JOURNAL OF VIROLOGY, Dec. 1997, p. 9782–9785 Vol. 71, No. 12
0022-538X/97/$04.00+0

Gain or Loss of Diabetogenicity Resulting from a Single Point Mutation in Recombinant Encephalomyocarditis Virus

HEE-SOOK JUN,[1] YUP KANG,[2] ABNER L. NOTKINS,[3] AND JI-WON YOON[1,2]*

Laboratory of Viral Immunopathogenesis of Diabetes, Julia McFarlane Diabetes Research Centre, Department of Microbiology and Infectious Diseases, Faculty of Medicine, The University of Calgary, Calgary, Alberta, Canada T2N 4N1[1]; Laboratory of Endocrinology, Institute for Medical Science, Department of Endocrinology and Metabolism, School of Medicine, Ajou University, Suwon, Korea[2]; and Oral Infection and Immunity Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20892-4322[3]

Received 9 July 1997/Accepted 28 August 1997

Molecular pathogenic mechanisms for virus-induced disease have received considerable attention. Encephalomyocarditis (EMC) virus-induced diabetes in mice has been extensively studied to elucidate the cellular and molecular mechanisms involved in the development of this disease. In this study, we report for the first time that a single point mutation at nucleotide position 3155 or 3156 of the recombinant EMC viral genome, located on the major capsid protein VP1, which causes an amino acid change, results in the gain or loss of viral diabetogenicity. A G base at nucleotide position 3155 (alanine at amino acid position 776 of the EMC virus polyprotein [Ala^{776}]; GCC) results in viral diabetogenicity, whereas the substitution of other bases at the same or next position results in a loss of viral diabetogenicity. This finding provides clear evidence that a point mutation at a critical site in a viral genome affects the ability of the virus to cause a cell-specific disease.

One of the best examples indicating that viruses play an etiological role in the pathogenesis of insulin-dependent diabetes mellitus (IDDM) comes from mice infected with encephalomyocarditis (EMC) virus (8, 13, 21, 22). The M variant of EMC (EMC-M) virus induces diabetes by infecting and destroying pancreatic beta cells in susceptible mice. However, this effect was found to be inconsistent (15, 24). Through plaque purification of EMC-M virus, we isolated two stable variants, the highly diabetogenic EMC-D virus and the nondiabetogenic EMC-B virus (22). We used these two variants to identify the viral gene or genes responsible for the induction of diabetes in mice and to study the long-term complications of virus-induced diabetes (25). Our initial examination of the genomes of these two variants revealed a total of 14 nucleotide differences between them (2). Further studies of 21 different nondiabetogenic and 15 different diabetogenic viruses derived from stocks of the EMC-B and EMC-D variants revealed that only the 776th amino acid, alanine (Ala^{776}), of the EMC virus polyprotein, located on the major capsid protein VP1, is common to all diabetogenic variants. In contrast, Thr at this position (Thr^{776}) is common to all nondiabetogenic variants (1). Identification of this difference between the diabetogenic and nondiabetogenic variants of EMC virus was not sufficient to determine whether Ala^{776} plays a critical role in the development of IDDM in EMC virus-infected mice.

We next needed to perform functional tests to determine whether the exchange of Ala^{776} for Thr^{776}, and vice versa, affects viral diabetogenicity. Previously, it was not possible to perform such experiments because of the characteristic long poly(C) tract, which makes it difficult to amplify viral cDNA in bacterial systems (1). We developed a new recombinant RNA technique involving the creation of a chimeric RNA by attaching the poly(C) region of the viral RNA to the 5′-truncated RNA transcript of the EMC viral cDNA (3). We used this technique and site-specific mutagenesis at nucleotide position 3155 of the EMC viral genome to investigate the role of Ala^{776} in the diabetogenicity of EMC virus in susceptible SJL/J mice.

