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Structural Analysis of Insulin Determinants Seen by T Cells Directed by *H-2* Genes

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Abstract. The magnitude of the immune response of lymphocytes to defined antigenic determinants of ungulate insulins is controlled by the products of *H-2* genes. Based on the crystallographically solved structure of porcine insulin, the close homology of all the ungulate insulins, and the amino acid substitutions, we built models of the different insulins. We analyzed the likely conformations of the antigenic determinants, and proposed structural specificities accounting for the immunologic phenomena observed.

Introduction

The immune system surveys the structure of macromolecules by virtue of the complementarity between antigenic determinants and antigen-sensitive receptors on T or B lymphocytes or antibodies. Immune response (*Ir*) genes linked to the major histocompatibility complex (MHC; *H-2* in the mouse) are important in regulating the nature of the immune response that follows the binding of the antigenic determinants to the complementary receptors. For example, mice with different *H-2* alleles demonstrate characteristically high or low degrees of T lymphocyte responses to insulin molecules of ungulates, although the molecules may vary by only one, two, or three amino acids at positions 8, 9, or 10 in the A chain loop of the molecule (Keck 1975). Table 1 shows the amino acids at those "A loop" positions for murine, ovine, equine, bovine, and porcine insulins, as well as three other "non-loop" positions: position 4 on the A chain and 3 and 30 on the B chain. Despite the fact that bovine and ovine insulins differ only by a Ser-Gly substitution at A9, $H\text{-}2^k$ mice respond well to ovine but not to bovine insulin, while $H\text{-}2^b$ mice respond more strongly to bovine insulin than they do to ovine insulin. Neither $H\text{-}2^k$ nor $H\text{-}2^b$ mice

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Table 1. Amino acid substitutions between murine, bovine, ovine, equine, and porcine insulins

Origin of insulin	Position					
	A4	A8	A9	A10	B3	B30
Murine	Asp	Thr	Ser	Ile	Lys	Ser
Bovine	Glu	Ala	Ser	Val	Asp	Ala
Ovine	Glu	Ala	Gly	Val	Asp	Ala
Equine	Glu	Thr	Gly	Ile	Asp	Ala
Porcine	Glu	Thr	Ser	Ile	Asp	Ala

respond to porcine insulin. *H-2*^{d} mice, by contrast, respond to ovine, bovine, or porcine insulin (Cohen and Talmon 1980).

The present work was undertaken to analyze the structure of the antigenic determinants of insulin that trigger the proliferative response of T lymphocytes controlled by *H-2* genes. Based on a highly refined 1.5 Å resolution of the three-dimensional structure of the porcine insulin molecule, we built molecular models of the other ungulate insulins. Our approach was to immunize in vivo mice of H-2^k, H-2^b, or H-2^d strains to ungulate insulins and to measure the secondary proliferative responses of their lymph-node T lymphocyte in vitro to the homologous or variant insulins. The experimental reactivities and cross-reactivities were then examined in the light of the models of the various insulins in an attempt to reveal the influence of the conformation of the antigenic determinant on the function of *H-2* genes in regulating the activation of T lymphocytes.

Materials and Methods

Mice. The Animal Breeding Center of this Institute supplied the inbred mouse strains C3H/eB (*H-2*^{k}), C3H.SW (*H-2*^{b}), and BALB/c (*H-2*^{d}). Male or female mice were used at the age of 2–3 months and were matched for age and sex in each experiment.

Antigens. Crystalline bovine, ovine, murine, and equine insulins were purchased from Sigma (Saint Louis, Missouri). Solutions were prepared by adding 10 mg of crystallized insulin to about 10 ml phosphate-buffered saline (PBS). Several drops of 0.01 N NaOH were added to dissolve the insulin without raising the pH above 8. The insulins at concentrations of 1 mg/ml were then filtered through a millipore filter 0.45 mM (Millipore, Bedford, Massachusetts). Porcine insulin in solution was obtained from Nordisk Insulin Laboratorium, Copenhagen (as insulin leo neutral). Purified protein derivative of tuberculin (PPD) was obtained from the Ministry of Health, Israel.

Immunization. Insulins were emulsified in complete Freund's adjuvant (Bacto – Adjuvant Complete H37 Ra, Difco, Detroit, Michigan), and mice were injected in each hind footpad with 0.05 ml of an emulsion containing 10 μg of insulin. Fourteen days later, the draining popliteal lymph nodes were removed and the proliferative response was measured in vitro.

