IMMUNE RESPONSE GENE CONTROL OF DETERMINANT SELECTION

I. Intramolecular Mapping of the Immunogenic Sites on Insulin Recognized by Guinea Pig T and B Cells

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There is considerable interest in identifying on structurally defined proteins, the precise amino acid sequence and conformation of those regions in a molecule that determine immunogenicity (1–3). Most studies have assessed the ability of antibodies generated by immunization of the animal with one molecular form to cross-react with either natural or synthetic antigen fragments, chemically modified immunogens, or naturally occurring protein mutants obtained from different species (4–6). Such approaches provide data necessarily restricted to identification of those areas of the molecule "seen" by T-helper cells (7, 8) or by antibody, the secretory product of differentiated B cells. In view of the controversy as to the nature and specificity of the receptor on the T lymphocyte, it would be of considerable value to determine in a complex antigen (*a*) whether the recognition of sequential or conformational determinants is controlled by distinct immune response genes and (*b*) whether those areas recognized by antibody are identical to those visualized by T-cell receptors. This latter issue is relevant to the functional role of shared idiotypic determinants between antibodies and T-cell receptors in rat (9), mouse (10), and guinea pig (11).

Both outbred and inbred strain 2 and 13 guinea pigs are known to be responders to the polypeptide hormone insulin as defined by delayed-type hypersensitivity (12) and specific antibody production (13). We show in the present study that, when T-lymphocyte proliferation and T-helper activity are assessed, different areas of the insulin molecule are recognized by strain 2 and 13 guinea pigs. Responsiveness of T cells to these distinctive regions of the molecule appears to be under the control of immune response genes, linked to the guinea pig major histocompatibility complex (GPLA).[1] By contrast, the specificity of antibodies elicited in strain 2 and 13 guinea pigs immunized with pork insulin were not correspondingly directed against distinct intramolecular regions despite the absolute disparity in the determinants recognized by their T cells. We

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[1] *Abbreviations used in this paper:* BUdR, 5-bromodeoxy uridine, CFA, complete Freund's adjuvant; FCS, fetal calf serum; GPLA, guinea pig major histocompatibility complex; HBSS, Hanks' balanced salt solution; MIF, migration inhibition factor; Mϕ, macrophages; OVA, ovalbumin; PECs, peritoneal exudate cells; PEG, polyethylene glycol; PELs, antigen-reactive T cells; PPD, purified protein derivative of tuberculin

have interpreted such data to indicate that relevant immune response genes function by "selecting" in a complex antigen those regions of the molecule to be recognized by the T lymphocyte for clonal expansion and expression of helper activity. The operation, at a macrophage-T-cell level of an *Ir* gene-dependent system for selection of antigenic determinants, does not appear to limit or define specificities, in a similar manner, at the level of the B cell.

Materials and Methods

Animals. Outbred and inbred strain 2 and 13 guinea pigs (Division of Research Services, NIH) weighing 300–500 g were used as sources of all cells and antisera preparations used in the study.

Media. Cell cultures were performed in medium RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with fresh L-glutamine (0.3 mg/ml), Gentamicin (10 μg/ml), penicillin (200 U/ml), 2-mercaptoethanol (2.5×10^{-5} M; Eastman Kodak Co , Rochester, N. Y.) and 5% fetal calf serum (FCS; Industrial Biological Laboratories, Rockville, Md.). All washing procedures were performed in Hanks' balanced salt solution (HBSS).

Antigens The following antigens were used, either for priming animals or for cell challenging "in vitro": beef, pork, chicken, fish, human, guinea pig, rabbit, rat, and sheep insulins (Eli Lilly & Co., Indianapolis, Ind.). Both beef and pork insulin contained less than 0.05% of pro-insulin contamination. DNP-pork insulin, containing 1.1 moles of DNP per molecule of insulin, was prepared as described (14). Pork insulin A and B chains (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N Y.) contained no detectable insulin contamination on a radioimmunoassay. DNP-ovalbumin (OVA) prepared from five times crystalized hen egg albumin and contained eight DNP groups per molecule of OVA. Purified protein derivative of tuberculin (PPD), was obtained from Connaught Medical Research Laboratory, Toronto, Canada.

Immunization. For the T-cell proliferative assay guinea pigs were immunized with antigen in saline emulsified with an equal volume of complete Freund's adjuvant (CFA) containing 0.5 mg/ml of killed *Mycobacterium tuberculosis*, H37 Ra (Difco Laboratories, Detroit, Mich.). Each animal received 0 1 ml emulsion in each foot pad for the total immunizing doses of antigen per animal as indicated in the text. Cells for culture and primary serum were collected 2–4 wk after immunization.

For the assessment of the T-helper function specificities, 30 strain 2 guinea pigs received 1 mg/day of DNP-OVA intraperitoneally (i.p.) for 3 consecutive days 7 days after the last injection the animals were divided into six groups and immunized with 10 μg/animal of pork, rabbit, rat, sheep, and beef insulin in CFA and one group with CFA alone. 3 wk later the animals were boosted with 100 μg/animal of DNP-pork insulin in incomplete Freund's adjuvant. Serum for the assessment of anti-DNP antibodies was collected immediately before boosting (day 0) and at days 7 and 21 after the last injection.

Cell Cultures Peritoneal exudate cells (PECs) were obtained by lavaging the peritoneal cavity of immune guinea pigs, 4 days after the injection of 25 ml of sterile oil (Marcol 52; Humble Oil & Refining Co., Houston, Tex.) The PECs were washed in HBSS two times and divided in two fractions, one of which was treated with mitomycin C (40 μg/ml; Sigma Chemical Co., St. Louis, Mo) for 60 min at 37°C at a concentration of $5\text{–}15 \times 10^6$/cells/ml. After four washes with HBSS this cell population was used as a source of macrophages (Mϕ). In situations where pulsed Mϕ were required, PECs obtained from virgin guinea pigs were incubated with the antigen under study (100 μg/ml) as previously described (15).

