Persistent Virus Infection Associated With Chemical Manifestations of Diabetes

II. Role of Viral Strain, Environmental Insult, and Host Genetics

ANTOINETTE TISHON and MICHAEL B. A. OLDSTONE

From the Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, California

Abnormal glucose metabolism (hyperglycemia and/or aberrant glucose tolerance test) occurred over the lifetimes of mice persistently infected with lymphocytic choriomeningitis virus (LCMV). Persistent infection could be initiated in both newborn and adult mice. For newborns, inoculation with any of several strains of LCMV (Armstrong, E350, Pasteur, Traub or WE) caused continuous infection, but such infection of adults required a selected lymphotropic variant of LCMV Armstrong (Clone 13). Throughout these animals' lives, viral materials (nucleic acid sequences and proteins) accumulated in multiple tissues, including the beta-cells of the islets of Langerhans; infectious virus was present in blood and tissues, and the LCMV-specific H-2 restricted CTL response was poor. Adult mice that had been inoculated with virus as newborns displayed neither histopathologic injury nor infiltrates of mononuclear cells in the islets of Langerhans despite moderate viral replication in beta-cells. In contrast, mice inoculated as adults with Clone 13 LCMV consistently developed inflammatory infiltrates in perivascular spaces of the islets of Langerhans, and their beta-cells expressed LCMV antigens. Addition of a subdiabetogenic dose of streptozotocin, a specific beta-cell toxin, magnified the virus-induced abnormality in glucose metabolism. This indicated a potentiating role between persistent virus infection initiated at birth or in adulthood and an environmental factor in causing abnormalities in glucose metabolism. (Am J Pathol 1987, 126:61-72)

PREVIOUSLY we reported that virus may persistently infect beta-cells of the pancreatic islets of Langerhans and, without causing their injury or destruction, lead to chemical manifestations of diabetes.[1,2] Only the lymphocytic choriomeningitis virus (LCMV) strains Armstrong (ARM) 1371 and WE were tested, and persistence was initiated in only one way, by inoculation of newborn mice. Our results were restricted to observing elevated blood glucose levels and abnormal glucose tolerance tests during the first 2 months of viral infection. We now extend these observations by reporting the establishment of persistent infection in adult immunocompetent mice in addition to the neonates. The beta-cells of these adults became infected, and the mice manifested chemical abnormalities in glucose metabolism. The abnormality in glucose metabolism associated with persistent virus infection was found throughout the animals' life courses. Further, the effect of virus on glucose metabolism in mice persistently infected as adults and as neonates could be enchanced by other environmental insults to beta-cells or a genetic defect in fat metabolism. For these studies, we utilized streptozotocin, a highly specific beta-cell toxin, and C57BL/6 ob/ob (C57 ob/ob) genetically obese mice.

Supported in part by USPHS Grant AG-04342.

This is Publication 4260-IMM from the Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, California.

Accepted for publication August 8, 1986.

Address reprint requests to Michael B. A. Oldstone, Scripps Clinic and Research Foundation, 10666 N. Torrey Pines Road, La Jolla, CA 92037.

Materials and Methods

Mice and the Persistent Virus Carrier State

BALB/WEHI, C3H/St, and SWR/J mice were bred in the vivarium of the Research Institute of Scripps Clinic; C57 ob/ob and ob/+ mice were obtained from Jackson Laboratory, Bar Harbor, Maine. Mice were inoculated either with 60–1000 PFU of virus intracerebrally within the first 18 hours of life or with 2×10^6 PFU of virus intravenously when 6–8 weeks of age. The details of inoculation, development of the LCMV carrier state (persistent infection), and the relevant assay have been reported elsewhere.[3,4]

Virus

For these experiments, six strains of LCMV were used: ARM CA 1371 Clone 53B, ARM CA 1371 Clone 13, ARM E-350, Pasteur CIPV 76001, Traub, and WE. The origins,[5,6] passage histories,[5,6] relatedness as determined by oligonucleotide mapping,[5] tryptic peptide analysis,[7] and recognition by cytotoxic T lymphocytes (CTLs)[8] and by monoclonal antibodies[9,10] have been published. Individual LCMV strains were cloned and plaque-purified three times in Vero cells. Thereafter, a stock was prepared after one passage in BHK cells. Such virus stocks were used throughout all experiments and had titers ranging from 1 to 8×10^7 PFU. Infectious LCMV was quantitated by assay on Vero cells as described.[4]

Localization of LCMV Antigens, Nucleic Acids, and Histopathologic Studies

Viral nucleic acids and antigens were determined in whole animal body sections.[11,12] Briefly, individual mice were anesthetized with ether, exsanguinated, embedded in 3.5% carboxy methylcellulose (CMC), and frozen by immersion in dry ice–ethanol. The CMC blocks were mounted, trimmed, and cut in an LKB cryomicrotome, Model 2258, with refrigeration altered by Haggarty of San Diego, until the appropriate plane for sectioning was obtained. At that point 40-μ sections were cut, collected on 3M Scotch Tape (No. 688), and used for either nucleic acid hybridization[11,12] or protein detection.[12] Hybridization was performed at 37 C for 24–72 hours, depending on the specific activity and base pair size of the probe used. ^{32}P-labeled nick-translated DNA probes of 200–1600 base pairs with specific activities of $1-10\times10^8$ cpm/μg of DNA were used.[11,13] To detect proteins, each 40-μ section stuck to Scotch Tape was transferred to a 0.2-μ Pall Biodyne nylon membrane. The strips were treated with buffers,[12] incubated for 2–4 hours with antibody, washed, and reacted with ^{125}I-staphylococcal protein A (5×10^5 cmp/ml) for 1 hour. Sections were subjected to autoradiography using XAR-5 (nucleic acids) or XRP-1 (proteins) Kodak X-ray film.

Purified restriction fragments of cloned LCMV cDNA corresponding to the nucleoprotein (NP) or glycoprotein (GP) coding region of the genomic S RNA segment were labeled *in vitro* by nick translation in the presence of ^{32}P-labeled deoxyribonucleotide triphosphate and used as described.[11,13] Guinea pig antibodies to the three major LCMV polypeptides GP1, GP2, and NP raised by inoculation of infectious virus were used. The procedures for chemical purification and analysis of the various reagents for purity have been detailed.[3,9]

Expression of viral antigens was also studied by fluorescence microscopy. For these observations, tissues obtained from individual mice were snap-frozen in liquid nitrogen; then 4-μ thin sections were cut on a cryostat, mounted on glass slides, fixed, and stained with monoclonal antibodies to various LCMV polypeptides as described.[3,5] The monoclonal antibody 113 used to detect the viral NP reacts equivalently with LCMV ARM, E-350, Pasteur, Traub, and WE (M. Buchmeier, manuscript in preparation). To mark LCMV glycoproteins, we used a mixture of monoclonal antibodies. Included were 2-11-10 directed to the GP1 of LCMV ARM, 2-WE 6.2, which binds equivalently to GPI of LCMV ARM, E-350, Pasteur, Traub, and WE, WE-40.3, which binds equivalently to GPI of LCMV Pasteur and WE, and 9-7.9, which reacts with GP2 of LCMV ARM, E-350, Pasteur, Traub, and WE.

Histopathologic studies were done either on tissues fixed in Bouin's solution or perfused with 4% paraformaldehyde. One to five 4-μ fixed sections were placed on glass slides and stained with methylene blue basic fuchsin or periodic acid–Schiff.

