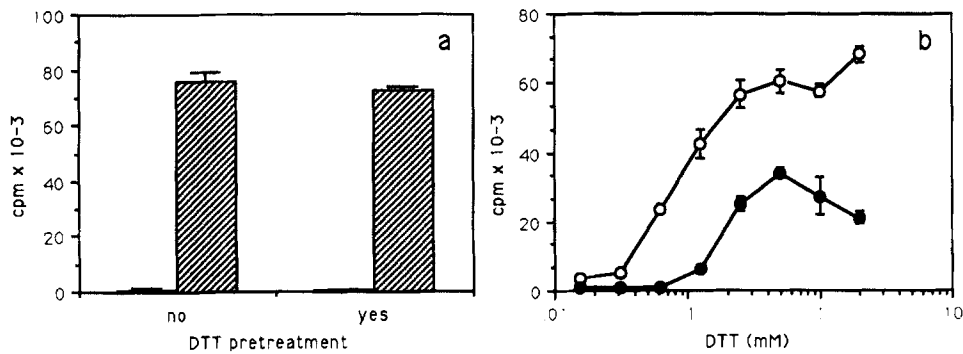
Presentation of A Chain Peptides by Fixed APC. The capacity of the reduced form of A(1-14) to form stable complexes with I-A^b and I-A^d was demonstrated in experiments with fixed APC (Fig. 3). Fixed TH2.2 B cells were pulsed with various concentrations of A(1-14) in the absence of serum, washed, and cultured with T cell hybridomas. The presence of DTT during the peptide pulse did not affect the capacity of the treated APC to stimulate T cells, in contrast to our results with insulin. This indicates that DTT does not mediate its effect on the presentation of intact insulin to Bb-6 and Bd-1.4 through alterations in the function of accessory molecules involved in antigen presentation. If this were the case,

Figure 1. Antigen presentation by fixed APC exposed to insulin and DTT. (a) Fixed TH2.2 were pretreated for 24 h at 37°C in PBS in the presence or absence of 2 mM DTT as indicated. Aliquots were washed and incubated for 18 h with 20 μM INS in the presence (hatched bars) or absence (open bars) of 2 mM DTT. After further washing, treated TH2.2 (10^5) were cultured with 10^5 Bd-1.4 T cells, and lymphokine production was measured as described in Materials and Methods. (b) Fixed TH2.2 were incubated for 18 h at 37°C in PBS containing 33 μM INS and various concentrations of DTT. After washing, treated TH2.2 (2 × 10^5) were cultured with Bd-1.4 (open symbols) or Bb-6 (closed symbols), and lymphokine was measured.
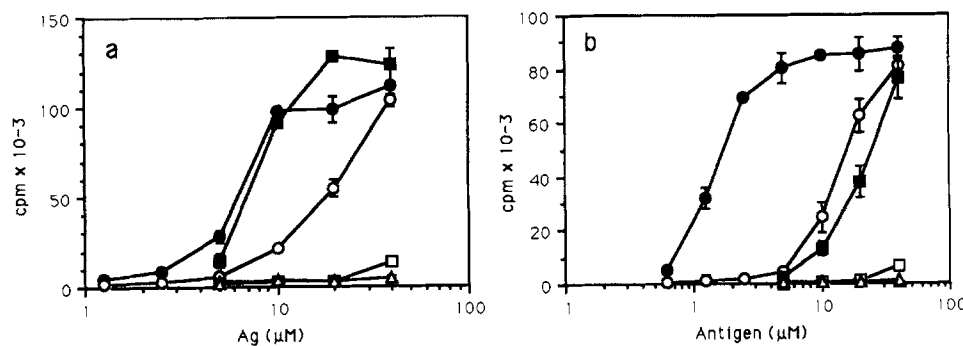

Figure 2. Specificity for an A chain determinant. Bb-6 (a) and Bd-1.4 (b) T cells (10^5) were cultured with 10^5 TH2.2 and various concentrations of antigen. INS ($\bullet$), A(SSO$_3$)$_4$ (O), oxA(1-14) ($\blacksquare$), A(1-14) ($\square$), rcamINS ($\blacktriangle$), oxA(1-14)cm7 ($\triangle$). Results reflect lymphokine production as described in Materials and Methods.

one would expect increased responses to the A(1-14) peptide after pulsing in the presence of DTT. Furthermore, it is evidence that the functional integrity of cell surface class II is not radically affected by DTT under the conditions used. However, this result does not exclude the potential activation of cell surface enzymes involved in processing insulin. The potency of reduced A(1-14) is equivalent to that of insulin/DTT in experiments with fixed APC (Fig. 3), confirming that all residues necessary for T cell recognition and MHC association are present within the peptide.

