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Immunology

Immune response genes have a variable influence on the selection of antigenic foreign and self determinants of insulin

(T lymphocytes/macrophages/major histocompatibility complex/*H-2* genes/autoimmunity)

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ABSTRACT **We studied the proliferative response of mouse T lymphocytes to determinants on ungulate insulins. The immunopotency of defined determinants on the molecule was found to be regulated by three factors: the immune response genes of the immunized mouse, the mode of presentation of insulin on cells or in adjuvant, and the intramolecular cooperativity between different determinants on the insulin molecule. Autosensitization against self determinants was observed under specific conditions. These findings emphasize the variable expression of immune response genes.**

Immune response (*Ir*) genes linked to the major histocompatibility complex (MHC) appear to restrict the response of T lymphocytes to certain determinants on immunogenic molecules (1). Recent studies of the secondary response of guinea pig T lymphocytes to ungulate insulins suggest that *Ir* genes may act through antigen-presenting macrophages (2, 3). To investigate further the selection of specific foreign and self determinants, we have studied the primary response of mouse T lymphocytes to determinants on beef and pork insulins.

These ungulate insulins differ from mouse insulin (Table 1) at several defined portions of the molecule (4, 5). Beef insulin (but not pork) differs from mouse at the A chain loop (A8–10). Both ungulate insulins differ from mouse insulin at A4, B3, and B30. It is likely that the A chain loop and the B3 and B30 portions of the molecule can contribute to foreign immunopotent determinants because they are accessible (6), do not affect or participate in binding to the hormone receptor (7), and influence the specificity of the immune response (8–10). Except for the substitution of glutamic acid for aspartic acid at A4, the other parts of ungulate insulins are identical to mouse insulin and may be considered to represent mouse self determinants.

Mice of the $H\text{-}2^b$ haplotype were found to respond to the A chain loop determinant present on beef insulin but not to the other determinants also present on pork insulin. $H\text{-}2^k$ strain mice did not respond to either beef or pork insulin injected with complete Freund's adjuvant. $H\text{-}2^d$ mice responded to both insulins (8).

Our approach was to compare the T lymphocyte proliferative response of $H\text{-}2^k$ and $H\text{-}2^b$ mice to ungulate insulins presented either in complete Freund's adjuvant or processed by mouse peritoneal exudate cells. The results described here indicate that MHC genes do not control the nature of the response to specific determinants in a fixed manner. Rather, different modes of presentation of insulin led to shifts in the responsiveness of *H-2* genotypes to specific foreign determinants. Furthermore, the mode of presentation of ungulate insulin and the nature of its foreign determinants controlled the response to self determinants on the molecule. Thus, selection of self as well as foreign determinants by the immune system was found to be dependent on three factors: the genotype of the responding mouse, the mode of presentation of the insulin, and intramolecular cooperation between different determinants. These findings have implications for current ideas about mechanisms of *Ir* gene control of the immune response.

Table 1. Amino acid substitution between mouse, beef, and pork insulins

	Substituted residues					
	A chain				B chain	
Insulin	4	8	9	10	3	30
Mouse	Asp	Thr	Ser	Ile	Lys	Ser
Pork	Glu	Thr	Ser	Ile	Asn	Ala
Beef	Glu	Ala	Ser	Val	Asn	Ala

The sequence differences relate to those present on both forms of mouse insulin (5). Positions 8–10 in the A chain are within the loop.

MATERIALS AND METHODS

Animals. Mice of strains C3H/eb ($H\text{-}2^k$), B10.BR ($H\text{-}2^k$), BALB.C3H ($H\text{-}2^k$), BALB.B10 ($H\text{-}2^b$), C57BL/6 ($H\text{-}2^b$), C57BL/10 (B10; $H\text{-}2^b$), B10.A(4R) ($H\text{-}2^{h4}$), B10.A(5R) ($H\text{-}2^{t5}$), HT1 ($H\text{-}2^t$) were supplied by the Experimental Animal Breeding Center of this Institute.

