Studies on the Mechanism of Processing and Presentation of Insulin by Antigen Presenting Cells[+]

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[+]This work was supported by grants from the Medical Research Council of Canada to T.L.D., M.L.P. and C.C.Y.
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ABSTRACT

We have analysed some of the mechanisms involved in the processing and presentation of an antigen by an antigen presenting cell (APC) to a T helper (T_H) cell. Five insulin-specific mouse T cell hybridomas and two antigen presenting B cell lines were used in these studies. We found that alteration of the metabolic activity (eg. membrane fluidity, level of expression of Ia antigens, binding of antigen to Ia, etc.) of an APC changes its ability to process and present insulin to a T_H cell. Chloroquine and leupeptin treatment of APC affected the responses of four T cell hybridomas differently; the responses of two of the clones were inhibited by these agents while the responses of two others were enhanced. Photoaffinity labelling experiments demonstrated that one APC protein which may be involved in the processing of insulin has an M_r of 104 kDa. This protein is not a subunit of the insulin receptor, does not consist of Ia or Ig determinants, binds insulin soon after exposure of the APC to antigen and thus may be involved in the initial binding or internalization of insulin. In vitro proteolysis of insulin by chymotrypsin showed that insulin may be cleaved into several immunogenic peptides, which differentially activate the four T cell hybridomas tested. The latter results and those obtained using chemically-modified insulins enabled us to identify the role of the hydrophilic and hydrophobic regions of an insulin molecule that may control its immunogenicity. Taken together, our analyses of the requirements for effective presentation by an APC and for the immunogenicity of insulin suggest that altered modes of processing of an antigen by APC might result in the activation of T cells with specificity for different epitopes of that antigen. This notion supports the hypothesis that antigen processing and presentation play an important role in regulating the development of the T cell repertoire.

Mediators of Immune Regulation and Immunotherapy
S.K. Singhal and T.L. Delovitch, Editors

INTRODUCTION

Recognition of a foreign antigen by T helper cells is preceded by a complex series of events mediated by antigen presenting cells. These events may include: (i) antigen-binding to the surface membrane of the APC, (ii) antigen internalization and proteolysis (i.e. processing), (iii) recycling and reinsertion of an immunogenic, enzymatically-cleaved antigen fragment(s) into the surface membrane and (iv) presentation of this antigen fragment to a clonally selected antigen reactive T cell. The mechanisms of antigen binding, processing and presentation by an APC are, however, not presently well understood [1].

We performed experiments to explore some of the mechanisms involved in the handling of an antigen by APC. Heterologous insulin was chosen as the foreign antigen for several reasons. First, the primary structures of insulins of various species are known and they exhibit limited amino acid sequence variability [2]. This facilitates analysis of the antigenic structure of the molecule. Second, H-2-linked Ir gene control of the response to this antigen in the mouse has been well characterized [3]. Third, some information concerning the internalization and degradation of insulin by non-lymphoid cells (eg. fibroblasts, hepatocytes, and adipocytes) is available. Enzymes such as glutathione insulin transhydrogenase, insulin protease and certain lysosomal enzymes have been implicated in these events [4]. Fourth, insulin responsive T_H cell hybridomas and B cell hybridomas which present insulin to these T cells, have been produced by Glimcher et al. [5, see Table I] and were made available to our laboratory. Finally, derivatives of this antigen including radioiodinated, photoactivatable insulins have been prepared and characterized by one of us (C.C.Y.) [6].

Is antigen processing required for T cell recognition of antigen? If so, does antigen processing by an APC occur at the plasma membrane and/or intracellularly? Most critically, how is the T_H cell repertoire affected by the mode(s) of antigen processing? To answer these questions, we have analysed some of the metabolic requirements for antigen processing by determining whether fixed APC present insulin to T cells and whether agents that inhibit intracellular metabolism affect the antigen presenting function of an APC. We have used the technique of photoaffinity labelling to identify surface membrane-associated and/or intracellularly located molecules of an APC that bind insulin, and we have attempted to mimic antigen processing in vitro by examining the effects of various structural alterations (enzymatic cleavage, charge and hydrophobicity changes) on the immunogenicity of insulin. The data obtained is discussed with reference to the known amino acid sequence, crystallographic structure and amphipathicity of the molecule.

MATERIALS AND METHODS

Cell lines. The five insulin-specific T hybridoma cell lines used and their antigen and MHC specificities are given in Table I. Also listed in Table I are the two B cell-B lymphoma hybridomas used as APC. These T and B cell hybridomas were generously provided by Drs. L. Glimcher and E. Shevach, NIAID, NIH.

Table I. Cell lines used

A. INSULIN RESPONSIVE T CELL HYBRIDOMAS

Cell Line	Abbreviation of Cell line	Parental Strain	Insulin Reactivity	MHC Reactivity	Reference
B8/C3X/6	B8/PB	(BALB/c x A/J)F_1	Pork, Beef	A^d_d	5
B8/C3X/B	B8/P	(BALB/c x A/J)F_1	Pork	A^d_d	-
A20-1	A20	(BALB/c x B10)F_1	Beef	A^d_b	-
AF3G7	AF	B10	Beef	A^b_b	7
DC33H5	DC	B10	Beef	A^b_b	7

