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II. Viral Reassortants Map Prevention of Insulin-dependent Diabetes Mellitus to the Small RNA of Lymphocytic Choriomeningitis Virus

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A relatively noncytopathic virus, lymphocytic choriomeningitis virus (LCMV),1 prevents the development of insulin-dependent diabetes mellitus (IDDM) in non-obese insulin-dependent diabetes (NOD) mice (1, 2). Typically, >90% of NOD female mice, by 9 mo of age, develop hyperglycemia, hypoinsulinemia, and lymphocytic infiltrates in the islets of Langerhans, with β cell destruction. However, their age- and sex-matched counterparts persistently infected since birth with LCMV are quite normal with respect to concentrations of blood sugar and pancreatic insulin.

LCMV is a negative-strand RNA virus with an ambisense coding strategy (3, 4). The viral genome is segmented into two strands, a large (L) and a small (S) RNA of 7.2 and 3.4 kb, respectively. The open reading frame (ORF) at the 3' end of the L RNA segment encodes a large (L) protein (molecular mass of ~200 kD) that is the virus transcriptase/replicase. The 5' ORF encodes a protein called Z (molecular mass of 10-12 kD) that contains a cysteine2-histidine2 zinc finger motif (4). The ORF for the L gene at the 3' end is complementary to the viral genome (negative sense), and the ORF of Z gene at the 5' end is of genomic sense (positive sense) (4). The S segment encodes three major structural proteins: from the 3' end, the internal nucleocapsid protein (NP; molecular mass, 63 kD) and from the 5' end, two surface glycoproteins (GPs) GP-1 (molecular mass, 43 kD) and GP-2 (molecular mass, 36 kD) that are derived from a common precursor polypeptide, GP-C (5, 6). The NP gene at the 3' ORF encoding the NP is complementary to the viral genome (negative sense), whereas the GP-C ORF at the 5' end is of positive sense (6-8).

We utilized this knowledge of the genomic structure of LCMV to determine whether the entire genome or, in contrast, a component part was needed for successful viral therapy to prevent IDDM. Towards that end, we first determined the differing abilities of individual LCMV strains to prevent the IDDM in NOD mice. We then used

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1 Abbreviations used in this paper: GP, glycoprotein; IDDM, insulin-dependent diabetes mellitus; LCMV, lymphocytic choriomeningitis virus; L RNA, large segment RNA; NOD, nonobese insulin-dependent diabetes; NP, nucleoprotein; ORF, open reading frame; PFU, plaque-forming unit; S RNA, small segment RNA.
these data to select viral reassortants for use in mapping the viral genes that inhibit the diabetes of NOD mice.

Materials and Methods

**Mice, Clinical Measurements, and Initiation of Virus Persistence.** NOD mice breeders were originally obtained from K. Lafferty, University of Colorado Medical School, Denver, CO. The NOD colony was established at Scripps Clinic from brother-sister matings of these breeders and was observed twice weekly for general health and length of survival. The incidence of IDDM, as defined by blood glucose of >300 mg/dl, was 90% or greater in all the mice by 9 mo of age or older. Blood glucose concentrations of individual mice were determined every 2 or 3 mo after birth (1, 2). For glucose tolerance testing, each animal’s blood was sampled immediately before administering 2 mg of glucose/kg of body weight, intraperitoneally. Blood samples were taken again 1 h later and tested for sugar concentrations; those that remained two and a half times the preglucose challenge levels were considered abnormal (9). Insulin was extracted from each pancreas and its concentration measured by RIA with rat insulin as a standard and A-14 125I-labeled bovine insulin as a tracer (2). To establish persistent infection, randomly selected mice were inoculated intracerebrally within the first 18 h of life with 10\(^3\) plaque-forming units (PFU) of either reassortants or the parental strains of LCMV. Details of the inoculation procedure, as well as the plaque assay, to measure LCMV persistent infection have been reported (2, 10). Deposition of viral nucleic acid sequences was studied in persistently infected mice by using sections of whole animals and LCMV \(^{32}\)P nucleic acid hybridization (11).