Proliferative response. To test the degree of sensitization to insulins, suspensions of cells from popliteal lymph nodes were incubated in RPMI 1640 medium containing 0.5% fresh, normal syngeneic mouse serum, 2-mercaptoethanol (5×10^{-5} M) and gentamycin (40 μg/ml) in flat-bottomed microtiter plates (Costar, Cambridge, Massachusetts). To each well, 5×10^5 cells were added in 0.2 ml medium containing 50 μg/ml of PPD or insulin, respectively. After 4 days, tritiated thymidine (2μCi = 74 KBq, specific activity

10 Ci/mmol, Nuclear Research Center, Negev, Israel) was added to each well. The cells were harvested onto filter paper 4 h later using an automatic harvester, and the incorporation of thymidine was measured as the delta cpm (cpm in test cultures minus cpm in control cultures without insulin). Each group represents the pooled lymph-node cells of 6–10 mice measured as the mean of three replicate cultures. Standard deviations were usually less than 10% of the mean cpm.

Control groups of mice were injected with PBS emulsified in adjuvant (1:1), and their lymph-node cells were used in the proliferative response in the presence of the test concentrations of insulins. The delta cpm of these control cultures was subtracted from the delta cpm of test mice that had been injected with insulin in adjuvant. This control was necessary because insulin was observed to stimulate the incorporation of thymidine into lymphocytes obtained from mice that had been injected with adjuvant alone. This control allowed us to separate the effect of insulin as an immunogen from its effect as a nonspecific mitogen. The mitogenic effect of insulin on adjuvant-stimulated lymph-node cells never exceeded 20% (it was usually less than 10%) of the specific response to insulin as an immunogen.

Procedure for model building. The 2-Zn porcine insulin coordinates, determined from a highly refined model based on 1.5 Å resolution crystallographic data (Dodson et al. 1979), were obtained from the protein data bank, Brookhaven, 1980. In this crystal form, the asymmetric unit contains two molecules of porcine insulin related by a noncrystallographic twofold axis. The best molecular fit (BMF; Nyburg 1974) program was used to compare the two molecules in the asymmetric unit and to insert long side-chains. The exposed surfaces of components of the insulins were computed using a program written by Richards and Richmond (1978). Since all the insulin molecules investigated had the same number of amino acids, it was possible to build models for the different ungulate insulins without changing the main chain conformation. A substitution of a long side-chain by a shorter one (Ala instead of Val for A8 and Asp instead of Glu in A4) was performed by removing the extra atoms. However, when a larger side-chain replaced a shorter one, the additional atoms were incorporated so that no short Van der Walls contacts were allowed. The details of model building for ovine and bovine insulins (Ranghino et al. 1981) were applied to equine insulin.

Results

Mice of H-2^d, H-2^b, or H-2^k strains were primed in vivo against either bovine, ovine, equine, or porcine insulins and 2 weeks later the T lymphocyte proliferative responses of the draining lymph-node cells were measured to the homologous or variant insulins.

Figure 1 summarizes the responses of BALB/c (*H-2^d*) mice. These mice responded well after immunization to each of the insulins. However, the cross-reactive responses showed two distinct patterns. Immunization to bovine, ovine, or equine insulins led to cross-reactive proliferative responses that were relatively strong to bovine, ovine, and equine insulins, but weak to porcine insulin. In contrast, immunization of *H-2^d* mice to porcine insulin led to a generally weaker response that was equally cross-reactive to the other insulins. Porcine insulin is identical with murine insulin at A8-A10 in the A chain "loop" (Table 1), but has the same "non-loop" substitutions (A4, B3, B30) as do the other ungulate insulins. Bovine, ovine, and equine insulins also differ from murine insulin at A8, A9, or A10 and hence may be considered to have both "loop" and "non-loop" determinants. The results shown here would suggest that the "loop" determinant is dominant over the "non-loop" determinant (Cohen and Talmon 1980).