The importance of free thiols in the T cell determinant was emphasized by the observation that disulfide reduction was required for presentation of air oxidized A(1-14) by fixed

APC (Fig. 4, a and b). The requirement for DTT in the presentation of oxidized A(1-14) strongly suggests that the effect of reducing agents on the presentation of insulin is a result of reduction of disulfide bonds in insulin rather than activation of processing enzymes on the APC surface. It is evident that one or more free thiols in A(1-14) are required for interaction with class II or recognition by the TCR. The small response observed with Bd-1.4 and oxidized A(1-14) may reflect a fraction of the peptide preparation bearing the appropriate free sulfhydryl groups. Our results with A(1-14)cm7 (Fig. 2) suggested that the A7 thiol is important. It is possible that the appropriate structure includes an intact loop disulfide in combination with a free thiol at A7. However, it seems
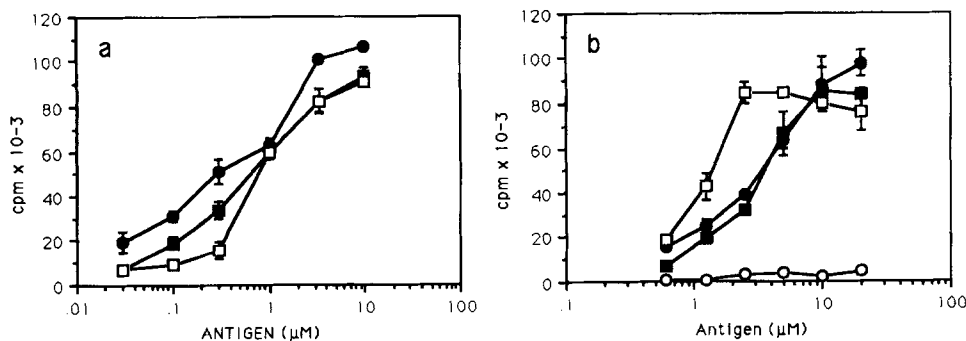
Figure 3. Reduced A(1-14) does not require processing. Fixed TH2.2 were incubated for 18 h at 37°C in PBS (a) or 0.15 M citrate/phosphate, pH 6 (b), with INS (O), INS + 2 mM DTT ($\bullet$), A(1-14) ($\square$), or A(1-14) + DTT ($\blacksquare$). Treated TH2.2 were washed and cultured with Bd-1.4 (a) or Bb-6 (b) T cells in separate experiments. The data represent lymphokine production.
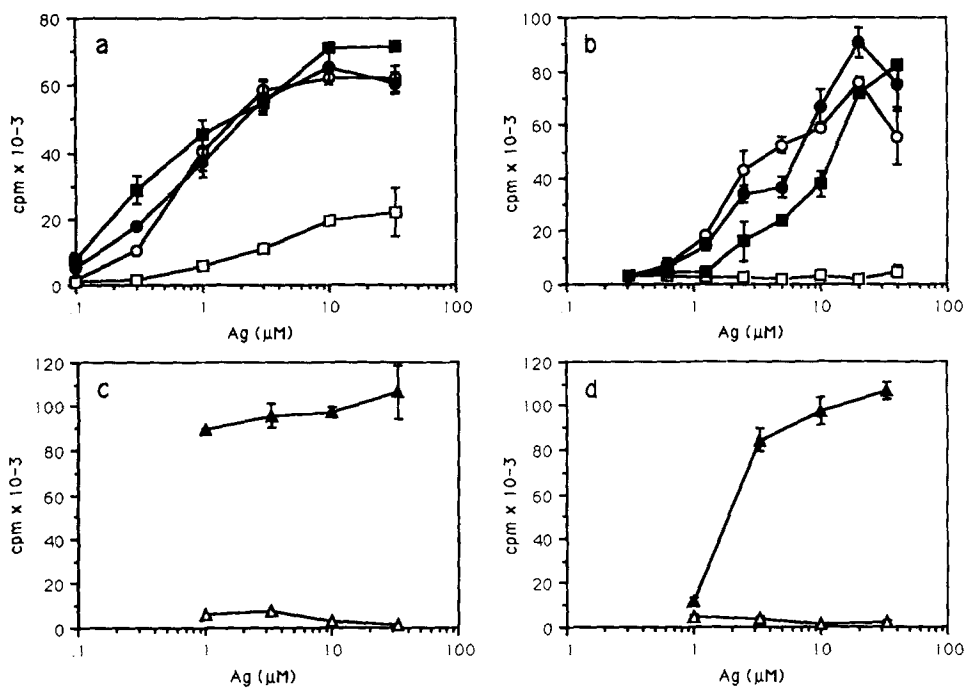
Figure 4. Processing requirements for reduced and oxidized A chain peptides. Fixed TH2.2 were incubated for 18 h at 37°C in 0.15 M citrate/phosphate, pH 5 (*a* and *b*) or pH 6 (*c* and *d*), with A(1-14) (○), A(1-14) + 2 mM DTT (●), oxA(1-14) (□), oxA(1-14) + DTT (■), A(SSO₃)₃)₄ (△), or A(SSO₃)₄ + DTT (▲). Treated TH2.2 (10⁵) were washed and cultured with 10⁵ Bd-1.4 (*a* and *c*) or Bb-6 (*b* and *d*), and lymphokine was measured.

unlikely that a disulfide bond between the loop cysteines, A6 and A11, would form in reduced A(1-14) in the presence of DTT. Results similar to those obtained with air-oxidized A(1-14) were observed in experiments using sulfonated A chain. Fixed APC pulsed with sulfonated A chain in the presence, but not the absence, of DTT were capable of stimulating Bd-1.4 and Bb-6 (Fig. 4, *c* and *d*).

