Autoimmune Diabetes Can Be Induced In Transgenic Major Histocompatibility Complex Class II-deficient Mice

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Summary

Insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease marked by hyperglycemia and mononuclear cell infiltration of insulin-producing β islet cells. Predisposition to IDDM in humans has been linked to the class II major histocompatibility complex (MHC), and islet cells often become aberrantly class II positive during the course of the disease. We have used two recently described transgenic lines to investigate the role of class II molecules and CD4$^+$ T cells in the onset of autoimmune insulitis. Mice that are class II deficient secondary to a targeted disruption of the Aβ^b gene were bred to mice carrying a transgene for the lymphocytic choriomenigitis virus (LCMV) glycoprotein (GP) targeted to the endocrine pancreas. Our results indicate that class II-deficient animals with and without the GP transgene produce a normal cytotoxic T lymphocyte response to whole LCMV. After infection with LCMV, GP-transgenic class II-deficient animals develop hyperglycemia as rapidly as their class II-positive littermates. Histologic examination of tissue sections from GP-transgenic class II-deficient animals reveals lymphocytic infiltrates of the pancreatic islets that are distinguishable from those of their class II-positive littermates only by the absence of infiltrating CD4$^+$ T cells. These results suggest that in this model of autoimmune diabetes, CD4$^+$ T cells and MHC class II molecules are not required for the development of disease.

Insulin-dependent diabetes mellitus (type I; IDDM)[1] is caused by the autoimmune destruction of insulin-producing β cells in pancreatic islets of Langerhans (1). The disease is associated with hypoinsulinemia, hyperglycemia, and mononuclear cell infiltration in the islets. The development of diabetes appears to be multifactorial and has both genetic and environmental contributions. Predisposition to type I diabetes is genetically determined and recent studies have linked multiple haplotypes of the MHC class II locus with either an increased risk of disease or a protective effect (2). As well, the aberrant expression of class II molecules on the β cells of diabetic individuals has focused attention on the role of the MHC in the development of diabetes (3). In susceptible individuals, the development of diabetes is associated with a loss of self-tolerance and immunologic destruction of pancreatic β cells (4).

Recently, transgenic animals expressing foreign genes under the control of the tissue-specific insulin promoter have been used as experimental models to study the development of insulin-dependent diabetes. Ectopic pancreatic expression of either MHC class I or II is not sufficient to produce autoimmune insulitis (5–8). Hyperglycemia does occur, presumably due to interference by the transgene with insulin gene expression or secretion (5). Diseased transgenic animals also lack histologic evidence of mononuclear cell infiltration of the pancreas. Ectopic IFN-γ expression in pancreatic islets does lead to mononuclear cell infiltration and diabetes mellitus (8, 9); however, hyperglycemia develops very rapidly and mononuclear cell infiltrates can be found in other endocrine glands. Thus, previous transgenic models do not accurately reproduce the pathophysiology of diabetes.

A transgenic model of insulin-dependent diabetes mellitus that mirrors the mononuclear cell infiltration and loss of self-

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[1] *Abbreviations used in this paper:* GP, glycoprotein; IDDM, insulin-dependent diabetes mellitus; LCMV, lymphocytic choriomeningitis virus; RIP, rat insulin promoter.

tolerance found in the human disease has been described (10). When the lymphocytic choriomeningitis virus (LCMV) glycoprotein was expressed in the islets of Langerhans of transgenic mice under the control of the rat insulin promoter (RIP), challenge with LCMV resulted in a mononuclear cell infiltrate isolated to islet cells and the onset of type I diabetes, i.e., hyperglycemia and hypoinsulinemia (10). In a similar model, the onset of hyperglycemia could be prevented by treating mice with mAb to either CD4 or CD8 before LCMV challenge (11).