The pattern of reactivity of *H-2^b* differed markedly from that of *H-2^d* mice (Fig. 2). Inoculation of *H-2^b* mice with porcine insulin failed to generate a response to any of the ungulate insulins. However, the *H-2^b* mice responded very well to immunization with bovine insulin, a response that showed no cross-reactivity to

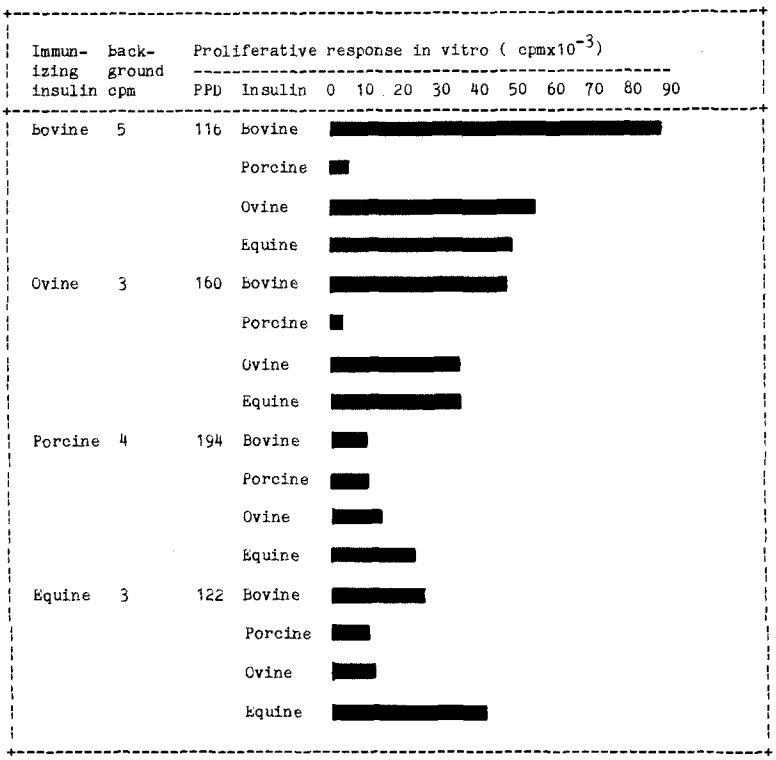

Fig. 1. Response of BALB/c ($H\text{-}2^d$) mice to bovine, ovine, porcine and equine insulins and the cross-reactions between them.

porcine insulin, modest cross-reactivity to ovine insulin and strong cross-reactivity to equine insulin. Note that immunization of $H\text{-}2^b$ mice to ovine or equine insulins led to proliferative responses that differed in magnitude but showed the same pattern of cross-reactivity as that obtained following immunization to bovine insulin. Despite primary immunization to ovine or equine insulin, the strongest response in vitro was to bovine insulin, while the response to equine insulin was relatively weaker and that to ovine was weakest. Thus, immunization with one insulin could prime mice for a secondary response in vitro that was stronger to a heterologous insulin than it was to the homologous insulin (Cohen and Talmon 1980).

Table 2 shows that $H\text{-}2^k$ mice responded to neither bovine nor porcine insulins, but immunization to ovine insulin led to a strong response to ovine, a moderately strong cross-reactive response to equine, and a weak cross-reactive response to bovine insulins. Immunization to equine insulin generated relatively weak responses to equine and ovine insulin and no response to either bovine or porcine insulins. Table 3 summarizes the patterns of response and cross-reactivity to the various ungulate insulins on a scale of relative magnitude.

