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Virus-Induced Diabetes in a Transgenic Model: Role of Cross-Reacting Viruses and Quantitation of Effector T Cells Needed To Cause Disease†

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Virus-specific cytotoxic T lymphocytes (CTL) at frequencies of >1/1,000 are sufficient to cause insulin-dependent diabetes mellitus (IDDM) in transgenic mice whose pancreatic β cells express as "self" antigen a protein from a virus later used to initiate infection. The inability to generate sufficient effector CTL for other cross-reacting viruses that fail to cause IDDM could be mapped to point mutations in the CTL epitope or its COO^- flanking region. These data indicate that IDDM and likely other autoimmune diseases are caused by a quantifiable number of T cells, that neither standard epidemiologic markers nor molecular analysis with nucleic acid probes reliably distinguishes between viruses that do or do not cause diabetes, and that a single-amino-acid change flanking a CTL epitope can interfere with antigen presentation and development of autoimmune disease in vivo.

Insulin-dependent diabetes mellitus (IDDM) develops after an individual's insulin-producing β cells in the pancreatic islets of Langerhans are destroyed by reactive T lymphocytes. This process is multifactorial, involving host genes, autoimmune responses, cytokines, and environmental factors (2, 8, 18). The evidence for environmental influence is several pronged. First, studies of monozygotic twins in which one has diabetes but the other does not show a discordance rate of approximately 30 to 50% (18, 25). Second, more than 80% of cases of IDDM occur in children with no family history of diabetes (18, 25). This evidence is reinforced by linking the aberrant immune responses of several autoimmune diseases, including IDDM, with somatic (antigen driven) rather than germline mutation (27, 40) and by analyzing epidemiologic surveys that associate multiple virus infections with IDDM (2, 9, 10, 30, 31).

For example, fulfilling Koch's postulates, coxsackievirus, which has been linked to diabetes (10, 31), was isolated from the pancreas of a human with acute-onset diabetes and, upon transfer, induced IDDM in an animal model (42). Several systemic viral infections in humans preceded destruction of islets of Langerhans accompanying mononuclear cell infiltration (12). In addition, 12 to 20% of children infected congenitally with rubella have IDDM (9, 19, 30). Finally, in several model systems, viruses directly or indirectly cause IDDM (9, 11, 19, 20, 22, 23, 30). However, despite this compelling evidence, in the vast majority of cases, no infectious agent (virus) has been uniformly identified.

This paper directly addresses the reasons for this dilemma. A transgenic mouse model is used in which a known viral gene (the nucleoprotein [NP]) of lymphocytic choriomeningitis virus (LCMV) is expressed in β cells (22). No injury to these cells occurs throughout an animal's life unless it is later exposed to the same virus. The kinetics of IDDM onset and severity of disease are also dependent on expression of the viral transgene in the thymus as well as in β cells (32), on the numbers and affinity of antiviral cells that escape negative selection and survive in the periphery (13, 32, 34, 36), on the host's major histocompatibility complex (MHC) background (32, 34), and on the expression of MHC molecules (35, 37) as well as T-cell activation molecules (36) in the islets' milieu. Although the events by which mononuclear cells are activated, infiltrate the islets, and destroy β cells, leading to hypoinsulinemia and hyperglycemia, are relatively clear in transgenic mice infected with the same virus, the role played by unrelated or other related viruses in causing IDDM is not.

This model allows us to address two fundamental issues. First, what is the number of effector cells required to cause disease? Second, what is the role played by unrelated or related viruses in causing IDDM? As expected, our results indicate that infections by vaccinia virus (VV) or Pichinde virus, representing viruses that do not generate cytotoxic T lymphocytes (CTL) cross-reactive with LCMV Armstrong (ARM) strain NP, the viral protein expressed on β cells, do not cause IDDM. Among the four strains of LCMV, a hierarchy of IDDM relatedness occurred: i.e., the LCMV strains E-350, Pasteur, and Traub elicited both CTL and antibody responses that cross-reacted with LCMV ARM and the LCMV ARM NP, but only ARM or E-350 infection elicited IDDM. The critical difference uncovered was that ARM and E-350 generated a higher CTL NP precursor (pCTL) frequency, of at least 1 or more $CD8^+$ CTL per 1,000 splenic lymphocytes, which were specific for the $H\text{-}2^d$ (L^d)-restricted LCMV NP epitope. In contrast, Traub and Pasteur generated at least 8- to 20-fold fewer pCTL, respectively, that recognized the same LCMV ARM NP epitope. Furthermore, the molecular basis of why the Pasteur and Traub strains failed to generate sufficient levels of anti-LCMV NP (self) CTL is uncovered. The major implications of our finding for the identification of etiologic agent(s) that may cause an autoimmune disease like IDDM, the molecular basis by which cross-reactive viruses may or may not cause autoimmune disease, the quantification of numbers of antigen-specific cells required to cause IDDM, and the implications for successful immunotherapy are discussed.

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MATERIALS AND METHODS

Transgenic mice. The generation and use of a transgenic mouse line (RIP NP 25-3) that expresses the full-length LCMV ARM NP in β cells of the islets of Langerhans have been described previously (22). Briefly, C57BL/6 ($H\text{-}2^b$) × BALB/c ($H\text{-}2^d$) mice were the source of oocytes, and eggs injected with the transgene under control of the rat insulin promoter (RIP) were implanted in pseudopregnant *bxd* females. The founder mouse contained integrated copies of the transgene, had the ability to express and pass the transgene to its offspring, and was crossed with BALB/c mice for at least 12 generations. Mice were obtained from and bred in the mouse hepatitis virus pathogen-free facility of The Scripps Research Institute (TSRI), La Jolla, Calif.

Virus stocks, reassortants, quantitation, and use. The LCMV ARM clone 53b, E-350, Pasteur, Traub, and ARM immunosuppressive variant clone 13 strains were obtained, triple plaque purified, and made into stocks as described previously (6, 21, 22, 29). The genome of LCMV is composed of short (S) and long (L) RNA strands. The complete genomic sequences of LCMV ARM and LCMV ARM clone 13 are published in reference 29. The RNA corresponding to the full-length NP of strains E-350, Traub, and Pasteur was amplified by reverse transcription (RT)-PCR with oligonucleotide primers 5′-GAGTGTCACAACA TTTGGGCCTCTAA-3′ (complementary to LCMV genomic positions 1642 to 1667) and 5′-CGCACAGTGGATCCTAGGC-3′ (corresponding to nucleotides 3357 to 3376 of the genomic RNA). The RT-PCR products were sequenced by the fmol method (Promega).

Pichinde virus, a member along with LCMV of the *Arenaviridae* family, was obtained from M. Buchmeier (TSRI) and triple plaque purified. All LCMV strains and Pichinde virus were quantified by plaquing on Vero cells (6, 22). VV recombinants expressing the full-length LCMV ARM clone 53b NP (VV/NP) or glycoprotein (GP) (VV/GP) and VV were generated, quantified by plaquing, and used as described previously (22, 38).