The second PEC fraction was used to obtain a population highly enriched for antigen-reactive T cells (PELs) by passing through a nylon wool adherence column (16). This population was used to assess cell proliferation by assay of a thymidine incorporation into DNA PELs resuspended in RPMI 1640 with 5% FCS were cultured in microtiter plates with round-bottomed wells (Cooke Laboratory Products Div., Dynatech Laboratories Inc., Alexandria, Va.) for 72 h in a 5% CO_2 humidified atmosphere at 37°C at a concentration of 1.2×10^6 PELs/ml and 3.6×10^5 mitomycin-treated Mϕ/ml in the presence of continuous antigen 18 h before termination, 1 μCi/well of tritiated thymidine (6.7 Ci/mmol; New England Nuclear, Boston, Mass.) was added. Cells were harvested onto glass fiber filter paper by the use of a semiautomated microharvesting device. Tritiated thymidine incorporation was then determined by liquid scintillation spectrometry and

the results reported as number of counts above the control (Δ counts per minute) unless otherwise stated

5-Bromodeoxy Uridine (BUdR) and Light Elimination Experiments For some clonal elimination experiments, immune PELs at a concentration of 1×10^6/ml and 3×10^5 mitomycin C-treated Mϕ were cultivated in the presence of continuous antigen for 48 h in 12 × 75 mm capped plastic tubes (Falcon 2058; Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). At the end of this time 2 μg/ml of freshly prepared BUdR (ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp , Cleveland, Ohio) was added to the cultures. 24 h later, the cell pellets were exposed to light by placing the culture tubes directly on an array of three fluorescent light bulbs (Cool-Ray; General Electric Co., Wilmington, Mass.) for 90 min. After four washes of the cell pellet with HBSS, the BUdR- and light-treated cells were cultured in microtiter plates, as described above, but in the presence of antigen-pulsed Mϕ instead of continuous antigen.

Antibody Assay. Anti-DNP antibodies were assayed by a radioimmunoassay using tritiated ϵ-DNP-L-lysine as the hapten (17). ϵ-DNP-L-lysine was prepared by reacting ^{3}H-DNFB with α-t-boc lysine as described (18).

Anti-insulin antibodies were assayed by a radioimmunoassay using ^{125}I-labeled insulin as the ligand. Pork insulin was iodinated by the stepwise procedure described by Gavin et al (19) using progressively increasing amounts of chloramine-T up to the point where the percent of incorporation reached the desired level. Antisera were incubated with ^{125}I-labeled pork insulin for 1 h at 4°C and the immune complex was precipitated with a mixture of 10% polyethylene glycol (PEG) and 40 μg/ml of human serum by centrifugation at 1,000 *g* for 45 min as described by Desbuquois and Aurbach (20) The precipitate was then rewashed with PEG and the amount of radioactivity counted on a gamma spectrometer. For the inhibition assay, antiserum obtained from individual animals were compared in their ability to bind to radioactive pork insulin The volume of the different antiserum that bound the same amount of radioactive antigen was then used to generate the inhibition curves. These latter curves were obtained by incubating the inhibitor (unlabeled insulins of different species) with the antiserum for 30 min at 4°C followed by 1 h incubation with the labeled pork insulin. The amount of radioactivity in the precipitate was counted and the result expressed as percent inhibition from a parallel sample to which no inhibitor was added. Inhibition curves generated by increasing amounts of inhibitors gave straight lines on a log scale. Regression lines were calculated by the method of least squares for each of the inhibitor, their slopes compared by means of an F test and the amount of inhibitor necessary to give 50% inhibition (Ia_{50}) was calculated. The consistency of the ordering of the Ia_{50} values was assessed by the Kendall coefficient of concordance (21) and by a chi-squared test

Serological Definition of Ia Specificities. Guinea pigs were serologically defined by the use of a chromium release assay in an antibody-dependent cytotoxic system as described by Shevach et al (22) using antisera raised by cross-immunizing inbred strain 2 and strain 13 guinea pigs (generous gifts of Dr. E. Shevach National Institute of Allergy and Infectious Diseases, Bethesda, Md.) Briefly, lymph node cells from immunized strain 2 and strain 13 guinea pigs were labeled with 100 μCi/ml of ^{51}Cr (sodium chromate, 7 μg/Cr/ml; Amersham/Searle Corp , Arlington Heights, Ill) and the release measured after the exposure of the cells for 30 min at 37°C to strain 2 anti-strain 13 serum and strain 13 anti-strain 2 serum plus guinea pig complement (Grand Island Biological Co) These antisera are able to recognize alloantigens that by cell distribution criteria and biochemical analysis are equivalent to the murine Ia antigens (23) Animals with cells giving a significant amount of ^{51}Cr release with a 2 anti-13 serum, 13 anti-2 serum, or both were classified, respectively, as 2^-13^+, 2^+13^-, and 2^+13^+.

Results

T-Cell Proliferative Response in Pork Insulin-Primed Animals. PELs from guinea pigs immunized with pork insulin proliferate when challenged "in vitro" with the immunizing antigen (Table I). The response observed in strain 2 guinea pigs ranged from 60 to 80% of that seen in strain 13 guinea pigs. Beef insulin induced a response equivalent to that of pork in strain 13 guinea pigs immunized to pork insulin but in strain 2 guinea pigs gave approximately 50% of the response of the same cells to pork insulin. Note that pork insulin immune

TABLE I

Proliferation Response to Insulin and Isolated Insulin A and B Chains by T-Lymphocytes from Inbred Strain 2 and Strain 13 Guinea Pigs Immunized to Pork Insulin

Addition (10 μg/ml)	Lymphocyte DNA synthesis ^{3}H-thymidine incorporation (Δ cpm × 10^{-3})	
	Strain 2	Strain 13
Pork insulin	39.5 ± 0.7*	70 6 ± 16.3
Beef insulin	15 7 ± 2.3	63.8 ± 7 1
Insulin A chain	3 2 ± 1.8	6.5 ± 1.8
Insulin B chain	2.5 ± 1.2	39.2 ± 7.2

* Mean ± SEM of four experiments.