Antigens. Crystalline beef insulin was purchased from Schwarz/Mann (Lot No. DZ-3133) and pork insulin in solution from Nordisk Insulinlaboratorium (Copenhagen) as Insulin Leo, Neutral. Beef insulin was prepared by adding 10 mg of crystallized material to about 5 ml of phosphate-buffered saline (P_i/NaCl). Several drops of 0.01 M NaOH were added to dissolve the insulin without raising the pH above 8. The beef insulin at 1 mg/ml was then filtered through a Millipore filter (0.45 μm pore diameter). Mouse insulin was purchased from Novo Industri (Copenhagen) and dissolved directly in the culture medium described below.

Purified protein derivative (PPD) was obtained from the Ministry of Health, Israel.

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

Immunization *In Vivo* with Peritoneal Exudate Cells. To stimulate peritoneal exudates, mice were injected intraperitoneally with 3 ml of sterile thioglycollate broth (Difco). Four or 5 days later, the exudate cells were collected by washing the

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Abbreviations: MHC, major histocompatibility complex; B10, C57BL/10 mouse strain; PPD, purified protein derivative; P_i/NaCl, phosphate-buffered saline.

peritoneum with P_i/NaCl containing heparin (5 units/ml; Evans Medical, Liverpool, England) and gentamycin (40 μg/ml; Teva, Jerusalem). The peritoneal exudate cells were used for presenting insulin either *in vivo* or *in vitro*.

For immunization *in vivo*, 10^8 cells (20×10^6/ml) were incubated at 37°C for 45–60 min with beef or pork insulin (50 μg/ml) in P_i/NaCl. No serum was added. The exudate cells were then washed by centrifugation three times in P_i/NaCl to remove free insulin, and 10^7 cells in 0.05 ml of P_i/NaCl were injected into each hind foot pad of syngeneic mice. Six or 7 days later, the draining popliteal lymph nodes were removed and the lymphocytes were tested for their response to insulins in the proliferative assay *in vitro*.

Immunization *In Vitro* with Macrophages (Adherent Peritoneal Exudate Cells). Suspensions of peritoneal exudate cells obtained as described above were incubated in RPMI 1640 medium plus 50 μM 2-mercaptoethanol without serum (RPMI medium) in 100-ml plastic tissue culture dishes (Nunc, Roskilde, Denmark), $10–15 \times 10^6$ cells per 10 ml per plate for 24 hr at 37°C in a humidified incubator in an air/10% CO_2 mixture. Nonadherent cells were removed by vigorous pipetting of the plates with P_i/NaCl until there remained only large, adherent, spreading cells that had the morphology of macrophages. These cells were pulsed with insulins by incubating them with 5 ml of RPMI medium containing insulin at 100 μg/ml. After incubation for 2 hr at 37°C, the cultures were washed three times with P_i/NaCl to remove any free insulin. Lymphocytes were immunized against insulins by incubating spleen cells from normal syngeneic mice (10^8 in 10 ml of medium) with insulin-treated adherent cells for 20 hr at 37°C. The lymphocytes were separated from the insulin-pulsed adherent cells by gently removing the nonadherent cells. The cells were then washed twice by centrifugation and resuspended in P_i/NaCl to a concentration of 5×10^6/0.05 ml. Syngeneic mice were then injected in each hind foot pad with 5×10^6 sensitized lymphocytes. The degree of sensitization of these lymphocytes was tested 6 days later by removing the draining popliteal lymph nodes and testing the proliferative response *in vitro* of suspensions of the lymph node lymphocytes. The procedure was similar to that described (11), except that sensitized lymphocytes were not irradiated before injection into the foot pads.