**Viruses and Generation of Reassortants.** The passage histories of LCMV strains Armstrong CA1371 53B (ARM), Pasteur, Traub, and WE, and the Clone 13 variant of ARM CA1371 53b, appear elsewhere (12-14). Individual LCMV strains were cloned and plaque purified three times on Vero cells. Thereafter, a stock was prepared through one passage in BHK-21 cells. Reassortants between Clone 13 and LCMV strain Pasteur CIPV 76001 (12) were generated, plaque purified, characterized by hybridization, and prepared as viral stocks (14). Stocks of the two parental genotypes (L RNA/S RNA, Clone 13/Clone 13, Pasteur/Pasteur) and reassortant genotypes (clone 13/Pasteur, Pasteur/Clone 13) had titers ranging from 10\(^7\) to 6 \times 10\(^7\) PFU/ml.

**Sequence Analysis.** Viral RNA was isolated from the Pasteur and ARM Clone 13 strains and from the Pasteur/ARM Clone 13 and ARM Clone 13/Pasteur reassortants for sequence analysis. Direct RNA sequence analysis using radioactive oligonucleotide primers and dideoxy chain termination has been described (4, 15). Oligonucleotides that anneal to both ARM and Pasteur viral RNA were chosen to identify the L and S RNAs of the reassortants. The oligonucleotide, CTGTTGATATCTTCAATCTG, chosen to identify the L RNA anneals to the 5' end (bases 310-329) (4). The oligonucleotide chosen to identify the S RNA, CATGAGTGTGTTGTTCAAAGGTC, anneals to the 5' end (bases 473-494) (16). Reactions were performed simultaneously with RNA from parental strains and reassortants and compared in parallel on buffer-gradient thin gels (17).

**In Situ Hybridization and Infective Center Analysis.** In situ hybridization studies of lymphocytes harvested from NOD mice using a \(^{35}\)S cDNA probe to the LCMV GP are described in the accompanying paper (2). Concurrently, infectious center analysis was done on materials from the same individual mice (10). Briefly, 2 \times 10\(^6\), 5 \times 10\(^5\), 2 \times 10\(^5\), or 2 \times 10\(^4\) lymphocytes were plated on confluent layers of Vero cells grown on petri dishes (Falcon Laboratories, Oxnard, CA) after absorption for 60 min at 37°C; 0.5% agarose was added, plates were incubated for 6–7 days at 37°C, and overlayed with crystal violet. The results were calculated as the number of infectious centers per 10\(^6\) viable PBL plated.

**Histopathology.** Pancreatic tissue was fixed in formalin, sectioned, and stained with hematoxylin and eosin. The percentage of normal islets of Langerhans present in each pancreas was calculated by counting at least 15 islets per pancreas and determining the number not destroyed by infiltrating lymphoid cells.
Results

Prevention of IDDM in NOD Mice by Distinct Strains of LCMV. Inoculation of groups of 15 or more newborn NOD mice, each with one of the four strains of LCMV, i.e., ARM, Pasteur, Traub, and WE, and a variant of LCMV ARM, Clone 13, resulted in persistent infection. The evidence was virus recovered from the blood and tissues and viral nucleic acid sequences visible in multiple tissues of animals killed and examined at increasing intervals of age. During the course of infection, the amount of virus carried and distribution of deposited LCMV sequences were apparently similar, regardless of the viral strain used to initiate infection. For example, testing of at least six individual sera from 4-mo-old mice inoculated with Clone 13, ARM, Pasteur, Traub, or WE showed titers that ranged from $4 \times 10^4$ to $5 \times 10^6$ PFU/ml. By whole mouse body sections, viral sequences in 4–6-mo-old persistently infected mice were routinely noted in brains, salivary glands, thymuses, lungs, livers, spleens, adrenals, and kidneys.

Infection with ARM, Pasteur, Traub, or WE was followed by near normal survival rates and the prevention of IDDM. Mice persistently infected with such viruses had survival rates that exceed 90% over the 12 mo of observation. Further, these mice had normal blood glucose and pancreatic insulin levels as well as normal morphology of the islets of Langerhans (Table I). In contrast, as also shown in Table I, uninfected NOD mice or those persistently infected with Clone 13 developed the elevated blood sugar and other manifestations of IDDM typical for this strain. At 9 mo of age, the incidence of IDDM in uninfected NOD mice was 90%, and, in Clone 13, persistently infected mice 76%. In contrast, NOD mice matched by age and gender, but persistently infected with LCMV ARM, Pasteur, Traub, or WE, had a <5% incidence of IDDM when 9 mo old.

