ANTIGEN-PRESENTING CELLS FROM NONRESPONDER STRAIN 2 GUINEA PIGS ARE FULLY COMPETENT TO PRESENT BOVINE INSULIN B CHAIN TO RESPONDER STRAIN 13 T CELLS

Evidence against a Determinant Selection Model and in Favor of a Clonal Deletion Model of Immune Response Gene Function

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The role of I region-encoded Ia antigens expressed at the level of the antigen-presenting macrophage has been firmly established as essential to the process of antigen recognition by T cells (1–3). The T cell proliferative response to natural species variants of insulin has proved to be a valuable tool to assess directly the role of the macrophage in antigen recognition by T cells and to locate the cellular site of the functional expression of immune response (Ir)[1] genes (4) in macrophage-T cell interaction. The results of these studies have suggested that antigen processing by the stimulator macrophage results in the selection of a distinct antigenic determinant, depending on the I region genotype carried by the presenting cell, and in the specific presentation of this particular determinant to the interacting T cell (the determinant selection theory [4, 5]). Thus, nonresponsiveness would result from either a failure of a given Ia-bearing macrophage to select and display an antigenic determinant to the T cell or a failure of a given antigen to associate with the Ia molecule during antigen recognition. It is implicit in this model that macrophages from nonresponder animals are genetically unable to present the relevant antigen to any population of antigen-specific T cells.

An alternative explanation for Ir gene-controlled nonresponsiveness (the clonal deletion theory [6, 7]) proposes that there are specific holes in the T cell repertoire that have been created during T cell ontogeny because of cross-reactions at the level of the T cell receptor between complexes of self-antigens and self-Ia, or self-Ia alone and a particular complex of a foreign antigen and self-Ia. Elimination of the self-reactive T cell population would therefore result in unresponsiveness to the cross-reacting foreign antigen. The recent demonstrations (8, 9) that macrophages from nonresponder animals can effectively present the relevant antigen to allogeneic, responder T lymphocytes are consistent with the clonal deletion model. However, one

[1] *Abbreviations used in this paper:* BUdR, bromodeoxyuridine; FCS, fetal calf serum; [^{3}H]TdR, tritiated thymidine; IL-2, interleukin 2; Ir, immune response; αMM, α-methyl-D-mannopyranoside; MLN, mesenteric node lymphocytes; NGPS, normal guinea pig serum; PEC, peritoneal exudate cells; PEL, peritoneal exudate lymphocytes; PPD, purified protein derivative of tuberculin.

difficulty encountered in the interpretation of these studies is that the antigens used were synthetic amino acid polymers, that is, antigens whose primary structure is largely unknown, and it is impossible to determine whether the responder T cells recognize the same antigenic determinant presented by nonresponder and responder macrophages.

It has previously been demonstrated (10) that strain 2 guinea pigs respond to the loop region of the bovine insulin A chain (residues A8–10) and are B chain nonresponders, whereas strain 13 guinea pigs recognize determinants on the bovine insulin B chain and are A chain loop nonresponders. One prediction that can be made from the determinant selection model is that irrespective of the Ir phenotype of the responder T cell population, strain 2 macrophages should only be capable of presenting A chain loop determinants and should be incapable of presenting B chain. In the present report, we have directly tested this hypothesis by priming strain 13 T lymphocytes in vitro with bovine insulin-pulsed allogeneic strain 2 macrophages. We will demonstrate that strain 2 macrophages are fully competent to present bovine insulin B chain to strain 13 T cells despite the fact that strain 2 guinea pigs are normally totally unresponsive to this antigen. In addition, we will show that insulin-specific, allo-Ia-restricted strain 13 T cells can be activated by the autologous guinea pig insulin molecule. These results strongly suggest that the nature of the antigenic determinant recognized by T cells is dependent not only on an associative interaction between antigen and Ia, but also on the availability of specific receptors in the T cell repertoire that may be altered or skewed during ontogeny by a clonal deletion mechanism of tolerance to self.

Materials and Methods

Animals. Inbred strain 2 and strain 13 guinea pigs were obtained from the Division of Research Services, National Institutes of Health, Bethesda, MD.

Immunizations. Guinea pigs were immunized with a 1:1 emulsion of 100 μg of the antigen in saline with complete Freund's adjuvant (containing 0.4 mg/ml mycobacterium tuberculosis H37Ra; Difco Laboratories Inc., Detroit, MI) injected in the four footpads.