To further examine the requirement for CD4 cells and MHC class II in the development of diabetes, mice bearing the LCMV glycoprotein transgene were bred to MHC class II–deficient animals. Mice deficient for MHC class II expression by virtue of a targeted disruption of the $A\beta^b$ gene have a profound reduction in the numbers of peripheral single-positive CD4 cells (12). This is associated with an elevated number of CD8-positive T cells in the thymus and in the periphery. Although they have normal, or elevated, levels of serum IgM, the animals are unable to make IgG antibodies to T-dependent protein antigens. We report here that class II–deficient animals have a normal CTL response to LCMV and develop diabetes after LCMV infection in the presence of a glycoprotein transgene.

Materials and Methods

Mouse Strains, Target Cells, and Viruses. The transgenic mouse line GP34-20 bearing LCMV glycoprotein (GP) under the control of the rat insulin promoter has been previously described (10). The class II–deficient mice have also been described (12). Founder mice in each case were crossed to C57Bl/6 mice; the F_3 generation was used in these experiments. Double transgenic mice were generated by mating homozygous class II–deficient ($A\beta^{-/-}$) mice to transgenic mice bearing an LCMV GP transgene under the control of the rat insulin promoter. F_1 generation mice transgenic for the glycoprotein and heterozygous at the class II locus were then backcrossed to class II–deficient mice to obtain the class II–deficient transgenic mice. In all experiments, mice were heterozygous at the GP transgene locus. Mice were housed in microisolator cages and appeared healthy. MC57 (H-2^b) fibroblasts were grown in RPMI 1640 supplemented with 7% FCS, 10^4 U penicillin/ml, 10 mg/ml streptomycin, and 1 mM L-glutamine. The LCMV ARM clone 53b has been described previously (13), as have recombinant vaccinia viruses expressing LCMV GP or the GP minimal CTL epitope peptide GP1 (glycoprotein amino acids 32–42) (14). Vaccinia virus was titered on 143 T4 cells and LCMV on Vero cells (13, 14).

Analysis of Blood Glucose. Blood samples were obtained from the retro-orbital eye plexus of mice at weekly intervals and blood glucose measurements were determined using Accu-Check II (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Cytotoxicity Assays. LCMV-specific CTL were induced by intraperitoneal injection of 10^5 PFU of virus. Hemi-splenectomies were performed 7 d after infection. Killing by cytotoxic T cells was quantitated using a 4–5-h ^{51}Cr release assay as described (13). Target cells were infected with either LCMV ARM or recombinant vaccinia virus expressing LCMV GP or polypeptides for the LCMV CTL epitope GP1. Cells infected with LCMV ARM were at a multiplicity of infection (MOI) of 1, and were incubated 1 h at 37°C with occasional rocking and harvested after 48 h. Cells infected with vaccinia viruses were at a MOI of 3 and incubated 45 min at 37°C. Cells were washed, placed in fresh media, and harvested

at 12 h. 2 × 10^6 target cells were labeled with 1 mCi ^{51}Cr and plated at 10^4 cells/100 μl. Splenocytes were resuspended at 10^7/ml after lysis of RBC with 0.84% NH_4Cl. E/T ratios of 50:1, 25:1, and 12:1 were used. Samples were processed in triplicate and standard deviation among triplicates was <5%.

Antibody Assays. Antibodies to whole LCMV were detected using an ELISA (15). Antibodies to LCMV GP were detected by staining for plasma membrane expression of LCMV in LCMV-infected MC57 cells with sera from infected animals as previously described (10). MC57 cells were used 48–72 h after infection with LCMV ARM 53b. Specific antibody was developed with fluorescein-conjugated mAb to mouse IgG. Control sera were normal mouse preimmune sera, anti-LCMV mAb, and hyperimmune serum obtained from a nontransgenic mouse 3 wk after infection with LCMV.

Histology and Immunohistochemistry. Tissues for histologic analysis were taken from animals 14 d after viral infection, fixed in 10% zinc formalin, and stained with hematoxylin and eosin (H&E). Immunohistochemistry was performed on 4–6-μm sections of liquid nitrogen frozen tissue cut with a microtome. Sections were fixed briefly in 1% paraformaldehyde. Tissue was blocked with avidin and biotin (Vector Labs, Burlingame, CA) and incubated with mAbs to class II, CD4 (L3T4; Pharmingen), or CD8 (Ly-2 and Ly-3; Pharmingen). Slides were incubated with biotinylated goat anti–rat IgG (Southern Biotec), followed by streptavidin horseradish peroxidase and developed with 3,3'-diaminobenzidine in the presence of 0.03% H_2O_2 for 5–20 min. Slides were then counterstained with hematoxylin.