Cytotoxic T-Lymphocyte (CTL) Assay

CLTs were generated by inoculating 1×10^5 to 2×10^6 PFU of LCMV intravenously or intraperitoneally into 6–8-week-old mice. Their spleens, harvested 6–8 days later, were the source of lymphocytes suspended in culture and added to ^{51}Cr-labeled H-2-restricted and non-H-2-restricted target cells. Target cells used were BALB/c1 7 (H-2^d), MC57 (H-2^b), SWR/J (H-2^q), and L929 (H-2^k). The CTL assay and calculations employed have been reported.[4]

Chemical Analysis

Glucose concentration in sera and glucose tolerance were tested as described.[1] Briefly, cortisol and

insulin concentrations were determined in a competitive inhibition radioimmunoassay.[1]

Results

Persistent Infection Following Inoculation of Neonatal Mice With Five LCMV Strains: Involvement of Beta Cells and Abnormal Glucose Metabolism

Any of five strains of LCMV, ARM 1371, E-350, Pasteur, Traub, or WE inoculated into newborn BALB/WEHI and SWR/J mice resulted in persistent infection, as reflected by the recovery of infectious LCMV from their blood and tissues and identification of viral nucleic acid sequences and viral protein in multiple organs of their whole body sections (Figure 1). Further, mice persistently infected with any of these virus strains developed hyperglycemia and were abnormal in response to glucose tolerance testing (Table 1). Hence, over 80% of LCMV-infected mice had blood glucose levels 3 SD above the mean value observed with age- and sex-matched uninfected mice. The virally infected mice became hyperglycemic either when they fasted overnight prior to morning

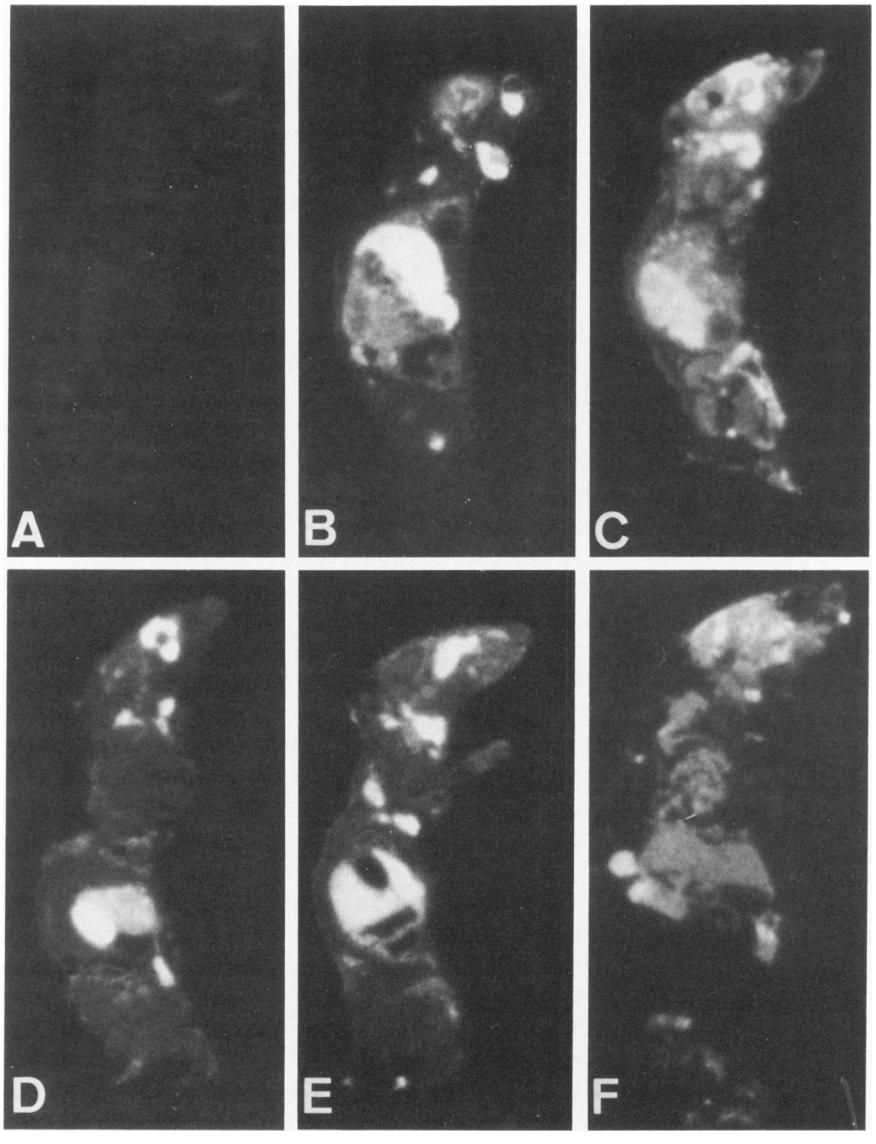

Figure 1 — Persistent viral infection shown as LCMV nuclei acid sequences expressed in 40-μ whole-body sections and visible after using a ^{32}P cDNA probe to LCMV GP. Mice represented in panels **A-E** were 3 months old and in panel **F** 6 months of age when sacrificed. **A** — Uninfected BALB/WEHI mouse. **B** — BALB/WEHI mouse infected with LCMV ARM at birth. **C** — BALB/WEHI mouse inoculated when 6 weeks old with the Clone 13 variant of LCMV ARM. **D** — SWR/J mouse inoculated at birth with LCMV ARM. **E** — SWR/J mouse inoculated when 6 weeks old with Clone 13. **F** — C57BL ob/ob mouse inoculated at birth with LCMV WE. Nucleic acid sequences are evident in the brain, salivary gland, liver, kidney, etc.

Table 1—A Variety of LCMV Strains Cause Persistent Noncytocidal Infection of Beta-Cells in Islets of Langerhans, Hyperglycemia, and Abnormal Glucose Tolerance

BALB/WEHI mice*	No. of mice	Glucose† (mg/dl)	Abnormal‡ GTT (%)	Islets of Langerhans§	
				LCMV Ag	Injury
Uninfected	33	140 ± 4‖	<10	Nil	Nil
LCMV ARM	28	191 ± 6‖	>70	2.5 ± 0.2	Nil
LCMV E-350	15	195 ± 7‖	>70	2.6 ± 0.2	Nil
LCMV Pasteur	21	178 ± 10‖	>70	1.7 ± 0.2	Nil
LCMV Traub	20	162 ± 6‖	ND	1.3 ± 0.3	Nil
LCMV WE	27	178 ± 7‖	>70	1.6 ± 0.2	Nil

*Twelve-week-old male and female BALB/WEHI mice were persistently infected with strains of LCMV listed after 60 PFU had been administered intracerebrally within the first 18 hours after birth.

†Blood glucose: numbers represent the mean value ± 1 SE.

‡Blood glucose tolerance test (GTT): 2 mg of glucose administered intravenously (time 0) per killogram of body weight. Individual mice were bled 1 hour later; a rise in blood glucose at that time two and a half times that at time 0 is considered abnormal. There was a minimum of 8 mice per test group. Numbers represent the percentage with abnormal GTT.

§LCMV antigen (Ag) demonstrated in beta-cells of the islets of Langerhans with the use of immunofluorescence. A score of 1, <33% of beta-cells involved; 2, 33–66% of beta-cells involved; 3, >66% of beta-cells involved. Cell injury or inflammation was noted by histologic examination. See Materials and Methods and Figure 2.