Antigens. Beef, sheep, and pork insulin as well as oxidized beef insulin B chain were purchased from Sigma Chemical Co., St. Louis, MO. The synthetic insulin fragments B(5-16), Asn-substituted B(5-16), Thr-substituted B(5-16), and the Asn-, Thr-substituted B(5-16) (11) were all kindly provided by Dr. Alan Rosenthal, Merck Institute for Therapeutic Research, Rahway, NJ. In some experiments, purified protein derivative of tuberculin (PPD; Connaught Medical Research Laboratory, Willowdale, Ontario, Canada) was used.

Preparation of Stimulator Cells. Nonimmune guinea pigs were injected intraperitoneally with 25 ml of sterile mineral oil (Marcol 52; Humble Oil and Refining Co., Houston, TX). Peritoneal exudate cells (PEC) were collected 3–7 d later and washed three times before use. This cell population, which contained 75% macrophages, 10% granulocytes, and 15% lymphocytes, was irradiated by γ irradiation (2,500 rad) and used without further purification as a source of antigen-presenting, stimulator cells.

Preparation of Responder T Lymphocytes from Immune Animals. Beef insulin-immune guinea pigs were injected intraperitoneally with 25 ml of sterile mineral oil. PEC were collected 4–6 d later, washed, and passed over a nylon wool column (made up by 3 g of acid-washed nylon wool; Fenwall Laboratories, Division of Travenal Laboratories, Morton Grove, IL) previously equilibrated at 37°C with Hanks' balanced salt solution (Biofluids, Inc., Rockville, MD) containing 5% heat-inactivated normal guinea pig serum (NGPS). After incubation for 1 h at 37°C, the nonadherent cells were collected. This population, referred to as peritoneal exudate lymphocytes (PEL), was used as the source of responding T lymphocytes from immune animals.

Antigen-dependent Induction of DNA Synthesis in PEL. For measurement of specific T lymphocyte

activation by different insulins, immune PEL were suspended (2×10^6 cells/ml) in medium RPMI 1640 supplemented with 5% NGPS, L-glutamine (300 μg/ml), 2-mercaptoethanol (5×10^{-5} M), gentamycin (5 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and 5-fluorocytosine (1 μg/ml). 0.1 ml of this cell suspension was added to round-bottomed, 96-well microtiter plates together with 0.1 ml of a suspension of irradiated, nonimmune PEC (1×10^6/ml) in complete medium. The specific antigens were added by dropping 10 μl of a 20-times-concentrated antigen solution to give the final desired concentrations. For stimulation of T lymphocytes by synthetic insulin fragments, PEL were cultured with PEC that had been previously pulsed with medium alone or with the synthetic peptides at 300 μg/ml for 1 h at 37°C. The pulsed PEC were washed three times to remove unbound antigen, counted, and adjusted to 1×10^6 cells/ml in complete medium before addition to PEL. Cell cultures were maintained for 3 d at 37°C, in a humidified atmosphere of 5% CO_2 in air. 18 h before harvesting, 1.0 μCi of tritiated thymidine ([^{3}H]TdR; sp act 6.7 Ci/mM; New England Nuclear, Boston, MA) was added to each well. The cultures were harvested with the aid of a semiautomated harvesting device (Mash II; Microbiological Associates, Walkersville, MD). The amount of radioactivity incorporated into cellular DNA was determined by liquid scintillation spectroscopy. The mean counts per minute of triplicate cultures is expressed as total counts per minute per well. The standard error of the mean was seldom >10% of the mean and is omitted for simplicity.

In Vitro Priming of T Cells with Antigen-pulsed Syngeneic PEC. Insulin-specific T cell lines, restricted to syngeneic macrophages were obtained by culturing 5×10^6 normal, unseparated, or nylon-wool purified mesenteric node lymphocytes (MLN) from nonimmune strain 13 guinea pigs, with 1×10^6 irradiated, beef insulin-pulsed syngeneic PEC in 2 ml of complete medium for 1–2 wk, in 24-well Costar culture vessels (Costar, Data Packaging, Cambridge, MA). The cultures were re-fed (1 ml) every 3–4 d with fresh medium. To test for antigen specificity, 10^5 responder lymphocytes were mixed with 10^5 irradiated, syngeneic PEC, either in the absence or in the presence of the various insulins or insulin peptide fragments in 0.2 ml of complete medium, in round-bottomed microtiter plates. Proliferation was assessed after 72 h in culture by [^{3}H]TdR uptake.

