

## THE *xid* GENE CONTROLS Ia.W39-ASSOCIATED IMMUNE RESPONSE GENE FUNCTION\*

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It has been shown that specific immune response (Ir) genes within the I region of the major histocompatibility complex (MHC)<sup>1</sup> of mammals play a major role in thymus dependent immune responses (1, 2). However, the mechanism of the expression of Ir gene function remains an area of intense research and great speculation. Over the years, an attractive hypothesis has emerged that suggests that the antigenic determinants involved in stimulating primed helper, delayed-type hypersensitivity, or proliferative T cell clones are a complex of antigen fragments in association with the glycoprotein alloantigens—the immune response-associated (Ia) molecules (3). This functional complex of nominal antigen and Ia is suitably displayed to T cells by antigen-presenting cells of variable origin, including those of mononuclear phagocyte (M $\phi$ ) lineage (4). To date, the information suggesting that Ia molecules are directly involved in Ir gene function has only been inferential; proof of such linkage would require experiments demonstrating the actual physicochemical association of antigen with Ia.

Previous work with defined protein antigens such as insulin has contributed much to our present understanding of the nature and mechanism of Ir gene function in antigen recognition (5–8). For example, MHC-linked Ir genes control the murine T cell proliferative response of H-2<sup>b</sup> mice to beef insulin (9). This control is dependent on a determinant selection process expressed by the Ia-positive antigen-presenting M $\phi$  and maps to the K<sup>b</sup> and/or I-A<sup>b</sup> subregion(s) of the H-2 gene complex.

Recently, Ia.W39, a new private specificity of I-A<sup>b</sup>, has been described (10). It is selectively expressed on a functional subset of B cells, which is absent in newborn normal and adult mutant mice carrying the *xid* gene. Furthermore, the molecule bearing this Ia specificity is synthesized in the cytoplasm of B cells in *xid*-defective and neonatal normal mice, although it is not expressed on the B cell membrane. Biochemical studies have revealed that Ia.W39 has a similar two-chain structure and two-dimensional gel pattern as the conventional Ia molecule, suggesting that Ia.W39 is a conformational specificity, expressed on a subset of molecules bearing the conventional Ia antigens.<sup>2</sup> This Ia molecule with its unique specificity and selective

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<sup>1</sup> Abbreviations used in this paper: AAC, adherent accessory cell; CFA, complete Freund's adjuvant; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; LNL, lymph node lymphocyte; M $\phi$ , mononuclear phagocyte; MHC, major histocompatibility complex; PC, peritoneal washout cells; NMS, normal mouse serum; PHA, phytohemagglutinin; PPD, purified protein derivative of tuberculin; TI-2, thymus independent antigen, type 2; TNP-OVA, trinitrophenylated ovalbumin.

<sup>2</sup> Huber, B. T., P. P. Jones, and D. A. Thorley-Lawson. Structural analysis of a new B cell differentiation antigen associated with products of the I-A subregion of H-2 complex. *Proc. Natl. Acad. Sci. U. S. A.* In press.

expression represents a potentially powerful probe in the dissection of the specific mechanism of Ir gene function for beef insulin in the H-2<sup>b</sup> mouse.

The experiments described in this report examine the role of Ia.W39 in the activation of insulin-specific H-2<sup>b</sup> T cells. They suggest that beef insulin related Ir gene function is dependent upon the expression of this Ia molecule at the level of the antigen-presenting Mφ. Furthermore, it is demonstrated for the first time that a non-MHC gene (*xid*) controls the optimal expression and execution of Ir gene function, possibly by regulating the membrane expression of an Ia molecule (Ia.W39) on antigen-presenting cells.

### Materials and Methods

**Animals.** C57BL6/J (B6) and (C57BL6/J × DBA2/J)F<sub>1</sub> (BDF<sub>1</sub>) mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. (CBA/N × C57BL6)F<sub>1</sub> [(N × B6)F<sub>1</sub>] and (C57BL6 × CBA/N)F<sub>1</sub> [(B6 × N)F<sub>1</sub>] mice were bred in the animal facilities at Tufts University School of Medicine, Boston, Mass. from breeding stock originally obtained from the National Institutes of Health, Bethesda, Md.