B. INSULIN PRESENTING B CELL-B LYMPHOMA HYBRIDOMAS

Cell Line	MHC Haplotype	
TA3	$I\text{-}A^{k/d}$	5
LB	$I\text{-}A^{b/d}$	8

Antigens. Beef and pork insulin were kindly supplied by Connaught Novo Ltd. (Toronto, Canada). The beef insulin derivatives in which either a lysine, methionine or arginine residue was coupled to the B-chain N-terminal phenylalanine were prepared by Drs. C. Yeung and C.C. Yip as previously described [9]. The N$^{\alpha}$B1-(monoazidobenzoyl) insulin (B_1-MABI) and N$^{\varepsilon}$B29-(monoazidobenzoyl) insulin (B_{29}-MABI) photoreactive analogs of pork and beef insulins were prepared as reported earlier [6]. Chymotryptic fragments of insulin were prepared by reacting insulin at a concentration of 1 mg/ml in 0.1 M Tris-HCl, pH 8, for 4 hr at 37°C with 0.25% (w/w) chymotrypsin (Worthington code CDS); the same amount of enzyme was again added to the reaction mix and the digestion was continued for a further 16 hr at 37°C. HPLC fractionation of the insulin peptides obtained was carried out using a Beckman model 334 HPLC system equipped with an Ultrasphere-ODS semipreparative reverse phase C18 column. Peptides were resolved using a gradient from 100% solvent A (0.1% trifluoroacetic acid in water) to 40% solvent A and 60% solvent B (0.1% trifluoroacetic acid in 80% acetonitrile) and their absorbance was monitored at 214 nm.

Photoaffinity labeling. 2×10^6 TA3 or LB APC and/or 3×10^6 insulin specific T hybridoma cells were incubated with 3×10^{-8} M ^{125}I-B_1-MABI or ^{125}I-B_{29}-MABI in PBS for 5 min to 6 hr at 37°C. Native insulin (10 μg/ml) was added as a competitive inhibitor of binding to demonstrate specificity of labeling in control cultures. Photolysis was carried out by exposing the sample for 30 sec to a focused light source of a 100-W high-pressure mercury lamp. The cells were washed 3X by centrifugation in PBS containing soybean trypsin inhibitor (100 μg/ml) and bacitracin (1.6 mg/ml). Cells were solubilized either by boiling for 20 min in 3% (w/v) sodium dodecyl sulfate (SDS), 0.0625 M Tris-HCl pH 6.8, and 10% glycerol or by resuspending in 1% Triton solubilization buffer [10] for 90 min at 4°C with stirring followed by centrifugation for 30 min at 18,000 x g to yield a supernatant used for immunoprecipitation.

Immunoprecipitation and gel electrophoresis. An aliquot (200 μl) of the solubilized supernatant was incubated with 10-15 μl of antiserum for 16 hr at 4°C. Glutaraldehyde-fixed Staphylococcus aureus cells (Staph A) were washed in a buffer of normal saline containing 0.5% NP-40, blocked in buffer containing 1% ovalbumin, then washed and resuspended in buffer to 20% (v/v). Staph A suspension (100 μl) was added to each sample and the incubation was continued for 1.5 hr. Samples were centrifuged at 8,000 x g, supernatants were removed, and the antigen-antibody-Staph A complexes were washed 3X in buffer containing NaCl (150 mM), HEPES (50 mM), PMSF (1 mM) and recovered by centrifugation. Antigens were dissociated from the complexes by boiling the samples for 20 min in the 3% SDS sample

buffer. Staph A was removed by centrifugation and the samples were analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was carried out on 3%-10% or 5%-10% gradient slab polyacrylamide gels for unreduced and reduced samples, respectively. Gel autoradiograms were obtained on Kodak X-OMat AR film using a DuPont Cronex Lightning-Plus intensifying screen during a 10-14 day exposure. M_r markers used for non-reducing gels were thyroglobulin (669 kDa), thyroglobulin intermediates (475 kDa, 280 kDa and 255 kDa), catalase dimer (120 kDa) and catalase monomer (60 kDa).

T cell response to insulin. $5x10^4$ insulin-specific T hybridoma cells and $5x10^3$ APC were cultured in the presence of 0 to 100 µg/ml of insulin or insulin derivatives in 0.15 ml of RPMI 1640 containing 5% FCS, $5x10^{-5}$M 2-mercaptoethanol and antibiotics for 48 hr. For photoreactive insulin derivatives, cultures were prepared and incubated in the dark. Supernatants from these cultures were assayed for IL-2 content in a secondary culture using the IL-2 dependent T cell line CTLL. 10^4 CTLL were cultured for 24 hr in the presence of 45% primary culture supernatant and the degree of stimulation was measured by incorporation of ^{3}H-TdR into DNA during the last 6 hr of culture.

Inhibition of antigen presentation. The effect of various inhibitors on antigen processing or presentation was tested by washing the APC in serum-free RPMI 1640 medium and then preincubating the APC for 30 min at 37°C in either the presence or absence of inhibitor (see Figs. 1 and 2). Insulin was added at a final concentration of 2 mg/ml and the APC were pulsed for 8-10 hr in the continued presence or absence of the inhibitor. A control APC population which was pretreated with inhibitor but not pulsed with antigen was routinely included in each experiment. APC populations were washed 3X with serum-free medium and were either not fixed or fixed for 30 sec with 0.1% glutaraldehyde [11]. To monitor the efficiency of glutaraldehyde fixation, the incorporation of ^{3}H-thymidine and ^{3}H-leucine by the treated cells during 48 hr was quantitated. Only those fixed APC populations in which the levels of incorporation were inhibited by >99% were used. Antigen pulsed and unfixed APC were added to cultures (0.15 ml) at $5x10^3$ cells/well whereas antigen pulsed and fixed APC were plated at $7x10^3$ cells/well. To evaluate the kinetics of recovery of antigen-presenting function of the APC after their treatment with a given inhibitor, APC that were neither pulsed with antigen nor glutaraldehyde fixed were also plated at $5x10^3$/cells well in the presence of various amounts of insulin. To monitor the effects of inhibitors and glutaraldehyde (which leach from treated cells during culture) on the responding T cells, APC which were fixed and treated with inhibitor but not pulsed with antigen were added to cultures containing T cells and unfixed, untreated and antigen-pulsed APC.