Results and Discussion

CTL Response to LCMV in MHC Class II–deficient Mice. In preliminary experiments, we examined primary CTL responses to LCMV in homozygous class II–deficient $(-/-)$ animals as compared to heterozygous class II–positive $(+/-)$ littermates. Table 1 presents the results of ^{51}Cr release assays performed on spleen cells obtained from class II–negative and class II–positive littermates 7 d after intraperitoneal injection of 10^5 PFU of virus. Targets were MC57 fibroblasts infected with LCMV ARM 53b or vaccinia virus recombinants that contain either intact LCMV GP (vvGP) or a peptide epitope of GP (vvGP1, GP amino acids 32–42) that elicits a strong cytotoxic response. At an E/T ratio of 50:1, LCMV ARM–infected targets are lysed equally efficiently by class II–positive (specific ^{51}Cr release, 36%) and class II–negative (specific ^{51}Cr release, 33%) splenic effectors. Class II–deficient animals also produce cytotoxic responses to glycoprotein (vvGP) and a minimal GP epitope (vvGP1) similar to that of both heterozygous littermates and wild-type C57Bl/6 H-2^b controls. These results were observed in multiple experiments.

We next asked whether this LCMV-specific immune response was also normal in class II–deficient mice carrying the RIP-GP transgene. As detailed in Table 1, the CTL response elicited to whole LCMV 7 d after infection is equivalent in class II–deficient (specific ^{51}Cr release at E/T of 50:1, 22%) and class II–positive GP-transgenic animals (specific ^{51}Cr release at E/T of 50:1, 24%). Both class II–positive and class II–negative GP-transgenic mice also display CTL cytotoxicity against H-2^b cells infected with vvGP and vvGP1 recombinants. However, in repeated experiments, transgenic mice consistently produce a slightly diminished CTL response

Table 1. *Primary CTL Reactivity 7 d after Infection with LCMV*

Source of day 7 splenic CTLs		Percent specific ⁵¹Cr released from H-2² targets infected with:			
Mouse	E/T ratio	LCMV ARM	vvGP	vvGP1	Uninfected
Class II⁻/⁻					
(n = 7)	50:1	33 ± 12	15 ± 6	30 ± 10	2 ± 2
	25:1	25 ± 8	15 ± 6	24 ± 8	1 ± 1
	12.5:1	20 ± 5	5 ± 3	8 ± 4	1 ± 0
Class II⁺/⁻					
(n = 2)	50:1	36 ± 4	26 ± 6	41 ± 10	1 ± 0
	25:1	18 ± 6	25 ± 5	26 ± 8	3 ± 1
RIP-GP class II⁻/⁻					
(n = 5)	50:1	22 ± 8	20 ± 6	16 ± 6	2 ± 2
	25:1	11 ± 4	16 ± 4	7 ± 3	1 ± 1
	12.5:1	5 ± 3	11 ± 5	5 ± 2	1 ± 1
RIP-GP class II⁺/⁻					
(n = 4)	50:1	24 ± 10	24 ± 10	21 ± 8	0 ± 0
	25:1	20 ± 8	16 ± 6	15 ± 6	1 ± 0
	12.5:1	10 ± 6	8 ± 2	7 ± 2	2 ± 1
Nontransgenic H-2ᵇ					
(n = 6)	50:1	32 ± 4	17 ± 8	33 ± 8	3 ± 2
	25:1	20 ± 2	10 ± 4	15 ± 4	2 ± 1