‖P <0.01.

bleeding or had no diet restriction. Glucose tolerance test scores were considered abnormal when the blood glucose was 2½ times or more over the baseline level measured 1 hour earlier, a minute or two before each mouse received 2 mg of glucose intravenously. As seen in Table 1, greater than 70% of mice infected with four different LCMV strains had a 2½-fold or greater increase in blood glucose 1 hour after a glucose challenge. These results were consistent and reproducible. Such tests were conducted concurrently on uninfected mice of which less than 10% had a 2½-fold or greater rise in blood glucose levels. Among the various viral strains, LCMV ARM and E-350 provoked the highest levels of hyperglycemia in BALB/WEHI mice, followed by Pasteur and WE, then Traub (Table 1); yet the virus titers carried in the sera of these mice were equivalent (data not shown). Persistently infected SWR/J mice responded similarly. Concentrations of blood cortisol and pituitary growth hormone were equivalent to those found in uninfected age- and sex-matched mice (not shown). Levels of blood insulin were elevated in most mice persistently infected with LCMV ARM 1371 or LCMV E-350 but not with Pasteur, WE, or Traub (the average ± 1 SE: BALB/WEHI females uninfected, 5.3 ± 0.4 μU/ml; LCMV ARM infected, 13.8 ± 0.9; P <0.01; LCMV E-350 infected, 14.1 ± 2.8, P <0.01; BALB/WEHI males uninfected, 16.3 ± 1.9; LCMV ARM infected, 22.7 ± 2.5, P <0.01; LCMV E-350 infected, 21.0 ± 2.5, P <0.05, 8–10 mice per group). These studies were done on the 12-week-old BALB/WEHI mice whose blood glucose and glucose tolerance test scores are recorded in Table 1. Immunofluorescence microscopy of the islets of Langerhans demonstrated the presence of LCMV NP antigen (Table 1), further identified as NP but not glycoprotein antigen, predominantly in beta-cells in Figure 2. A greater number of beta-cells contained LCMV antigens in islets of mice persistently infected with LCMV ARM and E-350, compared with those infected with LCMV WE, Pasteur, or Traub. These results were consistent in 5–10 mice studied from each experimental group (Table 1). In over 200 islets studied from 5 individual mice infected with the different viral strains, less than 5% of islets showed the inflammatory infiltrates pictured in Figure 2 or architectural destruction.

Persistent Infection Following Inoculation of Neonatal Mice With LCMV ARM 1371: Induction of Diabetes by the Cumulative Effect of Viral Infection and Subdiabetogenic Dose of Streptozotocin

Diabetes can be produced in many species by streptozotocin, a highly specific beta-cell toxin.[14,15] We gave BALB/WEHI and SWR/J mice concentrations of steptozotocin that did not produce diabetes, but reduced the beta-cell insulin reserve, as demonstrated by abnormalities in glucose tolerance. In preliminary experiments, several doses of streptozotocin were given intraperitoneally followed 12 days later by glucose tolerance testing. In these mice, a single dose of 2 mg did not elevate the blood glucose level nor cause abnormal results in glucose tolerance tests at 1 or 4 hours after a standard glucose challenge. Hence, for subsequent experiments, we used 2 mg streptozotocin inoculated intraperitoneally. As can be seen in Table 2, the cumulative effect of streptozotocin and persistent virus infection (LCMV ARM CA 1371) on beta-cells of the islets of Langerhans enhanced hypergly-

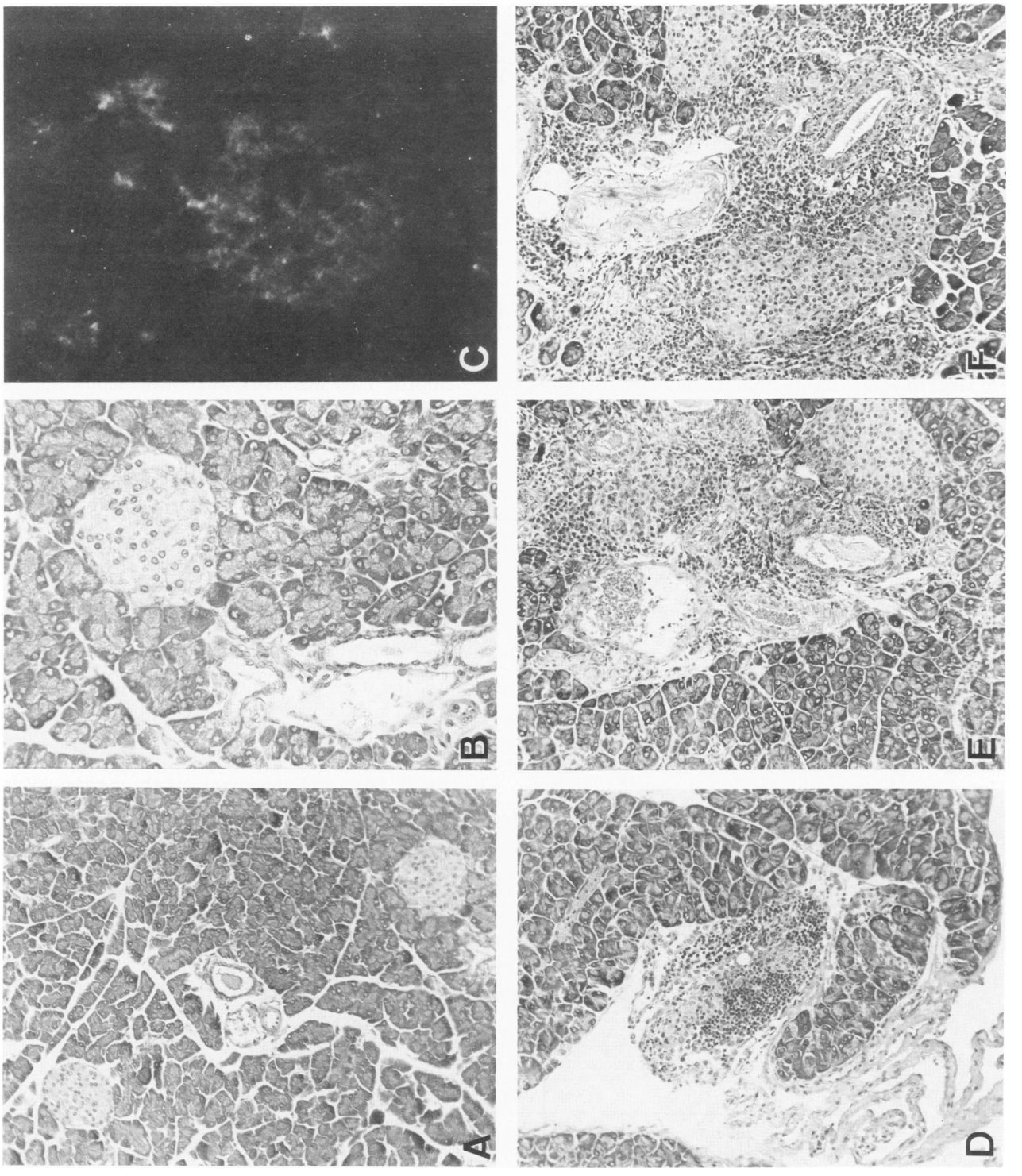

Figure 2 — Photomicrographs of islets of Langerhans in the pancreases of mice persistently infected by inoculation at birth with LCMV ARM wild type parental virus (WT) **(A – C)** or in adulthood with Clone 13 variant of LCMV ARM WT **(D – F).** Tissues from 4-month-old persistently infected BALB/WEHI **(A)** and SWR/J **(B)** mice are free from perivascular or islet infiltrates (contrast with **D – F**). Islets of Langerhans **(C)** show accumulated LCMV NP by immunochemical assay utilizing monoclonal antibody and rhodamine fluorochrome. Over 60% of beta-cells contained viral antigens. Staining of the same section with antibody to insulin coupled with fluorescein isothiocyanate located the viral NP primarily in beta-cells. Tissues **(D)** from a 4-month-old BALB/WEHI mouse and **(E** and **F)** from 2 SWR/J 4-month-old mice all had perivascular and islet infiltrates.

cemia over that caused by viral infection alone, and all these mice were abnormal in glucose tolerance tests. Further, Table 2 records that hyperglycemia in these persistently infected mice was not transient, but lasted continuously throughout the 7-month time span of testing.