RESULTS AND DISCUSSION

To examine the cellular processes involved in the presentation of an antigen by an APC to a T_H cell, we compared the antigen-presenting activities of untreated APC and APC treated with glutaraldehyde. Fixation of the TA3 and LB B cell hybridomas used as APC was carried out either before or after the cells were pulsed with insulin. Table II shows that all four of the T cell hybridomas tested recognize the relevant insulin when presented either in soluble form by the appropriate unfixed APC or by antigen-pulsed unfixed APC. By contrast, fixed APC that were not previously pulsed with antigen did not present insulin when used at a final concentration of 100 µg/ml or even 500 g/ml to any of the four T cell hybridomas. However, APC which were pulsed with insulin and then glutaraldehyde fixed retain their capacity to present insulin.

TABLE II. Fixed APC do not present insulin to T cell hybridomas.

T cell hybridomas	Unfixed APC[a] -insulin	+insulin	Pulsed	Fixed APC -insulin	+insulin		Pulsed
A20	11	1392[b]	1200	9	8[b]	6[c]	583
AF	9	255	154	7	5	4	144
DC	21	535	72	5	3	1	351
B8/PB	13	621	516	3	4	2	246

a) B8/PB and A20 T cells were cultured in the presence of TA3 APC and pork and beef insulin, respectively, and AF and DC T cells were co-cultured with LB APC and beef insulin. IL-2 secretion by T cells was assayed by stimulation of proliferation of 10^4 CTLL. Results are expressed as means of triplicates in cpm$\times 10^{-2}$.
b) Insulin was used at 100 μg/ml.
c) Insulin was used at 500 μg/ml.

Fixation of an APC may abrogate its antigen-presenting capacity due to changes in several of its cellular processes (metabolic or non-metabolic). We found that fixation of APC by glutaraldehyde reduced protein and DNA synthesis to ≤1% of that obtained in unfixed cells. Fixation of APC may also a) decrease the relative mobility of APC membrane-associated proteins perhaps by altering the net membrane fluidity, b) reduce the level of expression of Ia molecules below the threshold required for antigen presentation by an APC and c) cause the steric inhibition of antigen binding to Ia by crosslinking Ia to another APC cell surface-associated protein(s). Nonetheless, it is important to note (see Table II) that if an APC is fixed after it has been pulsed with antigen, it retains its antigen-presenting function. In this case, it is likely that the antigen is sufficiently processed by the APC before the activity of the APC is altered by treatment with glutaraldehyde.

It was previously shown that antigen presentation by macrophages and B cell lines could be abolished by exposure of these cells to either inhibitors of lysosomal function and cellular transport (chloroquine, ammonium chloride, monensin) [12] or to the specific leucine aminopeptidase protease inhibitor, leupeptin [13]. To determine whether the immunogenicity of insulin is dependent on its proteolysis by an APC, we tested the ability of TA3 and LB cells to present insulin after they were treated under various conditions with either chloroquine or leupeptin. Depending upon the stimulation of the particular T cell hybridoma being assayed, cultures contained either TA3 or LB B cells as APC and either pork insulin or beef insulin as antigen. The choices of optimum high and low concentrations of antigen used for a given T cell line were previously determined. Chloroquine did not significantly inhibit the responses of the B8/PB (Fig. 1A) and A20 (Fig. 1B) T cells to insulin, but rather enhanced these responses by 50-80%. However, a marked inhibition of the response of AF (Fig. 1C) T cells and a partial inhibition of the response of DC (Fig. 1D) T cells were observed. It is important to note that the same sample of LB B cells and batch of beef insulin were used for the experiments shown in Figs. 1B-1D. Thus, the variable effects of chloroquine on the responses of different T cells was not due to experimental variations in the treatment of APC or the preparation of antigen. Different patterns of inhibition were also noted in experiments in which the cationic ionophore monensin was used as an inhibitor of APC function (results not shown).

Similarly, we found that treatment of TA3 cells with leupeptin did not inhibit the response of B8/PB T cells to pork insulin, but rather enhanced this response by 2- to 3-fold over the range of concentration of inhibitor used (Fig. 2A).

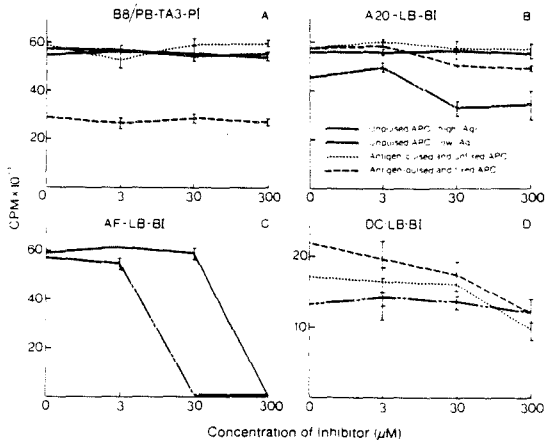

FIG. 1. Differential inhibition by chloroquine of presentation of insulin to T cells. T cells were cultured with TA3 or LB APC, which were either pulsed or not pulsed with insulin and were either fixed or not fixed with glutaraldehyde. Cultures contained either pork insulin (PI) or beef insulin (BI) and varying concentrations (0 to 300 uM) of chloroquine. High and low concentrations of insulin used were 50 g/ml and 25 g/ml for B8/PB (A), 50 g/ml and 5 g/ml for A20 (B), and 100 g/ml and 50 g/ml for AF(C) and DC(D). Results are expressed as cpmx10^{-3} $\pm$ standard error.