| Group of NOD female | No. of mice | 3 mo Blood glucose (mg/dl) | 6 mo Blood glucose (mg/dl) | 9 mo Blood glucose (mg/dl) | Normal islets (% of normal) |
|---------------------|-------------|---------------------------|---------------------------|---------------------------|-----------------------------|
| Uninfected          | 40          | 172 ± 6                   | 291 ± 35*                 | 412 ± 32*                 | <5                          |
| LCMV infected       |             |                           |                           |                           |                             |
| Pasteur             | 17          | 129 ± 4                   | 185 ± 9                   | 134 ± 4                   | >70                         |
| Traub               | 18          | 151 ± 5                   | 126 ± 5                   | 126 ± 5                   | ND                          |
| WE 54               | 16          | 132 ± 3                   | 137 ± 3                   | 116 ± 8                   | ND                          |
| ARM                 | 19          | 154 ± 4                   | 126 ± 6                   | 155 ± 11                  | >70                         |
| ARM Clone 13        | 18          | 194 ± 9                   | 247 ± 25*                 | 316 ± 31*                 | <10                         |

Mice were given $10^3$ PFU of LCMV within the first 18 h of birth. LCMV strains used were Pasteur, Traub, WE, Armstrong 1371, Clone 53b, or its variant ARM Clone (Cl) 13. Such mice carried infectious virus throughout their lives. Blood glucose concentrations were determined by the glucose oxidase method. The percent of normal islets of Langerhans was calculated by counting up to 15 islets per pancreas and determining the number not destroyed by infiltrating lymphoid cells.

* p < 0.01 compared with NOD mice infected at birth with LCMV Pasteur, Traub, WE, or ARM.
Reassortants between LCMV ARM Clone 13 and LCMV Pasteur Map the Prevention of IDDM to the S RNA of Pasteur. Reassortant between LCMV Pasteur, which prevents IDDM, and LCMV ARM Clone 13, which does not, were made from reported stocks (14), retested biochemically, and inoculated into newborn NOD mice. The identity of each reassortant was confirmed by using oligonucleotides that annealed to both Pasteur and Clone 13 L or S RNA. The results in Fig. 1 show that Pasteur/ARM Clone 13 contained the characteristic Pasteur L RNA and ARM Clone 13 S RNA sequences, while that of ARM Clone 13/Pasteur reassortant had ARM L and Pasteur S RNA sequences. The strain-specific sequences (Fig. 1) reveal 84% nucleotide identity and 92% amino acid identity in the region of the L RNA analyzed, and 84% nucleotide identity and 88% amino acid identity in the region of the S RNA analyzed.

The use of these reassortants showed that the L RNA Pasteur/S RNA Pasteur (parental Pasteur strain) and the L RNA of Clone 13/S RNA Pasteur (therapeutic reassortant) were associated with normal concentrations of blood sugar. In contrast, the L RNA Clone 13/S RNA Clone 13 (parental Clone 13) and the L RNA Pasteur/S RNA Clone 13 (nontherapeutic reassortant) left hyperglycemia unchecked, as Table II shows. In confirmation of these findings, lymphocytes infiltrated the islets of Langerhans in mice receiving the Clone 13 parent or the reassortant without Pasteur S RNA (Table II, Fig. 2). Yet, >75% of the islets were normal in NOD mice persistently infected with the parental Pasteur strain or the reassortant containing Pasteur S RNA and Clone 13 L RNA (Table II, Fig. 2).

The amounts of virus in sera of NOD mice persistently infected with Clone 13/Clone 13, Pasteur/Pasteur, Pasteur/Clone 13, and Clone 13/Pasteur were equivalent. Thus, in 3-mo-old animals, the titers ± 1 SE were 2.9 × 10^5 ± 0.7 for 10 mice injected with Pasteur/Pasteur; 2.1 × 10^5 ± 0.7 for nine mice given Clone 13/Clone 13; 1.7 × 10^5 ± 0.3 for 10 mice receiving Pasteur/Clone 13, and 2.5 × 10^5 ± 0.5 for 11 recipients of Clone 13/Pasteur.