We first changed the amino acid at position 776 (Ala; GCC) of the polyprotein of diabetogenic EMC virus to Thr (ACC) by site-specific mutagenesis with a Muta-Gene kit (Bio-Rad, Richmond, Calif.). In order to obtain a uracil-containing single-stranded template, an EMC-D viral cDNA fragment covering bases 1870 to 3190 from pD104 (1, 3) was subcloned to the *Sph*I and *Sst*I sites of a phagemid, pTZ19R, and then transfected into *Escherichia coli* CJ236 (Dut$^-$ Ung$^-$). The uracil-containing single-stranded recombinant phagemid was isolated by superinfection of the M13K07 helper phage. After annealing the mutagenic oligonucleotide containing the desired base (Thr^{776}-CCAACTGGAACGCCAACCAAGCCAA CCACTC) to the single-stranded template, a mutant strand was synthesized by T4 DNA polymerase and joined by T4 DNA ligase (3). This heteroduplex recombinant phagemid was introduced into *E. coli* MV1190 (Ung$^+$). Mutants were screened by the double-stranded dideoxy DNA sequencing method using the M13 universal primer (U.S. Biochemicals, Cleveland, Ohio). To obtain mutated transcript RNA, *Sph*I and *Sst*I fragments (nucleotides 1870 to 3190), mutated at nucleotide position 3155, were subcloned to the corresponding site of pEDfH (1, 3). In vitro transcription was carried out with T7 RNA polymerase after linearization of the plasmid with *Sal*I. The 5′ viral RNA (nucleotides 1 to 476) was isolated by gel elution after cutting of EMC-D viral RNA between nucleotide positions 476 and 477 with RNase H (17). This 5′ viral RNA (nucleotides 1 to 476) was joined to a mutated transcribed RNA [nucleotide 477 to the 3′ poly(A) tail] by using RNA ligase and a brace primer to construct chimeric RNA (3). The chimeric RNA was transfected into an L929 cell monolayer (6), and progeny viruses were isolated from the L929 cells. The sequence of the mutated region (i.e., GCC [Ala^{776}]→ACC [Thr^{776}]) of the progeny viruses was confirmed by direct RNA sequencing. Similarly, we changed the amino acid at position 776 (Thr; ACC) of the polyprotein of nondia-

* Corresponding author. Mailing address: Laboratory of Viral Immunopathogenesis of Diabetes, Julia McFarlane Diabetes Research Centre, Faculty of Medicine, The University of Calgary, 3330 Hospital Dr. NW, Calgary, Alberta, Canada T2N 4N1. Phone: (403) 220-4569. Fax: (403) 270-7526. E-mail: yoon@acs.ucalgary.ca.

TABLE 1. Effects of alanine and threonine at amino acid position 776 of the EMC virus polyprotein on induction of diabetes in EMC virus-infected SJL/J mice

Virus	No. of mice	Glucose index[a] (mg/dl; mean ± SD)	Pancreatic insulin level[b] (μg/g of pancreas; mean ± SD)	% with diabetes
None	32	129 ± 23	383 ± 47	0
EMC-D	21	436 ± 87	23 ± 8	100
Ala^{776} chimeric	17	406 ± 97	31 ± 9	100
Thr^{776} chimeric	20	153 ± 21	337 ± 36	0

[a] Blood glucose levels were measured in virus-infected (10^5 PFU/mouse, intraperitoneally) and PBS-injected mice by glucose oxidase assay with *o*-dianisidine dihydrochloride as the indicator dye, and the glucose index was calculated (22, 24). Briefly, for each mouse, nonfasting (NF) glucose levels were measured on days 7 and 14, and glucose tolerance tests (GTT) were performed on days 10 and 17. These four values were then combined to give the glucose index for each mouse in order to eliminate some of the variability associated with individual values which may occur when single glucose determinations are used: [(4 × NF day 14) + (3 × NF day 7) + (2 × GTT day 17) + (1 × GTT day 10)]/10. Any mouse with a glucose index greater than 244 mg/dl (5 standard deviations above the mean for nondiabetic control PBS-injected mice) was classified as diabetic (22).