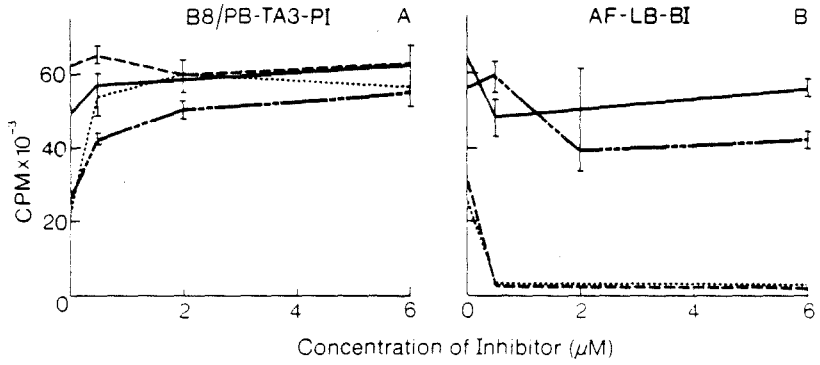

FIG. 2. Differential inhibition by leupeptin of presentation of insulin to T cells. Culture conditions were as described in Fig. 1, except that leupeptin (0 to 6 mM) was used.

Conversely, the response of A20 T cells to beef insulin presented by either LB or TA3 B cells was weakly inhibited (data not shown). Strong and intermediate levels of inhibition were obtained for the responses of AF (Fig. 2B) and DC (not shown) T cells to beef insulin presented by LB cells. In the latter two responses, the level of inhibition observed was enhanced when a low dose of antigen was used; this result is expected since leupeptin acts as a competitive inhibitor of enzymatic activity. Thus, treatment of APC with leupeptin inhibited their ability to present insulin to some of the T cell lines examined but not others.

Our observations that chloroquine and leupeptin effect the activation

of four insulin-responsive T cell hybridomas differently may be explained in several ways. First, it is known that a T_H cell must recognize both a processed foreign antigen and self Ia antigens on an APC in order to be activated and that the magnitude of a T cell response is dependent on the concentrations of both antigen and Ia on an APC [14]. Although the B8/PB, A20, AF and DC T cells were selected based on their ability to secrete IL-2 in response to insulin [5], different concentrations of insulin are required for their activation. We found that B8/PB and A20 cells may be stimulated by a relatively low antigen concentration, 1-5 μg/ml, while the activation of AF and DC requires a minimum concentration of 20 μg/ml. Despite the fact that the TA3 and LB B cell hybridomas were selected based on their expression of Ia antigens and their ability to present a variety of antigens in I-A restricted T cell responses [5], the relative levels of expression of I-A molecules by TA3 ($I\text{-}A^{k/d}$) and LB ($I\text{-}A^{b/d}$) cells has not yet been determined. TA3 cells present $I\text{-}A^{d}$ antigens and LB cells present both $I\text{-}A^{d}$ and $I\text{-}A^{b}$ antigens to the relevant MHC-restricted insulin-responsive T cell (see Table I). If TA3 and LB cells differ in their level of expression of surface I-A molecules and if treatment of these APC cell lines with chloroquine and leupeptin affect their surface density of I-A molecules, this might explain in part the observed variable effects of these inhibitors on T cell activation. Second, since B8/PB and A20 T cells may be activated by substantially lower doses of insulin than AF and DC T cells, B8/PB and A20 T cells likely possess a much higher relative avidity for their relevant insulin epitopes than do AF and DC T cells. Perhaps, this permits the stimulation of AF and DC T cells to be more strongly inhibited by chloroquine and leupeptin than the two other cell lines studied. Third, it is possible that the nature of the processed insulin antigenic determinants that activate our panel of four T cell hybridomas may differ both qualitatively and quantitatively. If this is the case, then chloroquine and leupeptin could differentially inhibit T cell responses to different insulin epitopes. Analysis of the immunogenicity of insulin peptides (see below) might resolve this issue.

The enhanced responses of B8/PB and A20 T cells obtained in the presence of chloroquine and leupeptin might be due to a "protective effect" of these inhibitors on the processing of insulin by APC. For example, it is conceivable that some insulin molecules after they are internalized by an APC may either not be processed or are processed in a subcellular compartment other than the lysosome before they are recycled to the membrane in an immunogenic form. Leupeptin causes the accumulation of insulin in Golgi-derived vesicles in adipocytes [15]. The contribution of lysosomal activity to insulin degradation in adipocytes and hepatocytes is relatively small [4]. It is possible that the relative contribution of lysosomes to insulin metabolism could vary under different conditions and in different cell types. This possibility is raised by the observations that in addition to the inhibition of lysosomal proteases, chloroquine blocks protein synthesis, binds to tissue macromolecules, alters prostaglandin activity, inhibits mitochondrial activity, decreases pinocytosis and interferes with some types of membrane binding [4]. Thus, treatment of APC with chloroquine and leupeptin might in some instances serve to protect the relevant immunogenic epitope of insulin. By contrast, the activation of AF and DC T cells likely requires that insulin be processed by proteolysis with both leucine aminopeptidase, which might be identical to the insulin protease that cleaves insulin between the B16 tyrosine and B17 leucine residues [4], and site non-specific lysosomal enzymes.

We propose that different modes of processing of an antigen by APC might result in the activation of T cells with specificity for different epitopes of that antigen. This idea supports the hypothesis that antigen processing and presentation play an important role in regulating the development of the T cell repertoire. It is possible that different processed fragments of antigen might be recognized by T cells in the context of either different regions of a given Ia molecule, different Ia

molecules encoded by a given haplotype, or allelic Ia molecules encoded by different haplotypes. This notion is especially applicable in the study of insulin as an antigen; while insulin may be recognized as an antigen by the immune system, it functions as a hormone in the endocrine system where non-lymphoid cells internalize insulin by the pathway of receptor-mediated endocytosis [4]. The pathway of internalization and proteolysis of insulin in non-lymphoid cells might differ from that which occurs in macrophages and lymphocytes and may give rise to different immunogenic forms of insulin. For example, it was recently reported that native beef insulin may be inserted into liposomes and be presented in this form to T cells [16]. While this result was interpreted to mean that insulin does not need to be processed in order to be immunogenic, it does not rule out the possibility that insulin is unfolded by denaturation and perhaps even proteolysed by residual proteases upon insertion into liposomes. Therefore, the possibility remains that diffent processed forms of insulin, eg. a denatured form, a proteolytically cleaved form and a form that is structurally altered by the binding to a membrane-associated and/or cytoplasmic cellular protein, are presented to T cells.