Persistent Infection Following Inoculation of Adult Mice With LCMV ARM 1371 Variant Clone 13: Long-Term Effect on Beta-Cells and Glucose Metabolism

Earlier studies indicated that injection of LCMV ARM 1371 Clone 13 to immunocompetent adult mice of the BALB/WEHI strain resulted in a persistent virus infection throughout the animals' life spans, as reflected by the recovery of infectious virus from their blood and tissues.[4] To determine whether other strains are susceptible, we inoculated 2×10^6 PFU intravenously into 6–8-week-old mice of the C3H/St, C57BL/6J, C57 ob/ob, C57 ob/+, SWR/J, and BALB/WEHI strains. The result was persistent infections with infectious virus present in the blood and tissues (Table 3) and viral nucleic acid sequences and proteins detectable in multiple organs of whole body sections (Figure 1). In over 80% of the mice (10–40 from each strain), virus titers in sera varied in individuals from 10^4 to 10^6 logs 15 days after infection and to 10^3 to $10^{4.5}$ logs of virus 180 days later. Table 3 also shows that such adult mice generated limited amounts of LCMV-specific H-2-restricted CTLs. However, these mice developed specific H-2-restricted CTL responses when challenged with a virus different from LCMV,[4] indicating a specific immune defect in CTLs directed against LCMV. In contrast, adult mice from the same or similar litters inoculated with LCMV ARM 1371, the wild type virus from which the variant Clone 13 originated,[4] generated high numbers of LCMV-specific H-2-restricted CTLs and cleared the virus. That is, 15 days after viral inoculation, none of these BALB/WEHI, CH3/St, or SWR/J mice still had virus in their sera (less than 50 PFU), and neither viral nucleic acid sequences nor viral proteins were noted in their lungs or kidneys as viewed in whole-body sections (Table 3).

Histopathologically, certain aspects of the LCMV persistent infection induced in adult mice with LCMV ARM 1371 Clone 13 resembled that in newborn mice inoculated with the wild type parental virus, LCMV ARM 1371. Kidneys showed endothelial and mesangial proliferation in glomerular capillaries. Later, varying gradations of basement membrane thickening and intracapillary hyalinization developed. This was associated with deposition of LCMV antigens, host immunoglobulin, and the third component of complement in the familiar pattern of immune complexes.[3] The liver contained scattered areas of focal necrosis and, on occasion, mixed inflammatory infiltrates. Moderate mononuclear interstitial infiltrates appeared in livers, kidneys, and lungs and to a lesser degree in heart and omental fat. Mononuclear infiltrates were also observed in Virchow-Robin spaces and meninges of the brain, but not in the brain parenchyma. In the two strains, BALB/WEHI and SWR/J, studied in detail (5–10 mice per group), the degree of mononuclear infiltration was markedly greater in mice 2, 4, and 6 months after infection with variant Clone 13 than with parental LCMV ARM 1371. This was most notable in the pancreatic islets of Langerhans, where mononuclear infiltration was common (80% of the mice studied) in mice inoculated with variant Clone 13 (Figure 2 D–F), but exceedingly rare (less than 5%) and mild in mice of the same strains inoculated as newborns with the parental virus, LCMV ARM 1371 (Figure 2A and B).

Table 2—Continuous Hyperglycemia Associated With LCMV Infection Is Enhanced by Streptozotocin

BALB/WEHI* mice—Age (days)	Blood glucose (mg/dl)†				
	No streptozotocin		Streptozotocin 2 mg intraperitoneally		
	Uninfected	LCMV ARM	Day‡	Uninfected	LCMV ARM
45	168 ± 4	212 ± 6§	ND‖	ND	ND
57	171 ± 6	244 ± 8§	+12	203 ± 7	215 ± 9§
78	167 ±4	211 ± 6§	+33	183 ± 8	240 ± 8§
87	143 ± 4	172 ± 5§	ND	ND	ND
115	160 ± 4	180 ± 5§	+70	162 ± 7	217 ± 7§
225	151 ± 4	186 ± 5§	ND	ND	ND

*Female BALB/WEHI mice were persistently infected with LCMV ARM after 60 PFU had been administered intracerebrally within the first 18 hours after birth. There was a minimum of 8 mice in each group.
†Blood glucose: numbers represent the mean value ± 1 SE. There were 8–10 mice per group.
‡Streptozotocin (2 mg) was inoculated intraperitoneally, once, into individual 45-day-old mice, and glucose levels were measured 12, 33, or 70 days later.
§$P < 0.01$.
‖ND, not determined.

Table 3—Establishment of Persistent Infection in Immunocompetent Adult Mice Inoculated With LCMV ARM Wild Type or Clone 13

Adult mice 6–8 weeks old (experimental group)*			CTL assay†								Virus persistence‡ or clearance			
			BALB/Cl 7		MC 57		SWR/J		L-929		PFU/ml sera		VNA in§ tissues	Viremia‖ +/total
			(H-2^d)		(H-2^b)		(H-2^q)		(H-2^k)					
Strain	(H-2)	LCMV	WT	Cl 13	WT	Cl 13	WT	Cl 13	WT	Cl 13	AVG	Range		
BALB/W	dd	WT	59 ± 3¶	56 ± 3	ND	ND	12 ± 3	8 ± 4	nil	<1	2.0×10^2	(4×10^2–<50)	Nil	2/20
BALB/W	dd	Cl 13	8 ± 2	9 ± 3	ND	ND	1 ± 0.1	<1	<1	<1	1.3×10^5	(3×10^5–2×10^3)	+++	20/20
C57BL/6	bb	WT	ND**	ND	58 ± 3	60 ± 5	ND	ND	10 ± 4	12 ± 1	2.0×10^2	(4×10^2–<50)	Nil	1/10
C57BL/6	bb	Cl 13	ND	ND	9 ± 4	10 ± 5	ND	ND	2 ±1	<1	1.2×10^4	(2×10^4–4×10^3)	+++	10/10
C57 ob/ob	bb	WT	5 ± 2	2 ± 1	25 ± 5	27 ± 4	ND	ND	ND	ND	<50	(2×10^2–<50)	Nil	0/5
C57 ob/ob	bb	Cl 13	4 ± 1	2 ± 1	9 ± 2	15 ± 4	ND	ND	ND	ND	5×10^5	(3×10^6–6×10^4)	+++	14/14
C57 ob/+	bb	WT	25 ± 4	19 ± 3	48 ± 7	33 ± 6	ND	ND	ND	ND	1.2×10^2	(3×10^2–<50)	Nil	0/5
C57 ob/+	bb	Cl 13	2 ± 2	1 ± 1	10 ± 3	12 ± 3	ND	ND	ND	ND	4×10^5	(6×10^5–2.5×10^5)	+++	12/15
C3H/St	kk	WT	2 ± 1	<1	<1	<1	ND	ND	35 ± 6	34 ± 2	<50		Nil	0/8
C3H/St	kk	Cl 13	<1	<1	<1	1 ± 0.3	ND	ND	14 ± 2	12 ± 1	8.1×10^4	(1×10^5–7×10^4)	+++	8/8
SWR/J	qq	WT	6 ± 3	5 ± 2	ND	ND	63 ± 4	78 ± 3	ND	ND	3.1×10^2	(6×10^2–<50)	Nil	2/18
SWR/J	qq	Cl 13	1 ± 0.9	<1	ND	ND	14 ± 1	17 ± 3	ND	ND	8.7×10^4	(2×10^5–4×10^3)	+++	20/20