In Vitro Priming of T Lymphocytes with Antigen-pulsed Allogeneic PEC. MLN from nonimmune strain 13 guinea pigs, either unfractionated or nylon wool column-purified, were primed with beef insulin-pulsed strain 2 PEC as previously described (12). Briefly, normal strain 13 T cells were first depleted of alloreactivity by culturing them (5×10^6) with normal strain 2 PEC (1×10^6) in a final volume of 2 ml of complete medium in 24-well vessels for 3 d. At the end of the 2nd d, the cultures were treated with 2 μg/ml 5-bromodeoxyuridine (BUdR; Sigma Chemical Co.). 20 h later, the cultures were illuminated for 90 min at 4°C with a fluorescent light source (12). The recovered lymphocytes were washed three times and resuspended at 5×10^6 cells/ml in complete culture medium. Nonimmune, irradiated strain 2 PEC were pulsed with beef insulin (300 μg/ml; 10×10^6 cells/ml) for 1 h at 37°C, washed, and adjusted to 1×10^6 cells/ml in complete medium. The recovered strain 13 T cells were then cultured (5×10^6) with beef insulin-pulsed strain 2 PEC (1×10^6) for 1 to 2 wk in 24-well vessels. 1 ml of the culture medium was decanted and replaced with fresh medium every3–4 d.

Soft Agar Cloning of Strain 13 T Cells Primed to Beef Insulin-pulsed Strain 2 PEC. After 1–2 wk in culture, in vitro-primed strain 13 T cells were cloned on a double layer of soft agar as previously described (13). The lower agar contained NGPS (10%), beef insulin (200 μg/ml), a source of interleukin 2 (IL-2) (25%) prepared as a concanavalin A-activated supernatant (13) and 20 mg/ml of α-methyl-D-mannopyranoside (αMM; Sigma Chemical Co.). The upper agar contained 10% NGPS, 1×10^6 primed T lymphocytes, and 2×10^5 fresh, irradiated strain 2 PEC. After 3–5 d, individual colonies developed on the agar surface and were picked with a sterile pasteur pipette. The recovered cells from each individual colony were divided between two flat-bottomed wells of a 96-well Costar plate. One well contained 0.2 ml of culture medium supplemented with 5% fetal calf serum (FCS), 25% IL-2, 20 mg/ml αMM, 10^4 irradiated strain 2 PEC; the other well was additionally supplemented with beef insulin (100 μg/ml). After a 10–14-d expansion culture, sufficient numbers of cells were available to be tested. Only those colonies visually demonstrating enhanced growth in the presence of antigen were selected for further testing.

Antigen-dependent Proliferation of Allo-sensitized T Cell Colonies. To measure specific activation of T cell colonies, we used a two-step proliferation assay as previously described (13). Briefly, 10^4 cells from individual colonies were cultured in flat-bottomed microwells together with 5×10^4 irradiated PEC in 0.3 ml of culture medium containing 5% FCS, 5% IL-2, and 20 mg/ml αMM. Individual wells received different antigens by adding 10 μl of the appropriate stocks. After 3 d in culture, each individual well was divided into three new wells by adding 0.1 ml of the original volume to the new wells and supplementing them with 0.1 ml of a suspension containing 5×10^4 PEC in complete medium containing FCS, IL-2, and αMM. The specific antigen was added again at this stage at the same final concentration. For testing the beef insulin B chain as well as the synthetic B chain fragments, PEC previously pulsed with each of these insulin fragments were used as stimulators. The microcultures were then cultured for an additional 3-d period, labeled with [^{3}H]TdR, and the incorporated radioactivity was determined.

Anti-Ia Sera. A strain 13 anti-strain 2 (anti-Ia.2,4) serum and a strain 2 anti-strain 13 (anti-Ia-1,3,7) serum were prepared as previously described (14).

Results

Distinct Insulin Epitopes Are Recognized by Strain 2 and 13 Guinea Pig T Cells. To compare the specificity of our reagents and priming regimen to that previously obtained in the guinea pig (15), we tested the proliferative response of in vivo-primed, beef insulin-immune strain 2 or 13 T cells to beef, sheep, and pork insulins and to the isolated oxidized beef insulin B chain. As shown in Fig. 1, strain 2 PEL demonstrated a vigorous proliferative response to beef insulin in the presence of syngeneic PEC. The response of strain 2 PEL to sheep insulin was much lower and no response to pork insulin was seen at the concentration used, although higher doses of pork insulin induced some proliferation of the PEL (data not shown). In addition, strain 2 PEC did not respond to the isolated B chain. These data are in complete agreement with the original finding of Barcinski and Rosenthal (15), and demonstrate that strain 2 T cells recognize a determinant on the A chain loop of the insulin molecule, because sheep and pork insulins differ by a single or a double amino acid change, respectively, from the beef insulin molecule in the A chain loop. On the other hand, as shown in