**Antigens.** Beef and pork insulin were obtained from Eli Lilly and Co., Indianapolis, Ind. and were the kind gifts of Dr. Alan S. Rosenthal of Merck, Sharp and Dohme, Rahway, N. J. Both beef and pork insulin contained <0.05% of proinsulin contamination. Ovalbumin five-times recrystallized (Sigma Chemical Co., St. Louis, Mo.), was coupled with 2,4,6-trinitrobenzene sulfonate (Pierce Chemical Co., Rockford, Ill.) as described elsewhere (11) to prepare trinitrophenylated ovalbumin (TNP-OVA). Purified protein derivative of tuberculin (PPD) was obtained from Connaught Medical Research Laboratory, Toronto, Ontario, Canada. Phytohemagglutinin (PHA) was obtained from Burroughs Wellcome Co., Research Triangle Park, N. C.

**Immunization.** Mice were immunized by injections of 0.05 ml in each hind footpad of an emulsion of antigen and complete Freund's adjuvant (CFA) containing 0.5 mg/ml of killed *Mycobacterium tuberculosis* (Grand Island Biological Co., Grand Island, N. Y.). 50 μg of beef or pork insulin and 10 μg of TNP-OVA were present in 0.1 ml of emulsion. In some instances, mice were boosted with equivalent amounts of antigen in CFA 14 d after primary immunization.

**Cell Culture Techniques.** The technique used for antigen-induced T cell proliferation represents a modification of previously described and well-characterized assays (9, 12). All washing procedures were performed in Hanks' balanced salt solution (HBSS; Grand Island Biological Co.) and cells were cultured in RPMI-1640 (Grand Island Biological Co.), supplemented with fresh L-glutamine (0.3 mg/ml), penicillin (100 μ/ml), and streptomycin (100 μg/ml), (all from Grand Island Biological Co.), 2-mercaptoethanol (5 × 10<sup>-5</sup> M) (Eastman Kodak Co., Rochester N. Y.), hepes buffer (10 mM) (Microbiological Associates, Walkersville, Md.), and 7.5% fetal calf serum (FCS; Hyclone Laboratories, Logan, Utah).

Briefly, the technique for cell culture is as follows: 14 d after immunization and in some instances, 14 d after boost immunization, femoral and popliteal lymph nodes were removed aseptically, teased apart, and separated by grinding between the frosted ends of two sterile microscope slides, followed by passage over sterile Nitex (Tetko Co., Elmsford, N. Y.) to eliminate solid debris. After two washings, these lymph node cells were incubated on nylon wool adherence columns bathed in media supplemented with 20% FCS for 60 min at 37°C. After this incubation, enriched, antigen-reactive lymph node T lymphocytes (LNL) were eluted from the nylon wool columns. Antigen-presenting adherent accessory cells (AAC) were obtained from two sources: spleens were aseptically removed from the animal donors, single-cell suspensions were made, and the spleen cells were allowed to adhere to sterile glass petri dishes for 2 h. Simultaneously, the peritoneal cavities of splenic donor mice were lavaged with heparinized HBSS (10 U heparin/ml), and these peritoneal washout cells (PC) were also allowed to adhere for 2 h. Then, the spleen-adherent cells and adherent PC cells were collected as described previously and pooled and treated with 40 μg/ml mitomycin C (Sigma Chemical Co.) for 60 min at 37°C. These cells were then washed vigorously five times to remove excess mitomycin C and then were used as accessory cells for T cell proliferation or as targets for antigen pulsing.

LNL T cells, and AAC, with or without antigen were then cultured for 96 h at densities of  $2 \times 10^5$  LNL T cells, and  $5 \times 10^4$  AAC in 200  $\mu$ l complete medium/well in round-bottomed microtiter plates (Cooke Engineering Co., Alexandria, Va.) at 37°C with an atmosphere of 5% CO<sub>2</sub> and 95% air. During the final 16–24 h of culture, each well was pulsed with 1  $\mu$ Ci of tritiated thymidine (<sup>3</sup>H]TdR; 6.6 Ci/mM, New England Nuclear, Boston Mass.). Cells were then harvested on glass fiber filter paper by the use of a microharvesting device (MASH II; Microbiological Associates). [<sup>3</sup>H]TdR incorporation was measured and all results reported as mean counts per minute SEM for triplicate determinations or as  $\Delta$ cpm = number of counts above control; e.g., experimental (with antigen) – control (no antigen).