Less time was required to generate functional antigen/class II complexes in the presence of DTT using A(1-14) as opposed to intact insulin (Fig. 5). This may in part reflect the time required to reduce disulfides and generate the appropriate structures necessary for binding class II (24). Binding was done at pH 6, a condition that does not favor rapid disulfide reduction. In addition, partial precipitation of insulin and reduced B chain occurs under these conditions. We have previously reported that peptide/class II complex formation is favored at acidic pH (20, 25). The observed kinetics of functional antigen/class II complex formation may reflect a balance between the effects of pH on antigen structure and on class II ionization states that may be important for peptide loading.

The A(1-14) Determinant Is Immunodominant in H-2^d and H-2^b Mice. The results described above using cloned Bd-1.4 and Bb-6 T cell hybridomas are representative of those obtained with several other I-A^d- and I-A^b-restricted T cell hybridomas (data not shown). Polyclonal lymph node proliferation assays were performed to determine whether the results obtained with the cloned T cells could be generalized to polyclonal populations. Beef insulin–immune H-2^d and H-2^b lymphocytes respond well to A chain peptides, but not to B chain (Fig. 6). The air-oxidized A(1-14) peptide was significantly more potent in stimulating secondary in vitro proliferation than was insulin. Reciprocal experiments with

A(1-14)-immune lymphocytes demonstrated that insulin and A(1-14) are fully cross-reactive. The results of these experiments clearly indicate that A(1-14), without additional B chain residues, serves as a major immunodominant determinant induced by immunization with insulin in these haplotypes. We and others have previously reported that performic acid–oxidized insulin and rcamINS do not stimulate insulin-immune T cells in proliferation assays (8, 12, 13, 17). Therefore, the results of experiments using polyclonal T cell populations are consistent with those obtained with T cell hybridomas.

Binding of Insulin to Purified I-A^d. The data presented above do not exclude potential thiol-dependent activation of processing enzymes on the surface of fixed APC that may act on intact insulin. To further address this possibility, the
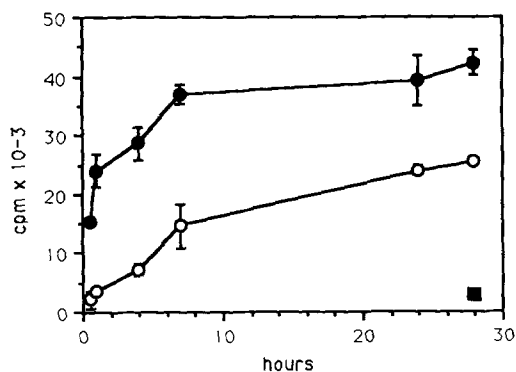
Figure 5. Kinetics of reduced peptide association. Fixed A20 B cells were incubated with 10 μM INS + 2 mM DTT (○), 10 μM A(1-14) + DTT (●), or DTT only (■) in 0.15 M citrate/phosphate, pH 6, at 37°C for the indicated time periods. The cells were washed, cultured with Bd-1.4, and T cell lymphokine production was measured.
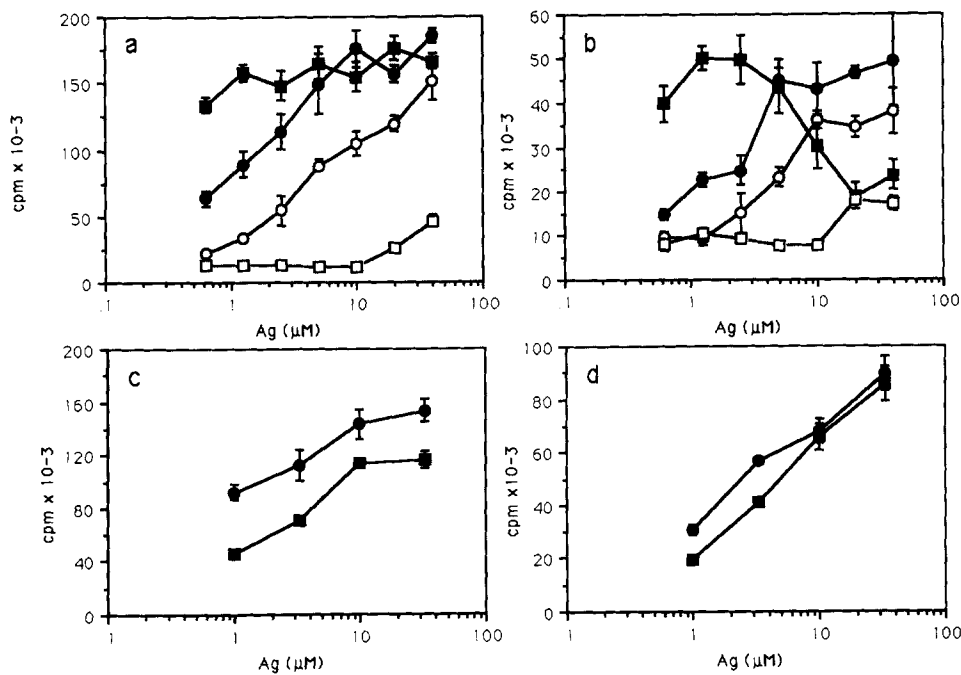
Figure 6. Response of polyclonal T cells to A chain peptides. Lymph node lymphocytes from INS-primed (*a* and *b*) or oxA(1-14)-primed (*c* and *d*) BALB/c (*a* and *c*) or C57BL/10 (*b* and *d*) donors were cultured with various concentrations of INS (●), A(SSO₃)₄ (O), oxA(1-14) (■), or B(1-30) (□) as described in Materials and Methods, and cell proliferation was measured.
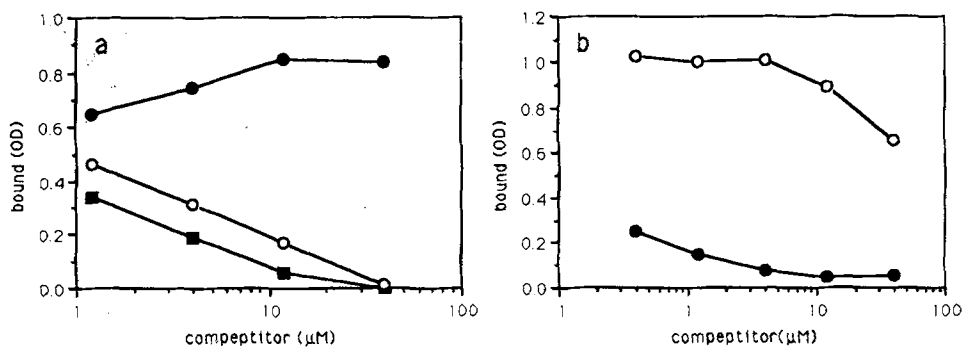
capacity of insulin to inhibit the binding of a biotinylated peptide to purified I-A^d was evaluated. We have recently described an immunoassay for measuring the binding of biotinylated peptide to purified class II glycoproteins (20). Binding, as measured by this assay, is saturable, inhibited by unlabeled peptide, and accurately reflects peptide specificity and pH dependence of complex formation as determined from functional assays. Insulin inhibited the binding of biotin-Myo(106-118) to purified I-A^d only in the presence of DTT (Fig. 7 *a*). DTT alone did not inhibit peptide binding under these experimental conditions, consistent with the previously documented stability of class II. No inhibition was observed in experiments where insulin was added after an initial incubation of biotin peptide with I-A^d (Fig. 7 *b*). This ruled out artifacts resulting from the potential effect of insulin/DTT in the immunoassay. DTT was neutralized with *N*-ethyl maleimide before immunoassay in these experiments. Control peptides did not inhibit binding, indicating that competition is specific (Fig. 7 *c*). These results support the interpretation that reduced insulin, but not intact insulin, interacts with the peptide-binding site in I-A^d.