Sequence Differences between LCMV Pasteur and ARM Clone 13 in GP and NP. The S RNA of LCMV encodes but two viral proteins, GP and NP (3, 5, 6). Since the S RNA of LCMV Pasteur aborted IDDM of NOD mice, but the corresponding S RNA of ARM Clone 13 did not (Table II), we compared the areas of homology

![Figure 1](image-url)
TABLE II
Prevention of IDDM Maps to the S RNA of LCMV Pasteur

| S RNA/L RNA | 3 mo | 5 mo | 7 mo | 9 mo |
|-------------|------|------|------|------|
| Pasteur/Pasteur | 156 ± 4 | 153 ± 8 | 164 ± 8 | 162 ± 6 | 55 ± 9 | >80 |
| Ci 13/Ci 13 | 188 ± 8 | 258 ± 12 | 312 ± 42 | 369 ± 49 | 17 ± 5 | <20 |
| Ci 13/Pasteur | 161 ± 5 | 284 ± 51 | 317 ± 50 | 335 ± 47 | 2 ± 8 | <15 |
| Pasteur/Ci 13 | 146 ± 3 | 132 ± 7 | 167 ± 5 | 155 ± 6 | 63 ± 13 | >75 |

10^9 PFU of parental and reassortment viruses were inoculated into newborn NOD mice <18 h old.

* Blood glucose was determined by the glucose oxidase method. The percent of normal islets of Langerhans was calculated by counting up to 15 islets per pancreas and determining the number not destroyed by infiltrating lymphoid cells.

\[ p < 0.005. \]

and divergence in both proteins encoded by these two viral strains. The GP of LCMV has 499 amino acids, of which, 474 are in similar positions in Clone 13 and Pasteur, whereas 25 amino acids are replaced, indicating a divergence of 5%. The disparate amino acids and their positions are noted in Fig. 3. For the NP, divergence is 3.9% (Fig. 3), with 22 amino acids replaced of the 558 total.

Replication of LCMV Pasteur and LCMV ARM Clone 13 in CD4+ Lymphocytes. The last series of experiments documented the replication of virus in lymphocytes and assessed the lymphocyte subset infected. Adult NOD mice persistently infected with either Pasteur or ARM Clone 13 since birth contained LCMV predominantly in their CD4+ lymphocytes. Fig. 4 records the presence of infective centers in CD4+ lymphocytes obtained from 7-mo-old NOD mice persistently infected with LCMV Pasteur. By FACS analysis, <1.7% of CD4+ lymphocytes were contaminated with CD4+ cells (Fig. 4). A similar tropism for these CD4+ cells was noted after infectious centers assayed of mice infected with ARM Clone 13. Approximately twice as many CD4+ cells from Pasteur-infected mice scored as infectious centers as from mice infected with ARM Clone 13.

Discussion

In this paper, we show that the S RNA segment of LCMV Pasteur is linked to the prevention of IDDM associated with NOD mice. This conclusion was determined by first showing that the LCMV ARM variant, Clone 13, failed to abort IDDM, whereas other LCMV strains, including LCMV Pasteur, prevented the disease. Second, using reassortants between ARM Clone 13 and Pasteur to produce persistent infection in NOD mice, we found that the S RNA of Pasteur prevented IDDM, but the L RNA of this virus did not.

Complete nucleotide sequencing of the S RNA of Pasteur and Clone 13 (16; Salvato, M. S., K. J. Schweighofer, E. M. Shimomaye, and M. B. A. Oldstone, manuscript submitted for publication) showed a 5% divergence in the GP molecules from both viruses. The cause was 25 different amino acids of the 499 that compose Pasteur
Figure 2. Islets of Langerhans from 9-mo-old NOD mice treated with LCMV Pasteur, LCMV Clone 13, or their reassortants, (×450). Upper row left and center show islets from LCMV Pasteur or L RNA Clone 13/S RNA Pasteur-infected mice, respectively. On occasion (<25% of islets), mild to moderate infiltration in or around the islet was observed as documented in upper row right on tissue from a LCMV Pasteur-infected mouse. Lower row shows islets from Clone 13 (left, center) and L RNA Pasteur/S RNA Clone 13 (right) infected mice.
FIGURE 3. Comparisons of amino acid divergence among proteins encoded by the S RNA of the LCMV ARM Clone 13 and LCMV Pasteur. The S RNA encodes two proteins, the GP from its 5' end, and the NP from the 3' end. The GPs of ARM Clone 13 and Pasteur contain 499 amino acids, and the NPs contain 558.