The following method was used to pulse beef insulin, TNP-OVA, or PPD onto antigen-presenting AAC: Mitomycin C-treated AAC were incubated in complete medium with or without 100  $\mu$ g/ml beef insulin, 100  $\mu$ g/ml TNP-OVA, or 100  $\mu$ g/ml PPD at 37°C for 60 min at a concentration of  $1 \times 10^6$ – $5 \times 10^6$  AAC/ml. After this, AAC were washed thoroughly five times to remove excess antigen and then readjusted to the appropriate cell concentration in complete medium.  $5 \times 10^4$  antigen- or media-pulsed AAC were then added to  $2 \times 10^6$  primed LNL T cells/well, cultured for 4 d as outlined above and proliferative activity measured by [<sup>3</sup>H]TdR uptake. The data are expressed as  $\Delta$ cpm = antigen-pulsed AAC activation of primed LNL T cells – media-pulsed AAC activation of primed LNL T cells.

*Alloantisera.* Anti I-A<sup>b</sup> alloantiserum, (A  $\times$  B10.A)F<sub>1</sub> anti-B10.A(5R) was obtained from the Research Resource Branch, National Institute of Allergy and Infectious Diseases, and its anti-K<sup>b</sup> activity was absorbed with E1-4 tumor cells. Anti-Ia.W39 was prepared by immunizing (N  $\times$  B<sub>6</sub>)F<sub>1</sub> male mice with B6 spleen cells, as previously described (10). Normal mouse sera (NMS) from nonimmune male (N  $\times$  B6)F<sub>1</sub> mice was used as a control.

*Blocking Experiments.* To assess the blocking effect of antisera, 1% NMS, conventional anti-I-A<sup>b</sup>, or anti-Ia.W39 were added at the start of culture and were present continuously in the cultures.

## Results

*Effect of Anti-Ia Sera on the Insulin-specific T Cell Proliferative Response.* To ascertain the functional role of Ia.W39 on antigen-presenting M $\phi$ , we tested the ability of anti-Ia.W39 serum to block insulin-specific T cell proliferation in B6 mice. As can be seen

TABLE I  
Effect of Anti-Ia Sera on Proliferative Responses of B6 T Cells\*

	Beef insulin (100 $\mu$ g/ml)		PPD (10 $\mu$ g/ml)	
	[ <sup>3</sup> H]TdR incorporation <sup>‡</sup>	Percent inhibition <sup>§</sup>	[ <sup>3</sup> H]TdR incorporation	Percent inhibition
	$\Delta$ cpm		$\Delta$ cpm	
Experiment 1				
1% NMS	11,901	—	10,055	—
1% anti-Ia.W39	1,287	89	3,786	62
Experiment 2				
1% NMS	22,509	—	8,229	—
1% anti-I-A <sup>b</sup>	2,580	89	0	100

\*  $2 \times 10^5$  LNL T cells,  $5 \times 10^4$  AAC/well cultured for 4 d in round-bottomed microtiter plates.

<sup>‡</sup>  $\Delta$ cpm = experimental (with antigen) – control (without antigen); baseline control = [<sup>3</sup>H]TdR incorporation of LNL and AAC without antigen: experiment 1 = 1,152  $\pm$  815 cpm, experiment 2 = 2,115  $\pm$  234 cpm.

<sup>§</sup> Percent inhibition =  $\left(1 - \frac{\Delta\text{cpm anti-Ia}}{\Delta\text{cpm NMS}}\right) \times 100$ .

TABLE II  
Effect of Anti-Ia Sera on Proliferative Response of Immune (N × B6)F<sub>1</sub>  
Female T Cells to Beef Insulin\*

	[ <sup>3</sup> H]TdR incorporation‡	Percent inhibition§
	<i>Δcpm</i>	
Experiment 1		
1% NMS	4,885	—
1% anti-Ia.W39	1,318	73
1% anti-I-A <sup>b</sup>	504	90
Experiment 2		
1% NMS	5,156	—
1% anti-Ia.W39	1,246	76
1% anti-I-A <sup>b</sup>	0	100

The proliferative technique was the same as in Table I.