*Mice were innoculated intravenously with 2×10^6 PFU of either LCMV ARM 1371 wild type (WT) or LCMV ARM 1371 Clone 13 (Cl 13). Six (BALB/W, C57BL6, C57 ob/ob, C57 ob/+, SWR/J) or 7 (C3H/ST) days later, a suspension of splenic lymphocytes in effector-to-target ratios of 50:1, 25:1 and 12.5:1 was added to a variety of virus infected H-2 target cells labeled with ^{51}Cr. Previous experiments showed that the effector cells were Th1.2$^+$, LYT2$^+$, LT34^{nil}.

†Percent specific ^{51}Cr release from H-2 and non-H-2-restricted, LCMV-infected target cells in a 5-hour assay. See Materials and Methods.

‡Viral persistence detected in mice 15 to 30 days after LCMV inoculation. Infectious virus in individual sera titered on Vero cells (PFU).

§LCMV nucleic acid (VNA) sequences in tissues detected after whole body sectioning of mice and testing 15 days after viral infection. Similar results were observed in mice tested 60–180 days after initiating viral infection.

‖Viremia (>50 PFU/ml). Number of mice with viremia over total population.

¶Mean value ± 1 SD. Five or more individual BALB/W, C57BL/6J, C3H/St, and SWR/J mice were tested. Three individual C57 ob/ob or or/+ mice assayed.

**ND, not determined.

Glucose metabolism was analyzed in mice 6 and 14 weeks after injection of the variant virus Clone 13. Blood glucose levels were not elevated (>85% of infected mice) when compared with those in age- and sex-matched uninfected mice, although glucose tolerance was abnormal in 60–70% of such persistently infected mice (Table 4). C3H/St and SWR/J mice reacted similarly when given injections at 4–6 weeks of age with LCMV ARM 1371 Clone 13. In these three strains, (C3H/St, BALB/WEHI, and SWR/J), viral antigen was found in one fourth or fewer of the beta or other islet cells by immunofluorescence microscopy, whereas infiltrates occurred in the islets (Table 5, Figure 2).

Table 5—Inflammation of Islets of Langerhans Is Associated With Persistent LCMV Infection Initiated in Adults by LCMV ARM Clone 13 Variant But Not in Neonatal Mice by LCMV ARM Wild Type Parental Strain*

Mouse		Experimental group	Inflammation islets of Langerhans
Strain	Age (mos)		
BALB/WEHI	6	Uninfected	0/10†
BALB/WEHI	6	LCMV ARM WT	0/6
BALB/WEHI	6	LCMV ARM Cl 13	4/6
C3H/St	6	Uninfected	0/10
C3H/St	6	LCMV ARM WT‡	0/10
C3H/St	6	LCMV ARM Cl 13	4/10
SWR/J	1	Uninfected	0/6
SWR/J	1	LCMV ARM WT	0/5
SWR/J	1	LCMV ARM Cl 13	4/6
SWR/J	6	Uninfected	0/10
SWR/J	6	LCMV ARM WT	0/5
SWR/J	6	LCMV ARM Cl 13	10/10

*Persistent LCMV infection initiated either by inoculating 5–7-week-old mice intravenously with LCMV ARM Clone 13 or by inoculating newborn mice with LCMV ARM wild type (WT).
†Number of mice with inflamed tissues over total number of mice studied.
‡C3H/St mice inoculated with LCMV ARM at birth usually die within 30 days of life (>95%) because of growth hormone insufficiency and hypoglycemia.[3] This group consists of sixth-generation congenitally infected mice bred from the <5% survivors.

Persistent Infection Following Inoculation of Adult Mice With LCMV ARM 1371 Clone 13: Induction of Hyperglycemia by Cumulative Effect of Persistent Infection of Beta-Cells and Subdiabetogenic Dose of Streptozotocin

Table 6 shows that mice persistently infected with Clone 13 had a significant increase in blood glucose concentration when treated with a subdiabetogenic dose of streptozotocin. Inoculation of streptozotocin alone into uninfected age- and sex-matched mice did not raise blood glucose above normal levels. Thus, hyperglycemia was maintained for 6 months after one injection of streptozotocin in persistently infected mice whose beta-cells contained the virus.

Persistent Infection Following Inoculation of Genetically Obese Adult Mice with LCMV ARM 1371 Clone 13: Effect on Glucose Metabolism and Obesity

Six- to 7-week-old genetically obese C57 ob/ob mice and their heterozygotic nondiabetic littermates (ob/+) were inoculated with LCMV ARM 1371 Clone 13 and became persistently infected, as typified by the infectious LCMV recovered from their blood (Table 6) and viral nucleic acid sequences and proteins in their whole-body sections (Figure 1). Analysis 2–24 weeks after infection failed to show any consistent abnormality in blood glucose levels (Table 7), but 60% of both groups had abnormal glucose tolerance test results (Figure 3). Surprisingly, persistent virus infection of ob/ob mice markedly reduced their weights, as noted in Figure 4. Thus, at 90 days after infection, there was a 40%, and at 180 days after infection, a 51% decrease in weight when compared with age- and sex-matched uninfected controls. By contrast, weight loss at similar times among LCMV-

Table 4—Abnormal Glucose Metabolism in BALB/WEHI Mice Infected During Adulthood With LCMV ARM Clone 13 Variant

Experimental group	Mice		Time of infection (weeks)	Glucose metabolism			Islets of Langerhans	
	No.	Age (weeks)		Blood† glucose	Blood‡ insulin	Abnormal§ GTT	LCMV Ag‖	Injury
Uninfected	27	12	6	142 ± 6	0.75	0/6	Nil	Nil
LCMV-infected	23	12	6	152 ± 11	1.57¶	6/9¶	+	+
Uninfected	29	20	14	178 ± 7	0.24	1/6	Nil	Nil
LCMV-infected	23	20	14	187 ± 5	0.95¶	6/9¶	+	+

*Six-week-old male and female BALB/WEHI mice developed persistent infection after receiving 2×10^6 PFU of LCMV administered intravenously.
†Blood glucose (mg/dl): Number represents the mean value ± 1 SE.
‡Blood insulin (ng/ml): number represents the mean value ± 1 SE.
§Blood glucose tolerance test (GTT): 2 mg of glucose administered intravenously (time 0) per kilogram of body weight. Individual mice were bled 1 hour later and a rise in blood glucose at that time of two and a half times time 0 considered abnormal. Minimum of 8 mice per test group. Numbers represent the total with abnormal GTT over the total mice in a group.
‖Moderate amounts of LCMV antigen (Ag) demonstrated in beta-cells of the islets of Langerhans with the use of immunofluorescence. +, <33% of beta-cells involved; ++, 33–66% of beta-cells involved; +++, >66% of beta-cells involved. Cell injury or inflammation noted by histologic examination. See Materials and Methods and Figure 2.
¶$P < 0.01$.