To initiate acute infection, 6- to 8-week-old mice were inoculated intraperitoneally (i.p.) with 10^5 PFU of plaque-purified LCMV. These mice generated antiviral CTL that cleared this virus from their blood and tissues within 14 days (15). Other mice were inoculated with 2×10^6 PFU of plaque-purified ARM or clone 13 virus intravenously (i.v.). Mice inoculated with ARM cleared virus by day 14; in contrast, mice inoculated with clone 13 failed to clear virus over a 4-month observation period (1, 3).

DNA, RNA, and protein analyses. Mice carrying the transgene were identified by hybridization of DNA extracted from tail biopsies by using LCMV-specific NP probes (22, 32). For RNA analysis, RNA was extracted from cells and organs with guanidinium-isothiocyanate, treated with RNase-free RQ1 DNase to eliminate contaminating DNA, and run for 40 cycles by PCR. Products were identified on 2% agarose, and the use of LCMV NP primers resulted in a 289-bp fragment. Expression of LCMV NP was determined by Western blot analysis, radioimmunoprecipitation, or immunofluorescence with an NP-specific monoclonal antibody, 113 (22, 32).

Analysis of blood glucose and pancreatic insulin levels. Blood samples were obtained from the retro-orbital eye plexus of each mouse at biweekly or monthly intervals. Glucose was measured by the glucose oxidase method (22), and mice with glucose values greater than 300 mg/dl were considered hyperglycemic. Mice with values exceeding 400 mg/dl were sacrificed. Insulin concentrations in the pancreas were determined by radioimmunoassay (22).

Construction of the recombinant plasmids. Oligonucleotides carrying the genes encoding ARM and Traub NP amino acids (aa) 116 to 140 were engineered behind a cytomegalovirus (CMV) immediate-early promoter (pCMV) and attached to ubiquitin (Ub) as described previously (28, 41). Briefly, the constructs used were based on the pCMV plasmid (Clontech, Palo Alto, Calif.). The fragments were either (i) cloned into the basic vector by using the *Not*I site, generating pCMV-Traub and pCMV-ARM, from which the minigene (MG) is expressed as a 26-aa fragment called pCMV-MG-Traub and pCMV-MG-ARM, respectively; or (ii) cloned in frame with the Ub gene to improve proteosome degradation of the product (28), generating the pCMV-Ub-MG-Traub or pCMV-Ub-MG-ARM. The fragments were amplified by PCR with two sets of primers: (i) the 5′ primer GGATCCATGTCTGAAAGGCCTCAAGCTTC and the 3′ primer GGATCCTTAAATTTGAGATCTTTGATC, both containing the restriction site *Bam*HI to facilitate the cloning downstream of Ub; (ii) the 5′ primer GCGGCCGCCATGTCTGAAAGGCCTCAAGCTTC and the 3′ primer GCGGCCGCTTAAATTTGAGATCTTTGATC, containing the *Not*I site for cloning into pCMV.

Alternatively, the full-length ARM NP was cloned into pCMV, as has been described before (41), and the full-length Traub NP was amplified by PCR with the 5′ primer GCGGCCGCCATGTCTCTGTCCAAAGAAGTC and the 3′ primer GCGGCCGCTTAGAGTGTCAAATGATTTGGGCG, both including the site *Not*I to facilitate the cloning into pCMV, resulting in pCMV-ARM NP and pCMV-Traub NP, respectively.

CTL, pCTL, and antibody assays. CTL activity was determined in a 5- to 6-h in vitro ^{51}Cr release assay (15, 22, 32). All samples were run in triplicate. Primary CTL were raised in vivo by inoculating 6- to 8-week-old mice i.p. with 10^5 PFU of virus and harvesting their spleens 6 to 9 days thereafter. The methods for obtaining lymphocytes from spleens, as well as for generating and characterizing CTL clones specific for LCMV GP or NP restricted by $H\text{-}2^d$ or $H\text{-}2^b$ haplotypes, were recorded previously (15, 22, 32, 38). The LCMV ARM NP sequence aa 118 to 127 is the sole immunodominant epitope for CTL from $H\text{-}2^d$ mice, is L^d restricted, and is recognized by primary CTL as well as CTL clone HD8 (14, 39). As a control, CTL clone NP-18, which is $H\text{-}2^b$ (D^b) restricted and recognizes LCMV ARM NP aa 397 to 406, was used (14). To judge CTL recognition, target cells were infected with LCMV ARM (multiplicity of infection [MOI] of 1) or recombinant VV expressing the LCMV ARM NP or LCMV ARM GP (MOI of 3); uninfected target cells were coated with LCMV ARM peptides NP aa 118 to 127 or NP aa 397 to 406, LCMV ARM NP aa 116 to 131, LCMV Traub NP aa 116 to 131, or LCMV Pasteur NP aa 118 to 127 (20 to 0.2 μg of peptide per 10^4 target cells). For some experiments, BALB cells (obtained from the American Type Culture Collection) were transfected with the various plasmids described (2 μg of DNA per 2×10^6 cells), by using Lipofectamine and Opti-MEM (Gibco-BRL) as the manufacturer recommended. Forty-eight hours later, transfected BALB cells were used as target cells to evaluate their recognition by primary CTL.

Assays using splenic lymphocytes employed effector/target (E/T) ratios of 50:1, 25:1, and 12.5:1, whereas those using CTL clones had ratios of 5:1 and 2.5:1. The $H\text{-}2^d$ target cells were BALB clone 7, and for $H\text{-}2^b$, we used MC57 cells. $CD8^+$ T cells were depleted from mice by using rat monoclonal antibody YTS 169.4 as described previously (32). Analysis by fluorescence-activated cell sorter (FACS) indicated >98% depletion of $CD8^+$ T cells from treated mice. The determination of LCMV-specific pCTL has been described elsewhere (5). Briefly, splenic lymphocytes from infected mice 6 to 9 days postinfection with LCMV were serially diluted and cultured in 96-well flat-bottom plates with LCMV-infected and irradiated (20 Gy) macrophages and irradiated spleen cells. After 8 days, cells from each well were split and tested in a 5-h ^{51}Cr release assay.

The intracellular cytokines gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) were detected in LCMV-specific $CD8^+$ T cells (17). Briefly, single-cell suspensions were prepared from spleens harvested 7 days after acute infection with LCMV. Cells were then stimulated for 5 h in the presence of 0.1 μg of peptide per ml from LCMV ARM or LCMV Traub and recombinant human interleukin 2 (IL-2 [50 U/ml]) or transfected fibroblasts (with pCMV-Ub-MG-ARM or pCMV-Ub-MG-Traub). Two micrograms of brefeldin A per ml was added to prevent cytokine secretion. After surface staining with an anti-CD8–allophycocyanin conjugate, cells were fixed and permeabilized with 1% fetal calf serum–4% paraformaldehyde–0.1% saponin buffer and stained with an anti-IFN-γ–phycoerythrin conjugate and anti-TNF-α–fluorescein isothiocyanate (FITC) conjugate. Cells were placed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, Calif.) and analyzed with Cell Quest software (Becton Dickinson).

LCMV-specific antibody in serum samples was detected by both solid-phase enzyme-linked immunosorbent assay and radioimmunoprecipitation assay (22).