The reduced A(1-14) peptide inhibited binding of biotin peptide to I-A^d in the absence of reducing agent (Fig. 8 *a*). No competition was observed with the thiol-containing control peptide, HEL(105-120). A(1-14) inhibited the binding of biotin-HEL(104-120) to I-E^d only at relatively high concentration, supporting the conclusion that A(1-14) interacts specifically with I-A^d (Fig. 8 *b*). T cells induced in H-2^d mice with beef insulin are generally restricted by IA^d and not IE^d (26). The role of sulfhydryls in MHC binding was further evaluated using alkylated forms of A(1-14). A (1-14)cm7 retained a capacity to efficiently inhibit binding of biotin-Myo(106-120) to I-A^d (Fig. 9). By contrast, A(1-14)cm6,7,11 was less effective in competition binding experiments. These
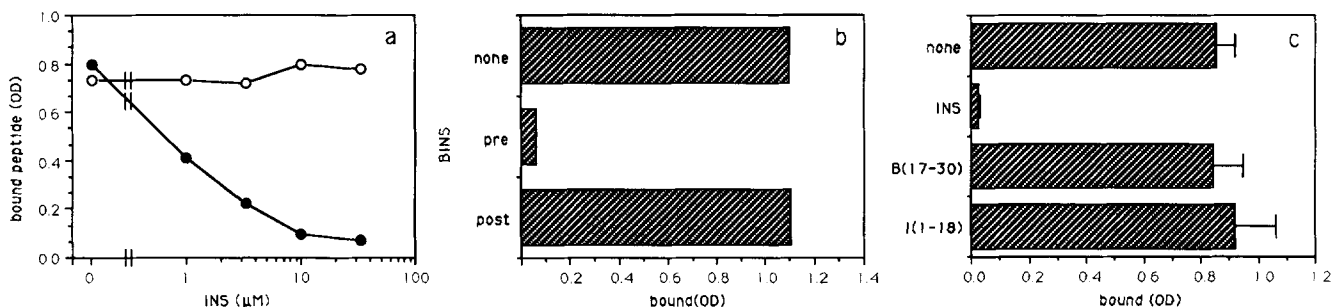
Figure 7. Reduced insulin inhibits binding of peptide antigen to purified I-A^d. (*a*) Purified I-A^d was incubated with 2 μM biotin-Myo(106-118) with various concentrations of INS in the presence (●) or absence (O) of 1 mM DTT for 48 h in duplicate, and biotin-peptide binding was measured by immunoassay as described in Materials and Methods. (*b*) Purified I-A^d was incubated with 2 μM biotin-Myo(106-118) and 1 mM DTT for 48 h in duplicate tubes. Insulin (40 μM) was added initially (pre) or 2 h before the immunoassay (post). (*c*) Purified I-A^d was incubated with 2 μM biotin-Myo(106-118), 1 mM DTT, and 40 μM INS, B(1-30), or I(1-18) as indicated for 48 h in triplicate tubes, and biotin peptide/I-A^d complexes were measured by immunoassay.

results, together with the functional experiments described above, suggest that the A7 cysteine thiol group may interact with the TCR, whereas the A6 or A11 (loop) thiols may be involved in interactions with class II.