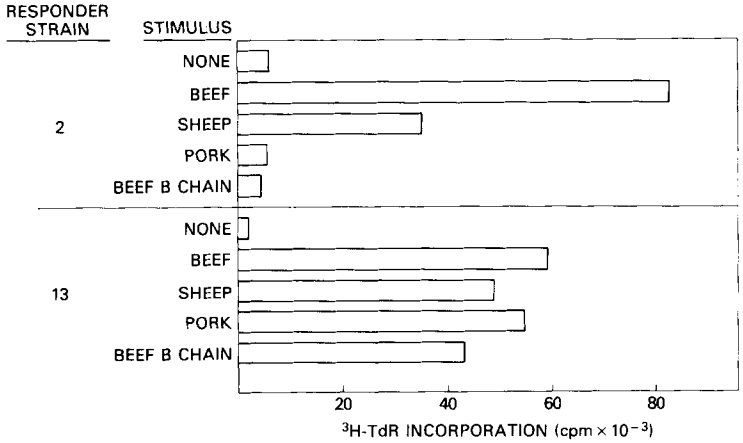

FIG. 1. Strain 2 and strain 13 insulin-immune T cells recognize distinct antigenic determinants on the insulin molecule. Beef insulin-immune PEL were cultured together with syngeneic irradiated PEC in the presence of the indicated insulin variants or beef insulin B chain. Proliferation was measured after 72 h in culture by [^{3}H]TdR uptake.

Fig. 1, in vivo-primed strain 13 PEL responded equally well to beef, sheep, and pork insulins, and also responded to the isolated B chain, thus localizing the antigenic epitope recognized by strain 13 T cells to the insulin B chain, which is also in accordance with the original findings in this system (15).

Production of Strain 13 T Cell Colonies Specific for Beef Insulin in Association with Allogeneic Strain 2 Ia Molecules. According to the determinant selection model (4, 5), strain 2 macrophages should fail to present the insulin B chain to responder strain 13 T cells because strain 2 macrophages are genetically committed to the presentation of the A chain loop determinant (10). To address this question directly, we generated strain 13 T cell colonies specific for beef insulin in association with strain 2 macrophages. We first depleted anti-strain 2 alloreactivity by culturing nonimmune 13 T cells with normal strain 2 PEC, followed by BUdR and light treatment. The resultant viable cells were then primed in vitro with beef insulin-pulsed strain 2 PEC for 1–2 wk. The primed T cell population was cloned in soft agar in the presence of beef insulin, strain 2 PEC, and IL-2. Individual colonies were expanded in liquid culture either with or without beef insulin, and colonies showing an enhanced growth in the presence of the antigen were selected for functional tests. Even under these selective conditions, a number of alloreactive 13 anti-2 T cell colonies were identified among the colonies tested. However, several colonies specific for beef insulin and restricted to allogeneic strain 2 PEC were found (Fig. 2). The antigen dose-response curve was similar to that seen with T cells primed in vivo (15). Approximately 50% of these allo-Ia-restricted T cell colonies showed minimal or no proliferation in the presence of allogeneic strain 2 macrophages, whereas the other 50% showed some degree of activation by strain 2 PEC alone, but gave much higher responses in the presence of the specific, but not of an unrelated, antigen.

We next determined the reactivity profile of these allo-Ia-restricted T cell colonies for beef, sheep, and pork insulins and the isolated oxidized B chain molecule. As shown in Table I (experiment 1), one of these colonies, I-14-E10, proliferated equally well to beef, sheep, and pork insulins in the presence of allogeneic, strain 2 PEC, which suggests that the antigenic determinant recognized was located in the B chain. This assumption was confirmed by showing that I-14-E10 could be activated by the

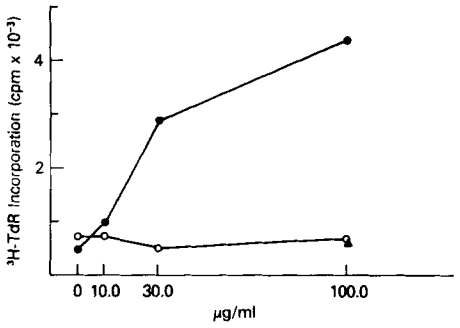

FIG. 2. Antigen specificity and I region restriction of an allo-Ia-restricted, insulin-specific strain 13 T cell colony. Colony I-32-F9, a strain 13 T cell colony primed with beef insulin-pulsed 2 PEC, was stimulated with various doses of beef insulin in the presence of syngeneic or allogeneic PEC, or with PPD in the presence of allogeneic PEC. Individual cultures were grown for 3 d and split, and proliferation was measured 72 h later by [^{3}H]TdR uptake.●, strain 2 plus beef insulin; ○, strain 13 plus beef insulin; ▲, strain 2 plus PPD.