Proliferative Response *In Vitro*. To test the degree of sensitization to insulins, suspensions of cells from popliteal lymph nodes were incubated in RPMI medium containing 0.5% fresh normal syngeneic mouse serum, 50 μM 2-mercaptoethanol, and gentamycin at 40 μg/ml in flat-bottomed microtiter plates (Costar, Cambridge, MA). Each well contained 5×10^5 cells in 0.2 ml of medium containing various concentrations of insulin. After 4 days, tritiated thymidine [2 μCi, specific activity 10 Ci/mmol, Nuclear Research Center, Negev, Israel (1 Ci = 3.7×10^{10} becquerels)] was added to each well. The cells were harvested onto filter paper 4 hr later by using an automatic harvester, and the incorporation of thymidine was measured as the Δcpm (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 P_i/NaCl emulsified in adjuvant (1:1), and the proliferative response of their lymph node cells was measured in the presence of the test concentrations of insulins. The Δcpm of these control cultures was subtracted from the Δcpm of test mice that had been injected with beef 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 was never more than 25%, and usually less than 20%, of the specific response to insulin as an immunogen. The injection of peritoneal exudate cells that had not been specifically exposed to insulin led to a much smaller nonspecific response to insulin. Insulin had no mitogenic influence on lymphocytes from unstimulated lymph nodes. There was no crossreactivity between insulin and other antigens such as thyroglobulin. These specificity controls are not shown in the tables.

RESULTS

H-2 genes influence proliferative response to beef insulin injected with adjuvant

Table 2 shows the results of injecting several strains of mice with beef insulin emulsified in complete Freund's adjuvant. Suspensions of lymphocytes from the draining popliteal lymph nodes were assayed for their proliferative response to the PPD antigen present in the adjuvant or to beef or pork insulins. A positive response in this type of assay depends largely on the presence of T lymphocytes sensitized specifically to the test antigens (11, 15). It can be seen that all the strains of mice responded to PPD. However, the response to beef insulin appeared to be a function of the alleles at the *K* end of the *H-2* complex. C57BL/6, HTI, and B10.A(5R) strains of mice, all with *b* alleles at *H-2K*, *I-A*, and *I-B*, responded to beef insulin. B10.A(5R) responded relatively less well than mice with $H\text{-}2^b$ alleles throughout the *I* region. It is possible that the strength

Table 2. Response to beef insulin injected with adjuvant is influenced by *H-2* genotype

Strain	*H-2* alleles: *I* region *K A B J E C D*	Background cpm	Proliferative response, Δcpm: PPD	Insulin: Source	1 μg/ml	10 μg/ml	100 μg/ml	250 μg/ml
C57BL/6	b b b b b b b	7499	17,100	Beef	<0	11,950	42,410	15,970
				Pork	<0	<0	<0	<0
HTI	b b b b b b | d	1125	23,360	Beef	907	6,912	31,161	37,921
B10.A(5R)	b b b | k k d d	4567	53,100	Beef	<0	758	13,819	<0
				Pork	<0	<0	<0	<0
B10.A(4R)	k k | b b b b b	4236	17,170	Beef	<0	<0	<0	<0
				Pork	505	<0	<0	<0
C3H/eb	k k k k k k k	1284	17,800	Beef	460	1,565	290	1,380
				Pork	<0	80	590	<0

Mice were injected with beef insulin in complete Freund's adjuvant and the proliferative response of lymphocytes from the draining lymph nodes was measured against PPD or beef or pork insulins.

Table 3. Immunization to insulin by injection of insulin-fed exudate cells reverses the relative responses of C57BL/6 and C3H/eb mice

Strain	*H-2*	Background cpm	Proliferative response to beef insulin, Δcpm 1 μg/ml	10 μg/ml	100 μg/ml	250 μg/ml
C57BL/6	*b*	402	125	188	2,164	3,150
C3H/eb	*k*	945	2212	1750	10,230	20,700

Mice were injected with beef insulin-fed syngeneic exudate cells and the proliferative response of lymphocytes from the draining lymph nodes was measured against beef insulin.

of the response is influenced by additional *I* region loci. B10.A(4R) mice failed to respond to beef insulin. These mice are congenic with responder B10.A(5R) but have *k* alleles at *H-2K* and *I-A* regions. C3H/eB mice (*H-2*^{k}) also did not respond to beef insulin. Thus, *b* alleles at *H-2K*, *I-A*, and *I-B* regions were associated with a T lymphocyte proliferative response to beef insulin and *k* alleles at *H-2K* and *I-A* regions were associated with no response. A similar conclusion was reached by using an assay of T lymphocyte helper function (12).