To further explore the mechanism of antigen uptake and internalization by lymphocytes, we determined whether insulin binds to protein(s) in addition to insulin receptors on TA3 and LB cells. Radioactive photoaffinity probes may be used to covalently crosslink and label specific functional sites on cell membranes. Yip and Moule [6] have used two photoreactive insulin analogues, NεB29-monoazidobenzoyl-insulin (B_{29}-MABI) and NαB1-monoazidobenzoyl-insulin (B_1-MABI) in the study of insulin receptors. We have used these same probes to search for cellular proteins which are involved in the processing, presentation and/or recognition of insulin as a foreign antigen.

In these studies we anticipated that the location of the photoreactive group on the insulin molecule would be critical in determining which cells and cellular proteins will be labeled. Positioning of the azidobenzoyl group proximal to a particular antigenic determinant might increase the likelihood of photocrosslinking specific recognition sites on an APC and T cells, but also increases the possibility that the determinant will be altered such that it can no longer be recognized by an antigen-specific T cell. If the location of the photoreactive group is distal to the antigenic determinant, the risk of altering the determinant is reduced but the possibility that the crosslinking reagent might be separated from the determinant during antigen processing is increased.

We first examined the ability of our photoreactive probes to be recognized as antigens by each of five insulin-specific T hybridoma cell lines. The ability of the cells to respond to pork or beef insulin probes labeled at either the B1 or B29 residues (P_{B1}-MABI, P_{B29}-MABI, B_{B1}-MABI, and B_{B29}-MABI) was tested in a functional assay in which either TA3 or LB B cells were used as APC. The assay was carried out under dark conditions.

TABLE III. Response of T cell hybridomas to photoreactive insulins.

T Hybridoma APC Antigen	B8/PB TA3	B8/P TA3	A20 LB	DC LB	AF LB
Beef Insulin	400[a]	11	1152	85	200
Beef B1-MABI	140	8	1060	232	600
Beef B29-MABI	842	6	1115	20	254
Pork Insulin	757	254	9	9	11
Pork B1-MABI	126	634	12	10	10
Pork B29-MABI	1411	25	7	10	10
No Antigen	116	8	9	12	11

a) Results are expressed as means of triplicates in cpm x 10^{-2}.

Table III shows that a good response was obtained for each clone using either pork or beef insulin in its native form. Derivatization of either pork or beef insulin in the B1 position destroyed the immunogenicity of the molecule for the B8/PB clone. This was not surprising since the determinant recognized by this clone involves both the A-chain loop and residue 3 or 4 of the B-chain of insulin [5], i.e., this determinant is quite close to the photoreactive group. Derivatization of either species of insulin at the B29 position, however, preserved the immunogenicity of the molecule and also increased the intensity of the response. The inverse result was seen with the B8/P cell line. This clone responded well to pork insulin derivatized at the B1 position but lost reactivity to P_{B29}-MABI. DC responded well to B_{B1}-MABI but poorly to B_{B29}-MABI. The response of AF to B_{B29}-MABI was enhanced and was unaffected by derivatization at the B29 position. The A20 hybridoma, which has a relatively high affinity for beef insulin, responded well to beef insulin modified at either position. Enhancement of the response of this clone by derivatized insulins could not be determined in this assay because the maximum response of the IL-2 dependent CTLL line was reached in the native insulin control.

These experiments clearly demonstrate that the position of the photoreactive group on the antigen is critical in determining the ability of the clones to recognize the probe. Each of the clones was shown to recognize at least one, and sometimes both, of the derivatized antigens. We may also conclude from such experiments that the TA3 and LB APC were able to present each of the four insulin derivatives when the appropriate responding cell was used to detect presentation.

Since the photoreactive insulin derivatives were found to be immunogenic, we proceeded with antigen-binding experiments in which cultures containing TA3 and B8/PB cells were reacted with each of the four radioiodinated, photoreactive insulin probes in the dark and were then photocrosslinked. Cell lysates were prepared and analysed by SDS-PAGE. P_{B1}-MABI, P_{B29}-MABI, B_{B1}-MABI and B_{B29}-MABI all labeled insulin receptors on these cell lines (Fig. 3A, lane 1) even though the labeling conditions were not optimized for hormone receptor identification [6]. P_{B29}-MABI (Fig. 3) and B_{B29}-MABI (data not shown) but neither P_{B1}-MABI nor B_{B1}-MABI (data not shown) bound to an additional 104 kDa protein under non-reducing conditions (Fig. 3A, lane 5). The specificity of binding for both this molecule and the hormone receptor bands is shown by displacement of the label by cold native insulin (Fig. 3A, lane 6). When the two cell lines were labeled independently, this 104 kDa molecule was found to be present on the TA3 APC in the absence of T cells (Fig. 3A, lane 9), and no bands attributable to the T cells or dependent upon the presence of both cell types could be found under these conditions. In experiments where photocrosslinking was carried out at different times from 5 min to 6 hr after addition of the MABI derivatives to cultures of TA3 cells, maximum detection of the 104 kDa band occurred at 20 min.