Table 6—Combination of Streptozotocin and Persistent LCMV Infection Causes Hyperglycemia in Adult Mice Inoculated With the Clone 13 Variant*

BALB/WEHI mice*	Number of mice	Age (days)	Glucose (mg/dl)†
Uninfected	25	82	160 ± 16
LCMV-infected	22	82	182 ± 13‡
Uninfected	26	102	150 ± 28
LCMV-infected	24	102	199 ± 10‡
Uninfected	27	156	164 ± 12
LCMV-infected	24	156	220 ± 7‡
Uninfected	29	189	128 ± 11
LCMV-infected	27	189	193 ± 8‡
Uninfected	31	222	200 ± 3
LCMV-infected	27	222	271 ± 12‡

*Male and female 7–8-week-old BALB/WEHI mice received 2×10^6 PFU of LCMV ARM Clone 13, intravenously. Persistent infection was documented by determining infectious virus in sera 15–30 days later. Mice were given streptozotocin 2 mg intraperitoneally one time when 47 days old and infected with virus 2 days later.

†Blood glucose: number represents the mean value ±1 SE.

‡$P < 0.01$.

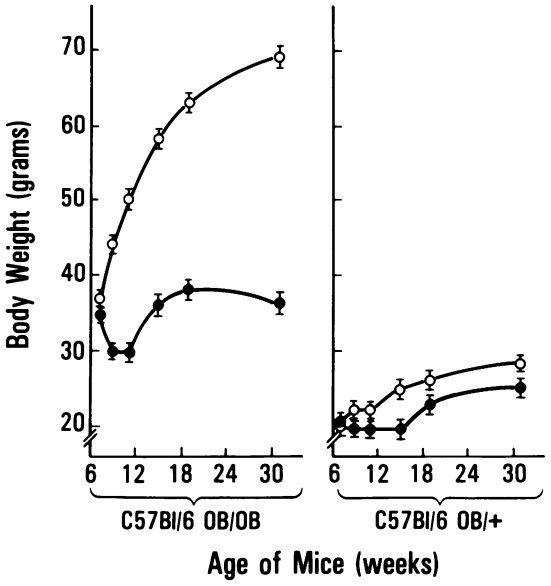

Figure 4—Persistent LCMV infection (LCMV ARM Clone 13 variant) reduces the weight of ob/ob mice handled and monitored as specified in Materials and Methods. •, LCMV infected; ○, age- and sex-matched control; ⊢⊣, 1 SD. There were over 20 mice per group.

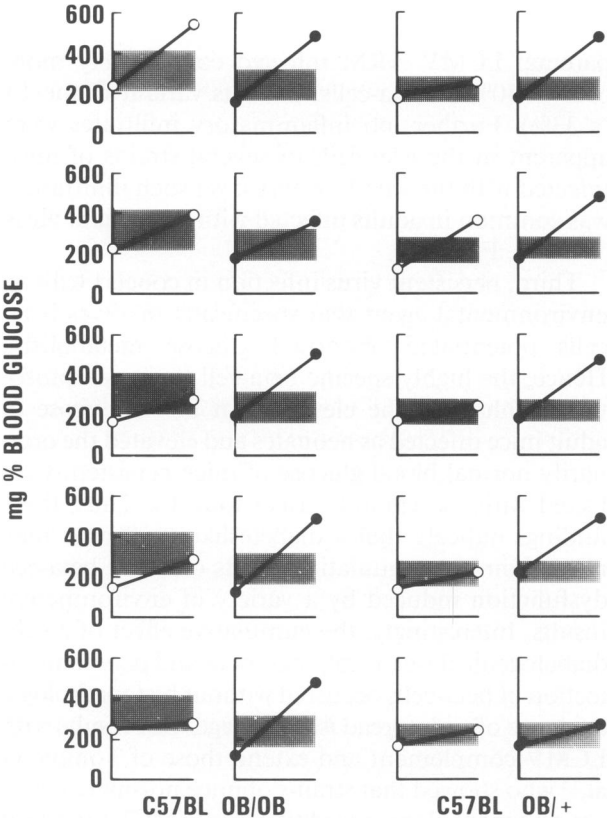

Figure 3—Abnormal glucose tolerance test in LCMV persistently infected C57BL ob/ob and C57BL ob/+ mice. Mice were infected with Clone 13 variant of LCMV ARM when 6 weeks of age. Thirty days later LCMV-infected (•—•) and uninfected age- and sex-matched controls (○—○) were pre-bled, inoculated, with 2 mg of glucose/kg intraperitoneally, and re-bled 1 hour later. *Shaded area* represents 2.5 SD above the mean blood glucose level.

infected ob/+, C3H/St, BALB/WEHI, or SWR/J mice varied slightly from that of matched uninfected controls (2–9% weight decrease when compared with uninfected mice). Histopathologic study of tissues taken from LCMV-infected ob/ob mice has, so far, revealed no reason for the severe weight loss. Inflammatory infiltrates were observed in several of their tissues, including islets of Langerhans, similar to those in BALB/WEHI, C3H/St, and SWR/J mice persistently infected with LCMV ARM 1371 Clone 13. Immunochemical study of the islets revealed small amounts of viral antigens in beta-cells of both ob/ob or ob/+ mice persistently infected with virus, ie, in less than 30% of their beta-cells (Table 7).

Discussion

This report makes four main points. First, our previous observations[1,2] that chemical manifestations of diabetes continue during 60 days' monitoring of persistent virus infection caused by LCMV ARM and WE strains in BALB/WEHI and SWR/J mice extend here to over 7 months of infection, the longest time of the experimental observation. Further, other strains of LCMV, ie, E350, Pasteur, and Traub, inoculated into newborns also cause abnormal glucose metabolism during adulthood. LCMV has a two-segment genome, and specific strains show unique tropisms and disease-causing properties.[6,16–18] In the appropriate setting for identifying pathogenic and nonpathogenic

Table 7—Persistent Viral Infection of Genetically Obese Mice Does Not Potentiate the Blood Glucose Level

Experimental group*	Age (weeks) Mouse	Age (weeks) After infection	Glucose† (mg/dl)	LCMV‡ (PFU)	Islets of Langerhans§ (viral Ag)
ob/ob uninfected	9	2	259 ± 21	ND‖	ND
ob/ob LCMV-infected	9	2	290 ± 6	2×10^5	ND
ob/+ uninfected	9	2	192 ± 5	ND	ND
ob/+ LCMV-infected	9	2	180 ± 12	1.5×10^5	ND
ob/ob uninfected	11	4	290 ± 11	ND	ND
ob/ob LCMV-infected	11	4	239 ± 13	ND	ND
ob/+ uninfected	11	4	173 ± 9	ND	ND
ob/+ LCMV-infected	11	4	186 ± 10	ND	ND
ob/ob uninfected	19	12	247 ± 15	ND	ND
ob/ob LCMV-infected	19	12	256 ± 24	ND	ND
ob/+ uninfected	19	12	184 ± 8	ND	ND
ob/+ LCMV-infected	19	12	190 → 17	ND	ND
ob/ob uninfected	31	24	217 ± 7	ND	Nil
ob/ob LCMV-infected	31	24	254 ± 19	2.3×10^4	+
ob/+ uninfected	31	24	164 ± 5	ND	Nil
ob/+ LCMV-infected	31	24	197 ± 5¶	1.4×10^4	+

*Male and female 6-week-old ob/ob and ob/+ mice received 2×10^6 PFU of LCMV ARM Clone 13, intravenously.
†Blood glucose: number represents the mean value ± 1 SE.
‡PFU of LCMV present in the sera (PFU/ml).
§LCMV antigen (Ag) demonstrated on beta-cells of the islets of Langerhans with the use of immunofluorescence: +, <33% of beta-cells involved; ++, 33–66% of beta-cells involved; +++, >66% of beta-cells involved.
‖ND, not determined.
¶$P < 0.01$.

viral strains, viral RNA segments from the individual strains can be reassorted to map the segment associated with a specific disease. This strategy has been successful in mapping viral genes associated with tropism for growth hormone-producing cells and resultant growth hormone deficiency disease (small 1.9 kb RNA segment on LCMV ARM),[16] acute death of guinea pigs (large 3.8 kb RNA segment of LCMV WE),[17] and CTL induction and recognition (encoded on the small RNA segment).[18] Unfortunately this strategy was not of value for determining the viral genes associated with the induction of chemical diabetes, because all the strains studied induced abnormalities in glucose metabolism.