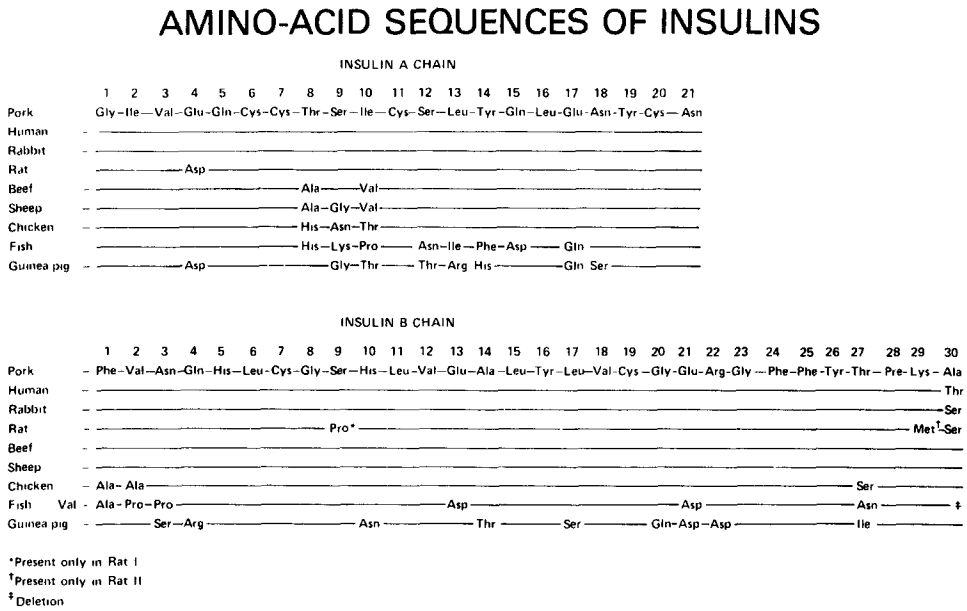

FIG. 1. The amino acid differences between porcine insulin and the several species variants of insulin used throughout the study are shown by the three letter amino acid abbreviations. Identities are shown by the solid lines.

strain 2 guinea pigs do not respond to either isolated oxidized insulin A or B chains while oxidized B but not A chain induced a significant DNA synthetic response in strain 13 guinea pigs. Pork and beef insulin have identical B chains but differ by amino acids 8 and 10 in the A chain (Fig. 1). The specificity of the immune responses observed is indicated by the fact that T cells from nonimmune animals or animals immunized to an irrelevant antigen do not respond to insulin (data not shown).

The T-Cell Proliferative Response to Oxidized Insulin B Chain is Under Genetic Control and Linked to the Major Histocompatibility Complex of Strain 13 Guinea Pigs. The experiments described above indicated that a determinant present in oxidized B chain and displayed in the intact insulin molecule is

TABLE II

T-Lymphocyte Proliferation in Response to Insulin B-Chain Determinants by Strain 2, 13, and F_1 (2 × 13) Hybrid Guinea Pigs Immunized to Isolated Insulin B Chain

Addition (10 μg/ml)	Lymphocyte DNA synthesis ^{3}H-thymidine incorporation (Δ cpm × 10^{-3})		
	Strain 2	Strain 13	F_1 (2 × 13)
Pork insulin	3.06 ± 1 56*	65.59 ± 5.90	22.67 ± 5 37
Beef insulin	2.26 ± 0 84	53 00 ± 3.77	27.11 ± 9 57
Insulin B chain	3.72 ± 1.31	119 02 ± 23.45	66.90 ± 23.69

* Mean ± SEM of three experiments

responsible for a significant fraction of the total DNA synthetic response of T cells in strain 13 but not of strain 2 guinea pigs. To determine whether or not this disparity might be explained by the presence in strain 13 and the absence in strain 2 of an appropriate immune response gene, we evaluated the response of strain 2, 13, F_1(2 × 13), and serologically defined backcrossed and outbred guinea pigs immunized with 10 μg of oxidized B chain in CFA. T cells from strain 13 and F_1 animals responded vigorously on in vitro exposure to oxidized B chain while no response to B chain was observed in strain 2 guinea pigs (Table II). Note that the B-chain immune strain 13 and F_1 (2 × 13) guinea pigs also proliferate when either beef or pork insulin is added to culture. Finally, of backcrossed and outbred guinea pigs, only T cells from those animals bearing the Ia-1,3 specificities recognized by strain 2 antiserum 13 serum [2^-13^+ and 2^+13^+ outbreds; 2^+13^+ (F_1 × 2) backcrosses; and all (F_1 × 13) backcrosses] responded to B chain while 2^+13^- outbreds and 2^+13^- (F_1 × 2) backcrosses did not (Fig. 2).

The B Chain Carries the Only Antigenic Determinant Recognized by T Cells from Strain 13 Guinea Pigs Immunized to Pork Insulin. In order to evaluate if strain 13 guinea pigs were able to recognize other regions of the insulin molecule besides the determinant present on insulin B chain, clonal elimination experiments were undertaken. BUdR is a thymidine analogue which if present in culture during the S phase of the cell cycle, is incorporated into newly synthesized DNA and induces the cross-linkage of the DNA strands upon light activation. Treatment of cells with BUdR and light leads to a permanent block in cell replication. We have been able to demonstrate that in strain 13 guinea pigs, the same clone of T cells which respond to pork insulin, recognizes isolated oxidized B chain (Table III). Thus pretreatment of PELs from guinea pigs immunized to pork insulin and PPD with B chain followed by BUdR and light eliminates 86% of the reactivity of these cells to pork insulin while the response of these same cells to PPD remains very high (Δ cpm > 200,000). Moreover, "negative selection" of pork insulin immune T cells with pork insulin eliminates 71% of the original response to oxidized B chain. Thus, a very low activity remained to pork insulin and B chain after cross-eliminating responsive cells with either of those polypeptides. These results strongly suggest that proliferating T cell of strain 13 guinea pigs immunized with pork insulin recognizes an antigenic determinant restricted to the B-chain portion of the molecule. The amino acids contributing to this determinant have not been further delineated.