CTL assays were performed 7 d after administering 10⁵ PFU LCMV intraperitoneally. Splenic lymphocyte effectors were used at E/T ratios of 50:1, 25:1, and 12.5:1. Targets were MC57 cells (H-2ᵇ) labeled with ⁵¹Cr and either uninfected or infected with LCMV-ARM or vaccinia virus (vv) recombinants containing LCMV GP or GP1 CTL epitopes. The values represent mean values ± SD. The difference in lysis between uninfected and infected MC57 cells was statistically significant (p ≤0.01).

to a given target when compared to their nontransgenic littermates (M. G. von Herrath, unpublished observation). For example, at an E/T ratio of 50:1, the percent specific ⁵¹Cr released from LCMV ARM-infected targets is 33% in class II–deficient animals and only 22% in class II–deficient animals with the GP transgene. The mechanism for this partial unresponsiveness remains unclear. T cells with transgenic TCRs specific for LCMV GP are not deleted in mice with RIP-GP transgenes (11). This decreased cytotoxicity presumably represents peripheral anergy in the presence of endogenous GP that can be overcome by the strong immune response elicited by viral infection.

These results demonstrate that CD8⁺ cells can mediate a normal anti-LCMV cytotoxic response in the absence of CD4⁺ cells and class II–positive APCs. Mice lacking CD4 by homologous recombination have a normal CTL response to LCMV (16). Our data indicate that class II–positive APCs, as well, are not required for normal CD8-mediated antiviral effector function. It has been reported that class II–deficient, mice housed in conventional animal facilities exhibit a marked

defect in their recall CTL response to influenza virus while mice housed in cleaner facilities have normal CTL responses (17). Our results are in agreement with the finding that class II–deficient animals housed in microisolator cages in a virus-free facility have efficient CTL responses to virus and raise the possibility that class II–deficient animals are unable to clear the routine environmental pathogens present in conventional facilities. Overinfection with these organisms may lead to generalized immunosuppression and the inability to appropriately respond to a nonroutine pathogen such as influenza.

In published studies, adult mice depleted in vivo of CD4 T cells by mAbs generate a CTL response to LCMV that is reduced by a factor of 10 when assayed by ⁵¹Cr release, suggesting a limited requirement for CD4 T cells to maintain CD8 function in this system (18–20). Anti-CD4 treatment does not alter the clearance of LCMV in vivo (21). The role of T cell help in the induction of antiviral cytotoxicity is, however, dependent on the virus studied. Thus, in vivo depletion of CD4 cells by antibody treatment quantitatively

diminishes the cytotoxic response to vaccinia (22) and sendai (23); whereas the effect of antibody treatment on the response to ectromelia (24) or herpes virus (25) is minimal.

Role of MHC Class II and CD4 Cells in LCMV-induced Insulitis. Transgenic mice expressing LCMV GP in the pancreas are not tolerant of the transgenic protein since they develop IDDM after infection with LCMV (10, 11). To evaluate the requirement for class II expression and CD4$^+$ cells in the development of autoimmune diabetes, four 6–8-wk-old class II–positive (+/−) and five class II–deficient (−/−) littermates carrying LCMV transgenes were challenged with 10^5 PFU of LCMV intraperitoneally and blood glucose levels were monitored weekly as detailed in Fig. 1. Those animals that developed hyperglycemia (blood sugar, >250 mg/dl) were killed. Class II deficiency is itself not associated with hyperglycemia or mononuclear infiltration of pancreatic tissue: four uninfected class II–deficient animals examined between the ages of 6 wk and 6 mo had normal blood glucose levels and normal pancreatic histology by routine H&E staining (T. Laufer, data not shown). LCMV challenge of 10^5 PFU into five control nontransgenic class II–deficient and three heterozygous littermates did not produce hyperglycemia and none of the infected animals in either group had a blood glucose level >225 mg/dl. In contrast, within 14 d of LCMV infection, the RIP-GP class II–deficient animals had blood glucoses that averaged 550 mg/dl and only one animal had a blood sugar <500 mg/dl. These values are not significantly different than the blood sugars of RIP-GP class II–positive littermates, whose blood glucose averaged 560 mg/dl on day 14. Thus, class II deficiency does not alter the development of hyperglycemia induced in transgenic animals by LCMV infection.