To immunochemically characterize the 104 kDa protein, immunoprecipitations were performed using lysates of B_{B29}-MABI labeled TA3 cells. Antibodies specific for the I-A, I-E, H-2K and H-2D mouse histocompatibility antigens did not immunoprecipitate the 104 kDa band. Because the TA3 cell line was derived from a B lymphocyte fusion we considered the possibility that our 104 kDa band might be an immunoglobulin heavy chain or immunoglobulin-like molecule. The band could not, however, be immunoprecipitated with a rabbit anti-mouse IgG,IgM,IgA (H and L chain) antibody. An antiserum specific for insulin receptors (ARS) also did not react with the 104 kDa band. It could, however, be effectively precipitated with guinea-pig anti-insulin serum (Fig. 3B, lane 1). Thus, the insulin photoprobe bound to our 104 kDa protein band retains a significant portion of its antigenic structure suggesting that the antigen is not processed by TA3 APC at this stage of antigen presentation.

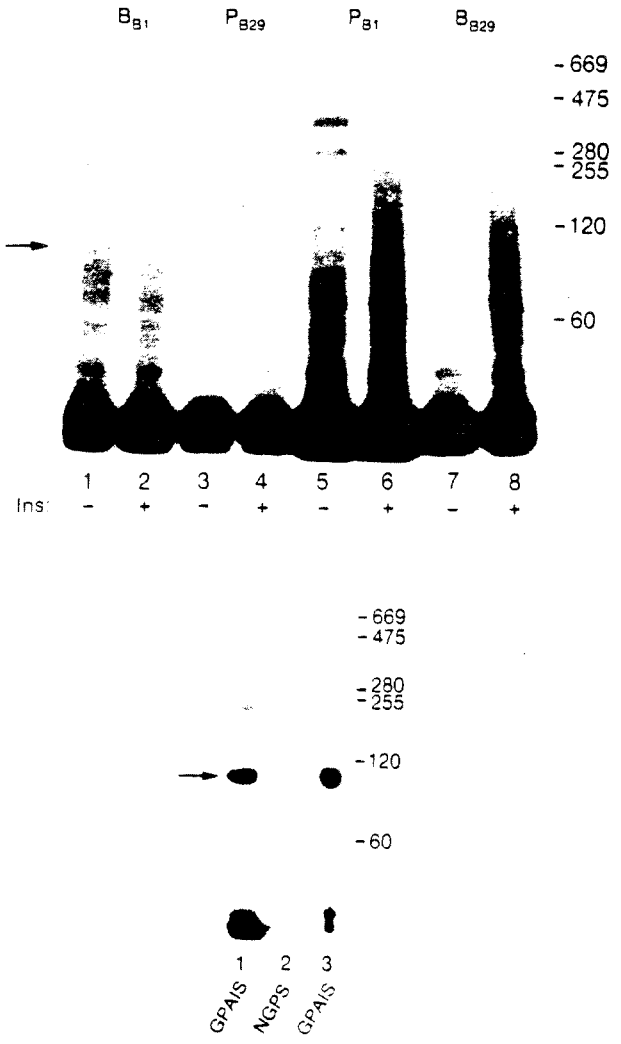

FIG. 3. Photoaffinity labeling of the p104 protein. (A) The TA3, B8/P and B8/PB cell lines were photoaffinity labeled with P_{B29}-MABI, washed solubilized and analysed by SDS-PAGE under nonreducing conditions. (B) TA3 cells were photoaffinity labeled with P_{B29}-MABI, and the solubilized membrane proteins were immunoprecipitated using guinea-pig anti-insulin serum (GPAIS) (lane 1) or normal guinea-pig serum (NGPS) (lane 2) and analysed by SDS-PAGE under nonreducing conditions. The p104 band was cut from lane 1, rehydrated in sample buffer containing 50 mM dithiothreitol and electrophoresed again under reducing conditions (lane 3).

Further evidence that the 104 kDa band does not consist of Ia molecules was acquired by electrophoresis under reducing conditions. The 104 kDa band was cut from a non-reducing SDS-PAGE gel, soaked in 50 mM dithiothreitol and re-electrophoresed on a 5-10% gradient gel. No change in the apparent M_r of the band could be seen other than that which would be accounted for by the loss of the A chain of the covalently linked insulin photoprobe, and no bands in the M_r range of I-A glycoproteins (32 kDa and 28 kDa) were seen (Fig. 3B, lane 5). We therefore believe that the 104 kDa band is composed of a single polypeptide chain (p104).

A variety of additional APC lines which either do or do not present insulin, were tested for the presence of p104. P_{B29}-MABI and B_{B29}-MABI but neither P_{B1}-MABI or B_{B1}-MABI bind to p104 on TA3 and LB cells. Both of these B cell lines are Ia positive. All four probes, including the B1 derivatives bind to the p104 on a subline of the mutant M12.4.1 B hybridoma cell line which had been selected for low expression of I-A gene products [17]. The appropriately restricted insulin-specific T hybridoma responds poorly to insulin presentation by this cell line presumbably because of its lack of I-A expression. J774.2 [18], P388D1 [19] and RAW 264.7 [20] macrophage-monocyte cell lines did not present insulin to $I\text{-}A^d$ restricted T responding cells and did not bind insulin probes. DEN-1, a dendritic cell "like" line [21] which is $I\text{-}A^b$ positive but does not present any soluble antigens including insulin to the appropriate T cell line, does not bind either insulin probe. Thus far, we have found that only those cell lines which express both $I\text{-}A^{b \text{ or } d}$ and p104 are capable of insulin presentation. The properties of the p104 protein described here suggest that it may be involved in the binding and/or internalization of an antigen by an APC.