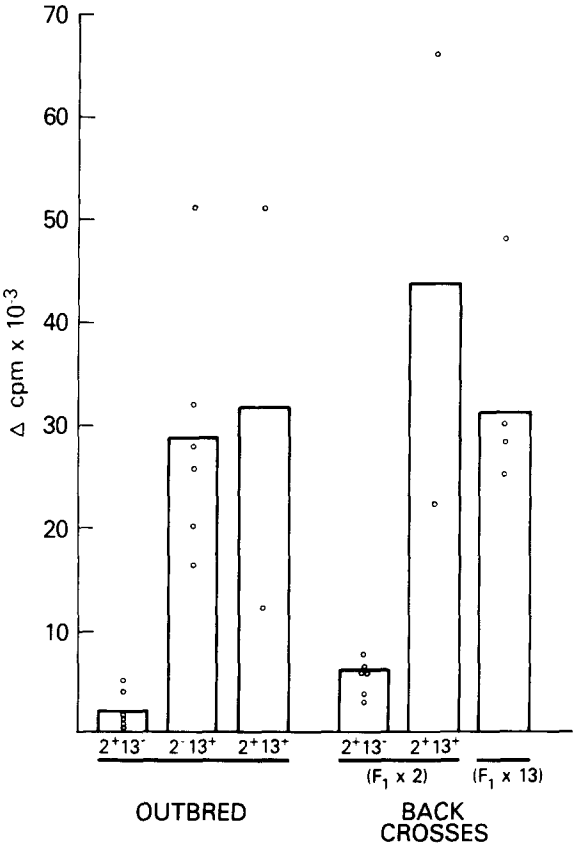

FIG. 2. Genetic analysis of T-lymphocyte proliferation in serologically defined backcrosses and outbred guinea pigs immunized to isolated insulin B chain. Thymidine incorporation into DNA in individual animals (open circles) and mean values (bars) for groups of serologically defined animals. All the 13^+ animals gave responses higher than the 13^- animals.

The Determinant Recognized by Strain 2 Guinea Pigs is on the A Chain of the Insulin Molecule. In order to more precisely define the determinant responsible for the response to insulin in strain 2 guinea pigs, strain 2 and 13 guinea pigs were immunized with intact native pork insulin and the T-cell proliferative response to a variety of purified species variants of insulin was measured and the pattern of cross-reactivity compared between strains. As shown in Fig. 3, the curves generated by addition of varying concentrations of antigen to strain 2 PELs (left panel) form three distinct "families." One family consisted of four insulins (sheep, chicken, guinea pig, and fish) which elicited no DNA synthetic response. A second family formed of four different insulins (rat, pork, rabbit, and human) gave a strong proliferative response. Lastly beef insulin consistently gave an intermediate level of DNA synthesis. The curve distribution pattern generated on strain 13 cells (right panel) differs completely from the one just described. No distinctive families are observed and all the different insulins tested are able to induce a proliferative response although of varying efficiency.

When the ability of the different insulins to mount a T-cell proliferative response in strain 2 guinea pigs are analyzed and compared with their amino

TABLE III

Evidence that a Region in the B Chain of Insulin is the Only Antigenic Determinant Recognized by T Cells from Pork Insulin Immune Strain 13 Guinea Pigs

Lymphocyte DNA synthesis*			
Stimulation by Mϕ pulsed with.	Stimulation (Δ cpm) of BUdR- and light-treated PELs precultured with.		
	PPD	Pork insulin	Insulin B chain
PPD	22,372	208,268	236,999
Pork insulin	61,587	32	13,755
Insulin B chain	49,689	6,518	3,739
Stimulation by Mϕ pulsed with·	Inhibition (% ± SE) of stimulation by BUdR and light treatment of pork insulin immune PELs precultured with:		
	PPD	Pork insulin	Insulin B chain
PPD	93.51 ± 2.81‡		
Pork insulin		99.98 ± 0.02	86.15 ± 0.74
Insulin B chain		71 45 ± 5 65	92 35 ± 1.32

* Typical experiment.

‡ Mean ± SE of three experiments

acid sequences, the following pattern emerges: the insulins that are able to elicit a response must share amino acids identities with pork insulin, in amino acids 8, 9, and 10 of the A-chain loop (α-loop) while insulins that fail to elicit a response need have differences with pork insulin only in this region. Beef insulin which gave an intermediate response shares identity with pork insulin in amino acid 9 (serine) of the α-loop but differs at amino acid 8 and 10 (Fig. 4). Three of the employed insulins are particularly instructive: sheep and beef because they share identities with pork insulin throughout the entire molecule, except for the area under consideration (amino acids A8, A9, and A10) and rat, which in spite of differences in amino acids A4, B10, B29, and B30 is still able to induce a response similar to pork insulin. The high discriminating power of the tested insulins thus permitted us to localize the determinant recognized by strain 2 guinea pig on the A-chain α-loop (amino acids A6 to A11 linked by a disulfide bridge) and indicates that this is the only determinant that defines responsiveness to insulin in this particular strain. Thus both strains 2 and 13 recognize insulin although they do so by recognizing distinct areas of the molecule.