TABLE I

An Allo-Ia-restricted Strain 13 T Cell Colony Recognizes a Determinant on the Beef Insulin B Chain

Experiment	T cell stimulus	I-14-E10 T cell colony [^{3}H]TdR incorporation	
		cpm	Δ *cpm*
1	13 PEC	1,763	
	13 PEC + beef	3,646	1,883
	2 PEC	11,063	
	2 PEC + beef	33,819	22,765
	2 PEC + sheep	27,915	16,852
	2 PEC + pork	37,117	26,054
2	2 PEC	21,149	
	2 PEC + beef	53,284	32,135
	2 PEC + beef B chain	45,598	24,449

TABLE II

Anti-Ia Serum Blocking of Antigen Presentation in Allo-Ia-restricted Strain 13 T Cell Colonies

T cell stimulus*	Added serum‡	I-14-E10 T cell colony [^{3}H]TdR incorporation
		Δ *cpm*
2 PEC + beef	NGPS	5,953
2 PEC + beef	Anti-Ia.2,4	949
2 PEC + beef	Anti-Ia.1,3,7	5,729

* Stimulator cells were beef insulin-pulsed strain 2 PEC.
‡ Final concentration in culture, 5%.

isolated beef B chain molecule (Table I, experiment 2). Several other colonies showed the same reactivity profile to natural variants of insulin (results not shown). These results are contrary to the determinant selection hypothesis, which would have predicted that strain 2 PEC should only have been capable of presenting A chain loop determinants and not B chain determinants.

In addition, a direct role of Ia antigens on the stimulator strain 2 macrophages was shown in these studies (Table II). I-14-E10 proliferated in the presence of beef insulin-pulsed strain 2 PEC. The proliferative response was blocked by addition to the cultures of an anti-Ia.2,4 serum directed to the stimulator PEC, but not by addition of anti-Ia.1,3,7 serum directed to the responder T cell.

Allo-Ia-restricted T Cell Colonies Recognize a Determinant on the B(5-16) Fragment of the Insulin B Chain. Because in vivo primed responder strain 13 T cells recognize the B(5-16) segment of the insulin B chain as the major, if not the only, antigenic determinant on the whole insulin molecule (11), it was of interest to determine the precise antigenic determinant recognized by the allo-Ia-restricted strain 13 T cell colonies. As shown in Fig. 3, colony I-24-B7 proliferated in a dose-related manner not only to the isolated B chain molecule in the presence of strain 2 macrophages, but also to the synthetic B(5-16) fragment of the insulin B chain; no response was seen to

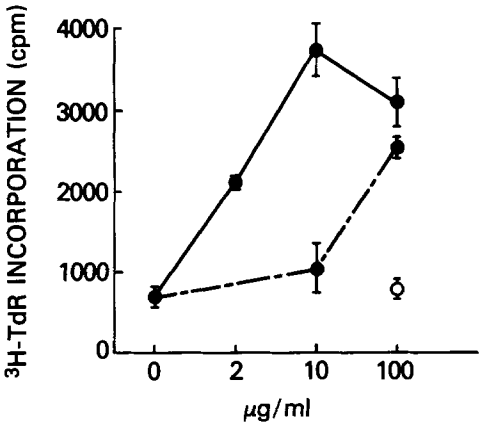

FIG. 3. Allo-Ia restricted strain 13 T cell colonies recognize an insulin determinant on the B(5-16) segment of the insulin B chain. A strain 13 allo-Ia-restricted T cell colony, I-27-B8, was stimulated with various doses of beef B chain (—●—), or the synthetic B(5-16) (- -●- -) fragment of the B chain, in the presence of allogeneic strain 2 PEC. As a control, I-27-B8 was stimulated with PPD (○) in the presence of strain 2 PEC. Individual cultures were grown for 3 d and split, and proliferation was measured 72 h later by [^{3}H]TdR uptake.

TABLE III

Amino Acid Sequence of the B(5-16) Synthetic Peptide Fragments

Fragment	Origin	5-	6-	7-	8-	9-	10-	11-	12-	13-	14-	15-	16
B(5-16)	Beef	His-	Leu-	Cys-	Gly-	Ser-	His-	Leu-	Val-	Glu-	Ala-	Leu-	Tyr-
B(5-16)His_{10}→Asn	—	----------					Asn						----------
B(5-16)Ala_{14}→Thr	—	----------									Thr		----------
B(5-16)His_{10},Ala_{14}→Asn,Thr	Guinea pig	----------					Asn				Thr		----------

an unrelated antigen, PPD. Thus far, all the strain 13 insulin-specific, allo-Ia-restricted colonies identified by this protocol have responded to the synthetic B(5-16) fragment of the beef B chain molecule. This finding indicates that responder strain 13 T cells recognize an insulin epitope presented by the allogeneic strain 2 PEC that is located in the same B chain segment that in vivo- or syngeneically primed strain 13 T cells recognize when insulin is presented to them on strain 13 PEC.