None of the strains of mice showed crossreactivity to pork insulin (Table 2). This indicates that the response of mice with suitable *b* alleles was to the A chain loop of beef insulin and not to the A4, B3, or B30 potential determinants or to undefined mouse self determinants shared by beef and pork insulins.

Reversal of *Ir* phenotype by injection of insulin-fed exudate cells

To study the function of antigen-presenting cells in *H-2* gene control of the response to beef insulin, we immunized mice by injecting them with exudate cells that had been fed with insulin *in vitro*. The results are shown in Table 3. Mice of the C3H/eb strain (*H-2*^{k} haplotype) demonstrated a relatively strong response to beef insulin after sensitization via insulin-fed syngeneic exudate cells. C57BL/6 (*H-2*^{b}) mice showed a relatively low response. These results, confirmed in seven experiments, appeared to be the opposite of those obtained after immunization via adjuvant (Table 2). Hence, the phenotype of the immune response of C3H/eb and C57BL/6 was reversed by injecting beef insulin-fed exudate cells rather than insulin emulsified in complete Freund's adjuvant. The results of preliminary experiments suggest that genes outside of the *H-2* complex were critical in the response of C3H/eb mice to injection of insulin-fed exudate cells.

Macrophages present insulin to *H-2*^{k} mice

To learn whether macrophages in the population of exudate cells were responsible for presenting beef insulin, we modified a system of sensitizing unprimed lymphocytes by antigen-fed macrophages *in vitro* (11, 13). The macrophages were isolated from the exudate cells by their adherence to culture dishes and fed with beef insulin. Spleen cells were sensitized to beef insulin by incubating them with the insulin-fed macrophages *in vitro*. The spleen cells were then injected into the hind foot pads of syngeneic mice and the draining popliteal lymph nodes were assayed 6 days later for lymphocytes responsive to beef or pork insulin.

Table 4 shows the results of experiments using congenic mice of *H-2*^{k} and *H-2*^{b} haplotypes on two different backgrounds. *H-2*^{k} mice on both the B10 and BALB backgrounds responded relatively well after injection of spleen cells that had been incubated with beef insulin-fed macrophages. This has been the consistent finding with other mice with *H-2*^{k} alleles (C3H.DiSn, C3H/eb, and B10.A). Mice of *H-2*^{b} strains tended to respond poorly, compared to *H-2*^{k} mice (experiment B); however, occasionally *H-2*^{b} mice did respond well (experiment A).

It is noteworthy that beef insulin-fed macrophages activated *H-2*^{k} lymphocytes that recognized both beef and pork insulin (Table 4). Thus, determinants in addition to the A chain loop were recognized. These could have included either the B chain determinant shared by pork and beef insulins or self determinants of mouse insulin also present on ungulate insulins.

Autosensitization to mouse insulin

We analyzed the nature of the determinants recognized after immunization with insulin-fed exudate cells by studying crossreactivity between beef, pork, and mouse insulins. We found (Table 5) that C57BL/6 mice (*H-2*^{b}) responded poorly to injection of exudate cells that had been fed with either beef or pork insulin.

C3H/eb mice (*H-2*^{k}), however, responded to injection of exudate cells fed with either insulin (Table 5). Cells fed with beef insulin sensitized a population of lymphocytes that recognized both beef and pork but not mouse insulins. This indicated that the C3H/eb mice responded to determinants common to beef and pork insulins (Table 1), but not to self determinants shared by ungulate and mouse insulins.