Recognition of the A-Chain α-Loop Determinant by Strain 2 Guinea Pigs Is At Least Partially Independent of Its Amino Acid Sequence. In order to verify if the T-cell α-loop recognition system that is operating on strain 2 guinea pigs is specific for the amino acid sequence Thr-Ser-Ile displayed on pork insulin, or if T cells from strain 2 guinea pig can recognize α-loops with different amino acid sequences, animals were injected with pork or sheep insulin and the T-cell proliferative response assessed. As shown in Table IV, sheep insulin elicited a strong T-cell proliferative response when added back to cells from animals immunized with the same antigen and the pattern of cross-reactivity indicated α-loop recognition. Thus sheep insulin immune animals gave an intermediate

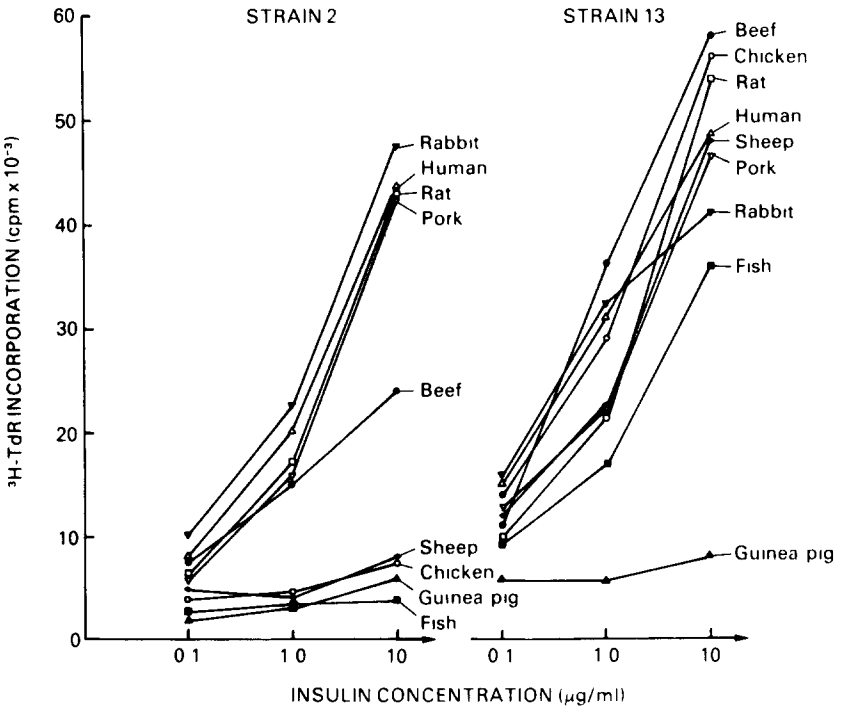

FIG. 3. Proliferative response to mutant insulins of T lymphocytes from strain 2 and 13 guinea pigs immunized to pork insulin. Thymidine incorporation (^{3}H-TdR) into DNA as a function of "in vitro" insulin concentration in strain 2 (left panel) and strain 13 (right panel) PELs from pork insulin immune guinea pig. Three distinct families of curves are generated on strain 2 PELs as opposed to the response shown with strain 13 PELs where no distinct families can be distinguished. Guinea pig insulin is used as a control for nonspecific effects of insulin.

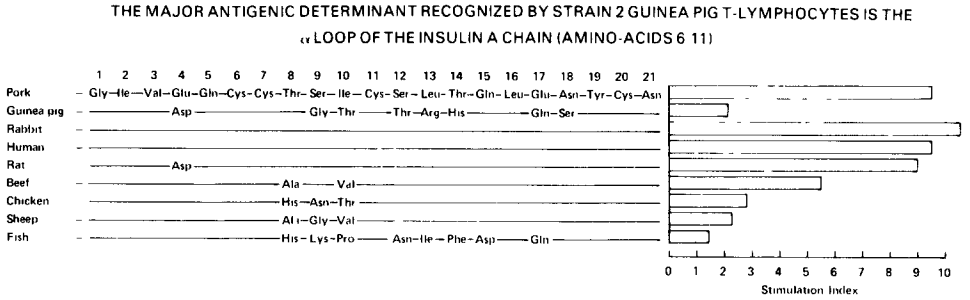

FIG. 4. Thymidine incorporation into DNA in different experiments normalized as a stimulation index (SI) as a function of the amino acid sequences of insulin A chain. Guinea pig insulin is used as a control. Rabbit, human, and rat insulin give SI similar to pork insulin. Beef insulin with substitutions on position A8 and A10 gives a lower SI ($P < 0.01$) and chicken, sheep (substitution on amino acids A8, A9, and A10), and fish (substitution on amino acids A8 to A10, A12 to A15, and A17) give SI no different from guinea pig insulin. The SI is a normalization procedure defined as the product of the Δ counts per minute by a coefficient obtained by dividing the highest and lowest Δ counts per minute obtained in one given experiment (range) by the number of different insulin mutants assessed in the same experiment

TABLE IV

Strain 2 Guinea Pigs Can Be Immunized with Insulins Having A-Chain α-Loops with Different Amino Acid Sequences

Animals immunized with	Lymphocyte DNA synthesis stimulation (Δ cpm) induced by the addition of insulin from:		
	Sheep	Pork	Beef
Sheep insulin	13,214	2,196	9,791
Pork insulin	4,021	38,745	20,870

type of response to beef insulin (beef and sheep insulins share two identities on the α-loop) and a negligible response to pork insulin (pork and sheep do not share identities at the α-loop).