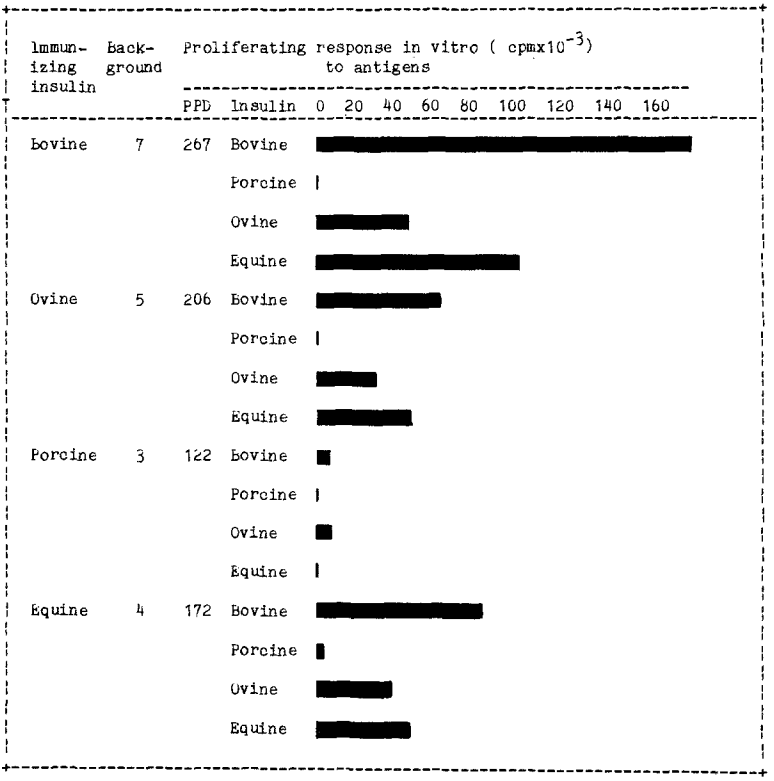

Fig. 2. Response of C3H.SW ($H\text{-}2^b$) mice to bovine, ovine, porcine, and equine insulins, and the cross-reactions among them.

Table 2. Responses of C3H/eB ($H\text{-}2^k$) mice to bovine, ovine, porcine, and equine insulins and the cross-reactions between them.

Immunizing insulins	Background cpm	Proliferative response in vitro to antigens ($\triangle$cpm $\times 10^{-3}$)				
		PPD	Bovine	Porcine	Ovine	Equine
Ovine	8	275	24	4	130	72
Equine	9	223	0	0	24	28
Porcine	10	232	10	0.5	0	8
Bovine	8	207	10	5	5	10

Structural analysis

The structural analysis was based on the assumption that all ungulate insulins have essentially the same conformation and that the main differences between the molecules can be localized to the side chains of the substituted amino acids. This assumption is supported by three observations: (1) antibodies to one ungulate

Table 3. Patterns of response and cross-reactivity to the various ungulate insulins on a scale of relative magnitude

H-2	Immunizing insulin	Proliferative response to insulin			
		Bovine	Ovine	Equine	Porcine
d	Bovine	+++	++	++	–
	Ovine	++	++	++	–
	Equine	++	+	+	–
	Porcine	+	+	+	+
b	Bovine	+++	+	+	–
	Ovine	++	+	+	–
	Equine	++	+	+	–
	Porcine	–	–	–	–
k	Bovine	–	–	–	–
	Ovine	–	+++	++	–
	Equine	–	++	++	–
	Porcine	–	–	–	–

insulin recognize other ungulate insulins (Keck 1975); (2) ungulate insulins bind equally well to the same hormone receptor (De Meyts et al. 1978); and (3) the crystallographically determined structures of porcine (Dodson et al. 1979) and bovine (T. L. Blundell, personal communication) insulins have been found to be very similar.

Mice and rats have two types of insulins that together differ from the ungulate insulins at residues A4, A8-A10, B3, and B30, all located in the proximity of the surface of the molecule (Fig. 3). Murine insulin I also differs from ungulate insulin at B9, which is isolated at a distance of at least 11 Å from the other substituted amino acids. The Pro at B9 makes no contact with any of the atoms of the other substituted residues and therefore it is unlikely that B9 Pro forms part of an antigenic determinant that includes the six other substituted amino acids.

The maximum distance between the edges of the region that includes the atoms of residues A4, A8-A10, and B3 is 21.7 Å. If we include residue B30, the length of the substituted region becomes 31 Å. The accessible surface of the region formed by A4, A8-10, and B3 is 114 $Å^2$. B30 is exposed; its accessible surface area is 40.0 $Å^2$. Important is knowing the size of an antigenic determinant that can be accommodated within the combining site of the receptor of a T lymphocyte. The antigen receptor of T lymphocytes has not been characterized chemically; however, the cross-reactivity between the idiotypes of antibodies and T lymphocytes suggests that the combining sites of the molecules may be of similar size and configuration (Binz and Wigzell 1975, Eichmann and Rajewsky 1975). The binding site of antibodies for haptens have been analyzed crystallographically (Amzel et al. 1974, Ely et al. 1973, Schiffer et al. 1973), and the area was found to be about 150 Å2. This would provide enough room to accomodate A4, A8-A10, and B3 within a single combining site. Therefore, B30 too could be accommodated within a binding site of this size. Hence, it is not unreasonable to assume that an antigenic determinant of