Histology and immunocytochemistry. Tissues taken for histological analysis were fixed in zinc-formalin (10%) and stained with hematoxylin and eosin. For immunocytochemical studies, 4- to 5-μm sections of liquid-nitrogen-frozen material were cut in a cryomicrotome and then fixed and stained with monoclonal antibodies to LCMV NP, CD8, or CD4 molecules as described previously (22, 32).

Western blotting. Immunoblotting was used to detect full-length NP in BALB cells transfected with pCMV-ARM NP or pCMV-Traub NP. Briefly, 10^6 cells were transfected as described before, and 48 h posttransfection, the cells were collected with lysis buffer (100 mM Tris-HCl [pH 7.6], 140 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate). Different concentrations of protein were separated on 10% polyacrylamide gels with sodium dodecyl sulfate, and proteins were detected with a 1:300 dilution of ascites fluid containing NP-specific monoclonal antibody 113 (4), by the ECL enhanced chemiluminescence procedure according to the manufacturer's instructions (Amersham, Buckinghamshire, England). The amount of NP expressed was quantitated by densitometry.

RESULTS

Comparison of NP sequences from LCMV ARM, E-350, Traub, Pasteur, and clone 13: $H\text{-}2^d$ mice generate CTL cross-reactive with LCMV ARM NP after challenge with E-350, Traub, and Pasteur LCMV strains, but not with VV and Pichinde virus. The complete NP sequences of all LCMV strains used were identified, as was the component within the ARM NP sequence recognized by CTL (Table 1 and Fig. 1). As also shown in Table 1, MHC-restricted CTL from all four LCMV strains recognized NP aa 1 to 201 and the single immunodominant CTL epitope for $H\text{-}2^d$ mice, NP peptide aa 118 to 127. Inoculation of VV ARM NP into mice did not produce a detectable primary CTL response on days 6 to 9 (data shown for day 7), although memory CTL specific for LCMV ARM NP were found in the spleen 45 days later after 5 days of culture. In contrast, neither primary nor secondary CTL responses to

TABLE 1. BALB ($H\text{-}2^d$) mice inoculated with LCMV strains ARM, E-350, Traub, and Pasteur recognize a CTL epitope at NP aa 1 to 210 and 118 to 127[a]

Infection group	E/T ratio	% ^{51}Cr release[b]								
		$H\text{-}2^d$ BALB clone 7 target cells								$H\text{-}2^b$ (MC57 ARM)
		LCMV ARM					VV	Pichinde virus	LCMV ARM (VV full-length GP)	
		LCMV ARM	VV full-length NP	VV/NP aa 1–321	VV/NP aa 1–201	Peptide aa 118–127				
ARM	50:1	34	51	50	54	64	2	4	1	2
	25:1	24	41	41	46	ND[c]	ND	ND	0	2
E-350	50:1	47	58	49	55	61	ND	ND	4	2
	25:1	37	48	43	50	ND	ND	ND	0	0
Traub	50:1	29	29	38	31	37	ND	ND	6	4
	25:1	24	30	33	30	ND	ND	ND	0	5
Pasteur	50:1	17	24	36	33	29	ND	ND	0	0
	25:1	13	10	21	21	ND	ND	ND	0	0
Recombinant VV ARM NP										
Day 7 primary immune response	50:1	9	27	ND	ND	7	38	ND	2	3
Day 45 secondary immune response	5:1	36	29	ND	ND	29	31	ND	1	6
VV day 7 primary immune response	50:1	4	ND	ND	ND	3	27	ND	ND	5
	25:1	7	ND	ND	ND	2	31	ND	ND	6
Pichinde virus day 7 primary immune response	50:1	5	ND	ND	ND	2	ND	36	ND	1
	25:1	3	ND	ND	ND	3	ND	19	ND	3
NP clones										
$H\text{-}2^d$ HD8	5:1	48	51	42	54	51	5	3	0	3
$H\text{-}2^b$ NP-18	5:1	4	2	1	3	2	4	3	2	56

[a] Six- to eight-week-old BALB mice of both genders were inoculated with 10^5 PFU of a specific virus. At 7 to 8 days postinoculation, CTL were analyzed with a suspension of single splenic lymphocytes at E/T ratios of 50:1, 25:1, and 12.5:1 (the data for 50:1 and 25:1 are shown). CTL clones $H\text{-}2^d$ HD8, which recognizes LCMV ARM NP aa 118 to 127, and $H\text{-}2^b$ NP-18, which recognizes LCMV ARM NP aa 396 to 405, were used at E/T ratios of 5:1. Targets were infected with LCMV ARM clone 53b (MOI of 1 for 2 days); VV recombinants expressing LCMV ARM NP aa 1 to 558, 1 to 321, or 1 to 201 at an MOI of 3 overnight; or uninfected cells coated with LCMV ARM NP peptide aa 118 to 127. Target cells ($H\text{-}2^d$, BALB clone 7; $H\text{-}2^b$, MC57 fibroblasts) were labeled with ^{51}Cr.

[b] The values shown are the mean percentages of specific ^{51}Cr released from targets assayed in triplicate. Variation was <10%. The data were similar in two additional experiments.

[c] ND, not done.

LCMV ARM or LCMV ARM NP developed after inoculation of VV or Pichinde virus. These observations were confirmed in two additional assays. Analysis of the NP sequence for ARM, E-350, Traub, and clone 13 showed complete homology at NP aa 118 to 127. However, Pasteur had 3 aa substitutions: aa 119 Pro→Leu, 120 Gln→Lys, and 121 Ala→Thr (Fig. 1). Although the flanking sequences of the CTL epitope were similar for ARM, E-350, and clone 13, the COO^- flanking sequence of Traub displayed a single-amino-acid change at NP amino acid residue 131 Thr→Ala (Fig. 1). Overall, in comparison to the amino acid sequence of ARM, NP homology was >99% for E-350 NP (557 similar residues out of a total of 558) and 95% for both Traub NP (532 residues out of 558) and Pasteur NP (537 residues out of 558). For clone 13, the 558 NP residues were identical to those of ARM (homology of 100%) (data not shown, and see reference 6).

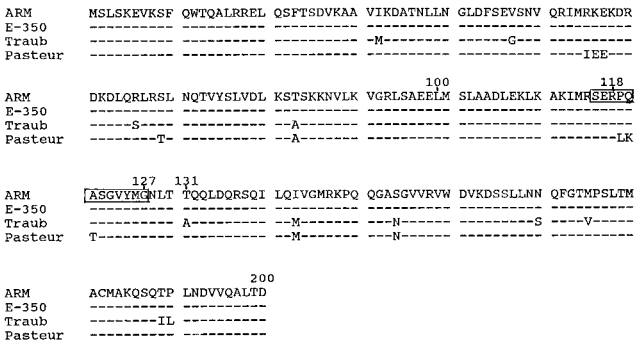

FIG. 1. Sequences of NP aa 1 to 201 of various LCMV strains. The sequences are presented in the single-letter code. See Materials and Methods for details.