[b] Insulin was extracted from the pancreas and measured by radioimmunoassay at 17 days after infection (22).

betogenic EMC virus to Ala (GCC) with the appropriate oligonucleotide (Ala^{776}-CCAACTGGAACGCCAGCCAAGCC AACCACTC) using pB104 (1), the EMC-B viral cDNA clone containing nucleotide bases 1120 to 3530. The *Sph*I-to-*Sst*I fragment containing the mutated nucleotide was substituted into the corresponding site of pEBfH, and the RNA transcript was synthesized after linearization with *Sal*I. The 5′ viral RNA fragment was isolated from EMC-B viral RNA after cutting with RNase H and joined to a mutated transcribed RNA (3).

To determine whether each mutated chimeric virus, containing either Ala^{776} or Thr^{776}, induced diabetes in SJL/J mice, we infected each animal with 10^5 PFU of recombinant progeny virus intraperitoneally (21, 22). Phosphate-buffered saline (PBS)-injected and wild-type EMC-D virus-infected (10^5 PFU/mouse) mice were used as negative and positive controls, respectively. Nonfasting blood glucose levels were measured at 7 and 14 days after virus infection, and glucose tolerance tests were conducted at 10 and 17 days after infection. These four values were then combined to determine a glucose index for each mouse as described previously (15, 22, 24). All of the EMC-D virus-infected mice became diabetic, as indicated by their mean blood glucose index of 436 ± 87 mg/dl. Similarly, all Ala^{776} chimeric virus-infected mice became diabetic, with a mean blood glucose index of 406 ± 97 mg/dl. In contrast, none of the Thr^{776} chimeric virus-infected mice became diabetic, as indicated by their mean blood glucose index of 153 ± 21 mg/dl, not significantly different from that of the PBS-injected control mice (129 ± 23 mg/dl) (Table 1).

We also measured pancreatic insulin content by radioimmunoassay (22). Pancreatic insulin concentrations were low for both EMC-D virus-infected and Ala^{776} chimeric virus-infected mice, with levels of 23 ± 8 and 31 ± 9 μg/g of pancreas, respectively. In contrast, pancreatic insulin concentrations were significantly higher in Thr^{776} chimeric virus-infected and PBS-injected control animals, with levels of 337 ± 36 and 383 ± 47 μg/g of pancreas, respectively (Table 1).

To determine whether the differences in blood glucose levels and pancreatic insulin content were reflected in the histopathology of the pancreas, groups of experimental and control mice, treated as described above, were sacrificed at 17 days after infection. Microscopic examination of pancreatic sections revealed extensive beta-cell destruction in Ala^{776} chimeric virus-infected mice (Fig. 1B). Approximately 94% of examined islets showed lymphocytic infiltration, with 25% of the islets showing mild to moderate lymphocytic infiltration, 51% showing severe pathological alterations, and 18% showing atrophied, small, retracted islets with severe beta-cell necrosis, with or without residual lymphocytic infiltrate (Table 2). In contrast, no pathological changes were found in the islets of Thr^{776} chimeric virus-infected mice (Fig. 1A; Table 2) or PBS-injected control mice. On the basis of blood glucose levels, pancreatic insulin content, and islet histopathology, we conclude that the chimeric virus containing Ala^{776} induces IDDM as effectively as does the wild-type EMC-D virus. In contrast, the chimeric virus containing Thr^{776} in place of Ala^{776} shows no diabetogenicity.