\* Beef insulin, 100 μg/ml for in vitro challenge.

‡ Δcpm = experimental (with antigen) – control (without antigen); baseline control = [<sup>3</sup>H]TdR incorporation of LNL and AAC without antigen: experiment 1 = 567 ± 103 cpm, experiment 2 = 259 ± 86 cpm.

$$\text{§ Percent inhibition} = \left(1 - \frac{\Delta\text{cpm anti-Ia}}{\Delta\text{cpm NMS}}\right) \times 100.$$

TABLE III  
Effect of Anti-Ia Sera on Proliferative Responses of Pork Insulin-immune BDF<sub>1</sub> T  
Cells

	[ <sup>3</sup> H]TdR incorporation*			Percent inhibition‡
	Pork insulin (100 μg/ml)	Beef insulin (100 μg/ml)	TNP-OVA (200 μg/ml)	
	<i>Δcpm</i>			
Experiment 1				
1% NMS	8,700	7,287	74,208	—
1% anti-Ia.W39	7,248	6,583	41,279	44
1% anti-I-A <sup>b</sup>	7,862	6,444	42,440	42
Experiment 2				
1% NMS	5,679	6,694	34,667	—
1% anti-Ia.W39	6,435	5,979	23,066	33
1% anti-I-A <sup>b</sup>	5,972	5,336	18,769	45

The proliferative technique was the same as in Table I.

\* Δcpm = experimental (with antigen) – control (without antigen); baseline control = [<sup>3</sup>H]TdR incorporation of LNL and AAC without antigen: experiment 1 = 1,821 ± 807 cpm, experiment 2 = 573 ± 84 cpm.

$$\text{‡ Percent inhibition} = \left(1 - \frac{\Delta\text{cpm anti-Ia}}{\Delta\text{cpm NMS}}\right) \times 100 \text{ for TNP-OVA.}$$

in Table I, experiment 1, 1% anti-Ia.W39 serum markedly inhibited beef insulin-induced proliferation (89%) and, to a lesser, extent PPD-induced cell division (62%). Anti-I-A<sup>b</sup> serum also significantly blocked both beef insulin- (89%) and PPD- (100%) induced activation. The same results were obtained using (N × B6)F<sub>1</sub> female T cells

TABLE IV  
Proliferative Response of Immune  $F_1$  T Cells

	$[^3\text{H}]\text{TdR}$ incorporation*		
	(N × B6) $F_1$ ♀	(N × B6) $F_1$ ♂	(B6 × N) $F_1$ ♂
	$(\Delta\text{cpm} \pm \text{SEM}) \times 10^{-3}$		
Beef insulin (100 $\mu\text{g}/\text{ml}$ )	12.28 ± 2.74	2.30 ± 0.69	17.45 ± 1.84‡
Pork insulin (100 $\mu\text{g}/\text{ml}$ )	2.51 ± 0.91	1.33 ± 0.47	2.64 ± 1.32
TNP-OVA (200 $\mu\text{g}/\text{ml}$ )	49.68 ± 3.73	42.08 ± 5.69	ND§
PPD (10 $\mu\text{g}/\text{ml}$ )	6.98 ± 1.10	4.91 ± 1.29	7.58 ± 2.72
PHA (1 $\mu\text{g}/\text{ml}$ )	63.96 ± 16.39	48.05 ± 10.55	39.55 ± 4.03

The proliferative technique was the same as in Table I.

\* Mean  $(\Delta\text{cpm} \pm \text{SEM}) \times 10^{-3}$  for four experiments.  $\Delta\text{cpm}$  = experimental (with antigen) – control (no antigen); control = spontaneous incorporation of  $[^3\text{H}]\text{TdR}$  in cultures of AAC and LNL T cells without antigen; control baselines: (N × B6) $F_1$  ♀: 3.45 ± 1.16, (N × B6) $F_1$  ♂: 3.37 ± 1.66, (B6 × N) $F_1$  ♂: 2.12 ± 1.19.