Helper T Cells and Proliferating T Cells Recognize the Same Antigenic Specificities. Since a fine T-cell specificity, with sensitivity for one amino acid difference, has been shown in strain 2 guinea pigs, this strain was used to compare the recognition specificity of helper T cells with that of the proliferating cell. A hapten-carrier system was employed to measure T-cell helper function. Strain 2 guinea pigs were primed i.p. with DNP-OVA and subsequently immunized with different insulins in CFA. The amount of anti-DNP antibodies was measured immediately before (day 0) and on days 7 and 21 after the boosting with DNP insulin. Two insulins that cross-react (rabbit and rat) with pork insulin at the level of T-cell proliferation and two which did not (sheep and fish) were assessed for their ability to prime T cells to function as helpers to DNP-OVA-primed B cells. Only those insulins that share A-chain α-loop identity with pork insulin are able to elicit a T-helper function similar to that elicited by pork insulin (Fig. 5). Insulins different from pork insulin in the A-chain α-loop gave no significant response. Evidence that differences in this specific area of the molecule are enough to impair the ability of an insulin to help, is provided by the nonresponse observed with sheep insulin, which differs from pork insulin exclusively in amino acids A8, A9, and A10. Although these experiments do not discriminate whether or not T-helper and proliferating activities are the function of a single cell clone or distinct subpopulations of antigen-recognizing T cells, they do establish that they bear receptor with the same fine specificity and discriminating capacity at the inductive phase of their responses.

Evidence that Strain 2 and Strain 13 Anti-Pork Insulin Antibodies Share Specificities. Having defined the specificity for T-cell proliferation in both inbred strains of guinea pig, as two dinstinct sites on the pork insulin molecule, operationally under the control of relevant *Ir* genes, and having shown that at least in one strain the same specificities initiate T-cell helper function, we next examined the fine specificity of anti-insulin antibodies generated in strain 2 and 13 guinea pigs. Both antibody populations present similar isoelectric-focusing patterns, revealing very restricted heterogeneity (I. Zitron, personal communication). Inhibition assays of the binding of ^{125}I-labeled pork insulin to strain 2 and 13 anti-pork insulin antibodies by different insulin variants were performed. All the variants tested were able to inhibit the above mentioned reactions. The amount of the different Ia_{50} were calculated for five different sera

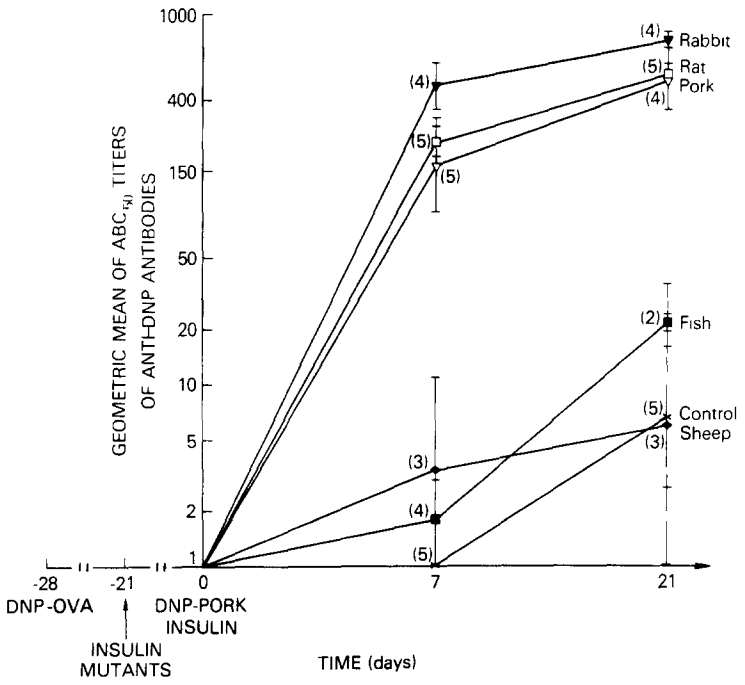

FIG. 5 Determinant specificity of T-helper function. evidence that only insulins sharing identities at the A-chain α-loop cross-react to generate T-helper function activity in strain 2 guinea pigs Levels of anti-DNP antibodies on strain 2 guinea pig serum collected after an immunization schedule designed to assess T-helper function. Rabbit and rat insulins were able to prime T-helper cells as efficiently as pork insulin, on DNP-OVA-primed and DNP-pork insulin-boosted animals Sheep and fish insulin were unable to prime T cells for help on the same animals.

from individual animals and are shown on Table V. In all the five different sera the amount of different insulins required to inhibit 50% of the antigen/antibody reaction are of the same order of magnitude, and no obvious strain-specific clustering of different insulins into distinguishable families is observed. If rank coefficients are given to each individual insulin within each of the five different sera, and the ranking is compared by means of the Kendall coefficient of concordance ($0 < W < 1$) a value of 0.874 is found. This high coefficient of concordance indicates that all the five different sera are indistinguishable in their requirements for binding inhibition and thus indicate that strain 2 and strain 13 guinea pigs generate antibodies to pork insulin that share identical specificities. When the five different sera are compared by means of a χ^2 test, the null hypothesis cannot be rejected, indicating again similarity between the five analyzed sera.

One can however see in the sera from both strains of animals that insulins sharing identities in the entire A chain (pork, human, and rabbit) are better inhibitors than the rest of the insulins. Indeed, if one does a partition of the 0.874 value found for the Kendall coefficient of concordance, one finds that 0.714 of the value is due to the difference between two groups of insulins, mainly group I pork, human, and rabbit and group II beef, chicken, fish, rat, and sheep. This

TABLE V

A Comparison of Strain 2 and Strain 13 Anti-Pork Insulin Antibody Specificities

Insulin used as inhibitor	Strain 2 antiserum			Strain 13 antiserum	
	Serum no			Serum no.	
	1	2	3	1	2
Beef	42.97*	34.41	36.11	23.52	22.83
Chicken	42.20	34 93	54.45	23 63	28.44
Fish	122 77	40.00	55 81	40.54	40.85
Human	38 38	30.76	34.46	16.31	21 54
Pork	37.88	32 25	35.54	18.26	20.23
Rabbit	39.49	19.64	4.54	<10.00	<10 00
Rat	69.87	36.21	41 05	20.98	24 55
Sheep	62.18	36 97	36.59	19 21	25.00
	$W\ddagger_{\text{strain 2}} = 0.894$			$W_{\text{strain 13}} = 0\ 941$	
	$W_{\text{total}} = 0.874$				
	$\chi^2 = 6.02\ (P > 0.30)$				

* Amount (nanograms) of unlabeled insulin necessary to give 50% inhibition of the binding of strain 2 and strain 13 guinea pig anti-pork insulin to ^{131}I-labeled pork insulin.