The data presented in Table III showed that the conjugation of a hydrophobic photoreactive group to insulin clearly changed the immunogenicity of the molecule. This observation may be attributed to a conformational change which altered the relative accessibility of hydrophobic and hydrophilic surfaces of insulin to APC and T cells. It has been suggested that the hydrophobic and hydrophilic regions of an antigen contribute to the binding of an antigen to APC and T cells, respectively (see below). We examined the relationship between the hydrophathicity and immunogenicity of insulin by inducing changes in the molecule that result from the addition of either hydrophobic or hydrophilic residues. The B1 residue is situated proximal to one of the antigenic epitopes of insulin. B1 binds to residue A14 and this binding contributes to the conformational stability of the molecule [22]. Beef insulins derivatized at the NH_2-terminus of the B-chain change their conformation, state of aggregation, hormonal activity, and receptor and antibody binding activities [9]. We therefore assayed the T cell response to three derivatives of beef insulin prepared by the addition of a single amino acid with a hydrophobic or hydrophilic side chain to the B1 position. Addition of a hydrophilic lysine residue NH_2-terminal to B1 increased the response to this derivative of the A20, AF and DC clones 3- to 10-fold over the response to native insulin (Fig. 4). Introduction of a hydrophobic methionine residue at the NH_2-terminus increased the response of all three clones, particularly A20, but the effect was less dramatic than that seen for lysine. Addition of a hydrophilic arginine residue at B1 had little or no effect on the response of any of the three beef insulin-specific clones. Thus, the insertion of some but not other hydrophilic and hydrophobic residues at position B1 enhances the immunogenicity of insulin. This suggests that not only the hydropathicity but also the overall conformation of an antigen determines its immunogenicity.

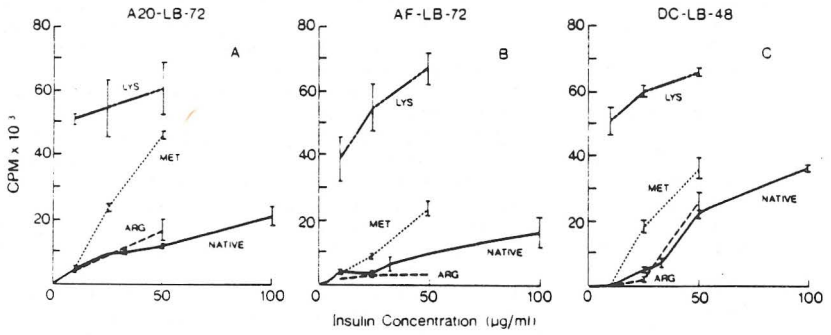

FIG. 4. T cell responses to beef insulins derivatized by the addition of an amino acid at position B1.

To understand the role of antigen processing in the development of the T cell repertoire for that antigen, we analyzed the proliferative responses of our panel of insulin-specific T cells to different fragments of insulin generated by in vitro proteolysis. This approach of mimicking antigen processing in vitro by either enzymatic digestion or chemical cleavage was previously used to study the immunogenicity of other proteins such as lysozyme [23,24], ovalbumin [25], cytochrome C [14] and myoglobin [13]. Our approach was guided by several previously reported findings on the structure and immunogenicity of insulin. First, the amino acid sequences as well as many sites of enzymatic cleavage of both beef and pork insulins are known [2,26]. Second, antibody [27] and T cell [5] reponses to beef and pork insulin in $H\text{-}2^b$ and $H\text{-}2^d$ mouse strains are elicited to two dominant insulin epitopes, one consisting of residues A8, A9, A10 and B3 and the other consisting of residues A1, A4, B29 and B30. Both of these epitopes are situated in a hydrophilic region on a surface of the molecule that is topographically distinct from a hydrophobic region comprised of residues B22 to B27 which binds to the insulin receptor [28]. Third, the sites of cleavage of insulin by chymotrypsin are located such that one would expect the structure of these two insulin epitopes to remain intact after digestion of insulin by this enzyme. Chymotrypsin is known to cleave insulin at the carboxy-terminus of four residues, A14, B16, B25 and B26; these cleavages should yield three peptides, the first consisting of residues A1 to A14 disulfide-linked to residues B1 to B16, the second consisting of residues A15 to A21 disulfide-linked to residues B17 to B25 and the third consisting of residues B26 to B30 [29].

We assayed the response of B8/PB, A20, DC and AF T cells to a series of beef and pork insulin-derived chymotryptic peptides that were fractionated by HPLC chromatography on a reverse phase C18 column.

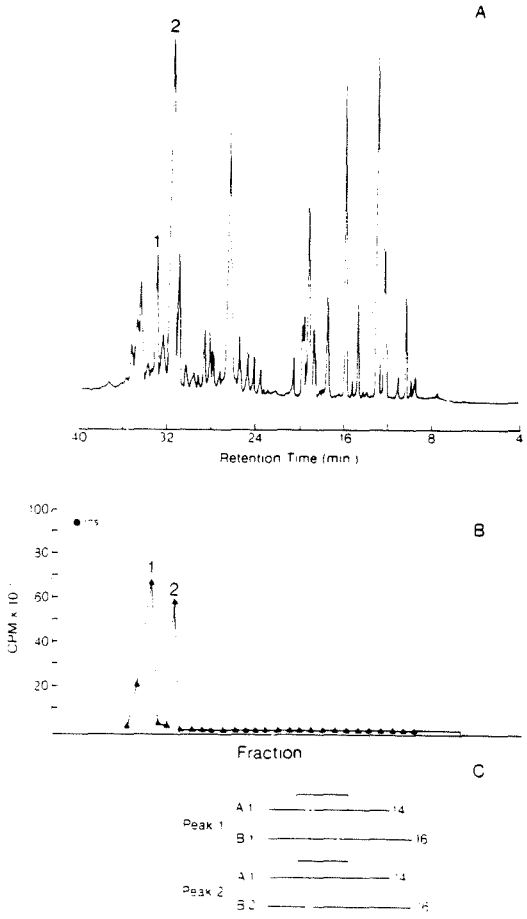

FIG. 5. Fractionation and assay of beef insulin chymotryptic peptides. (A) The relative OD_{214} is plotted versus the retention time. (B) Each of the peptides resolved in (A) were tested at a concentration of about 1-10 μg/ml for their ability to be presented by LB APC to A20 T cells. Only peptides 1 and 2 displayed this activity. The control response to beef insulin (o) used at 5 μg/ml is shown. (C) The structures of peptides 1 and 2 determined from amino acid composition analyses are indicated.