‡ Mean  $(\Delta\text{cpm} \pm \text{SEM}) \times 10^{-3}$  for three experiments.

§ Not done.

TABLE V  
Response of Immune (N × B6) $F_1$  Female T Cells to Antigen-pulsed Adherent Presenting Cells\*

Antigen-pulsed $\text{M}\phi$ ‡ (AAC)	$[^3\text{H}]\text{TdR}$ incorporation§
	$(\Delta\text{cpm} \pm \text{SEM}) \times 10^{-3}$
$F_1$ ♀ Beef insulin	11.09 ± 0.58
$F_1$ ♀ PPD	15.10 ± 1.94
$F_1$ ♀ TNP-OVA	24.28 ± 2.65
$F_1$ ♂ Beef insulin	1.77 ± 0.62
$F_1$ ♂ PPD	6.68 ± 0.23
$F_1$ ♂ TNP-OVA	19.32 ± 1.51

\*  $5 \times 10^4$   $\text{M}\phi$  (AAC) ± antigen pulse mixed with  $2 \times 10^5$  LNL T cells/well cultured for 4 d in round-bottomed microtiter plates.

‡  $\text{M}\phi$  (AAC) -pulsed with 100  $\mu\text{g}/\text{ml}$  beef insulin or 100  $\mu\text{g}/\text{ml}$  PPD in some instances, as outlined in Materials and Methods.

§  $\Delta\text{cpm}$  = antigen-pulsed  $\text{M}\phi$  activation of LNL T cells – media-pulsed  $\text{M}\phi$  activation of LNL T cells. Baseline: spontaneous incorporation of  $[^3\text{H}]\text{TdR}$  in culture of  $\text{M}\phi$  (AAC) and LNL T cells without antigen pulse ( $\Delta\text{cpm}$ ). Control baseline:  $F_1$  ♀ AAC: 2.94 ± 0.15;  $F_1$  ♂ AAC: 2.36 ± 0.55; mean  $(\Delta\text{cpm} \pm \text{SEM}) \times 10^{-3}$  for three experiments.

for proliferation (Table II, experiments 1 and 2); namely, anti-Ia.W39 and anti-I-A<sup>b</sup> sera caused a 75 and 95% inhibition of beef insulin-induced activation, respectively.

As a specificity control for the activity of anti-Ia.W39 and anti-I-A<sup>b</sup> sera, BDF<sub>1</sub> mice were primed with pork insulin and TNP-OVA (Table III). Only the DBA/2 (H-2<sup>d</sup>) parent of BDF<sub>1</sub> mice can respond to the antigenic determinants in pork insulin which are on the B chain. Because the B chains of beef and pork insulin are identical, there is a complete cross-reaction between beef and pork insulin in the H-2<sup>d</sup> haplotype. As can be seen in Table III, BDF<sub>1</sub> mice made equal proliferative responses to beef and pork insulin, and moreover these responses were unaffected by the addition of anti-Ia.W39 and anti-I-A<sup>b</sup> sera. However, both antisera reduced the response of BDF<sub>1</sub>

TABLE VI  
*Response of Immune Adult (N × B6)F<sub>1</sub> Female T Cells to Antigen-pulsed Adherent Presenting Cells\**

Antigen-pulsed M $\phi$ (AAC)‡	[ <sup>3</sup> H]TdR incorporation§
	$\Delta$ cpm
Adult F <sub>1</sub> ♀ beef insulin	9,064
Adult F <sub>1</sub> ♀ TNP-OVA	20,339
Neonatal F <sub>1</sub> ♀ beef insulin	0
Neonatal F <sub>1</sub> ♀ TNP-OVA	21,753

\* Usual proliferative technique as described in Table V. Results of one typical experiment out of three are shown here.

‡  $\Delta$ cpm = antigen-pulsed M $\phi$  activation of LNL T cells – media-pulsed M $\phi$  activation of LNL T cells, baseline control: spontaneous incorporation of [<sup>3</sup>H]TdR in cultures of M $\phi$  (AAC) and LNL T cells without antigen pulse (cpm). Control baseline: adult F<sub>1</sub> ♀ M $\phi$  (AAC): 1,157 ± 398, neonatal F<sub>1</sub> ♀ M $\phi$  (AAC): 883 ± 243.