Discussion

The data presented in this study demonstrate that a requirement for antigen processing can be bypassed by exposing fixed APC to insulin in the presence of reducing agent. Several experimental findings support the interpretation that the effect is mediated by reduction of insulin rather than APC membrane components. Pretreatment of fixed APC with DTT had no effect on a subsequent capacity to form insulin/class II complexes in the absence of reducing agent. An air-oxidized preparation of the minimal peptide, A(1-14), was not presented by fixed APC and therefore required further processing for functional association with class II. As with intact insulin, reduction of disulfides in oxidized A(1-14) with DTT was sufficient to bypass its processing requirement. No reducing agent was required for presentation for the reduced form of A(1-14) by fixed APC. More complex potential mechanisms were excluded in experiments using purified I-A^d. Intact insulin inhibited the binding of a biotinylated peptide antigen to purified I-A^d in the presence, but not the absence, of reducing agent. Under these experimental conditions, DTT can only act on insulin or class II. Results from functional

and binding experiments indicated that the reduced form of A(1-14) could interact directly with class II in the absence of reducing agent to form stable complexes that could be recognized by T cell hybridomas. We conclude that reduction of disulfide bonds in insulin is both necessary and sufficient to allow formation of functional insulin/class II complexes.

It is evident that reducing agents, present in the concentration range used in this study, have no major effect on the function of class II glycoproteins. This is not surprising given the previous demonstration of the unusual stability of the class II molecule. The α and β subunits remain associated even in the presence of SDS and 2-ME. Recent evidence suggests that peptide antigen remains associated with the class II complex under these conditions (27, 28). It is possible that class II disulfides are not reduced under the experimental conditions used in this study. Alternatively, class II conformation and function may be preserved after reduction of disulfide bonds. We cannot exclude the possibility that insulin peptides form covalent bonds that reduce cysteine in class II. This possibility seems unlikely because of the observed capacity of A(1-14) to bind class II in the absence of reducing agents, coupled with the lack of non–disulphide-linked cysteines in the extracellular domains of I-A^d and I-A^b (29, 30).

The common feature among insulin derivatives capable of stimulating I-A^d- and I-A^b-restricted T cells in the presence of live APC is the status of the cysteine thiols. Derivatives with disulfide bonds or sulfonated thiols are antigenic, whereas those with irreversibly protected sulfhydryl groups are not. We argue that previous results (8, 31), interpreted to suggest that disulfide-dependent conformation is an essential feature of the insulin determinant recognized by loop-reactive T cells, actually reflect a requirement for the potential to regenerate free sulfhydryl groups after reduction in antigen processing compartments. Results from polyclonal lymph node proliferation assays support the conclusion that the major immunodominant determinant recognized by beef insulin–immune H-2^b and H-2^d T cells is within A(1-14) with no requirement for disulfide-linked B chain residues. This conclusion is consistent with the results of Falcioni et al. (13), who demonstrated that >90% of a large panel of I-A^b-restricted beef insulin–reactive T cell clones responded to sulfonated A chain but not performic acid–oxidized insulin. Gradehandt et al. (11, 32) have demonstrated that A$_\alpha^b$A$_\beta^k$-restricted clones recognize an A(1-14) determinant that is sensitive to performic acid oxidation. It was proposed that processing of insulin
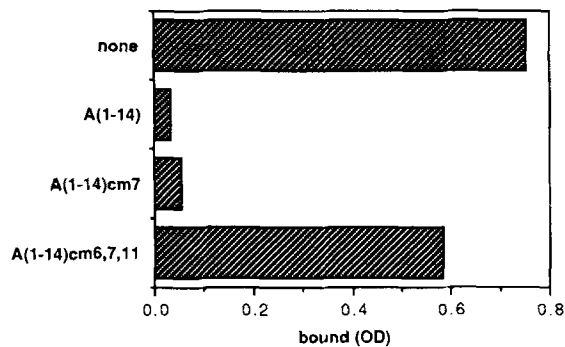
is not dependent on proteolysis, but rather on modification or conversion of disulfide bridges. Miller et al. (12) have described loop-reactive human T cell lines that respond to disulfide forms, but not alkylated derivatives, of isolated A chain.

By contrast, Naquet et al. (8) described a minimal peptide determinant, composed of A(1-14) disulfide linked to B(7-15), capable of stimulating loop-specific T cell hybridomas. Reduction or further proteolysis of this peptide markedly decreased its antigenicity. The data presented by these authors supported the interpretation that the T cells used in their study recognized a conformational determinant requiring residues from both the A chain and the B chain of insulin. Given the current findings, we suggest that the status of cysteine sulfhydryls, rather than conformation maintained by disulfide bonds, is the critical element for antigenicity. A chain peptides with reversibly protected thiols were not used in their study. One finding reported in that study is inconsistent with this alternative interpretation. The peptide A(1-14) disulfide linked to B(7-13) was inactive in antigen presentation assays. This indicates that residues B14 or B15 are essential components of the minimal determinant. It is possible that the T cell hybridomas used in the study of Naquet et al. (8) have a different specificity than those used in the present study. However, we argue that the majority of T cells from H-2^d and H-2^b animals recognize a determinant that is completely contained within the A chain.