Pancreatic sections were taken from hyperglycemic mice killed 14 d postinfection. The tissue sections reveal a lymphocytic infiltrate restricted to the islets and sparing the exocrine pancreas, and are indistinguishable in class II–positive and class II–negative littermates (Fig. 2, *b* and *c*). To further characterize the pathologic process within the islets of Langerhans, additional pancreatic sections were stained with mAbs

to CD4 and CD8 (Fig. 3). In class II–positive RIP-GP mice, the lymphocytic infiltrate was composed of both CD4 and CD8 cells (Fig. 3, *a–c*). Conversely (Fig. 3, *d–f*), the lymphocytic infiltrate present in the islets of RIP-GP class II–negative animals was uniformly CD8$^+$. Thus, in RIP-GP mice that are class II deficient, CD8$^+$ cells are sufficient to mediate the development of an islet-restricted lymphocytic infiltrate and hyperglycemia after LCMV infection.

Antibody Response to LCMV in Class II–deficient RIP-GP Mice. It has been previously described that class II–deficient animals fail to produce antigen-specific antibodies to soluble protein antigen (12). Fig. 4 *a* demonstrates that despite normal CTL reactivity to LCMV in the absence of CD4 cells and class II, the anti-LCMV antibody response remained dependent on the class II status of the animal. Three nontransgenic class II–positive animals assayed before LCMV infection had anti-LCMV titers of <1:10 plasma dilution. When serum obtained 14 d after challenge with 10^5 PFU of LCMV intraperitoneally was assayed in an ELISA with whole sonicated virus, all class II heterozygous mice had significant antibody responses with titers averaging 1:3,500 and ranging from 1:150 to 1:10,000. In contrast, seven class II–deficient animals failed to produce LCMV-specific antibodies and had titers in the ELISA that averaged 1:30 and ranged from 1:1 to 1:50. Antibody specific to LCMV GP was assayed by immunofluorescent staining of LCMV-infected MC57 cells with sera at 1:10 dilutions from transgenic animals 14 d after viral infection. Serum from class II–positive animals had evidence of fluorescent staining of the MC57 cells (Fig. 4 *c*). Class II–negative animals, in contrast, failed to produce detectable antibody (Fig. 4 *d*).

Our results indicate that in the absence of a functional population of class II–restricted CD4$^+$ helper cells, class II–restricted CD8$^+$ cells can provide help for the generation of cytotoxicity. This situation has some analogy to the development of LCMV-specific class II–restricted CD4$^+$ CTLs identified by Muller et al. (26) in β_2-microglobulin-deficient mice that lack class I and, therefore, CD8$^+$ cells. In that system, CD4 depletion with antibody had minimal effect on
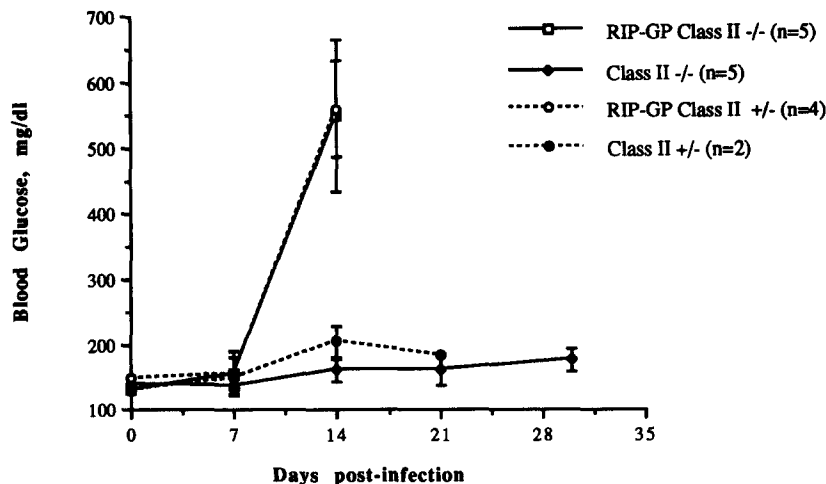