§ M $\phi$  (AAC) -pulsed with 100  $\mu$ g/ml beef insulin or 200  $\mu$ g/ml. TNP-OVA in some instances, as outlined in Materials and Methods.

|| Neonatal M $\phi$  (AAC) prepared as described in Materials and Methods from mice that were 8-d old.

mice to TNP-OVA, suggesting that at least some TNP-OVA response may be associated with Ia.W39.

*Immune Proliferative Response of Normal and *xid*-defective F<sub>1</sub> Mice to Beef Insulin.* A series of experiments is summarized in Table IV that depict the comparative ability of beef insulin-primed T cells from normal (N × B6)F<sub>1</sub> female and (B6 × N)F<sub>1</sub> male mice and from defective (N × B6)F<sub>1</sub> male mice to respond to beef and pork insulin and other immunogens. Both normal female (N × B6)F<sub>1</sub> and male (B6 × N)F<sub>1</sub> mice mounted good T cell responses to beef insulin with very little cross-reaction to pork insulin stimulation (indicative of H-2<sup>b</sup> type recognition of the A chain loop determinant within beef insulin). The *xid*-defective male (N × B6)F<sub>1</sub> mice, however, could not develop a significant primed T cell proliferative response to beef insulin. All three groups of mice made comparable responses to TNP-OVA, PPD, and PHA. Because the defective F<sub>1</sub> male mice express all conventional Ia<sup>b</sup> specificities but lack Ia.W39, the striking conclusion offered by these data is that beef insulin responsiveness in the H-2<sup>b</sup> mouse is associated with the expression of Ia.W39.

*Response of Immune F<sub>1</sub> Female T Cells to Antigen-pulsed Presenting Cells.* To test potential cellular defects in the beef insulin response of (N × B6)F<sub>1</sub> male mice, we compared the ability of antigen (beef insulin or PPD) -pulsed M $\phi$  from *xid* defective F<sub>1</sub> male and normal F<sub>1</sub> female mice to activate syngeneic F<sub>1</sub> female T cells, which are immune to beef insulin and CFA. As is shown in Table V, the F<sub>1</sub> female M $\phi$  presented beef insulin to the immune T cells, whereas the F<sub>1</sub> male M $\phi$ , pulsed with beef insulin, were unable to induce proliferation in the same group of T cells. Although a T cell defect cannot be ruled out, these data imply that the *xid*-associated unresponsiveness to beef insulin is at the level of the antigen-presenting cell, which lacks Ia.W39 determinants. In controls included in Table V, both male and female F<sub>1</sub> M $\phi$ , pulsed with PPD and TNP-OVA, activated the primed T cells significantly, although the F<sub>1</sub> female M $\phi$  were slightly more efficient than the F<sub>1</sub> male. Although this difference may not be statistically significant, it may imply that some degree of PPD and TNP-OVA responsiveness in the H-2<sup>b</sup> haplotype is also associated with Ia.W39.

*Ontogeny of Presenting Cell Competence for Beef Insulin Is Ia.W39 Related.* Expression of Ia.W39 on the B cell membrane is not evident until 21 d after birth, whereas conventional Ia is present on B cells from neonatal mice. With this in mind, we compared the ability of M $\phi$  from neonatal (8 d old) and adult (N  $\times$  B6)F<sub>1</sub> female mice to present beef insulin to adult primed syngeneic T cells. As can be seen in one typical experiment out of a total of three in Table VI, the neonatal M $\phi$  were specifically deficient in presenting beef insulin, although they presented TNP-OVA efficiently. The adult M $\phi$  were able to present both antigens. These results highlight once again the association of Ia.W39 with beef insulin responsiveness at the level of the antigen-presenting M $\phi$ .

### Discussion

Our understanding of the I region of the MHC has increased through the functional and genetic analysis of recombinant mice and by serological and structural definition of the Ia antigens (13–15). However, progress has been considerably slower in the study of the mechanism, cellular site of action, and regulation of expression of Ir gene function.