The Reactivity Profile for Synthetic B(5-16) Peptides of Allo-Ia-restricted T Cells Is Distinct from That of Self-Ia-restricted T Cells. Previous studies (11) have demonstrated that in vivo-primed, self-Ia-restricted strain 13 T cells recognize the His amino acid residue at position 10 on the B chain molecule as a critical portion of the relevant antigenic epitope. Table III shows the amino acid sequence of the B(5-16) segment of the B chain molecule in comparison with the autologous guinea pig B(5-16) amino acid sequence. The beef B(5-16) sequence differs from the guinea pig sequence at two residues: His at position 10, which becomes Asn in the guinea pig sequence, and Ala at position 14, which becomes Thr in the guinea pig sequence. In addition, two synthetic B(5-16) fragments with single amino acid changes at positions 10 and 14, are shown in Table III. B(5-16) His_{10}→Asn has the guinea pig Asn residue at position 10, instead of His, whereas B(5-16) Ala_{14}→Thr has the guinea pig residue Thr at position 14, and not Ala.

Strain 13 T cells primed in vivo or in vitro to beef insulin-pulsed syngeneic PEC

recognize His_{10} in the B(5-16) peptide but not Ala_{14}, because these T cell populations can be stimulated by B(5-16) $Ala_{14}\rightarrow$Thr but not by B(5-16) $His_{10}\rightarrow$Asn (Table IV). This result is identical to that obtained (11) with strain 13 T cells primed in vivo with pork insulin. We next used these same fragments to stimulate the allo-Ia-restricted stain 13 T cell colonies (Table V). Surprisingly, we found that these colonies could be specifically activated not only by the beef B(5-16) fragment but also by the B(5-16) $Ala_{14}\rightarrow$Thr and B(5-16) $His_{10}\rightarrow$Asn synthetic fragments. These results demonstrate that the reactivity profile of allo-Ia-restricted T cell colonies is distinct from the profile of self-Ia-restricted cells, although both populations are activated by the same B chain segment. Furthermore, neither His_{10} nor Ala_{14} is critical to the antigenic specificity recognized by the allo-Ia-restricted T cells as vigorous proliferative responses were also seen with the guinea pig B(5-16) sequence.

Discussion

According to the determinant selection model of Ir gene function (4, 5), Ia antigens on antigen-presenting cells would function as a class of receptors of broad specificity that would focus or orient distinct regions of the antigen for presentation to the T cell. Nonresponsiveness would result because macrophages from the nonresponder animal would be defective in their ability to generate such a complex either in the absolute

TABLE IV

Recognition of the His_{10} Determinant on the B Chain by In Vivo and In Vitro Self-Ia-Primed, Strain 13 T Lymphocytes

T cell stimulus	[^{3}H]TdR incorporation	
	PEL*	I-37-B1‡
	Δ *cpm*	
13 PEC-B(5-16)	27,741	6,602
13-PEC-B(5-16)$His_{10}\rightarrow$Asn	1,472	957
13 PEC-B(5-16)$Ala_{14}\rightarrow$Thr	23,836	5,456
13 PEC-B(5-16)His_{10},$Ala_{14}\rightarrow$Asn,Thr	0	0

* Responder T cells were in vivo-primed beef insulin immune strain 13 PEL.
‡ Responder I-37-B1 T cells were from an in vitro-primed, uncloned, short-term beef insulin-specific strain 13 T cell line.

TABLE V

Allo-Ia-restricted Strain 13 T Cell Colonies Recognize a Determinant Present on the Autologous Guinea Pig Insulin B(5-16) Peptide Sequence

T cell stimulation by	[^{3}H]TdR incorporation by colony			
	I-30-D4	I-32-F5	I-32-D8	I-32-D9
	cpm		*cpm*	
13 PEC	628	330	340	573
13 PEC + B(5-16)	228	550	531	442
2 PEC	1,496	1,012	644	638
2 PEC + B(5-16)	16,546	6,242	4,115	4,342
2 PEC + B(5-16)$His_{10}\rightarrow$Asn	10,162	5,739	3,156	4,170
2 PEC + B(5-16)$Ala_{14}\rightarrow$Thr	9,684	5,514	4,450	5,543
2 PEC + B(5-16)His_{10},$Ala_{14}\rightarrow$Asn,Thr	10,200	8,650	4,239	6,165