According to an enzyme-linked immunosorbent assay with whole inactivated virus, none of the $H\text{-}2^d$ mice had antibody to LCMV preceding inoculation of the various viruses. However, after viral inoculation, recipients of LCMV strains ARM, E-350, Traub, and Pasteur and Pichinde virus all displayed equivalent titers of antibodies that reacted with LCMV ARM at day 30 and thereafter. Titers ranged from a low of 1/640 to a high of 1/10,240. Testing by radioimmunoprecipitation documented that mice making antibodies against whole inactivated LCMV also made antibodies against LCMV ARM NP (data not shown).

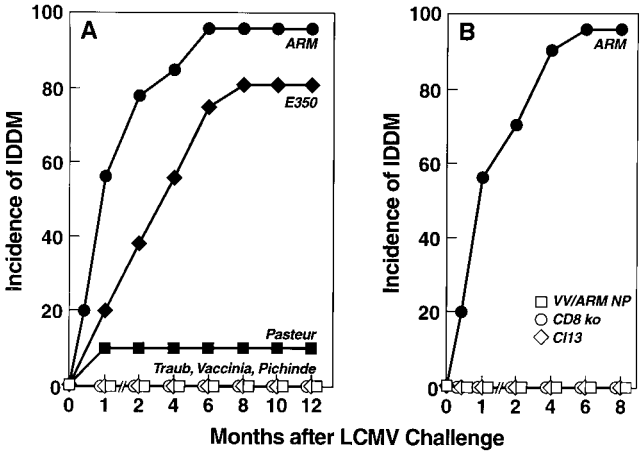

FIG. 2. (A) Incidence of IDDM in transgenic mice expressing LCMV ARM NP in their β cells after inoculation (10^5 PFU i.p.) with LCMV strains ARM 53b, E-350, Traub, or Pasteur; VV; or Pichinde virus. A minimum of 20 mice of both genders were inoculated with LCMV strains, and 7 to 10 mice were infected with VV or Pichinde virus, all when the mice were 8 weeks of age. IDDM is defined as a blood glucose level of >300 mg/dl, a pancreatic insulin level of <15 μg of insulin/mg of pancreatic tissue, and mononuclear cell infiltration into islets of Langerhans with destruction of β cells. (B) Incidence of IDDM in transgenic mice expressing LCMV ARM NP in their β cells after inoculation with LCMV ARM 53b or LCMV ARM clone 13 (C113) (2×10^6 PFU i.v.), VV recombinant expressing LCMV ARM NP (10^5 PFU i.p.), or LCMV ARM (10^5 PFU i.p.) into CD8-deficient mice (CD8 knockout [ko]). Mice in groups of 7 to 10 were injected when 8 weeks old. A similar lack of IDDM was observed in mice whose $CD8^+$ T-cell population was removed by antibodies (see Materials and Methods) given 10^5 PFU of LCMV ARM i.p.

Some LCMV strains that generate cross-reactive CTL to ARM NP do not induce IDDM in RIP LCMV ARM NP transgenic mice. To evaluate several viruses for the biological consequences of infection in transgenic mice whose β cells express LCMV NP, groups of at least 20 mice were inoculated with LCMV ARM, LCMV E-350, LCMV Traub, or LCMV Pasteur, and groups of 7 to 10 mice were inoculated with LCMV clone 13, VV ARM NP, VV, or Pichinde virus. During the 12-month period that followed, only transgenic mice inoculated with LCMV ARM (incidence, >95%) or E-350 (incidence, >80%) developed IDDM (Fig. 2A), which was defined as blood glucose levels above 300 mg/dl, less than 15 μg of insulin/mg of pancreatic tissue, and histologic evidence of mononuclear cell infiltration into the islets with β-cell destruction (Table 2). In contrast, none of the 24 mice inoculated with Traub developed IDDM, whereas 2 of 26 mice inoculated with Pasteur (8%) did so (blood glucose levels of 315 and 360 mg/dl), yet both of these viruses generated CTL that cross-reacted with LCMV ARM NP (Table 1). As expected, viruses that failed to generate CTL cross-reactive with LCMV NP, VV, and Pichinde virus also failed to elicit IDDM in the transgenic mice (Fig. 2).

LCMV clone 13 is a variant of LCMV ARM that differs from the parental virus by a mutation in two open reading frames: GP aa 260 $Phe^{ARM} \rightarrow Leu^{Clone\ 13}$ and L protein (polymerase) aa 1079 $Lys^{ARM} \rightarrow Gln^{Clone\ 13}$ (25). The NP sequences are identical for both viruses (29). When 2×10^6 PFU of LCMV ARM was injected i.v. into several strains of mice, including *H-2*^{d} BALB, the same amount of virus-specific CTL formed as that produced by 10^5 PFU given i.p. In contrast, 2×10^6 PFU of clone 13 given i.v. caused a profound generalized immunosuppression (1, 3) documented by the failure to generate LCMV-specific CTL at day 7 due to selective immunopathologic destruction of interdigitating dendritic cells (3). As shown in Fig. 2B, when we administered LCMV ARM i.v. at 2×10^6 PFU, IDDM followed. In contrast, the same dosage and route of clone 13 administration failed to induce IDDM over a 12-month observation period. To confirm the essential role of $CD8^+$ CTL in causing IDDM (22, 32), LCMV ARM inoculated into transgenic mice depleted of CD8 cells by antibodies or in mice whose CD8 gene had been disrupted did not cause IDDM (Fig. 2B). Inoculation of VV ARM NP (10^5 PFU i.p.) also failed to induce IDDM (Fig. 2B).

Figure 3 documents the histopathologic analysis of islets of Langerhans from the mice depicted in Fig. 2. An abundance of mononuclear cells infiltrated pancreatic tissue from ARM- and E-350-inoculated transgenic mice. In contrast, infiltration of mononuclear cells was negligible in pancreatic tissues from transgenic mice inoculated i.p. with Pasteur, Traub, or VV/NP or in CD8-depleted mice inoculated with LCMV ARM or given i.v. inoculation of clone 13. These observations were

TABLE 2. Summary of results for LCMV ARM NP *H-2*^{d} RIP 25-3 transgenic mice[a]

Mouse group	Blood glucose level (mg/dl)[b]		Pancreatic insulin level (μg/mg)[b]	Mononuclear infiltration into islets at 10 mo
	1 mo p.i.[c]	10 mo p.i.	10 mo p.i.	
Not infected	169 ± 6	180 ± 5	52 ± 5	0
Infected (10^5 PFU i.p.)				
LCMV ARM	402 ± 26	462 ± 12	4 ± 3	++++
LCMV E-350	281 ± 9	385 ± 25	7 ± 6	++++
LCMV Traub	164 ± 22	182 ± 8	39 ± 7	0 to +
LCMV Pasteur	151 ± 7	205 ± 18	38 ± 3	0 to +
VV ARM NP	153 ± 9	171 ± 11	43 ± 1	0 to +
Infected CD8-depleted				
10^5 PFU of LCMV ARM i.p.	174 ± 11	189 ± 39	48 ± 6	0
2×10^6 PFU i.v.				
LCMV ARM	347 ± 42	455 ± 28	5 ± 2	++++
LCMV clone 13	142 ± 13	145 ± 16	44 ± 12	0 to +

[a] Data were from age- and sex-matched mice. At least 20 mice per group were infected with ARM, E-350, Pasteur, and Traub, and 7 to 10 mice were infected with VV/NP, were CD8 depleted, or were given 2×10^6 PFU of virus i.v.
[b] Each value represents the mean ± standard error.
[c] p.i., postinoculation.