In contrast, injection of C3H/eb mice with exudate cells that had been fed with pork insulin led to the generation of lymphocytes that responded well to mouse insulin and also to the ungulate insulins (Table 5). Hence, injection of pork insulin-fed exudate cells was more stimulatory of an autoimmune response

Table 4. Sensitization *in vitro* by using beef insulin-fed macrophages leads to response with crossreaction to pork insulin

Exp.	Strain	*H-2*	Background cpm	Insulin source	Proliferative response, Δcpm 1 μg/ml	10 μg/ml	100 μg/ml
A	B10	*b*	10,677	Beef	<0	659	12,638
			7,215	Pork	1794	1789	<0
	B10.BR	*k*	6,404	Beef	<0	895	11,956
			3,943	Pork	1931	8551	<0
B	BALB.B10	*b*	352	Beef	380	712	985
			128	Pork	710	436	<0
	BALB.C3H	*k*	773	Beef	1188	2105	4,875
			530	Pork	1960	2755	1,101

Spleen cells were sensitized *in vitro* by culture with beef insulin-fed syngeneic macrophages. The sensitized spleen cells were then injected into syngeneic mice. The proliferative response of lymphocytes from the draining lymph nodes was measured against beef or pork insulins.

Table 5. Autosensitization to self determinants on insulin is influenced by genotype of responding mouse strain and foreign determinants on insulin molecule

Strain	*H-2*	Sensitization	Background cpm	Proliferative response, Δcpm		
				Beef	Pork	Mouse
C57BL/6	*b*	Beef	1454	510	364	<0
		Pork	2699	310	49	<0
C3H/eb	*k*	Beef	4144	24,020	36,323	<0
		Pork	4814	12,910	6,530	24,580

Mice were injected with syngeneic beef or pork insulin-fed exudate cells and the proliferative response of lymphocytes from the draining lymph nodes was measured against beef, pork, or mouse insulins at 50 μg/ml.

to mouse insulin determinants than was injection of beef insulin-fed exudate cells. Because the only difference between these ungulate insulins is in the A chain loop region, this specific foreign determinant regulated the immunogenicity of self determinants present on the insulin molecule. Therefore, provided that the mode of presentation (exudate cells rather than adjuvant) and the strain of mouse (C3H/eb rather than C57BL/6) were permissive, an autoimmune response was contingent upon a suitable array of foreign and self determinants on the insulin molecule.

DISCUSSION

It has been suggested that MHC-linked *Ir* genes regulate the immune response through the agency of macrophages by controlling the manner in which macrophages process and present immunogens to lymphocytes (2, 14, 15). In this way macrophages define the determinants available to activate those lymphocytes possessing complementary antibody-like receptors. A fundamental question is how *Ir* gene products in macrophages might choose determinants for presentation. One suggestion is that *Ir* gene products actually recognize specific amino acid sequences on fragments of protein antigens (2, 14). Another idea is that *Ir* gene products might be related to families of enzymes that metabolize antigens in very specific ways so that only selected determinants remain intact for presentation to lymphocytes (2). These concepts assume that *Ir* gene products themselves can distinguish between determinants.

However, the findings presented here cannot easily be explained by the notion that *Ir* genes code for structures with specific recognition sites. If *Ir* gene products themselves see specific determinants, how do $H\text{-}2^k$ and $H\text{-}2^b$ mice change their immune response phenotypes so readily under the different circumstances we observed? It would seem more reasonable to propose that other factors present in both responders and nonresponders actually see specific determinants and that *Ir* genes regulate the expression of these factors. Thus, both $H\text{-}2^k$ and $H\text{-}2^b$ mice would have the machinery to recognize particular ungulate insulin determinants, but *Ir* genes would regulate variable expression of this machinery under different conditions of immunization with adjuvant or exudate cells, or combinations of cooperating determinants.