We emphasize that the data with fixed APC reported by Naquet et al. (8) suggest that even the minimal A(1-14)/B(7-15) peptide requires further processing. Evidence for further processing requirements of sulfonated and disulfide forms of A chain peptides were also provided by Miller et al. (12) in studies with human loop-specific T cell clones and those of Gradehandt et al. (11, 32) using A$_\alpha^b$A$_\beta^k$-restricted clones. The first demonstration of efficient presentation of insulin peptides by fixed APC is provided by the current study. Results from functional experiments and binding studies suggest that the interchain A7 cysteine thiol is required for T cell recognition, but not for binding to I-A^d. By contrast, the loop thiols, B6 and/or A11, appear to interact with class II.

It is known that the interchain disulfides are more readily reduced than is the loop disulfide. It is therefore conceivable that partially reduced peptides containing an intact loop disulfide are the major substrates for formation of stable class II complexes. Observations that make this possibility less likely include the formation of functional complexes between reduced A(1-14) and class II in the continued presence of DTT. Similarly, one would need to propose that loop structures are formed in sulfonated A chain during incubation with reducing agents. However, the potential role of loop disulfides in class II binding cannot be excluded from the current data, and further experiments are in progress to address this issue.

Phillips et al. (33) have demonstrated the binding of labeled photoreactive bovine insulin to APC-associated class II glycoproteins. Binding was inhibited by unlabeled insulin, indicating that interaction with class II is not dependent on chemical modification of insulin. Insulin interacted with class

II in a haplotype-unrestricted manner in their study. By contrast, intact insulin did not inhibit the binding of labeled peptide antigen to purified I-A^d in the experiments described in the current study. Reduction of insulin disulfides was required for interaction with the putative peptide-binding groove. Preliminary studies from our laboratory (data not shown) suggest that biotinylated insulin can bind to I-A^d and I-E^d in the absence of reduction. However, this binding is not inhibited by peptides known to form functional complexes with these class II glycoproteins and may reflect stable interaction of intact insulin with a monomorphic site on class II that is distinct from the peptide-binding groove. The functional significance of this interaction is unclear. We have previously described T cells that recognize insulin in an MHC-unrestricted, class II–dependent fashion, and proposed that these cells recognize insulin associated with monomorphic determinants in class II (34). We have also measured binding of biotin insulin to I-A^d in the presence of 1 mM DTT. Binding under these conditions, however, is inhibited by peptide antigens.

Of the T cell determinants described in the literature, there are none with a well-defined requirement for an intact disulfide structure despite study of many disulfide-containing antigens. A number of linear determinants have been described that include cysteines involved in disulfide bridges present in the parent protein. It is therefore logical to suggest that a mechanism exists to reduce protein disulfide bonds during antigen processing. This mechanism must exist in a variety of cell types since insulin can be efficiently processed and presented to the T cells used in the current study by macrophages, B cells and transformed B cells. Studies with the disulfide-bonded, 18–amino acid peptide, apamin, indicate that disruption of the disulfide bonds is generally sufficient to bypass the antigen processing requirement and allows presentation of apamin to T cells by fixed APC (35). Rare (3/16) T cells fail to recognize apamin after reduction and alkylation, in the presence of live APC. This situation is analogous to that observed with loop-reactive insulin-specific T cells. We would predict that reduction of disulfide bonds would allow presentation of apamin to the disulfide-dependent T cell clones by fixed APC. For most situations, the status of a cysteine thiols in T cell determinants does not appear to be critical. For example, alkylation of the sulfhydryl in HEL(104-120) does not affect its capacity to bind purified I-E^d or to be recognized by the appropriate T cells (data not shown). In some situations, the chemical form of the thiol group may be important for establishing appropriate interactions with the class II molecule or the TCR. This is analogous to the situation where amino acid substitutions are tolerated in some, but not all, positions in peptide determinants.

The data provided in this communication provide strong evidence that disulfide bonds can be reduced during antigen processing. The mechanism and subcellular site of reduction remain to be determined. Very little information is available concerning the fate of protein disulfide bonds in proteins trafficking through the endocytic pathway. A number of studies using disulfide conjugates support the conclusion that

disulfide bonds remain oxidized during transport with recycling membrane proteins, including class II and the transferrin receptor (36–38). By contrast, other reports provide evidence for reducing activity in endocytic organelles. Shen et al. (39) provided evidence for the release of methotrexate from endocytosed poly(D-lysine) conjugates using a disulfide spacer but not a triglycine spacer. The reduction of a labeled poly(D-lysine) disulfide conjugate after endocytosis was directly demonstrated in a subsequent study (40). The site of reduction was proposed to be the Golgi apparatus based on the results of subcellular fractionation experiments. Collins et al. (41) have recently demonstrated reductive cleavage of α_2-macroglobulin/transferrin conjugates in a compartment in macrophages that comigrates with lysosomes in density gradients. The potential importance of lysosomes in protein disulfide reduction is given further weight by the demon-

stration of a lysosomal transport system that efficiently delivers cysteine from the cytoplasm into this compartment (42). The existence of this pathway was postulated earlier by Lloyd (43). The potential role of enzymes with protein disulfide reductase activity (24, 44–46) remains undetermined. Convincing evidence for the localization of enzymes with this activity in vesicles in the endocytic pathway is not available.