‡ *W*, Kendall coefficient of concordance (see results)

result strongly suggests that the main specificity recognized by the antibody produced in both inbred strains of guinea pig is in the A chain of the insulin molecule or at least is dependent on the A-chain amino acid sequence. It is important to notice that rat insulin presenting one single amino acid difference in A chain, outside the α-loop, belongs to group II and not I of insulins, thus strongly suggesting that amino acid identities restricted to A-chain α-loop were not enough to produce cross-reacting antibodies. Identities are necessary at least throughout the entire amino terminus of the insulin A chain.

Discussion

The present data establishes that for at least one naturally occurring protein antigen recognition by T cells involves specific selection of distinct epitopes by recognitive mechanisms whose functions are linked to immune response genes. Both strain 2 and 13 guinea pigs respond to insulin in a T-cell proliferation assay when immunized with pork insulin although they do so by mounting responses directed against separate determinants on the same molecule. Strain 13 guinea pigs and outbred and backcrossed animals bearing the Ia-1,3 antigens recognize insulin through a B-chain determinant that is not recognizd by strain 2 guinea pigs. This was shown by the ability of isolated oxidized insulin B chain to induce a strong proliferative response in strain 13 but not strain 2 guinea pigs immunized to native pork insulin, and by the capacity of this same antigen to prime strain 13 but not strain 2 guinea pigs to insulin. The BUdR-induced clonal elimination experiments further showed that after exclusion of B-chain-responsive T cells from the total proliferating cell pool only a very small residual responsiveness to native insulin remains. This would strongly indicate that the

B chain exclusively bears the determinant in insulin recognized by strain 13 guinea pigs.

Strain 2 guinea pigs instead respond to a well defined area on the insulin A chain constituted by amino acids A8-A9-A10 present in the surface of the insulin monomer (24). Definition of strain 2 insulin recognition structure was made possible by comparing the ability of different species variants of insulin to induce T-cell proliferation. The same approach enabled us to demonstrate a receptor sensitivity to one amino acid difference. This area, called A-chain loop or α-loop, is formed by an intrachain disulfide bridge between Cys 6 and Cys 11. It constitutes an evolutionary hypervariable region distinct from the receptor-binding (25) dimer formation (26) and negative cooperativity surfaces described in the insulin molecule (P. deMeyts, personal communication).

From the precise definition of the determinant seen by strain 2 T cells on the insulin molecule some interesting features of the T-cell recognition system emerge. A high degree of specificity and sensitivity exists as a single amino acid identity between beef and pork insulin in the α-loop still enables beef insulin to elicit a response in pork insulin immune animals, while sheep insulin which share two substitutions presented by beef insulin is rendered a complete nonstimulatory protein by one additional substitution in this area. Comparable resolution by T-cell receptors has been described. Gell and Silverstein (27) found by skin testing guinea pigs that delayed-type hypersensitivity responses can discriminate between different points of attachment of the same hapten to the carrier protein. Silverstein and Gell (28) studying the degree of cross-reactivity in delayed-type hypersensitivity reactions in guinea pigs among guinea pig albumin conjugates of *ortho*-, *meta*-, and *para*-substituted haptens were able to show that the reactions are sensitive to the spatial position of the ionic substituent of the hapten. Similar results were obtained by Alkan and El-Khateeb (29) studying delayed skin tests and migration inhibition factor (MIF) production in guinea pigs. Janeway et al. (30), studying delayed-type hypersensitivity, T-cell proliferation, and MIF production in response to nitrophenyl haptens coupled directly to mycobacteria showed that guinea pig T cells were able to discriminate between haptens with *para*-nitro and *ortho*-nitro groups. In T-helper assays in the mouse both Hill and Sercarz (7) by comparing different lysozymes and Keck (31) by comparing pork and beef insulin found a high discriminating power for these cells operating under an *Ir* gene control. Keck (31) observed that C57BL mice are responders to beef insulin and nonresponders to pork insulin. Since the only difference in both insulins lies in the A-chain loop, he concluded that this area in the bovine insulin is acting as the carrier determinant recognized by $H\text{-}2^b$ mice. Our studies suggest that the haplotype present in strain 2 guinea pig is recognizing the same determinant that the $H\text{-}2^b$ haplotype in the mice. It is of interest to note that strain 2 guinea pig as opposed to C57BL mice are responders to both beef and pork insulin. This is perhaps due to the fact that guinea pig insulin differs from pork and beef in A-chain loop while mouse insulin is identical to pork in this area. Waterfield et al. (32) observed two antigenic determinants on oxidized ferrodoxin which were able to elicit MIF production but not T-lymphocyte proliferation when isolated from the intact molecule. Later, Kelly et al. (33) with the same antigen noted that lymphocyte

proliferation could be induced by only one of the determinants but that the induction was much more efficient if the two determinants were simultaneously present.

The exquisite sensitivity of this system for a conformation-dependent determinant is indicated by the fact that pork or beef immune strain 2 guinea pigs do not respond to isolated pork or beef insulin A-chain which although possessing the same A8, A9, and A10 amino acids, no longer has an α-loop conformation as the isolation procedure (performic acid oxidation) forms cysteic acid and destroys disulfide bridges. The additional information that strain 2 guinea pigs can be immunized by different species variants of insulin having a variety of A-chain α-loop amino acids such as pork, beef, and sheep insulins, infers that the structure of the loop contributes significantly to the overall mechanisms by which the α-loop recognition system operates in strain 2 guinea pig. This latter statement should not be taken to mean however that the nature of the amino acids comprising the loop are without consequence in the function of the structures involved in such a recognition system.