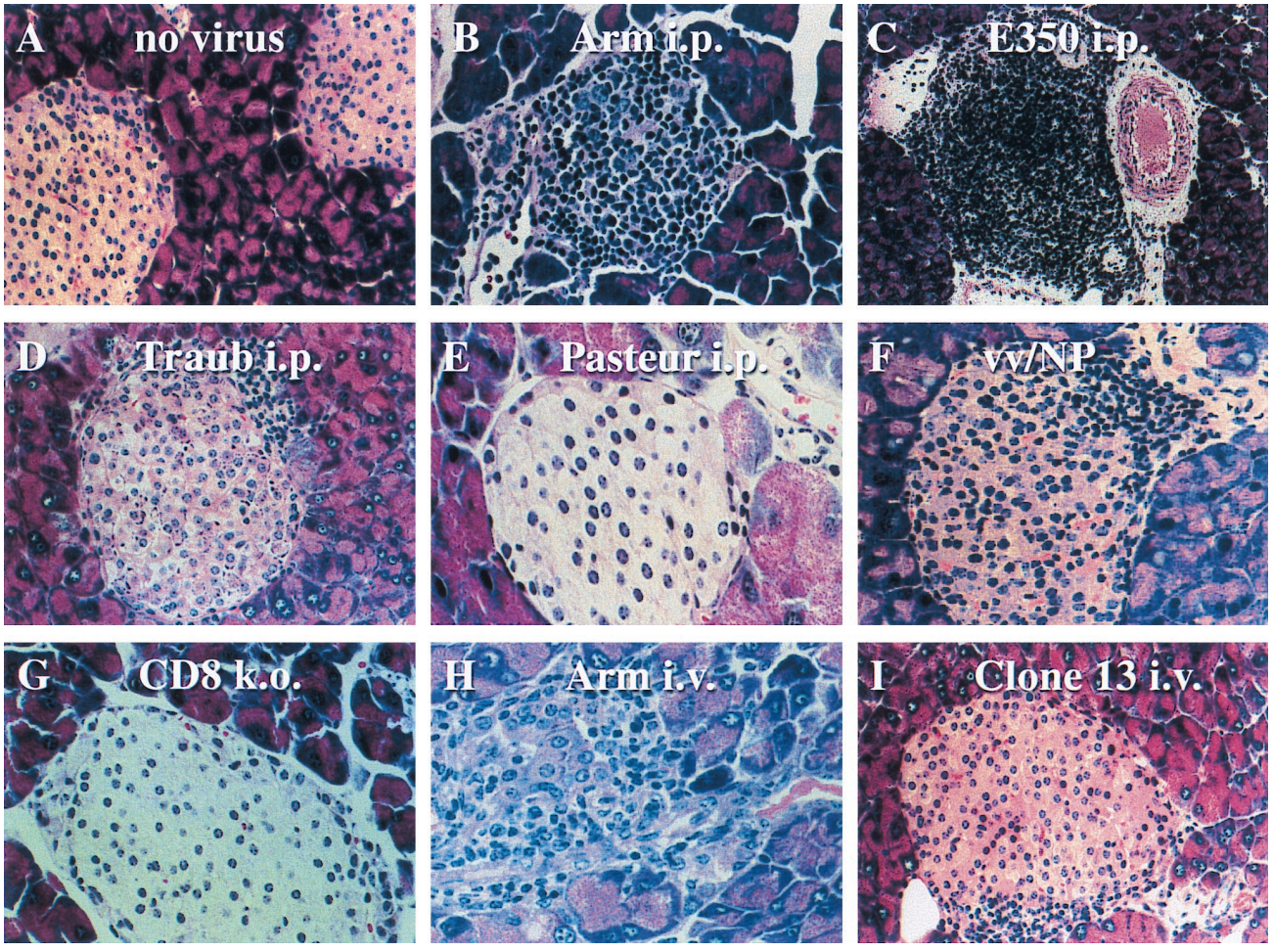

FIG. 3. Histopathologic analysis of islets of Langerhans from pancreatic tissue taken from representative transgenic mice at 6 months of age (Fig. 2 and Table 2). (A) Uninfected mouse. (B to G) Mice inoculated i.p. with 10^5 PFU of ARM (B), E-350 (C), Traub (D), Pasteur (E), VV recombinant expressing LCMV ARM NP (VV/NP) (F), and ARM in a mouse deficient in $CD8^+$ lymphocytes (G [compare panels B and G]). (H) Tissue from a mouse inoculated with 2×10^6 PFU of LCMV ARM i.v. (I) Tissue from a mouse inoculated with 2×10^6 PFU of ARM variant clone 13 i.v. Similar data were obtained from 7 to 20 individual mice from each experimental group analyzed. In addition, islets studied from 10-month-old uninfected mice; mice infected i.p. with 10^5 PFU of Traub, Pasteur, or VV/NP; CD8-deficient mice given ARM, or mice of a similar age given 2×10^6 PFU of clone 13 i.v. when 8 weeks old failed to show lymphocytic infiltrates.

consistent in six mice from each experimental group examined. When mononuclear cell infiltrates occurred, they were composed primarily of $CD8^+$ and $CD4^+$ T cells with some B lymphocytes as described earlier (32). Table 2 summarizes the results of infection of transgenic mice with these viruses.

Precursor frequency and affinity of CTL generated in transgenic mice infected with multiple LCMV strains. To determine why ARM and E-350 viral infections caused IDDM, but neither Traub, Pasteur, VV ARM, nor clone 13 (given i.v.) did, we analyzed frequencies of NP-specific $CD8^+$ T cells in transgenic and nontransgenic mice. As shown in Table 3, strains of virus generating approximately 1 pCTL per 1,000 splenocytes (ARM and E-350) yielded IDDM. In contrast, pCTL frequencies of less than 1/8,000 failed to induce disease (Traub, Pasteur, VV/NP, and clone 13). The affinities of LCMV ARM and Pasteur CTL for the immunodominant NP peptide were evaluated by serial dilutions of the appropriate NP peptide. While *H-2*^{d} mice infected with LCMV Pasteur generated 6 (nontransgenic mice)- or 24 (transgenic mice)-fold fewer pCTL than ARM-infected mice in the limiting dilution analysis, all CTL directed to the LCMV ARM NP epitope had binding affinities equivalent to those for LCMV ARM (10^{-8} to 10^{-9} M) (Fig. 4).