To determine whether the substitution of other amino acids for Ala^{776} has any effect on the diabetogenicity of EMC virus, we constructed all possible base permutations of either the first or second base position of codon 776, using site-specific mutation with appropriate oligonucleotides (Ala^{776}-CCAACTGG

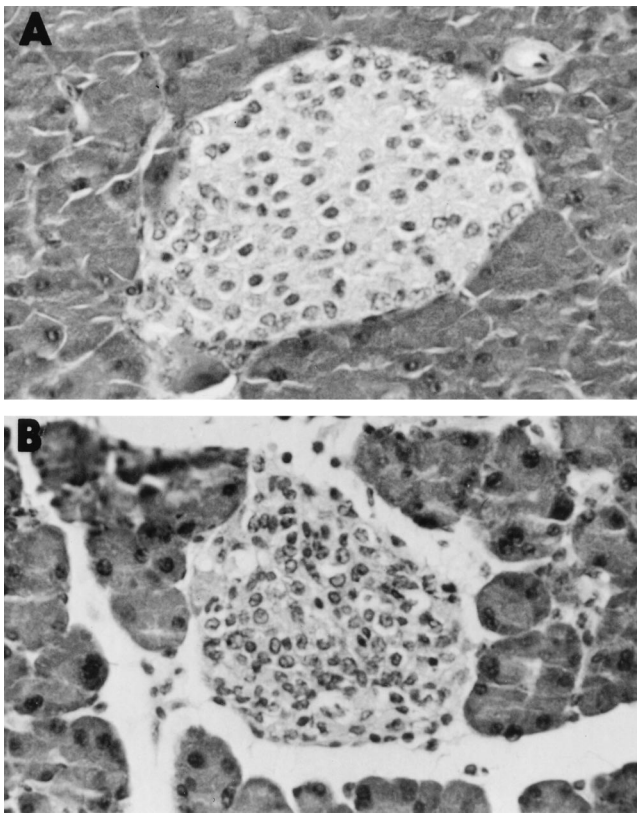

FIG. 1. (A) A pancreatic section from mice infected with Ala^{776} chimeric virus shows extensive inflammatory infiltration of the islets and beta-cell necrosis. (B) A pancreatic section from mice infected with Thr^{776} chimeric virus shows no inflammation. Original magnification, ×400.

TABLE 2. Histological changes in the islets of Langerhans from SJL/J mice infected with recombinant chimeric viruses[a]

Recombinant chimeric virus	Development of diabetes	Total no. of islets examined	No. of islets (%) with the indicated histological changes[b]			
			N	M	S	A
Ala^{776}	Yes	88	5 (5.7)	22 (25.0)	45 (51.1)	16 (18.2)
Thr^{776}	No	95	95 (100)	0 (0)	0 (0)	0 (0)
Ser^{776}	No	87	87 (100)	0 (0)	0 (0)	0 (0)
Pro^{776}	No	103	103 (100)	0 (0)	0 (0)	0 (0)
Asp^{776}	No	92	92 (100)	0 (0)	0 (0)	0 (0)
Val^{776}	No	112	112 (100)	0 (0)	0 (0)	0 (0)

[a] Five- to six-week-old SJL/J mice were infected with recombinant chimeric viruses (10^5 PFU/mouse, intraperitoneally).
[b] N, normal islets with normal morphology; M, mild to moderate degree of insulitis with lymphocytic infiltration of 1 to 49% of islets and well-preserved islet architecture; S, severe insulitis with lymphocytic infiltration of 50 to 100% of islets; A, atrophied, small, retracted islets showing severe beta-cell necrosis, with or without residual lymphocytic infiltrate (11). Results are based on 15 to 20 islets per mouse and five to seven mice per group.

AACGCCAGCCAAGCCAACCACTC, Thr^{776}-CCAACTGG AACGCCAACCAAGCCAACCACTC, Ser^{776}-CCAACTGG AACGCCATCCAAGCCAACCACTC, Pro^{776}-CCAACTGG AACGCCACCCAAGCCAACCACTC, Asp^{776}-CCAACTGG AACGCCAGACAAGCCAACCACTC, and Val^{776}-CCAAC TGGAACGCCAGTCAAGCCAACCACTC). These manipulations resulted in EMC viruses with alanine, threonine, serine, proline, aspartic acid, or valine at position 776 of the EMC virus polyprotein. None of these viruses, with the exception of those containing Ala^{776}, produced insulitis (Table 2) or diabetes (Table 3) in infected SJL/J mice, indicating the importance of alanine in EMC virus diabetogenicity. We cannot, however, exclude the possibility that other, untested amino acid substitutions, requiring site-specific mutation at two or more base positions in the codon, produce diabetogenic viruses. We are currently investigating this possibility.