GP and Clone 13 GP. For the NP, the difference was 22 of 558 amino acids that characterized the protein of both viruses. Divergence occurred at random throughout both molecules. No specific type of mutation was noted such as the C to U transitions recently reported with another negative strand virus that causes persistent in-

FIGURE 4. LCMV Pasteur (PAST) and LCMV Clone (Cl) 13 replicate preferentially in CD4* lymphocytes. The upper panel illustrates an infectious center assay on CD4+ and CD4* lymphocytes recovered from 7-mo-old NOD mice persistently infected with PAST. From left to right, 10^5, 10^4, and 10^3 PBL were incubated with a confluent monolayer of Vero cells. Plaques were read 5 d later. The lower panel records the purity of the lymphocyte populations used in the infectious center assay. Cells were obtained from the portion of the display.
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Infection (18, 19). Hence, the S RNA is clearly linked to prevention of IDDM, but we are, at present, unable to identify the region more precisely.

LCMV Pasteur and LCMV ARM Clone 53B both prevent IDDM (Table I). Because of sequence variability between LCMV Pasteur and ARM Clone 13 variant, it was possible to easily detect reassortants between these two viruses. In contrast, LCMV ARM and Clone 13 show high sequence conservation with complete homology in the NP and just two base substitutions in the GP (16; Salvato et al., manuscript submitted for publication). Reassortants between these two strains have, so far, not been generated. However, sequencing shows two mutations in the GP, one leading to a significant amino acid substitution. Such data (16; Salvato et al., manuscript submitted for publication), in conjunction with results reported here, suggest that the structural determinant capable of blocking IDDM likely maps to the GP of LCMV. However, the precise mapping of the IDDM-preventing trait of these viruses awaits the ability to make recombinants of the S RNA or the acquisition of infectious cDNA. None of these have yet been accomplished. The recent ability to make cDNA from another negative strand virus, influenza (20), suggests that a similar approach may be applied to LCMV. With this technology, the precise gene and its component needed to prevent IDDM can be mapped.

Virusescaninfect, and have profound effects on, cells that participate in immune responses, with resultant immunosuppression or immune enhancement (reviewed in references 21 and 22). Several studies have focused on manipulating immune responses of autoimmune disorders, and a variety of these diseases have been successfully controlled. Examples are use of LCMV to abrogate IDDM in both NOD mice (1) and BB diabetes-prone rats (23), as well as leukemia/lymphoma of MRL/lpr mice (M. B. A. Oldstone and R. Ahmed, unpublished data); lactic dehydrogenase virus to inhibit experimental allergic encephalomyelitis (24) and measles virus to prevent nephrotic nephrosis (25). Bacterial and fungal products have long been manipulated to provide therapeutic agents. It should be possible to similarly harness viruses or their products towards that goal.

Summary

Nonobese diabetic (NOD) mice are the experimental prototype of type 1 insulin-dependent diabetes mellitus (IDDM). These mice develop a characteristic autoimmune lesion in the pancreatic islets of Langerhans, where infiltrating lymphocytes destroy β cells, resulting in hypoinsulinemia, hyperglycemia, ketoacidosis, and death. This IDDM, which closely resembles that in humans, is prevented by infecting NOD mice with particular strains of lymphocytic choriomeningitis virus (LCMV), including Armstrong 53b, Traub, WE, and Pasteur. In contrast, the LCMV Armstrong 53b variant, Clone 13, fails to abort IDDM. Hence, although Clone 13 establishes a persistent infection that endures throughout the life spans of NOD mice, their hyperglycemia, hypoinsulinemia, and lymphocytic infiltration into the islets of Langerhans still occur. Genetic reassortant viruses generated between the IDDM therapeutic strain of LCMV Pasteur and the nontherapeutic variant, LCMV Clone 13, were used to treat NOD mice. By using such reassortants and both parental strains of virus to infect NOD mice, the prevention of IDDM was mapped to the S RNA segment of LCMV Pasteur.
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