Further analysis of NP-specific $CD8^+$ T-cell frequencies after restimulation with NP aa 118 to 127 and staining for intracellular IFN-γ showed that ARM-, E-350-, and Traub-infected nontransgenic *H-2*^{d} mice generated approximately one in three $CD8^+$ T cells specific for NP aa 118 to 127, while Pasteur-infected mice had 1 in 10 specific $CD8^+$ T cells. These results correlate well with the results of CTL assays using bulk splenocyte effectors ex vivo (Table 1). In transgenic mice, frequencies of NP-specific $CD8^+$ T cells were three- to sixfold reduced.

To determine the affinity of LCMV Traub CTL for ARM NP, we used peptide NP aa 116 to 131 to include the COO^- flanking region of the immunodominant epitope. As shown in Fig. 5 (left panel), over a wide dose range, externally added LCMV ARM and Traub NP aa 116 to 131 showed equivalent effector CTL affinities. Also, numbers of LCMV $CD8^+$ T cells expressing cytokines were similar when reacted with target cells coated externally with peptide NP 116 to 131 from either Traub or ARM LCMV (Fig. 6, lower panels).

Role of antigen processing in the failure of LCMV Traub to elicit sufficient CTL cross-reactive with LCMV ARM NP. To determine why LCMV Traub infection stimulated substantially fewer pCTL for ARM NP than E-350 or ARM infection, we

TABLE 3. Frequencies of pCTL to LCMV ARM NP generated in LCMV ARM NP transgenic *H-2*^{d} RIP 25-3 mice inoculated with diabetes- and nondiabetes-associated strains of LCMV[a]

Infection group	% Specific ^{51}Cr release for[b]:			Reciprocal precursor frequency of *H-2*^{d} ARM NP[c]	IDDM status[d]
	H-2^d target		*H-2*^{b} ARM		
	ARM	ARM NP			
10^5 PFU					
ARM	30 ± 12	29 ± 8	2 ± 1	560, 810, 850, 920	++++
E-350	30 ± 10	25 ± 4	3 ± 1	625, 1,078, 910, 1,401	++++
Traub	12 ± 3	8 ± 2	1 ± 1	8,500, 9,600, 5,900, 10,200	0
Pasteur	16 ± 2	4 ± 3	2 ± 1	15,800, 21,000, 16,600, 24,000	0
VV/NP	4 ± 2	1 ± 1	4 ± 2	<50,000	0
2×10^6 PFU i.v.					
ARM	28 ± 5	25 ± 2	2 ± 1	810, 690, 910	++++
Clone 13	3 ± 1	2 ± 1	2 ± 1	<50,000	0

[a] The CTL assay was performed 7 days after infection. Single splenic lymphocyte E/T ratios of 50:1 (shown here) and 25:1 were used. The targets were *H-2*^{d} BALB clone 7 or *H-2*^{b} MC57 fibroblasts. See Materials and Methods for details of the CTL and pCTL frequency assays.

[b] Data reflect the mean ± 1 standard error for four mice.

[c] Data from four individual mice are shown separately.

[d] IDDM is defined here as a blood glucose level above 300 mg/dl, a low pancreatic insulin level (<15 μg of insulin/mg of pancreas), and mononuclear infiltration into the islets of Langerhans with β-cell destruction.

compared the ability of cytosolic, internally processed Traub NP and ARM NP aa 116 to 140 to present antigen for CTL recognition. Because ubiquitination of proteins facilitates their entry into MHC class I pathways (28), we transfected BALB cells with pCMV-Ub-MG-ARM or pCMV-Ub-MG-Traub and evaluated the CTL recognition by two criteria. First, we judged the ability of day 7 primary LCMV-specific CTL to lyse syngeneic MHC-matched targets and, second, quantitated the intracellular expression of IFN-γ and TNF-α for these T lymphocytes after stimulation with target cells transfected with pCMV-Ub-MG-ARM or pCMV-Ub-MG-Traub. Processing of Traub NP aa 116 to 140 was markedly inferior to that of ARM NP, as assayed by CTL ^{51}Cr release assay (Fig. 5, right panel) or by intracellular expression of IFN-γ- and TNF-α-activated CD8$^+$ LCMV-specific CTL (Fig. 6, upper panels). In contrast, there was no difference in cytokine-producing CD8$^+$ T cells (Fig. 6, lower panel) or in ^{51}Cr release (Fig. 5, left panel) when such peptides were added externally.

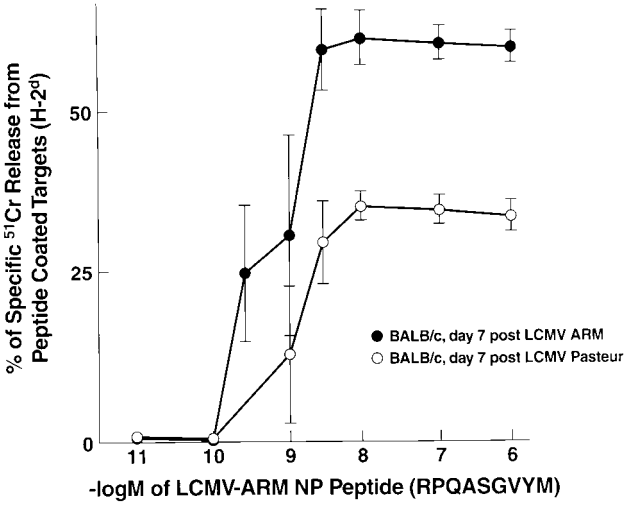

FIG. 4. Relative affinities of CTL harvested from LCMV ARM (IDDM inducer) or LCMV Pasteur (non-IDDM inducer). LCMV NP peptide aa 118 to 127 was used to coat BALB clone (BALB/c) 7 targets over a range of 10^{-6} to 10^{-10} M. The data are recorded as means ± 1 standard error of triplicate determinations (see Materials and Methods and reference 19 for experimental details).

To ensure that ARM and Traub were expressed equivalently in transfected cells, DNA plasmids expressing full-length ARM and Traub NP (pCMV-ARM NP and pCMV-Traub NP, respectively) were employed. Full-length NP constructs were used because the antibody (monoclonal antibody 113 or polyclonal guinea pig sera) (4) detection system does not recognize NP aa 116 to 140. The ability of full-length Traub NP to be processed was markedly reduced compared to that of ARM NP. Insufficient MHC NP complex was present on transfected BALB cells to cause CTL recognition and lysis (Fig. 7, top). In contrast, similarly transfected full-length ARM NP was processed and recognized by LCMV NP-specific CTL. Next we assayed whether the levels of expression of pCMV-ARM NP and pCMV-Traub NP in such transfected BALB cells were equivalent. As shown in Fig. 7 (bottom) by Western immunoblotting, there was no difference between the amounts of protein expressed inside the cell by the two plasmids.