sense, as seen in the case of amino acid copolymers, or in a restricted sense, as is seen with insulin where only certain determinants would be displayed on the surface of the nonresponder antigen-presenting cell. The recent experiments of Ishii et al. (8) as well as our own studies with the copolymer L-glutamic acid,L-lysine (9) offered a strong challenge to the concept of determinant selection in that macrophages from nonresponder animals were fully capable of presenting the relevant antigen to an allogeneic responder T cell population. However, these studies did not address the possibility that the nonresponder macrophage was presenting a different determinant to the T cell from the one that was recognized in the syngeneic combination of T cells and macrophages. The studies described in this report were designed to test the determinant selection model and to compare the nature of the antigenic determinant presented by the syngeneic macrophage with the determinant recognized in the context of allogeneic Ia. We have used the same experimental model from which the determinant selection concept was derived, the selective recognition of pork or beef insulin by guinea pig T lymphocytes (15).

After elimination of alloreactive T cells, nonimmune strain 13 T cells were primed to allogeneic strain 2 beef insulin-pulsed macrophages, cloned in soft agar, expanded, and analyzed by a T cell proliferation assay. We could generate several colonies that failed to respond to syngeneic macrophages either in the presence or in the absence of beef insulin, but that could be specifically stimulated by beef insulin in the presence of allogeneic strain 2 macrophages. These colonies could be directly activated by isolated oxidized beef insulin B chain, which demonstrates that the determinant being presented by the macrophage from the B chain nonresponder strain 2 animal resides in the B chain molecule. Moreover, allo-Ia-restricted strain 13 T cell colonies could also be stimulated by the synthetic beef B(5-16) peptide sequence, which is the same B chain segment that was shown to contain the determinant recognized by self-Ia-primed strain 13 T cells (11). The fact that beef insulin B chain and the beef B(5-16) fragment were capable of association with strain 2 Ia antigens to create an immunogenic complex that was capable of efficiently activating strain 13 T cells is difficult to reconcile with a strict interpretation of the determinant selection hypothesis, which would have predicted that strain 2 macrophages would have been restricted to the presentation of A chain loop determinants.

A second aspect of these studies involved a comparison of the reactivity profiles of self-Ia- and allo-Ia-primed strain 13 T cells to a series of B(5-16) peptides with single amino acid substitutions. Thomas et al. (11) have previously shown that a single histidine residue in the 10th position of the B chain was critical for activation of strain 13 T cells primed in vivo to pork insulin. We were able to confirm this observation when strain 13 T cells were primed in vivo with beef insulin. Furthermore, we extended these observations and showed that in vitro primed, self-Ia-restricted strain 13 T cell lines also recognize the His_{10} residue on the beef B(5-16) peptide. These results showed that in vitro priming per se does not alter the fine specificity of the beef insulin epitope being recognized by T cells, and does not lead to the recognition of the autologous guinea pig insulin molecule. Moreover, we have been unable to prime strain 13 T cells in vitro with guinea pig insulin B(5-16) peptide (results not shown). When the B(5-16) synthetic peptides were then used to assess the nature of the antigenic determinant recognized by the allo-Ia-restricted T cell colonies, we identified a number of T cell colonies that responded equally well to the beef B(5-

16) peptide as well as the autologous guinea pig B(5-16) peptide, which suggests that the specificity recognized by these beef insulin-primed allo-Ia-restricted T cells was different from the specificity recognized by self-Ia-primed T cells. In fact, several allo-Ia-restricted colonies could be directly activated by guinea pig insulin in association with strain 2 PEC (results not shown).

The observation that the complex of strain 2 Ia and the beef B(5-16) peptide is distinct from the complex of strain 13 Ia and the B(5-16) peptide is not surprising and is consistent with the view that the nature of the antigenic epitope seen by the T cell is strongly influenced by the nature of the restricting Ia molecule on the antigen-presenting cell. This interpretation is also compatible with a more liberal interpretation of the determinant selection concept. Similar conclusions have been drawn from the studies of Heber-Katz et al. (16) and Hedrick et al. (17) in the murine cytochrome *c* system in which certain responder and nonresponder strains appeared to have the same antigen-specific T cell repertoire, but could not be activated by peptides in association with nonresponder Ia antigens, presumably because of a failure of the interaction of the antigen with nonresponder Ia. However, it is also apparent that none of these experimental systems allows one to distinguish easily whether the Ia molecule associates with and modifies in a specific manner the orientation of the nominal antigen to be presented before encounter with the specific T cell or whether the change in the epitope being presented is caused by the need to accommodate the antigen and the distinct Ia molecule into a single complex to be bound by the interacting T cell receptor.