To determine the infectivity of recombinant EMC viruses with different amino acid substitutions at position 776 of the polyprotein, we measured antiviral antibodies in the serum of infected SJL/J mice. Antibody titers were high in all groups, ranging from 1,280 to 2,560, and were not significantly different among the groups. However, only those animals infected with Ala^{776} chimeric virus showed EMC virus-specific antigens in their beta cells (Table 3).

The significance of the 776th amino acid on the EMC virus polyprotein in the early stages of EMC virus infection and the pathogenesis of IDDM is not clear. By analogy to the structurally similar mengovirus (12), the 776th amino acid lies in the pit area, the proposed attachment site for mengovirus. Furthermore, this residue is in a highly conserved hydrophilic patch of the EMC virus major capsid protein, VP1, containing three proximal prolines (Pro-Thr-Gly-Thr-Pro-Ala^{776}-Lys-Pro). Such sites are proposed as viral attachment sites in picornaviruses (16). Our earlier study showed that an amino acid change from Thr^{776} (nondiabetogenic EMC-B virus) to Ala^{776} (diabetogenic EMC-D virus) reduced the hydrophilicity of this region by 37% (9). We speculate that a shift away from optimal hydrophilicity may affect the initial interactions between the virus and the beta cells. Thus, more hydrophobic (valine) or more hydrophilic (threonine, serine, proline, and aspartic acid) amino acid substitutions may adversely affect the attachment of the virus to beta cells. In addition, alanine may play an important role in viral penetration, uncoating of hydrophobic patches buried inside the capsid, or release of viral RNA from the capsid. These possibilities remain to be investigated.

Finally, genetic factors may exert a major influence on the development of IDDM (4). However, much evidence suggests that environmental factors are also important, influencing the penetrance of diabetes susceptibility genes (19). For example, the concordance rate for IDDM between monozygotic, genetically identical twins is 40% or less (5). As an environmental factor affecting the induction of IDDM, the virus may be considered a primary agent injurious to pancreatic beta cells and/or a triggering agent of beta-cell-specific autoimmunity, resulting in the onset of IDDM (7, 10, 18). The development of diabetes in mice infected with the EMC virus provides the best evidence that viruses are primary injurious agents inducing diabetes (8, 13, 22). For the past 30 years, EMC virus-induced diabetes has been extensively studied (18). However, the molecular pathogenic mechanisms involved in the development of this disease have not been elucidated. In this study, we have demonstrated for the first time that a single amino acid at

TABLE 3. Effects of amino acid substitutions at position 776 of the EMC virus polyprotein on the infection of beta cells and the induction of diabetes in SJL/J mice

Amino acid at position 776	Nucleotides[a]	Antiviral antibody titer[b]	Presence of viral antigen[c]	Glucose index[d] (mg/dl; mean ± SD)	Pancreatic insulin level (μg/g of pancreas; mean ± SD)	% with diabetes
Alanine	GCC	1,280	+++	407 ± 98	30 ± 91	90
Threonine	ACC	1,280	−	149 ± 19	391 ± 36	0
Serine	TCC	2,560	−	159 ± 21	387 ± 41	0
Proline	CCC	1,280	−	142 ± 19	396 ± 43	0
Aspartic acid	GAC	2,560	−	169 ± 21	379 ± 37	0
Valine	GTC	2,560	−	172 ± 18	398 ± 45	0