The experiments in this report have unequivocally shown, using different approaches, that the responsiveness to beef insulin in the H-2<sup>b</sup> haplotype is dependent on the expression of Ia.W39 on antigen-presenting M $\phi$ . Moreover, we have demonstrated for the first time a non-MHC gene (*xid*)-linked regulation of an Ir gene function.

Mutant CBA/N mice possess an X-linked recessive defect, which has been recently mapped (*xid*) (16). This *xid* gene regulates the expression of the late-appearing B cell differentiation antigens Lyb-3 (17), Lyb-5 (18), Lyb-7 (19), and the newly defined Ia.W39 (10). As this latter alloantigen is encoded by the I-A<sup>b</sup> subregion of the H-2 gene complex, it seemed reasonable to ask two questions about its function: First, is Ia.W39 expressed functionally by antigen-presenting M $\phi$ ? Second, what is the relationship of Ia.W39 to I-A<sup>b</sup>-linked Ir gene function? The response of H-2<sup>b</sup> mice to beef insulin represented an ideal situation to analyze these two questions.

We have shown that monospecific anti-Ia.W39 serum could block beef insulin-induced T cell proliferation in H-2<sup>b</sup> mice. The conventional anti-I-A<sup>b</sup> serum also blocked beef insulin-induced T cell proliferation; however, it should be noted that the anti-I-A<sup>b</sup> antisera may contain anti-Ia.W39 antibodies. This is a likely possibility, because it was found in immunoprecipitation experiments that anti-I-A<sup>b</sup> serum could preclear Ia.W39, but not vice versa.<sup>2</sup> Over the years, on the basis of multiple studies in both mice and guinea pigs, it has been implied that alloantisera blockade is at the level of the antigen-presenting M $\phi$ , although no data absolutely prove this point (20, 21). More recent experiments utilizing monoclonal antibodies and parent  $\rightarrow$  F<sub>1</sub> chimeras as a source of lymphoid cells reconfirm the suspicion that inhibition of response by anti-Ia reagents occurs at the level of the antigen-presenting accessory cell (23). It therefore follows from these blocking experiments that Ia.W39, a B cell differentiation marker expressed on a subpopulation of B cells, is also functionally expressed by antigen-presenting M $\phi$ .

With a different approach, we have shown that beef insulin responsiveness is associated with the expression of Ia.W39, by demonstrating that normal (N  $\times$  B6)F<sub>1</sub> female and (B6  $\times$  N)F<sub>1</sub> male mice as opposed to defective (N  $\times$  B6)F<sub>1</sub> male mice

could mount a T cell proliferative response to beef insulin. The same pattern was seen in the secondary IgG plaque-forming cell (PFC) response to beef insulin, measured in these groups of mice (B. T. Huber and L. J. Rosenwasser, manuscript in preparation). Furthermore, we have shown that defective F<sub>1</sub> male M $\phi$  cannot present beef insulin to responder F<sub>1</sub> female beef insulin-primed T cells.

In sum, we have found in (N  $\times$  B6)F<sub>1</sub> male mice a defect in insulin-related Ir gene function and expression of Ia.W39 on antigen presenting M $\phi$ . In the *xid* defective (N  $\times$  B6)F<sub>1</sub> male B cells, it is felt that lack of Ia.W39 represents a maturation arrest in the differentiation pathway, whereby certain subsets of B cells do not develop. By analogy, the mechanism of *xid*-regulated defective Ir gene function may be a result of deletion or faulty maturation of a subpopulation of antigen-presenting M $\phi$  or alternatively, a selective defect in Ia.W39 expression on the M $\phi$  membrane may be evident on all Ia-bearing M $\phi$ . Experiments to assess those possibilities are in progress. Additionally, T cell proliferation experiments in (N  $\times$  B6)F<sub>1</sub> males and females with beef collagen and poly(L-Tyr, L-Glu)-poly(DL-Ala)-poly(L-Lys); both under the control of genes located in the I-A<sup>b</sup> subregion are in progress, to see if these responses are also dependent on expression of Ia.W39. The molecular mechanism of this *xid*-regulated Ir gene deficit is unknown and investigation in this area may provide a key to the precise workings of Ir genes. Another point of interest that is immediately apparent is whether this *xid* regulatory gene is operative in other haplotypes as a general regulatory phenomenon; and for that reason, experiments examining the insulin B chain determinant responses in (N  $\times$  BALB)F<sub>1</sub> males and females are in progress. (H-2<sup>d</sup> mice recognize a determinant in the B chain of insulin, whereas H-2<sup>b</sup> mice recognize a determinant in the A chain loop).