Of the various beef insulin peptides resolved in Fig. 5A, only two peptides, designated peptides 1 and 2, respectively, activated A20 T cells (Fig. 5B) but neither AF nor DC T cells (data not shown). Amino acid composition analyses of these peptides demonstrated that they possess the structures shown in Fig. 5C. These peptides differ by the presence of a single amino acid, B1, which is present in peptide 1 and absent from peptide 2. Thus, we found that chymotrypsin can also cleave insulin at position B1. It is curious that both peptides are immunogenic, since it has been determined from X-ray crystallographic studies of insulin that the binding of residue B1 to residue A14 confers rotational strength to the molecule [22]. Notwithstanding this fact, the absence of amino acid B1

from peptide 2 seems to enhance its specific activity as compared with that of peptide 1; the amount of protein present in peptide 2 was estimated from optical density measurements to be less than that present in peptide 1 (Fig. 5A) and the response of A20 cells to peak 2 was slightly greater than that to peak 1 (Fig. 5B). It is possible that the absence of B1 phenylalanine, a hydrophobic amino acid, in peptide 2 induces a conformational alteration that enhances the immunogenicity of this peptide perhaps by either augmenting or changing its mode of binding to both Ia molecules on TA3 APC and the antigen receptor for insulin on A20 T cells.

It is interesting to note that a pork insulin-derived chymotryptic peptide, which is comprised of residues A1 to A14 disulfide-linked to residues B1 to B16 and is a homologue of the beef insulin peptide 1 shown in Fig. 4C, stimulates the response of B8 T cells but not the responses of A20, AF or DC T cells. This result is compatible with the finding that B8/P T cells recognize pork insulin in the context of I-A^d molecules presented by TA3 cells [5, see Table III]. Beef and pork insulins differ only at positions A8 and A10; pork insulin contains a threonine for alanine interchange at residue A8 and an isoleucine for valine interchange at residue A10. These A-chain loop associated residues map within one of the two insulin antigenic determinants described above and therefore may be involved in the binding of insulin to distinct allelic Ia molecules and/or T cell antigen receptors. Thus, an interaction of the beef and pork insulin epitope which contains these residues with I-A^d molecules on TA3 cells may result in the activation of A20 and B8/P (I-A^d restricted) T cells. If this is the case, it suggests two possible interpretations of our data; first, this same beef insulin epitope does not bind to I-A^d or I-A^b molecules on LB cells in a manner that can be recognized by AF and DC (I-A^b restricted) T cells and second, the beef insulin epitopes seen by AF and DC T cells in association with I-A^b molecules differ from those recognized by B8/P and A20 T cells recognized in association with I-A^d molecules. Further experimentation with insulin peptides which are smaller in size than peptides 1 and 2 analysed here are required to test these possibilities.

Since the A-chain intra-disulfide loop structure and the A7-B7 interchain disulfide bond are preserved in insulin chymotryptic peptides 1 and 2, it is likely that these peptides also possess the three-dimensional conformations of both the A-chain loop and the A1-A9 and B9-B16 α-helical regions found in the intact insulin molecule [30,31]. Retention of these conformations by an insulin peptide may permit hydrophilic and hydrophobic regions of insulin to be expressed on different surfaces of the molecule. This notion supports the hypothesis of DeLisi and Berzofsky [32] who recently proposed that T cell antigenic sites are amphipathic structures, i.e. structures which have separated hydrophilic and hydrophobic surfaces and display a periodicity in hydrophilic residues. Interestingly, these investigators observed that the A4-A17 and B5-B16 regions of beef and pork insulin are amphipathic structures [32].

Thus, it is apparent from data obtained with both chemically modified insulins and insulin peptides that a) the immunogenicity of insulin depends on its overall conformation and hydropathicity and b) there is a good correlation between the location of the amphipathic structures and the immunogenic epitopes of insulin. However, it should be cautioned that in addition to amphipathicity, other host-related attributes such as self-tolerance and the regulation of Ir gene expression may also be major contributing factors to the immunogenicity of a protein [1,3,14,32,33]. For example, T cell recognition of the various amphipathic sites of an antigen may differ between animals and individuals of different haplotypes, i.e. not all amphipathic sites may be immunogenic. Hence, it is important to extend our studies on the role of antigen processing in the development of the T cell repertoire to an analysis of the insulin-specific T cell response at the whole T cell population level using several additional insulin peptides. It is conceivable that within the T cell repertoire, the

selection of clones that become activated is hierarchical and is strongly influenced by the conformation, relative amphipathicity and binding capacity to both Ia and T cell receptor of the antigenic determinant being recognized [33].

ACKNOWLEDGMENTS

We thank Drs. L. Glimcher and E. Shevach (NIAID, NIH) for generously providing the T and B cell hybridomas, Dr. M. Pierres (Centre d'Immunologie de Marseille-Luminy) for kindly supplying the CTLL cell line, Dr. C. Yeung for synthesizing and supplying the lysine, methionine and arginine coupled beef insulin derivatives, Dr. J. Berzofsky (NCI, NIH) for his advice on the use of chloroquine and leupeptin, Ms. C. Hirstwood, Ms. K. Kokolic and Ms. J. Ellis for their expert technical assistance, and Ms. F. Teodoro for her excellent assistance with the preparation of this manucript.

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