DISCUSSION

The results reported here establish that the generation of CTL capable of cross-reacting with viral ("self") antigens in pancreatic β cells is necessary but not sufficient to initiate IDDM. Disease followed only when the quantity of cross-reactive CTL exceeded a critical threshold, which, in this model, was 1/1,000 pCTL (or 1/50 to 1/100 LCMV-specific CD8$^+$ T cells). IDDM did not occur with 8- to 10-fold-fewer pCTL or LCMV-specific CD8$^+$ T cells. To understand the rules by which viruses can cause IDDM, we devised an in vivo murine model in which a viral gene was expressed in pancreatic β cells and passed on to progeny mice (i.e., the viral transgene became a self antigen). Previous experiments determined that expression of the transgene, per se, failed to initiate β-cell injury and resultant IDDM, unless either a specific cytokine like IFN-γ (13, 37) or an activation molecule like B7.1 (36) was coexpressed in the islet milieu in β cells or a systemic infection was initiated by the same virus from which the β-cell-expressing transgene originated (22, 32). Our interest here was to

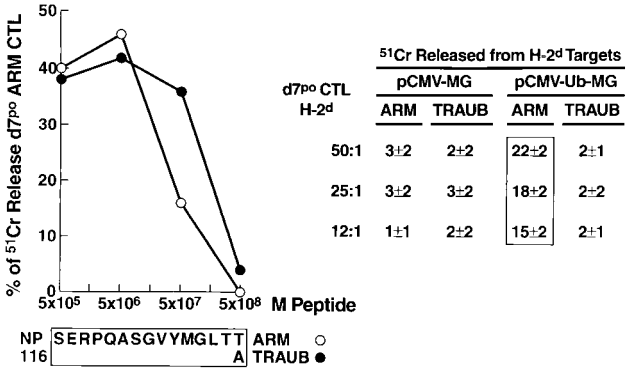

FIG. 5. Defect in intracellular processing of Traub NP aa 116 to 131, but not this peptide's ability to coat target cells for lysis by anti-LCMV ARM CTL. The panel on the left shows that equivalent molar amounts of ARM or Traub peptides NP aa 116 to 131 coated *H-2*^{d} target cells for corresponding CTL lysis. Similar data were observed in two other experiments. The panel on the right shows that the transfected ubiquitinated ARM NP oligomer that encodes NP aa 116 to 140 (pCMV-U-MG-ARM) is processed inside BALB cells and traffics to and is expressed on the surface of BALB cells for recognition by day 7 primary (d7^{po}) MHC-restricted LCMV-specific CTL. In contrast, a similar transfection with ubiquitinated Traub NP (NP aa 116 to 140) (pCMV-U-MG-Traub) fails to present LCMV Traub NP to the cell's surface for CTL recognition. Percentages of ^{51}Cr released at E/T ratios of 50:1, 25:1, and 12:1 are given.

determine if other viruses, closely or distantly related or unrelated could also cause IDDM.

First we analyzed four LCMV strains selected for their structural homology, CTL-generating capacity, and IDDM production. We found that only LCMV strain E-350, which shared 557 out of 558 aa with LCMV ARM NP (>99% homology) (Fig. 1) caused IDDM. Within the LCMV ARM NP used as the self antigen was the 10-aa NP aa 118 to 127 peptide that constituted the single immunodominant epitope recognized by *H-2*^{d} BALB mice. LCMV E-350 shared all 10 of these NP amino acids with LCMV ARM. Transgenic mice expressing the NP of LCMV ARM, when challenged with E-350, generated CTL that recognized NP aa 118 to 127. Furthermore, these E-350-specific CTL were generally equivalent in number to the CTL generated by LCMV ARM. After either infection, IDDM was evident from the characteristic hyperglycemia, hypoinsulinemia, and mononuclear cells infiltrating into the islets of Langerhans and participating in β-cell destruction (Fig. 3 and Table 2). Although sequence comparison between the Traub and ARM LCMV strains showed complete homology at the CTL epitope NP aa 118 to 127 (Fig. 1), and this LCMV ARM NP epitope was recognized by CTL generated in response to Traub infection (Table 1), Traub generated eightfold-fewer pCTL than ARM. Traub infection produced no IDDM, because this smaller number was not sufficient to cause the disease (Fig. 2 and 3). In agreement with our findings, a sevenfold reduction in pCTL frequency following DNA immunization for LCMV leads to vaccine failure (28). Additionally, Traub differed from ARM at the flanking sequence NP aa 131 Thr→Ala. This suggested that the substitution in position NP aa 131 may alter antigen processing of the LCMV NP epitope. Experiments with intracellular cytoplasmic antigen processing showed that the single change at NP aa 131 diminished the presentation of antigen, although the amounts of transfected and expressed protein were equivalent for ARM and Traub. It is likely that the biologic consequence of this point mutation was the inability of Traub to cause IDDM. The importance of flanking sequences in antigen processing has been shown previously (7, 16, 26); however, the model described here provides evidence for an in vivo biologic consequence.

Comparison of NP aa 118 to 127 (Fig. 1) of Pasteur with that of ARM showed 3 aa substitutions: the NP immunodominant epitope, aa 119 Pro→Leu, 120 Gln→Lys, and 121 Ala→Thr. Undoubtedly, these differences accounted for the 20-fold discrepancy in pCTL frequency between LCMV ARM and LCMV Pasteur and the inability of LCMV Pasteur infection to cause IDDM in transgenic mice expressing LCMV ARM NP (Fig. 2 and 3 and Table 2).

From the studies recorded here, two rules for the initiation of IDDM emerged. First, CTL must cross-react with a gene expressed in β cells, and, second, a sufficient number of CTL must be present. VV, a DNA virus with no structural relationship to LCMV ARM, failed to generate CTL that recognized the LCMV ARM NP transgene and, consequently, did not cause IDDM when inoculated into the transgenic mice (Fig. 2). *Pichinde virus* is distantly related to LCMV ARM, since both viruses are members of the *Arenaviridae* family, but Pichinde virus did not generate CTL that cross-reacted with LCMV ARM NP, despite inciting antibodies that recognized ARM NP. Pichinde virus infection did not produce IDDM (Fig. 2). Clone 13, an LCMV variant derived from the ARM strain (1), shares complete homology with ARM NP (29). However, clone 13, when inoculated (2×10^6 PFU i.v.) into mice, suppressed humoral and cellular immune responses because of its selected tropism for and associated destruction of interdigitating dendritic cells in the white pulp (3). In comparison, ARM is tropic for F4/80-positive macrophages in the red pulp and does not cause injury of professional antigen-presenting cells (3). In our experiments here, 7 days after clone 13 infection, no CTL were detected, although low levels (less than 1 per 50,000