Two further points should be made concerning the results reported here. First, the data in this paper highlight the strong association of the Ia.W39 specificity with Ir gene function for beef insulin in H-2<sup>b</sup> mice. Other examples of the association of Ia antigens with Ir gene functions have been documented in previous studies in both guinea pigs and mice (23, 24). More recently, work with a monoclonal antibody directed against Ia antigens containing the combinatorial determinant A<sub>e</sub><sup>b,k,r,s</sup>-E <sub>$\alpha$</sub>  has shown a clear association of these hybrid Ia antigens with Ir gene function for pigeon cytochrome *c* in B10.A mice and for poly(Glu, Lys, Phe)<sup>9</sup> in B10.A(5R) mice (25). Second, the findings of this report document for the first time a potential M $\phi$  defect associated with mutant CBA/N mice. These mice make defective responses to thymus-independent type 2 (TI-2) antigens such as TNP-Ficoll; Boswell et al. (26) have shown that the in vitro response of B cells to thymus-independent type 2 (TI-2) antigens is M $\phi$  dependent, however, those same workers felt that the defect in CBA/N mice to TI-2 antigens rested with a lack of the Lyb-5<sup>+</sup> subset of B cells and that CBA/N M $\phi$  could present TI-2 antigens to an appropriate B cell (27).

Finally, we have shown that neonatal, antigen-presenting M $\phi$ , which presumably do not acquire Ia.W39 until 21 d after birth but have conventional Ia antigens analogous to neonatal B cells, are deficient in presenting beef insulin to primed adult T cells, again suggesting the association of Ia.W39 with beef insulin responsiveness at the level of the M $\phi$ . Recently Nadler et al. (28) showed that neonatal (<14 d old) antigen presenting M $\phi$  are incompetent at inducing in vitro primary TNP-KLH PFC responses and Lu et al. (29) showed that neonatal M $\phi$  are deficient at presenting *Listeria monocytogenes* antigen to immune T cells. Our findings on the incompetency of



normal neonatal M $\phi$  to present insulin to primed, adult T cells is in accord with those studies. However, we do not find a generalized defect in Ia-dependent T cell activation in neonatal M $\phi$ , because they can present TNP-OVA to immune T cells. It is reasonable to infer from our studies that neonatal M $\phi$  acquire the ability to present insulin as they acquire Ia.W39; therefore, it may be likely that functional maturation and acquisition of Ia.W39 and perhaps all Ia antigens by antigen-presenting M $\phi$  may be under the influence of the X-linked regulatory gene(s).

Thus we have shown that beef insulin responsiveness in the H-2<sup>b</sup> mouse is dependent on the expression of Ia.W39 by antigen-presenting M $\phi$ . The defect in beef insulin reactivity in mutant (N  $\times$  B6)F<sub>1</sub> male mice is a result of lack of expression of Ia.W39, regulated by the *xid* gene. The discovery of an X-linked regulatory gene for Ir gene function is a new and provocative finding and should provide the basis for future studies on the regulation and mechanism of antigen responsiveness.

### Summary

Immune response (Ir) genes are encoded for by the I region of the major histocompatibility complex (MHC). A class of serologically defined specificities, Ia antigens, is also encoded for by genes within this region. A new Ia specificity, Ia.W39, has recently been defined. It is private for I-A<sup>b</sup> and its expression is controlled by a gene on the X-chromosome. Using different approaches, the role of Ia.W39 in the immune response of H-2<sup>b</sup> mice to beef insulin was examined in a macrophage-dependent T cell proliferation assay. It was found that beef insulin-related Ir gene function was associated with the expression of Ia.W39 by antigen-presenting macrophages and that control of this Ir gene function was X-linked (*xid* gene).

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