The second point established here is that persistent infection could be initiated in adult immunocompetent mice of many different strains (Table 3). This occurred through parenteral administration of a LCMV lymphotropic variant derived from LCMV ARM.[4] Infection persisted primarily because the virus aborted generation of LCMV-specific H-2-restricted CTLs. This was associated with accumulation of viral materials (nucleic acids and proteins) in tissues and infectious virus in blood and tissues, just as in mice inoculated as newborns for initiation of persistent infection. However, these two models differed quantitatively and qualitatively with respect to virus in the islets of Langerhans and the beta-cells. Both virus strains infected beta-cells, although the wild type parental LCMV ARM infected considerably more cells (>50% of beta-cells) than its variant Clone 13 (<33%). Further, no inflammatory infiltrates were apparent in the islet cells of several strains of mice infected with the wild type virus; yet such infiltration was common in adults infected with the variant virus (Table 5, Figure 2).

Third, persistent virus infection in concert with an environmental agent that specifically involves beta-cells potentiated abnormal glucose metabolism. Hence, the highly specific beta-cell toxin, streptozotocin, enhanced the elevation in blood glucose of adult mice infected as neonates and elevated the ordinarily normal blood glucose of mice persistently infected with the variant virus Clone 13. Thus, these findings indicate that a diabeteslike syndrome may result from the cumulative effects of subtle beta-cell dysfunction induced by a variety of environmental insults. Interestingly, the cumulative effect of a subdiabetogenic dose of streptozotocin and persistent infection of beta-cells occurred without histopathologic evidence of widespread islet damage. Our results with LCMV complement and extend those of Toniolo et al,[15] who showed that strains of mice normally resistant to encephalomyocarditis virus (EMCV)-induced diabetes, after treatment with a subdiabetogenic dose of streptozotocin, then infection with EMCV, developed diabetes. The same investigators found[15,19] that mice infected with other viruses such as Coxsackie B3

and B5, which ordinarily produce little if any beta-cell damage, became diabetic when pretreated with a subdiabetogenic dose of streptozotocin.

Beta-cells of the pancreatic islets of Langerhans are believed to be the sole source of insulin production in mammals. Destruction of beta-cells by any means, including acute cytolytic viral infection,[19,20] leads to chemical manifestations of diabetes, ie, hypoinsulinemia, hyperglycemia, abnormal glucose metabolism, and such pathologic states as glomerulosclerosis, thickening of capillary basement membranes, and retinal disease. The severity of this diabetes depends on the number of beta-cells destroyed. Recently, viruses have been shown to alter the function of differentiated cells without killing them, ie, by altering differentiation or luxury functions but not housekeeping or vital functions.[21-30] Such subtle alterations can fail to elicit cytomorphologic injury,[1,2,21] yet cause cellular dysfunction leading to altered homeostasis and disease.[1,21,22] In our studies the chemical abnormality in glucose metabolism was associated, in part, with persistent infection of beta-cells. It occurred commonly in mice infected with any of several strains of LCMV (ARM 1371, E350, Pasteur, Traub, WE) and paralleled the infection of beta-cells. In support of this observation, the variant LCMV Clone 13 infected fewer beta-cells and rarely caused any elevation in blood glucose, although glucose tolerance test results were abnormal for approximately 65% (30/46) of these mice, compared with less than 10% of age- and sex-matched uninfected mice. However, other events associated with persistent LCMV infection may also play a part in abnormal glucose metabolism. For example, in other tissues associated with glucose regulation, like the liver and kidney, viral nucleic acid sequences and proteins accumulate during persistent infection. However, neither the amount of viral material deposited in such tissues nor the amount of infectious virus carried in the blood seems to correlate with the extent of the abnormality in glucose metabolism (unpublished data). Other hormones that regulate glucose metabolism, like growth hormone and cortisol, were found to be within normal limits,[1] although the role of glycogen was not evaluated and the assays measured immunochemical binding of hormones, rather than their functional abilities. The abnormalities of blood insulin levels in persistently infected mice occurred consistently; yet why they are elevated above levels found in age- and sex-matched controls remains unclear. Perhaps the hyperplasia of islet cells during persistent virus infection[2] indicates enhanced synthesis of insulin and a very early manifestation of diabetes.

Fourth and finally, the marked loss of weight in genetically ob/ob mice (Figure 4) and the inflammatory infiltrates observed in several tissues, including the islets of Langerhans, of adult mice of this and other strains after infection with LCMV ARM variant Clone 13 was unexpected and of interest. This loss was far greater (40-50% weight loss) than that in other strains of mice persistently infected with LCMV in adulthood whose weight loss is comparatively trivial (average of 5% weight reduction with a range of 2-9%). Thus, it is also unlikely that weight loss *per se* was an important component in the glucose abnormalities associated with persistent viral infection. Whether the loss of weight in the ob/ob mouse strain is caused by the effect of virus on an additional differentiation pathway is unknown. Interestingly, canine distemper virus has been shown to induce obesity in mice, presumably by interfering with catecholamine synthesis or release.[29,30] Alternatively, the reduction in weight might result from the activation of macrophages, cells that can be persistently infected,[31] and their postulated release of cachectin.[32] Cachectin is a monokine that inhibits production of lipogenic enzymes in cultured adipocytes.[33,34]

The inflammatory infiltrates observed in several tissues, including the islets of Langerhans of adult mice infected with LCMV Clone 13 are also of interest. Because such mice develop few, if any, LCMV-specific H-2-restricted CTLs (Table 3), it is unlikely that the infiltration represents a virus-specific CTL response against LCMV antigens. Recently, a number of reports have shown that viruses can induce autoimmune responses by several mechanisms.[35] Our preliminary studies indicate that autoantibodies to numerous host tissues and antigens may be induced by persistent infection with LCMV. The identities of such autoantibodies and the structural differences between LCMV ARM 1371 wild type and its variant, Clone 13, are matters currently under investigation.