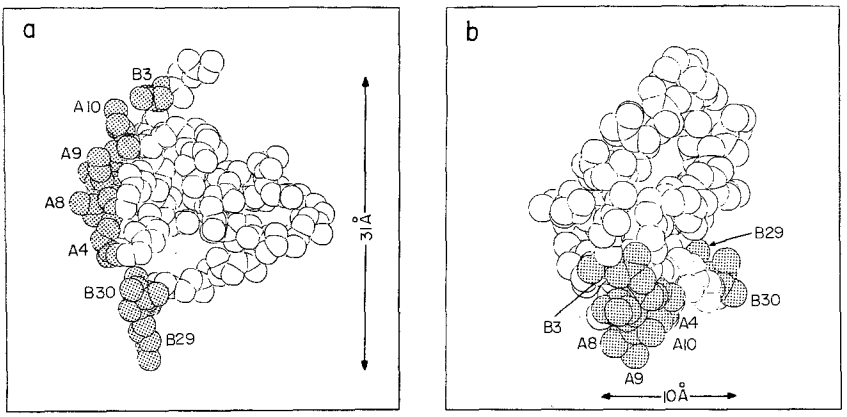

Fig. 3a and b. Van der Waals spheres (radius 1.4 Å) of the backbone atoms of pork insulin and of the side chains of residues A4, A8-A10, B3, B29, B30, which are shaded. The views looking down the x and y axes are drawn on the *right* and *left* sides of the figure, respectively. The coordinates for this figure as well as for Fig. 4 were kindly supplied by G. Dodson.

insulin could consist of five or six residues (A4, A8-A10, B3 and perhaps also B30). This is consistent with the number of residues proposed for the antigenic determinants of myoglobin (Atassi 1979a) and lysozyme (Atassi 1979b). Thus, from a structural point of view, it is not unreasonable to include the A4, B3, B30 "non-loop" substitutions together with the A8-A10 "loop" substitution in a single antigenic determinant. Hypervariable regions appear in the form of clefts with dimensions of about 30×40 Å (Givol 1979, Davies 1975).

The intramolecular Van der Waals contacts and H-bonds that could be made by components of this antigenic determinant are shown in Table 4. Note that: (a) the side chain of Lys B29 does not interact with any other residue and it exists in two conformations; (b) B30, the c-terminal amino acid, interacts only with A1 and is partially disordered in the crystaline state. One of the two molecules that compose the asymmetric unit in the crystals of porcine insulin (Dodson et al. 1979) has two conformations. (c) Residue A9 interacts with A4 through its backbone (Fig. 4). (d) B3 is Lys in murine insulin and Asn in the ungulate insulins. This substitution alone may not generate a foreign antigenic determinant as the side chain of B3 interacts with the rest of the molecule mainly through two atoms: $C\beta$ and $C\gamma$, which make hydrophobic interactions with the side chain of A10 (Fig. 4). The positions of these two atoms can be identical for both Lys and Asn. However, these interactions are missing when Ile (murine or porcine insulins) is replaced by Val (bovine and ovine insulins). Thus, the substitution of Ile by a similar amino acid Val at A10 is probably more consequential than the substitution of Lys by the very different amino acid. It is of interest that the conformation of the side chain of B3 of murine insulin is found to be very similar in three of the four insulin molecules in the 2-Zn crystal forms (Bentley et al. 1976, Dodson et al. 1979). This might be due to the interaction between $C\beta$ and $C\gamma$ of B3 and the side chain of Ile A10. In the fourth molecule, B3 is close to the Zn binding site, which may be the cause for conformational change. (e) $O\gamma$ of Ser A9, and $O\gamma$ of Thr A8 can make H-bonds with a carbonyl and an NH

Table 4. Intramolecular contacts (less than 3.6 Å)