Figure 1. Hyperglycemia after LCMV Infection. The occurrence of IDDM in RIP-LCMV transgenic mice after LCMV infection. Littermates at 6–8 wk of age received 10^5 PFU of LCMV intraperitoneally. Mice were subsequently bled and blood glucose levels were measured as described in Materials and Methods.
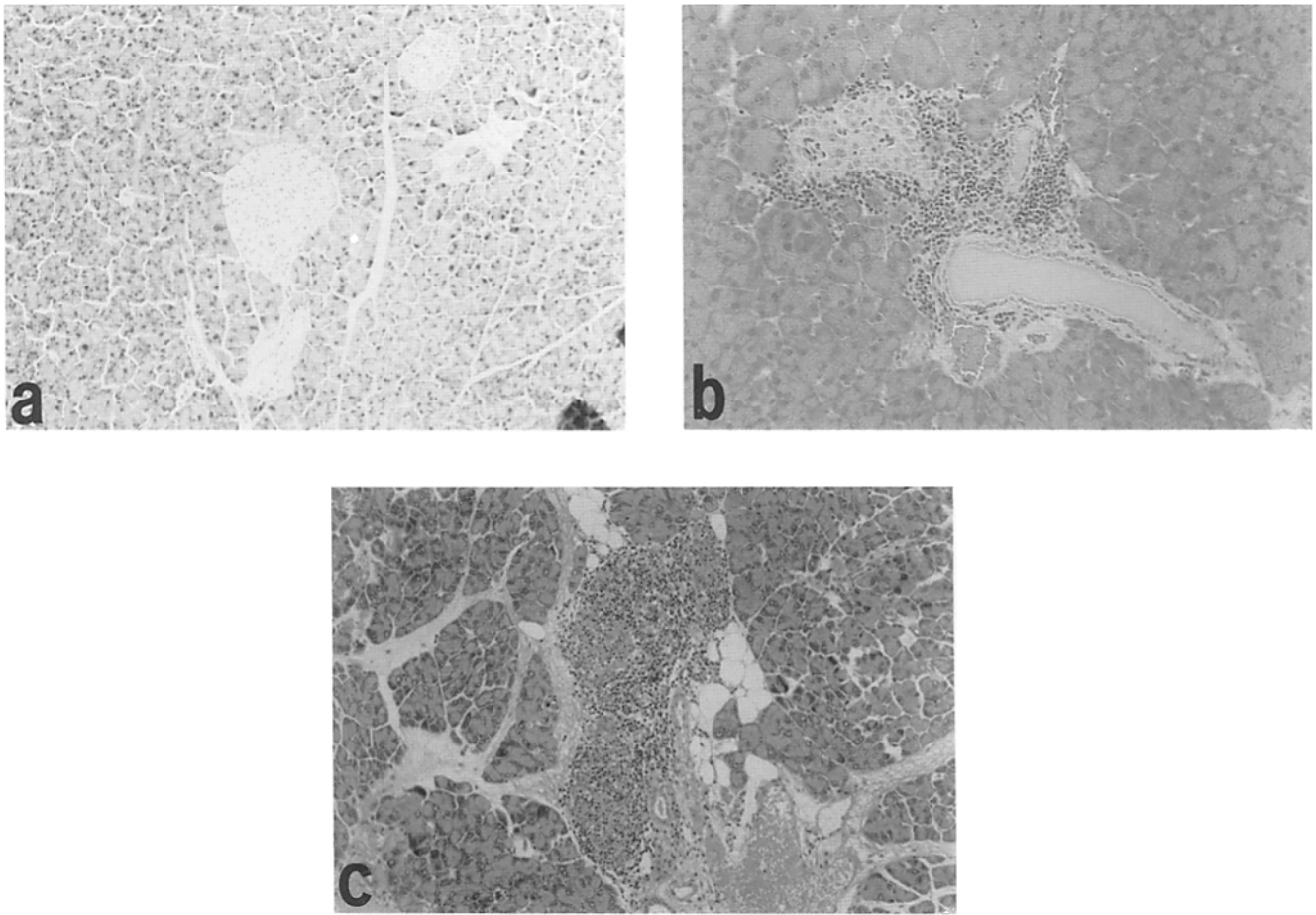