[a] Point mutations at nucleotide position 3155 or 3156 were made with appropriate synthetic oligonucleotides.
[b] Sera were obtained from mice 17 days after infection, and the titer of neutralizing anti-EMC antibodies was determined. Values are expressed as the mean reciprocal of the highest dilution of serum that inhibited plaque formation by 50%.
[c] At 3 days after infection, sections of pancreas from three randomly selected mice were stained with fluorescein-labelled anti-EMC virus antibody and rhodamine-labelled anti-insulin antibody to determine the presence of viral antigens in the beta cells (14, 20, 23). −, no colocalized fluorescence in the examined islets; +++, colocalized fluorescence in over 50% of the examined islets.
[d] Ten mice per group; 10^5 PFU of recombinant virus/mouse.

position 776 of the EMC virus polyprotein determines viral diabetogenicity. The change of a single amino acid (Ala→Thr) at amino acid position 776 of the polyprotein in diabetogenic EMC virus results in a loss of viral diabetogenicity. Similarly, the change of a single amino acid (Thr→Ala) at the same position in nondiabetogenic EMC virus results in a gain of viral diabetogenicity.

This work was supported by grants MA9584 and MTI3224 from the Medical Research Council of Canada and the Canadian Diabetes Association to J.W.Y. J.W.Y. is a Heritage Medical Scientist awardee of the Alberta Heritage Foundation for Medical Research.

We gratefully acknowledge the editorial assistance of Karen Clarke and the secretarial assistance of Alannah Ireland.