Atom	To atom	Type	Distance (Å)
Thr A8 $O\gamma 1$	A8 N	Porcine, murine, equine	3.0
	A9 N	Porcine, murine, equine	3.1
Thr A8 $C\gamma 2$	A4 O	Porcine, equine, bovine, ovine, murine	3.4
Ser A9 $C\beta$	A5 O	Porcine, murine, bovine	3.3
Ser A9 $O\gamma$	A10 N	Porcine, murine, bovine	2.9
	A10 O	Porcine, murine, bovine	3.0
Ile A10 $C\delta 1$	B3 $N\delta 2$	Porcine, equine, bovine, ovine	3.3
	B3 $C\beta$	Murine, porcine, equine, bovine, ovine	3.6
	B3 $C\gamma$	Murine	3.3

group of the neighboring main chain (Fig. 4). Substitution of Gly, for Ser (ovine and equine insulins) leaves room for a molecule of water in place of the CH_2-OH. This water molecule is so situated that it makes the same H-bonds with the carbonyl and amino groups as would the OH of Ser (bovine insulin). Therefore the combination of Gly and a water molecule can mimic the Ser side-chain.

Discussion

Although the conformations of a molecule in the crystalline state do not represent the range of conformations that the molecule can assume in solution, relating the immunologic properties of insulin to its structure in crystal form may help us appreciate how the receptors of lymphocytes recognize structure. The immunologic cross-reactivities between substituted insulins have been used to define the foreign antigenic determinants of ungulate insulins that are recognized by mouse T lymphocytes under the direction of Ir genes. An A chain "loop" determinant was recognized by mice of $H\text{-}2^d$, $H\text{-}2^b$, and $H\text{-}2^k$ genotypes (Table 3), while a "non-loop" determinant was recognized only by $H\text{-}2^d$ mice (Rosenwasser et al. 1979). Moreover, the "non-loop" determinant was immunologically recessive to the "loop" determinant (Cohen and Talmon 1980). The response of $H\text{-}2^d$ mice to bovine and ovine insulins was similar. Since the only difference between these two insulins is at A9 (Ser and Gly, respectively), and since the difference between murine and bovine insulin is at A8 (Thr and Ala, respectively), one may assume that the pair Ala-Ser is more similar to Ala-Gly than to Thr-Ser or Thr-Gly, that is, Ala-Ser = Ala-Gly $\pm$ Thr-Ser. Similarly, since the interaction with equine and ovine insulin is on the same order of magnitude, then Thr-Gly = Ala-Gly $\pm$ Thr-Ser. Thus, Ala-Ser = Ala-Gly = Thr-Gly $\pm$ Thr-Ser. In other words, A8 Ala can appear like A8 Thr provided that A9 is Gly and not Ser.

$H\text{-}2^b$ mice differed in their pattern of reactivity from $H\text{-}2^d$ mice. The $H\text{-}2^b$ mice immunized with bovine insulin did not respond in vitro to porcine insulin, but did to

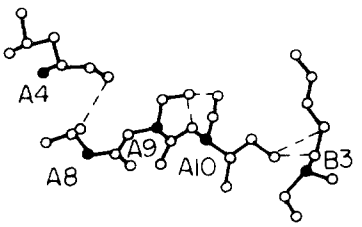

Fig. 4. The conformation of A8-A10, A4 and B3 in pork insulin molecule (Dodson et al. 1979). The alpha carbons are *black*. Possible hydrogen bonds and van der Waals contacts are shown in *broken lines*.

equine insulin, while those immunized to ovine insulin did not respond to porcine insulin but did to bovine and equine insulins. Moreover, immunization with ovine insulin primed a response to bovine insulin that was greater than the response to the homologous ovine insulin. The response of $H\text{-}2^b$ mice to equine insulin was similar to their response to ovine insulin. In contrast to $H\text{-}2^d$ mice, $H\text{-}2^b$ mice distinguished between porcine and equine insulins, responding only to the latter, although the molecules differ only by one amino acid (A9 Ser-Gly). In summary, $H\text{-}2^b$ mice seemed to recognize the "loop" as the major component of an antigenic determinant. Within the "loop" there was a hierarchy of responses to the amino acid pairs

Ala-Ser > Ala-Gly
Ala-Ser > Thr-Gly

and

Thr-Ser ≠ Thr-Gly

The "loop" substitutions were also dominant over the "non-loop" amino acids for the T cells of $H\text{-}2^k$ mice. The response of $H\text{-}2^k$ mice to equine and ovine insulin was almost identical but there were marked differences between the responses to equine and porcine insulins. Thus, the critical amino acid was A9-Gly.