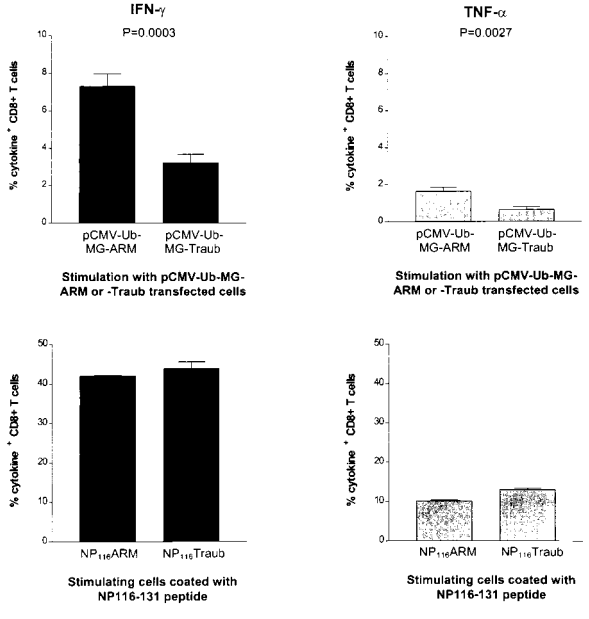

FIG. 6. Intracellular cytokine staining (IFN-γ and TNF-α) of LCMV-specific CD8$^+$ T cells indicates that transfection of *H-2*^{d} BALB cells with ubiquitinated Traub NP aa 116 to 140 leads to activation of significantly less CD8$^+$ T cells than does activation by ubiquitinated ARM NP aa 116 to 140 (*P* value for IFN-γ expression, 0.0003; *P* value for TNF-α expression, 0.0027). However, when either peptide is externally added to coat target cells (Traub and ARM NP aa 116 to 131), equivalent numbers of cytokine-containing CD8$^+$ T cells (i.e., 43 and 42% IFN-γ-expressing CD8$^+$ T cells, respectively) are generated. The data are representative of three independent experiments.

D7 p° spl CTL H-2d (BALB) E:T ratio	% ^{51}Cr released from BALB/clone 7 targets			
		Infected	Transfected with:	
	Uninfected	LCMV ARM	pCMV-ARM NP	pCMV-Traub NP
50:1	2	84	32	8
25:1	3	70	28	6
12.5:1	4	61	22	6
6:1	1	35	15	2

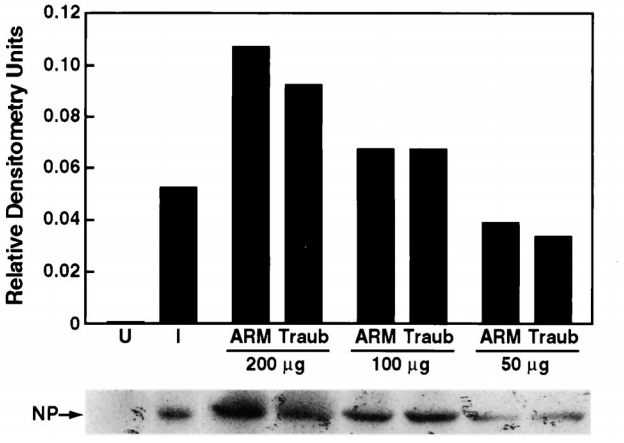

FIG. 7. Defect in the intracellular processing of full-length NP Traub despite equivalent levels of cytosolic expression of Traub and ARM NP. The top panel shows that whereas transfected full-length ARM NP (pCMV-ARM [see Fig. 5 legend]) is processed correctly as judged by recognition of LCMV-specific CTL over several E/T ratios, there is no recognition of similarly transfected full-length Traub NP (pCMV-Traub). The numbers shown represent the mean value of triplicate samples. Variance was <10% in the 5-h ^{51}Cr release assay. D7 p°, day 7 primary immune response; splenic lymphocytes. The lower panels show that cytosolic extracts from the full-length ARM- or Traub-transfected constructs (above) express similar levels of NP by Western blotting and by densitometry analysis. The negative control (U) was processed with cytosolic extract from uninfected BALB cells, and the positive control (I) is from LCMV ARM-infected BALB cells. ARM, pCMV-ARM NP; Traub, pCMV-Traub NP (constructs used to transfect BALB cells). Different concentrations of protein from each sample were loaded, and detection was with monoclonal antibody to LCMV ARM NP (see reference 14 and Materials and Methods).

pCTL by frequency analysis) appeared several weeks later, when progenitor cells from the bone marrow had repopulated the spleen and lymph nodes and differentiated into dendritic cells (M. B. A. Oldstone, A. Tishon, and P. Borrow, unpublished data). Additionally, inoculation of the transgenic or normal control mice with VV ARM NP failed to elicit a primary CTL response to LCMV ARM, although by days 45 to 60, we identified secondary (memory) CTL and pCTL frequencies of 1/15,500. Thus, although the Traub and Pasteur strains of LCMV generated primary CTL responses, the numbers of CTL generated (1/8,550 to 1/19,300 functional CTL) were insufficient to initiate significant β-cell destruction for development of IDDM. In contrast, the E-350 strain made, on average, 1/1,003 functional CTL able to cross-react with the LCMV ARM NP transgene in β cells and destroyed sufficient β cells to cause IDDM. Our findings indicated that the same generic virus, i.e., LCMV, causing infection in a genetically identical inbred mouse strain may (LCMV ARM or LCMV E-350) or may not (LCMV Traub or LCMV Pasteur) cause IDDM, depending on the strain or variant of virus involved. This suggests that one might note apparently similar viruses in two genetically identical people, i.e., monozygotic twins, but the viruses would cause IDDM in one and not the other because they are not the same.

Several other factors might have underlain the inhibition of IDDM seen here. First, the β-cell target could have had few or no MHC class I molecules and failed to present the autoantigen. Such a scenario has been reported in the RIP LCMV NP transgenic model when the IFN-γ gene was disrupted or when MHC molecules were retained in the endoplasmic reticulum due to the E3 transcriptional unit of adenovirus (35, 37). This possibility is not likely in our current studies, since sufficient MHC and transgenic peptides were expressed on the β cells to allow recognition by CTL primed against LCMV ARM NP. A second possibility is that sufficient CTL were available, but their affinity was too low for activation and the resultant target cell lysis. This possibility is also unlikely, because the doses of peptide required for activation and lysis of CTL generated by Pasteur and Traub infections, which did not induce IDDM, were similar (10^{-8} to 10^{-9} M) to that for CTL from the LCMV ARM strain (10^{-8} to 10^{-9} M), which did cause IDDM. The third possibility, and the one that proved true, was the need for a sufficient threshold of effector CTL to cause IDDM.

The fact that a finite number of CTL rather than an all-or-nothing response is required to cause an autoimmune disease has important ramifications. Within a viral family like LCMV, some strains cause autoimmune disease and others do not. Yet, neither serologic markers nor cell proliferation assays necessarily distinguish the strains causing disease from those not causing disease. In the studies performed here, virus strains that caused or did not cause IDDM all generated antibodies and CTL that cross-reacted with the self (viral) antigen in the β cells responsible for IDDM. Furthermore, high homology (>96%) was noted between such viruses. Therefore, the interpretation of previous and current epidemiologic data utilizing serology or proliferation assays or molecular probes to detect regions shared among viral family members and assign a cause for IDDM or other autoimmune diseases is of questionable reliability.

Finally, to be successful in the treatment of autoimmune disease, reduction of the effector T cells rather than their elimination may be all that is necessary. Recently, using adoptive transfers and peptide blockers (24, 33), we determined that IDDM did not occur when precursor frequency was lowered from 1/800 to 1/5,000 virus (self)-specific biologically active lytic CTL. We are currently quantitating the precise number of effector CTL required and evaluating several strategies to lower the number to a level that would circumvent autoimmune disease.

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