Reduction may play a major role as an initial step in the processing of disulfide-containing protein antigens. Reduction of small polypeptides, such as insulin and apamin, can be sufficient to allow stable interaction with class II during antigen processing. With more complex proteins, reduction may help to unfold proteins allowing interaction of buried determinants with class II (4, 47, 48) and facilitating cleavage by endopeptidases (41, 49, 50) to generate linear peptides capable of stable association with class II glycoproteins.

Address correspondence to Peter E. Jensen, Department of Pathology, Room 775 WMB, Emory University School of Medicine, Atlanta, GA 30322.

References

1. Germain, R.N. 1986. The ins and outs of antigen processing and presentation. Nature (Lond.). 322:687.
2. Unanue, E.R., and P.M. Allen. 1987. The basis for the immunoregulatory role of macrophages and other accessory cells. Science (Wash. DC). 236:551.
3. Shimonkevitz, R., J. Kappler, P. Marrack, and H. Grey. 1983. Antigen recognition by H-2-restricted T cells. I. Cell-free antigen processing. J. Exp. Med. 158:303.
4. Allen, P.M., and E.R. Unanue. 1984. Differential requirements for antigen processing by macrophages for lysozyme-specific T cell hybridomas. J. Immunol. 132:1077.
5. Kovac, F., and R.H. Schwartz. 1985. The molecular basis of the requirement for antigen processing of pigeon cytochrome c prior to T cell activation. J. Immunol. 134:3233.
6. Babbitt, B.P., P.M. Allen, G. Matsueda, E. Haber, and E.R. Unanue. 1985. Binding of immunogenic peptides to Ia histocompatibility molecules. Nature (Lond.). 317:359.
7. Buus, S., A. Sette, S.M. Colon, D.M. Jenis, and H.M. Grey. 1986. Isolation and characterization of antigen-Ia complexes involved in T cell recognition. Cell. 47:1071.
8. Naquet, P., J. Ellis, B. Singh, R.S. Hodges, and T.L. Delovitch. 1987. Processing and presentation of insulin. Analysis of immunogenic peptides and processing requirements for insulin A loop-specific T cells. J. Immunol. 139:3955.
9. Spaeth, E., and E. Rude. 1983. Epitope specificity and Ia restriction of T cell responses to insulin in a system of com-

plementing Ir genes: analysis with primed lymph node T cells and a long-term cultured T cell line. Eur. J. Immunol. 13:756.
10. Spaeth, E., and E. Rude. 1985. Development of T cell clones reactive to two defined restriction elements in conjunction with two defined epitopes of antigen. Eur. J. Immunol. 15:1177.
11. Gradehandt, G., J. Hampl, D. Plachov, K. Reske, and E. Rude. 1988. Processing requirements for the recognition of insulin fragments by murine T cells. Immunol. Rev. 106:59.
12. Miller, G.G., J.F. Hoy, L.J. Nell, and J.W. Thomas. 1988. Antigen processing and the human T cell receptor repertoire for insulin. J. Immunol. 141:3293.
13. Falcioni, F., Z. Dembic, S. Muller, P.V. Lehmann, and Z.A. Nagy. 1990. Flexibility of the T cell repertoire. Self tolerance causes a shift of T cell receptor gene usage in response to insulin. J. Exp. Med. 171:1665.
14. Hamano, T., K. Jin Kim, W.M. Leiserson, and R. Asofsky. 1982. Establishment of B cell hybridomas with B cell surface antigen. J. Immunol. 129:1403.
15. Jensen, P.E. 1988. Protein synthesis in antigen processing. J. Immunol. 141:2545.
16. Kappler, J.W., B. Skidmore, J. White, and P. Marrack. 1981. Antigen inducible H-2-restricted, interleukin 2-producing T cell hybridomas. Lack of independent antigen and H-2 recognition. J. Exp. Med. 153:1198.
17. Jensen, P.E. 1990. Immunogenicity of B chain in insulin responder and nonresponder mice. Cell. Immunol. 130:129.

18. Bailey, J.L., and R.D. Cole. 1959. Studies on the reaction of sulfite with proteins. *J. Biol. Chem.* 234:1733.

19. Jensen, P.E. 1989. Stable association of processed antigen with antigen-presenting cell membranes. *J. Immunol.* 143:420.

20. Jensen, P.E. 1991. Enhanced binding of peptide antigen to purified class II major histocompatibility glycoproteins at acidic pH. *J. Exp. Med.* 174:1111.

21. Ozato, K., L. Epstein, P. Henkart, T.H. Hansen, and D.H. Sachs. 1981. Studies on monoclonal antibodies to mouse MHC products. *Transplant. Proc.* 13:958.

22. Gorga, J.C., V. Horejsit, D.R. Johnson, R. Raghupathy, and J.L. Strominger. 1987. Purification and characterization of class II histocompatibility antigens from a homozygous human B cell line. *J. Biol. Chem.* 262:16087.

23. Keck, K. 1975. Ir-gene control of immunogenicity of insulin and A-chain loop as a carrier determinant. *Nature (Lond.).* 254:78.

24. Lundstrom, J., and A. Holmgren. 1990. Protein disulfide-isomerase is a substrate for thioredoxin reductase and has thioredoxin-like activity. *J. Biol. Chem.* 265:9114.