The above results support the conclusion that the amino acid residues at positions A8-A10 in the "loop" have a major influence on the reactivity of murine T lymphocytes to the ungulate insulins. Is the "loop" itself seen as an antigenic determinant by T lymphocytes? Although only the substituted residues in the "loop" (A8-A10) are sequentially attached, the three-dimensional structure of insulin places the other substitutions (A4, B3, and B30) in contiguity with A8-A10 across the surface of the molecule (Fig. 4). Moreover, the contribution of A8-A10 to the surface conformation is not as an independent loop but rather as a part of the continuum or patch of the substituted residues. According to its surface area, the bulk of this variable patch of six amino acid residues could be accommodated within a combining site of a single antibody, or presumably, of a T lymphocyte. This raises the possibility that the entire patch (A4, A8, A9, A10, B3, and B30) serves as a single antigenic determinant, of which the immunologically critical portion is A8-A9-A10. If this is the case, then the so-called "non-loop" determinant recognized in porcine insulin by $H\text{-}2^d$ mice might reside on an unsubstituted part of the molecule and constitute a self-antigenic determinant. In this light, we could understand the immunologically recessive nature of such a self-determinant when it is combined with a foreign determinant defined by substitutions at A8 and A10 in ovine or bovine insulins. In fact, we have observed (Cohen et al. 1979) that porcine insulin

and not bovine insulin sometimes can induce an autoimmune response to mouse insulin.

If the so-called "non-loop" determinant is nevertheless part of the variable patch, the contact between A10 and B3 could explain the immunodominant influence of a residue within the "loop" on the conformation of a "non-loop" side chain. Whether or not the "non-loop" determinant is a self or a substituted structure, it seems that its conformation remains intact in the isolated reduced B chain as it has been shown that porcine insulin and its isolated B chain cross-react for T lymphocytes of $H\text{-}2^d$ mice (Rosenwasser et al. 1979).

Our model building suggests a novel hypothesis to explain the immunologic mimicry between A9 Gly (ovine and equine) and A9 Ser (bovine) insulin variants. It appears that the OH of A9 Ser can make good H-bonds with carbonyl and NH groups of A10 (Fig. 4). These H-bond donors would be available to the OH of a water molecule that could fit in the space provided by the A9 Gly substitution. Therefore, the contour of A9 Gly might look similar to that of A9 Ser under particular conditions of hydration.

As T lymphocytes recognize antigen that is processed by antigen-presenting cells (APC), it is likely that the influence of *H-2* genes on the response to insulin is expressed at the level of the interaction between T lymphocytes and the APC that present insulin. Recently it has been shown that APC originating from *H-2* low responder strains can present antigen to T lymphocytes of responder strains (Ishii et al. 1981). This indicates that low responder APC are able to process and present antigen adequately, and implies that low responder strains may have a defective repertoire of T lymphocytes. However, we have shown that $H\text{-}2^k$ mice can produce T-cell responses to pork or beef insulin if primary immunization is carried out using insulin-pulsed APC rather than insulin in adjuvant (Cohen et al. 1979). Hence $H\text{-}2^k$ mice must possess T lymphocytes capable of recognizing these insulins. Hence, there would not seem to be any absolute defect in either the APC or the T lymphocytes that could explain the low responder phenotype. This has led us to propose that the Ir phenotype is generated by population interactions between competing lymphocyte clones that are quantitative, statistical, and nondiscrete (Grossman and Cohen 1980). Our analysis shows that very slight quantitative differences in affinities could produce what looks like all-or-none immune response, responses that could vary as a result of small changes in initial conditions. The hydration hypothesis viewed in this way would suggest that the arrangement of water molecules over the surface of an immunogen, by modifying the contour of the molecule, could influence the affinity of binding of antigenic determinants to particular lymphocyte receptors. This would, in turn, influence the identity or behavior of competing clones of lymphocytes activated by contact with the immunogen (Grossman and Cohen 1980). Ir gene products might regulate the response of the different clones of lymphocytes by physically associating with the immunogen so as to control its orientation and state of hydration during presentation. To view *Ir* gene control from another vantage point, we have begun to investigate molecular events in the immunologic processing and presentation of the avidin molecule. The results of these studies confirm that T lymphocytes can see conformational determinants and that processed antigen is associated with I region gene products (Friedman et al. 1983).

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