Figure 2. Islets of Langerhans from mice 14 d after viral infection. Pancreatic sections from individual class II–deficient nontransgenic (*a*), RIP-GP class II–positive (*b*), and RIP-GP class II–negative animals stained with H&E. Note the mononuclear cell infiltrates restricted to the islets in *b* and *c*.
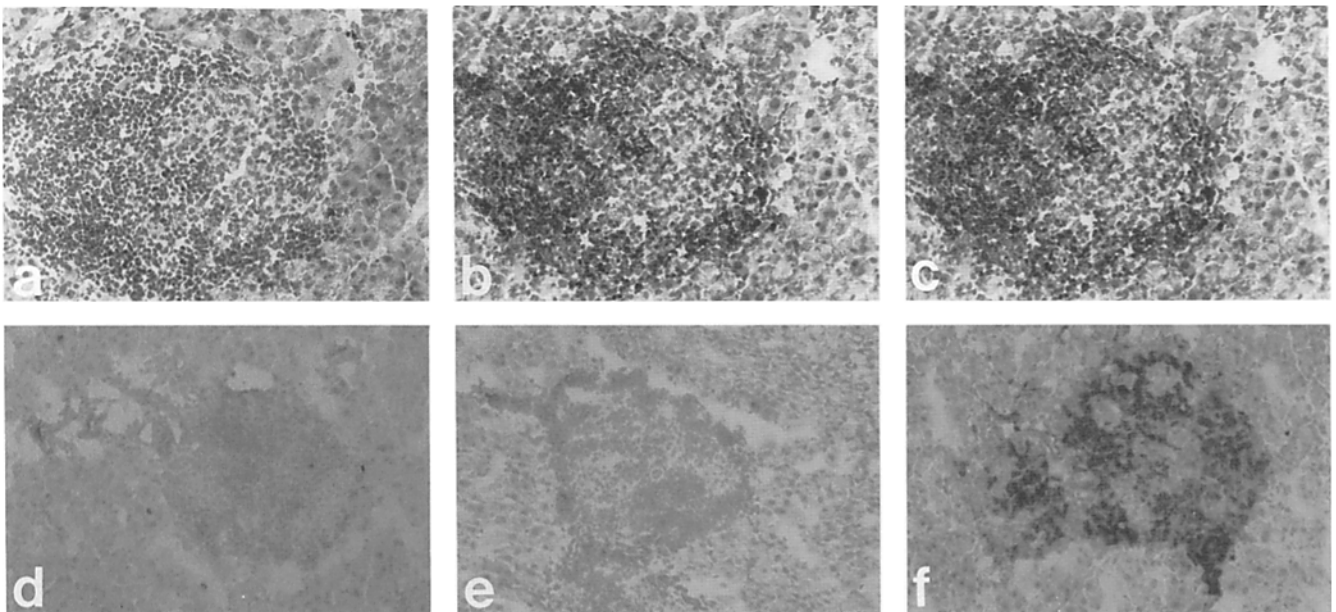