25. Jensen, P.E. 1990. Regulation of antigen presentation by acidic pH. *J. Exp. Med.* 171:1779.

26. Rosenwasser, L.J., M.A. Barcinski, R.H. Schwartz, and A.S. Rosenthal. 1979. Immune response gene control of determinant selection. II. Genetic control of the murine T lymphocyte proliferative response to insulin. *J. Immunol.* 123:471.

27. Roche, P.A., and P. Cresswell. 1990. High-affinity binding of an influenza hemagglutinin-derived peptide to purified HLA-DR. *J. Immunol.* 144:1849.

28. Rothenhausler, B., K. Dornmair, and H.M. McConnell. 1990. Specific binding of antigenic peptides to separate α and β chains of class II molecules of the major histocompatibility complex. *Proc. Natl. Acad. Sci. USA.* 87:352.

29. Choi, E., K. McIntyre, R.N. Germain, and J.G. Seidman. 1983. Murine I-Aβ chain polymorphism: nucleotide sequences of three allelic I-Aβ genes. *Science (Wash. DC).* 221:283.

30. Benoist, C.O., D.J. Mathis, M.R. Kanter, V.E. Williams II, and H.O. McDevitt. 1983. Regions of allelic hypervariability in the murine A immune response gene. *Cell.* 34:169.

31. Glimcher, L.H., J.A. Schroer, C. Chan, and E.M. Shevach. 1983. Fine specificity of cloned insulin-specific T cell hybridomas: evidence supporting a role for tertiary conformation. *J. Immunol.* 131:2868.

32. Gradehandt, G., J. Hampl, S. Milbradt, and E. Rude. 1990. Processing without proteolytic cleavage is required for recognition of insulin by T cells. *Eur. J. Immunol.* 20:2637.

33. Phillips, M.L., C.C. Yip, E.M. Shevach, and T.L. Delovitch. 1986. Photoaffinity labeling demonstrates binding between Ia molecules and nominal antigen on antigen-presenting cells. *Proc. Natl. Acad. Sci. USA.* 83:5634.

34. Jensen, P.E., and A.O. Kraft. 1990. T cells from nonresponder mice. MHC restricted and unrestricted recognition of insulin. *J. Immunol.* 145:3985.

35. Regnier-Vigouroux, A., M.E. Ayeb, M. Defendini, C. Granier, and M. Pierres. 1988. Processing by accessory cells for presentation to murine T cells of Apamin, A disulfide-bonded 18 amino acid peptide. *J. Immunol.* 140:1069.

36. Cresswell, P. 1985. Intracellular class II HLA antigens are accessible to transferrin-neuraminidase conjugates internalized by receptor-mediated endocytosis. *Proc. Natl. Acad. Sci. USA.* 82:8188.

37. Reid, P.A., and C. Watts. 1990. Cycling of cell-surface MHC glycoproteins through primaquine-sensitive intracellular compartments. *Nature (Lond.).* 346:655.

38. Bretscher, M.S., and R. Lutter. 1988. A new method for detecting endocytosed proteins. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:1077.

39. Shen, W.-C., H.J.-P. Ryser, and L. LaManna. 1985. Disulfide spacer between methotrexate and poly(D-lysine). *J. Biol. Chem.* 260:10905.

40. Feener, E.P., W. Shen, and H.J. Rysler. 1990. Cleavage of disulfide bonds in endocytosed macromolecules. *J. Biol. Chem.* 265:18780.

41. Collins, D., E.R. Unanue, and C.V. Harding. 1992. Reduction of disulfide bonds within lysosomes is a key step in antigen processing. *J. Immunol.* In press.

42. Pisoni, R.L., T.L. Acker, K.M. Lisowski, R.M. Lemons, and J.G. Thoene. 1990. A cysteine-specific lysosomal transport system provides a major route for the delivery of thiol to human fibroblast lysosomes: possible role in supporting lysosomal proteolysis. *J. Cell Biol.* 110:327.

43. Lloyd, J.B. 1986. Disulphide reduction in lysosomes. *Biochem. J.* 237:271.

44. Duckworth, W.C. 1988. Insulin degradation: mechanisms, products, and significance. *Endocrine Rev.* 9:319.

45. Holmgren, A. 1985. Thioredoxin. *Annu. Rev. Biochem.* 54:237.

46. Freedman, R.B. 1989. Protein disulfide isomerase: multiple roles in the modification of nascent secretory proteins. *Cell.* 57:1069.

47. Streicher, H.Z., I.J. Berkower, M. Busch, F.R.N. Gurd, and J.A. Berzofsky. 1984. Antigen conformation determines processing requirements for T cell activation. *Proc. Natl. Acad. Sci. USA.* 81:6831.

48. Sette, A., L. Adorini, S.M. Colon, S. Buus, and H.M. Grey. 1989. Capacity of intact proteins to bind to MHC class II molecules. *J. Immunol.* 143:1265.

49. Griffiths, P.A., and J.B. Lloyd. 1979. Evidence for lysosomal reduction of cystine residues. *Biochem. Biophys. Res. Commun.* 89:428.

50. Mego, J.L. 1984. Role of thiols, pH and cathepsin D in the lysosomal catabolism of serum albumin. *J. Biochem.* 218:775.