References

1. Oldstone MBA, Southern P, Rodriguez M, Lampert P: Virus persists in beta cells of islets of Langerhans and is associated with chemical manifestations of diabetes. Science 1984, 224:1440-1443
2. Rodriguez M, Garrett RS, Raitt M, Lampert PW, Oldstone MBA: Virus persists in beta cells of islets of Langerhans and infection is associated with chemical manifestations of diabetes: II. Morphologic observations. Am J Pathol 1985, 121:497-504
3. Oldstone MBA, Dixon FJ: Pathogenesis of chronic disease associated with persistent lymphocytic choriomeningitis viral infection: I. Relationship of antibody production to disease in neonatally infected mice. J Exp Med 1969, 129:483-505.
4. Ahmed R, Salmi A, Butler LD, Chiller JM, Oldstone MBA: Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently in-

fected mice: Role in suppression of cytotoxic T lymphocyte response and viral persistence. J Exp med 1984, 60:521-540
5. Oldstone MBA, Ahmed R, Buchmeier MJ, Blount P, Tishon A: Perturbation of differentiated functions during viral infection *in vivo*: I. Relationship of lymphocytic choriomeningitis virus and host strains to growth hormone deficiency. Virology 1985, 142:158-174
6. Dutko FJ, Oldstone MBA: Genomic and biological variation among commonly used lymphocytic choriomeningitis virus strains. J Gen Virol 1983, 64:1689-1698
7. Buchmeier MJ: Antigenic and structural studies on the glycoproteins of lymphocytic choriomeningitis virus, Negative Strand Viruses. Edited by R Compans, D Bishop. New York, Academic Press 1984, pp 193-200
8. Ahmed R, Byrne JA, Oldstone MBA: Virus specificity of cytotoxic T lymphocytes generated during acute lymphocytic choriomeningitis virus infection: Role of the H-2 region in determining cross-reactivity for different lymphocytic choriomeningitis virus strains. J Virol 1984, 51:34-41
9. Buchmeier MJ, Lewicki HA, Tomori O, Oldstone MBA: Monoclonal antibodies to lymphocytic choriomeningitis viruses: Generation, characterization and cross-reactivity with other arenaviruses. Virology 1981, 113:73-85
10. Oldstone MBA, Buchmeier MJ: Restricted expression of viral glycoprotein in cells of persistently infected mice. Nature 1982, 300:360-362
11. Southern PJ, Blount P, Oldstone MBA: Analysis of persistent virus infections by *in situ* hybridization to whole-mouse sections. Nature 1984, 312:555-558
12. Blount P, Elder J, Lipkin WI, Southern PJ, Buchmeier MJ, Oldstone MBA: Analysis of endogenous and exogenous antigens in the nervous system using whole animal sections. Brain Res 1986, 382:259-265
13. Southern PJ, Buchmeier MJ, Ahmed R, Francis SJ, Parekh B, Riviere Y, Singh MK, Oldstone MBA: Molecular pathogenesis of arenavirus infections, In Vaccines 86: Modern Approaches to Vaccines. Edited by RA Lerner, RM Chanock, F Brown. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory, (In press)
14. Junod A, Lampert AE, Stauffacher W, Renold AE: Diabetogenic action of steptozotocin: Relationship of dose to metabolic response. J Clin Invest 1969, 48:2129-2139
15. Toniolo A, Onodera T, Yoon, JW Notkins AL: Induction of diabetes by cumulative environmental insults from viruses and chemicals. Nature 1980, 288:383-385
16. Riviere Y, Ahmed R, Southern PJ, Oldstone MBA: Perturbation of differentiated functions during viral infection *in vivo*. II. Viral reassortants map growth hormone defect to the S RNA of the lymphocytic choriomeningitis virus genome. Virology 1985, 142:175-182
17. Riviere Y, Ahmed R, Southern PJ, Buchmeier MJ, Oldstone MBA: Genetic mapping of lymphocytic choriomeningitis virus pathogenicity: Virulence in guinea pigs is associated with the L RNA segment. J Virol 1985, 55:704-709
18. Riviere Y, Southern PJ, Ahmed R, Oldstone MBA: Biology of cloned cytotoxic T lymphocytes specific for lymphocytic choriomeningitis virus. V. Recognition is restricted to gene products encoded by the viral S RNA segment. J Immunol 1986, 136:304-307
19. Notkins AL, Yoon JW: Virus-induced diabetes mellitus, Concepts in Viral Pathogenesis. Edited by AL Notkins, MBA Oldstone. New York, Springer-Verlag, 1984, pp 241-247
20. Craighead JE: The role of viruses in the pathogenesis of pancreatic disease and diabetes mellitus, Progress in Medical Virology. Vol 19. Edited by JL Melnick. Basel, S. Karger, 1976, pp 162-207
21. Oldstone MBA: Virus can alter cell function without causing cell pathology: Disordered function leads to imbalance of homeostasis and disease,[19] pp 269-276
22. Oldstone MBA, Sinha YN, Blount P, Tishon A, Rodriguez M, von Wedel R, Lampert PW: Virus-induced alterations in homeostasis: Alterations in differentiated functions of infected cells *in vivo*. Science 1982, 218:1125-1127
23. Casali P, Rice GPA, Oldstone MBA: Viruses disrupt functions of human lymphocytes: Effects of measles virus and influenza virus on lymphocyte-mediated killing and antibody production. J Exp Med 1984, 159:1322-1337
24. Oldstone MBA, Holmstoen J, Welsh RM: Alterations on acetylcholine enzymes in neuroblastoma cells persistently infected with lymphocytic choriomeningitis virus. J Cell Physiol 1977, 91:459-472
25. Arbogast B, Yoshimura M, Kefalides N, Holtzer H, Kaji A: Failure of cultured chick embryo fibroblasts to incorporate collagen into their extra-cellular matrix when transformed by Rous sarcoma virus. J Biol Chem 1977, 252:8863-8868
26. Anderton P, Wild TF, Zwingelstein G: Measles-virus-persistent infection in BGM cells. Biochem J 1983, 214:665-670
27. Rice GPA, Schrier RD, Oldstone MBA: Cytomegalovirus infects human lymphocytes and monocytes: Virus expression is restricted to immediate-early gene products. Proc Natl Acad Sci USA 1984, 81:6134-6138
28. Schrier RD, Oldstone MBA: Recent clinical isolates of cytomegalovirus suppress human cytomegalovirus specific HLA-restricted cytotoxic T lymphocyte activity. J Virol 1986, 59:127-131
29. Lyons MJ, Faust IM, Hemmes RB, Buskirk DR, Hirsch J, Zabriskie JB: A virally induced obesity syndrome in mice. Science 1982, 216:82-85
30. Bernard A, Wild TF, Tripier MF: Canine distemper infection in mice: Characterization of a neuro-adapted virus strain and its long-term evolution in the mouse. J Gen Virol 1983, 64:1571-1579
31. Buchmeier MJ, Welsh RM, Dutko FJ, Oldstone MBA: The virology and immunobiology of lymphocytic choriomeningitis virus infection. Adv Immunol 1980, 30:275-331
32. Beutler B, Greenwald D, Hulmes JD, Chang M, Pan YCE, Mathison J, Ulevitch R, Cerami A: Identity of tumor necrosis factor and the macrophage-secreted factor cachectin. Nature 1985, 316:552-554
33. Beutler B, Mahoney J, LeTrang N, Pekala P, Cerami A: Purification of cachectin, a lipoprotein lipase-suppressing hormone secreted by endolokin-induced raw 264, 7 cells. J Exp Med 1985, 161:984-995
34. Torti FM, Dieckmann B, Beutler B, Cerami A, Ringold GM: A macrophage factor inhibits adipocyte gene expressions: An *in vitro* model of cachexia. Science 1985, 229:867-859
35. Notkins AL, Onodera T, Prabhakar B: Virus-induced autoimmunity,[19] pp 211-215

Acknowledgments

The authors would like to thank Sandy Shyp and Paul Blount for technical assistance and Kathy Nasif and Phyllis Minick for manuscript preparation.