The results of experiments done by Keck (9) also support the conclusion that mice of the C3H $H\text{-}2^k$ "low responder" haplotype possess the immune machinery for responding, even in adjuvant, to determinants on pork insulin. Keck injected C3H/He mice with adjuvant including a mixture of pork insulin and sheep insulin, to which $H\text{-}2^k$ mice normally respond (9). He found that the expected response to sheep insulin was markedly reduced, while that to pork insulin was increased. This showed that C3H/He mice were able to recognize the pork insulin determinant, and in addition that a cooperative interaction between insulin determinants on separate molecules could take place.

Our results indicated that a cooperative interaction between determinants on the same insulin molecule could control the immunopotency of particular self determinants. We found that the presence or absence of the beef A chain determinant influenced the immune expression of mouse self determinants on the ungulate insulin molecule (Table 5).

The triggering of autosensitization to mouse insulin has implications for our understanding of autoimmune processes. Our results suggest that mice have lymphocytes capable of recognizing self determinants on insulin despite the fact that the immune system has a lifelong exposure to circulating insulin. This extends our earlier observations that healthy rats (16) and guinea pigs (13) have lymphocytes that can recognize brain antigens and that the immunogenicity of such self antigens is controlled by the mode of presentation.

The mode of presentation has also been found to be critical in regulating the immune response to synthetic antigens under *Ir* gene control. Pierce and coworkers (17) studied the secondary antibody response *in vitro* to GAT, a polymer of glutamic acid, alanine, and tyrosine under MHC-linked control. They found that *H-2* nonresponder macrophages could present GAT to F_1 hybrid (responder × nonresponder) lymphocytes for a secondary response only when the F_1 hybrids had been injected earlier with GAT-fed nonresponder macrophages. There was no secondary response to GAT-fed nonresponder macrophages after immunization of F_1 hybrids with GAT in adjuvant. Other studies demonstrated that nonresponder *H-2* haplotype mice developed suppressor T lymphocytes when injected with GAT in adjuvant (18).

Exudate macrophages fed with a polyproline synthetic antigen have been found to stimulate an antibody response in syngeneic mice that responded poorly to the same antigen injected with adjuvant (19). Genetic control of this response was not linked to MHC genes.

Intramolecular coorperativity between different determinants involving suppressor cells has been described in responses against the immunogens β-galactosidase and fowl lysozymes (20). The response to lysozymes, like that to insulins, is under *H-2* genetic control. It appeared that suppressor cells were activated by a particular determinant on a lysozyme molecule and such suppressor cells exerted a negative effect on the response to other lysozyme determinants.

Intramolecular competition between determinants on multichain synthetic polypeptides has been described by Taussig and coworkers (21). *Ir* gene-regulated selection of the dominant determinant could be modified in one case by injection of poly(adenylic acid)·poly(uridylic acid), an agent that can stimulate macrophages. The response to the dominant determinant was decreased, while that to the previously weak determinant was augmented (22).

Whether or not suppressor cells or some other mechanism is involved in selecting determinants on insulin, the results presented here indicate that the selection is sensitive to a

number of variables; *Ir* genotype, mode of presentation, and intramolecular cooperativity between determinants. Can we postulate a single feature of the T cell immune response that could serve as a final common pathway for these disparate influences?

One way to approach this question is to consider the fact that presentation and recognition of an immunogen take place at the cell membrane. Therefore, it is conceivable that selection of determinants is influenced by the orientation of the immunogenic molecule at the interface between an antigen-presenting cell and a responding lymphocyte. The manner in which an immunogen sits in or floats on the membrane could decide whether a particular determinant or set of determinants was displayed or buried. T lymphocytes have been shown to recognize determinants in association with MHC products (23). Hence, it is not unlikely that products of MHC genes are components of the patch of membrane specializing in antigen presentation. The probability of specific orientation of an immunogen floating at anchor in the antigen-presenting patch would be influenced by the integrated physicochemical properties of the immunogen itself, as well as the lipid and MHC glycoprotein components of the membrane patch. In this way, orientation of an immunogen might be influenced by chemical substitutions or additions of other determinants to the immunogen itself, by small allelic differences in MHC gene products, or by changes in the state of the membrane of the interacting cells.

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