Strain 13 guinea pigs on the other hand recognize one specific molecular site under the genetic control of an autosomal dominant gene linked to Ia-1,3 antigen of the GPLA. This was demonstrated by finding a positive B-chain response only in backcrosses ($F_1 \times 2$) and outbred animals bearing the Ia-1,3 alloantigen. Thus responsiveness to complex antigen by different strains of animals with different haplotypes cannot be taken as evidence to mean that the response is controlled by the same *Ir* gene, since relevant immune response genes do not necessarily function by defining clear-cut distinctions between responders and nonresponder animals to complex antigens.

The possibility that macrophages play some role in the specificity of determinant selection in strain 2 and 13 guinea pigs is reinforced by experiments of epitope selection by antigen-bearing parental macrophages used to activate $F_1(2 \times 13)$ guinea pigs immunized to pork insulin.[2] T cells from such animals were able to respond to sheep insulin or to isolated insulin B chain only when presented on strain 13 Mϕ. Although strain 2 Mϕ were unable to present sheep insulin or B chain to pork immune F_1 T cells, strain 2 macrophages were fully active in presentation of pork or other insulins having α-loop identities. In addition, $F_1(2 \times 13)$ guinea pigs immunized to isolated insulin B chain respond only to insulin presented on strain 13 Mϕ. This situation resembles the one described by Shevach and Rosenthal (34) where F_1 guinea pig T cells primed with the artificial polymers GL and GT, respond only when Mϕ from the responder parental strain "presents" the antigen. The important difference is that in the present situation the same Mϕ presentation function distinguishes between two different determinants in the same antigen molecule.

Having defined two distinct immunogenic sites on the pork insulin molecule recognized by proliferating T cells and the specificity of the T-helper function, we next examined the specificity of anti-pork insulin antibodies generated in strain 2 and 13 guinea pigs. Several reports in the literature exist comparing delayed-type with immediate-type hypersensitivity (35, 36) and more specifically

[2] Rosenthal, A. S., M A Barcinski, and J. T. Blake Manuscript submitted for publication

T cells cross-reactivity with cross-reactivity at the antibody level (37, 38). Results are still controversial as far as size and range of specificities in both T- and B-cell libraries. In our system we found that T cells effecting a helper function display the same fine specificity as the ones operating on the proliferating T cells. Only strain 2 T cells primed to insulins sharing identities at the A-chain loop were able to help B cells primed to DNP-OVA and boosted with DNP-pork insulin to produce anti-DNP antibodies. It would appear that proliferating MIF producers and skin test-reactive T cells (39), as well as helper T cells, exhibit the same fine specificity.

In contrast to T-cell proliferation and helper activity, strain 2 and 13 guinea pig antibody specificities against pork insulin could not be distinguished. Specificity was assessed in our system by the ability of different insulin mutants to inhibit the binding of strain 2 and strain 13 guinea pig anti-pork insulin antibodies to radiolabeled pork insulin. Arquilla and Finn by means of an hemolytic assay (40) showed differences in the ability of strain 2 and strain 13 anti-beef insulin to combine to insulin coupled to sheep to which rabbit anti-insulin had been previously coupled. These authors concluded that configurational differences existed between the antibodies produced by the two inbred strains of guinea pig. The apparent disagreement with our results could be due to the fact that those authors used as their indicator the binding of antibodies to the insulin hexamer, while in our assay the insulin is present in monomeric form. A recent study of that same group (41) has shown a striking difference in the assessment of anti-insulin antibodies when comparing the hemolytic and radioimmunoassay.

Our results suggest that the T-cell helper specificity degenerates at the B-cell level. The selection of specificities from the B-cell library would thus appear to be independent of the specific T-cell recognition event that triggers the helper activity. Thus, cross-reactivity in an antibody assay, dependent on T help cannot be taken as evidence that help is the result of recognition of the same immunogenic moiety in the carrier molecule. Senyk et al. (42) studying the immunogenicity of glucagon found that the amino-terminal heptadecapeptide of the molecule was able to bind to anti-glucagon antibodies and that the carboxy-terminal amino acid were able to induce T-cell responses. With insulin, the same type of intramolecular hapten-carrier system may be operating with the addition of a discriminating *Ir* gene functioning at the macrophage or T cell, such that the recognition of distinct carrier determinants in each of the two inbred strains nonetheless supports the common recognition, by B cells, of diverse haptenic determinants on the same molecule.

We are encouraged to conclude that mapping of antigenic determinants for T- and B-cell recognition may provide important insights into the nature and specificity of T-cell receptors and the role of accessory cells in the genetic control of antigen recognition.

Summary

T-cell DNA synthesis and T-helper cell function in response to isolated insulin chains and naturally occurring insulin variants was assessed in insulin immune guinea pigs. Two distinct antigenic determinants, recognized by T cells, were defined. One localized in the B chain and the other one constituted by amino

acids A8, A9, and A10 of the insulin A-chain loop. Recognition of the B-chain determinant is under the control of *Ir* genes linked to the strain 13 major histocompatibility complex. This was shown by studying the response to isolated insulin B chain in $F_1(2 \times 13)$ guinea pigs, as well as serologically defined backcrosses and outbred animals. Insulin recognition through the A-chain loop determinant is specific for strain 2 guinea pigs. These animals recognize this region of the molecule even when displaying different amino acid sequences. The strain differences observed in those antigenic sites eliciting T-cell recognition was not found at an antibody level. No differences could be detected in the ability of the different insulin variants to inhibit the binding of ^{125}I-labeled pork insulin to strain 2 guinea pig anti-pork insulin or to strain 13 guinea pig anti-pork insulin.

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