REFERENCES

1. **Bae, Y. S., and J. W. Yoon.** 1993. Determination of diabetogenicity attributable to a single amino acid, Ala-776, on the polyprotein of encephalomyocarditis virus. Diabetes **42:**435–443.
2. **Bae, Y. S., H. M. Eun, and J. W. Yoon.** 1989. Genomic differences between the diabetogenic and nondiabetogenic variants of encephalomyocarditis virus. Virology **170:**282–287.
3. **Bae, Y. S., Y. Kang, E. Ohtsuka, and J. W. Yoon.** 1993. Development of a recombinant RNA technique for the construction of chimeric RNA with a long poly(C) tract. Nucleic Acids Res. **21:**2703–2708.
4. **Bain, S. C., C. H. Mijovic, and A. H. Barnett.** 1997. Genetic factors in the pathogenesis of insulin-dependent diabetes mellitus, p. 13.1–13.13. *In* J. Pickup and G. Williams (ed.), Textbook of diabetes. Blackwell Scientific Publications, London, United Kingdom.
5. **Barnett, A. H., C. Eff, R. D. G. Leslie, and D. A. Pyke.** 1981. Diabetes in identical twins. A study of 200 pairs. Diabetologia **20:**87–93.
6. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7:**2745–2752.
7. **Chung, Y. H., H. S. Jun, Y. Kang, K. Hirasawa, B. R. Lee, N. Van Rooijen, and J. W. Yoon.** 1997. Role of macrophages and macrophage-derived cytokines in the pathogenesis of Kilham rat virus-induced diabetes in diabetes-resistant BB rats. J. Immunol. **159:**466–471.
8. **Craighead, J. E., and M. F. McLane.** 1968. Diabetes mellitus: induction in mice by encephalomyocarditis virus. Science **162:**913–914.
9. **Eun, H. M., Y. S. Bae, and J. W. Yoon.** 1988. Amino acid differences in the capsid protein, VP1, between diabetogenic and nondiabetogenic variants of encephalomyocarditis virus. Virology **163:**369–373.
10. **Guberski, K. L., V. A. Thomas, W. R. Shek, A. A. Like, E. S. Handler, A. A. Rossini, J. E. Wallace, and R. M. Welsh.** 1991. Induction of type 1 diabetes by Kilham's rat virus in diabetes-resistant BB/Wor rats. Science **254:**1010–1013.
11. **Jun, H. S., S. W. Yoon, Y. Kang, C. Y. Pak, M. C. Lee, and J. W. Yoon.** 1995. Cloning and expression of the VP1 major capsid protein of diabetogenic encephalomyocarditis (EMC) virus and prevention of EMC virus-induced diabetes by immunization with the recombinant VP1 protein. J. Gen. Virol. **76:**2557–2566.
12. **Luo, M., G. Vriend, G. Kamer, I. Minor, E. Arnold, M. G. Rossman, U. Boege, D. G. Scraba, G. M. Duke, and A. C. Palmenberg.** 1987. The atomic structure of mengo virus at 3.0 Å resolution. Science **235:**182–191.
13. **Notkins, A. L., J. W. Yoon, T. Onodera, and A. B. Jenson.** 1981. Virus-induced diabetes. Perspect. Virol. **XI:**141–162.
14. **Onodera, T., A. B. Jenson, J. W. Yoon, and A. L. Notkins.** 1978. Virus-induced diabetes mellitus: reovirus infection of pancreatic beta cells in mice. Science **301:**529–531.
15. **Ross, M. E., T. Onodera, K. S. Brown, and A. L. Notkins.** 1976. Virus-induced diabetes mellitus. IV. Genetic and environmental factors influencing the development of diabetes after infection with the M variant of encephalomyocarditis virus. Diabetes **25:**190–197.
16. **Rossmann, M. G., E. Arnord, J. W. Erickson, E. A. Frankenberger, J. P. Griffith, H. J. Hecht, J. E. Johnson, G. Kamer, M. Luo, A. G. Mosser, R. R. Lueckert, B. Sherry, and G. Vriend.** 1985. Structure of human common cold virus and functional relationship to other picornaviruses. Nature **317:**145–153.
17. **Shibahara, S., S. Mukai, T. Nishihara, H. Inoue, E. Ohtsuka, and H. Morisawa.** 1987. Site-directed cleavage of RNA. Nucleic Acids Res. **15:**4403–4415.
18. **Yoon, J. W.** 1995. A new look at viruses in type I diabetes. Diabetes Metab. Rev. **11:**83–107.
19. **Yoon, J. W.** 1997. Pathogenesis of IDDM: environmental factors, p. 14.1–14.14. *In* J. Pickup and G. Williams (ed.), Textbook of diabetes. Blackwell Scientific Publications, London, United Kingdom.
20. **Yoon, J. W., and C. J. Bachurski.** 1984. Double labelled immunofluorescent techniques for the screening of diabetogenic viruses, p. 313–326. *In* S. L. Pohl and J. Larner (ed.), Methods in diabetes, vol. 1A. John Wiley and Sons, New York, N.Y.
21. **Yoon, J. W., M. A. Lesniak, R. Fussganger, and A. L. Notkins.** 1976. Genetic differences in susceptibility of pancreatic β-cells to virus-induced diabetes mellitus. Nature **264:**178–180.
22. **Yoon, J. W., P. R. McClintock, T. Onodera, and A. L. Notkins.** 1980. Virus-induced diabetes mellitus. XVIII. Inhibition by a nondiabetogenic variant of encephalomyocarditis virus. J. Exp. Med. **152:**878–892.
23. **Yoon, J. W., T. Onodera, B. Jenson, and A. L. Notkins.** 1978. Virus-induced diabetes mellitus. XI. Replication of coxsackie B3 virus in human pancreatic beta cell culture. Diabetes **27:**778–781.
24. **Yoon, J. W., T. Onodera, and A. L. Notkins.** 1977. Virus-induced diabetes mellitus. VIII. Passage of encephalomyocarditis virus and severity of diabetes in susceptible and resistant strains of mice. J. Gen. Virol. **37:**225–232.
25. **Yoon, J. W., M. M. Rodrigues, C. Currier, and A. L. Notkins.** 1982. Long-term complications of virus-induced diabetes mellitus in mice. Nature **296:**566–569.