Figure 3. Immunohistochemistry of islets of Langerhans after viral infection. RIP-GP class II–positive (*a–c*) or negative (*d–f*) animals received 10⁵ pfu of LCMV intraperitoneally. 14 d after infection, pancreases were removed and sectioned. Immunohistochemistry was performed using no primary antibody (*a* and *d*), anti-CD4 (*b* and *e*), or anti-CD8 (*c* and *f*).

a

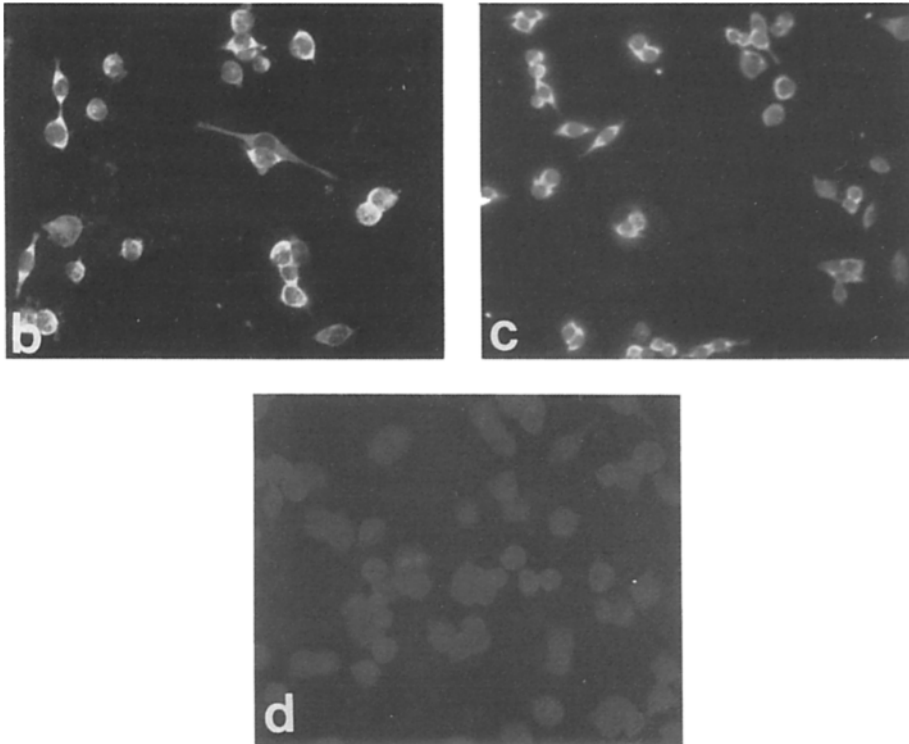| Sample	Antibody titer
Normal mouse Preimmune serum (n=2)	<1:10±10
Class II +/- (n=5)	1:3500±1700
Class II -/- (n=7)	1:30±11
Normal mouse Hyperimmune serum (n=1)	1:7600±1600

Figure 4. Sera from class II–deficient animals challenged with LCMV lack specific antibodies (a). Sera from infected mice were assayed in an ELISA to whole sonicated LCMV. The reactions were developed with a secondary anti–mouse IgG-peroxidase conjugate. Titers represent the dilution at which the OD_{492} = 0.3 ± SD. (b–d) Antibodies to LCMV GP from class II–heterozygous (c) or –deficient (d) animals were detected by immunofluorescence of LCMV-infected MC57 cells with sera diluted 1:10. The positive control panel (b) is serum obtained from a wild-type H-2^b mouse 3 wk after LCMV challenge.

the LCMV-specific CTL response in normal B6 mice, suggesting a very low frequency of CD4$^+$ CTL precursors normally. In class II–deficient animals, a population of class I–restricted, CD8$^+$ cells that can produce cytokines may develop to substitute for the CD4$^+$ help typically generated. However, such a population may not normally exist. This may account for the reduction of anti-LCMV cytotoxicity in normal mice by treatment with anti-CD4 antibody, which is not seen in mice that are CD4- or class II–deficient from birth. It may also explain Ohashi et al.'s (11) finding that wild-type H-2^b RIP-GP transgenics fail to develop hyperglycemia if anti-CD4 antibody is given before LCMV infection. Alternatively, the cytotoxic response to LCMV may be an independent response that does not require cytokine-mediated help. We favor the first possibility, as evidence exists in other murine systems for production of cytokines by CD8$^+$ cells in response to "allo" differences (27, 28), mitogenic stimulation (29), and stimulation with immobilized anti-CD3 (30), and preliminary experiments suggest that IL-2 is elaborated by primary LCMV CTL cultures from class II–deficient mice (M. G. von Herrath, unpublished observation).

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