There are several aspects of the experimental protocol we have used that should be noted. First, several of the isolated colonies possessed a certain degree of alloreactivity, as defined by their capacity to proliferate to allogeneic strain 2 macrophages alone in the absence of antigen, whereas others had no significant alloreactivity. Because our studies have been performed with soft agar colonies that have not as yet been recloned and because non-antigen-specific, purely alloreactive colonies could also be detected after BUdR and light treatment (data not shown), those colonies with high background response may well have resulted from contamination by an alloreactive T cell of an insulin-specific clone, whereas the low background colonies may represent pure insulin-specific clones. An alternative possibility is that the partially alloreactive colonies actually represent unique T cells with low-affinity receptors for allogeneic Ia molecules, in addition to being antigen specific. Whatever the reason for the residual alloreactivity, the antigen-specific responses manifested by these colonies were strong and insulin specific. Moreover, since both types of colonies yielded essentially the same results, there is no indication that the residual alloreactivity interfered with the response patterns we have analyzed. Second, our ability to prime T cells across an I region barrier is consistent with our own previous studies (9, 12), as well as the experience of others (8). However, we must caution that we have not as yet compared the frequency of allo-Ia-restricted T cells to the frequency of self-Ia-restricted T cells.

Why is the strain 2 guinea pig a nonresponder to bovine insulin B chain? As pointed out above, it is unlikely that nonresponsiveness results from a failure of association of strain 2 Ia and B chain. Our results are most consistent with the possibility that the complex of strain 2 Ia and bovine insulin B chain resembles a complex of strain 2 Ia and guinea pig insulin. The strain 2 T cell clones capable of responding to both of these complexes are deleted during T cell ontogeny.

Although our results do not allow us to definitively rule out determinant selection models of Ir gene action, our demonstration that strain 13 T cells that have been sensitized to beef insulin pulsed strain 2 macrophages also react to autologous guinea pig insulin also strongly favors a clonal deletion model. Furthermore, although the possibility has been raised in the past (18), this is the first direct demonstration that clonal deletion of self-reactive T cells is likely to be I region restricted. Thus, as expected, we were unable to find any T cells reacting with guinea pig insulin plus self-Ia, but all the allo-Ia-restricted T cell colonies we have so far analyzed react with the guinea pig insulin molecule. It should also be pointed out that the insulin system may be somewhat unique in that the self-antigen that leads to cross-reactive tolerance to foreign insulins is likely to be insulin itself. In other cases of nonresponsiveness where the foreign antigen in question does not resemble any known self-protein, we would propose that although the self-resemblance of the antigen determinants is not apparent, the combination of foreign antigen and self-Ia may well cross-react at the level of the T cell receptor with a complex of self-antigen plus self-Ia as originally suggested by Schwartz (6). Thus, nonresponsiveness to any protein antigen can be ultimately traced to holes in the T cell repertoire caused by cross-reactions with self.

Finally, we would like to propose that some of the concepts of an I region-restricted clonal deletion mechanism may be helpful in understanding certain autoimmune phenomena. The concept that clonal deletion is I region restricted leaves open the possibility that in vivo a T cell may become sensitized to self-antigens in the context of Ia molecules that are slightly different from self-Ia. Although one can only speculate how Ia molecules may be somatically altered, it is possible that such alterations may occur during the course of a viral infection or because of environmental changes in a localized inflammatory site. Such changes in self-Ia on antigen-presenting cells would permit activation of T cells with specificity for self-antigens and might allow the initiation of an autoimmune reaction. Needless to say, we would have to postulate that such antigen-presenting cells with altered or mutant Ia specificities would also be able to escape the normal control mechanisms that recognize alterations on self-Ia molecules.

Summary

To test directly the determinant selection hypothesis of immune response gene function, we primed strain 13 T lymphocytes in vitro with allogeneic bovine insulin pulsed strain 2 macrophages. Strain 2 macrophages were found to be fully competent to present bovine insulin B chain to strain 13 T cells despite the fact that strain 2 guinea pigs are normally totally unresponsive to this antigen. These results are incompatible with a strict interpretation of the determinant selection hypothesis, which would have predicted that strain 2 macrophages would have been restricted to the presentation of A chain loop determinants. In addition, a comparison of the reactivity profiles of self-Ia- and allo-Ia-restricted strain 13 T cells to a series of synthetic B chain peptide fragments revealed that the allo-Ia-restricted populations could be activated by autologous guinea pig insulin. Taken together, these observations strongly suggest that the clonal deletion of self-reactive cells is likely to be I region restricted and that nonresponsiveness to any protein antigen may result from a restriction in the T cell repertoire that is generated during ontogeny by a clonal deletion mechanism of tolerance to self.

We wish to thank Dr. W. E. Paul for helpful discussions and careful review of the manuscript and Ms. Shirley Starnes for preparation of the manuscript.

Received for publication 2 December 1982.

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