5-1988

Studies on Encephalomyocarditis Virus-Induced Diabetes in Mice

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STUDIES ON ENCEPHALOMYOCARDITIS VIRUS-INDUCED DIABETES IN MICE

by

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A dissertation submitted in partial fulfillment of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Biology

(Virology)

UTAH STATE UNIVERSITY
Logan, Utah

1988

ACKNOWLEDGMENTS

It is with great appreciation that I acknowledge the helpful suggestions of those who so willingly served on my graduate committee. Dr. Reed P. Warren, chairman of the committee, and Dr. Robert W. Sidwell provided invaluable help during the early writing. I especially express my thanks to my wife, Mary, who encouraged me and provided a great deal of technical help in accomplishing some of the experiments.

John Henry Huffman

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ABSTRACT

Studies on Encephalomyocarditis Virus-Induced Diabetes in Mice

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The D-variant of encephalomyocarditis (EMC-D) virus was given to SJL/J mice by the artificial, intraperitoneal (ip) route of infection, or by the natural routes of infection per os (po) or intranasal (i.n.), in comparable concentrations. The po route of infection was found to be ineffective. Mice given virus by either the ip or i.n. routes of infection became diabetic. Mice were more resistant to the infectious and diabetogenic properties of the virus when given by the i.n. than when given by the ip route. Glycosuria in mice given virus i.n. usually lagged one day behind that in mice given virus ip.

Measurement of glucose in mouse urine by use of Diastix® reagent strips was found to be a reliable indicator of diabetes. This test was easily and quickly accomplished without harm or pain to the mice.

Crude virus preparations were compared to purified virus preparations for their diabetogenic and infectious properties in mice. No statistically significant differences in either parameter were observed. Virus prepared by a

single passage in BHK-21 cells was fully diabetogenic, contrary to a previously published report.

Male SJL/J mice were infected with a diabetogenic dose of EMC-D virus by the i.n. route. Relative times of development of virus infection and mouse resistance parameters were compared to the time of development of signs of diabetes in the mice. A rapid decrease of plasma interferon titer corresponded to the time of development of signs of diabetes in the infected mice. Whether this was coincidental or has some significance in development of diabetes is unknown.

Tissue sections from pancreas, spleen, kidney, liver, lung, heart, and thymus were studied by immunohistochemical staining techniques for the presence of virus antigen, insulin, and the three types of mouse interferon (α, β, and γ).

Glucose was excreted in the saliva of mice with glycosuria. Previous reports of this excretion in diabetic mice have not been found in the literature. Mice without glycosuria did not excrete measurable (by Clinistix® or Diastix®) glucose in saliva.

Some mice were able to control the polyuria, polydipsia, and polyphagia normally seen in diabetes mellitus. These mice eventually reverted from having signs of diabetes to a normal state of plasma glucose and urine devoid of glucose. The mechanisms by which the mice were able to do this are unknown at this time.

(133 pages)

CHAPTER I

INTRODUCTION

The disease known as diabetes is actually a collection of many different disorders, all of which cause excessive excretion of urine. The definition of diabetes given in Webster's Ninth New Collegiate Dictionary (1, p. 349) is

> [L, fr. Gk *diabetes* , lit., siphon, fr. *diabainein*] (15c) : any of various abnormal conditions characterized by the secretion and excretion of excessive amounts of urine; *esp* : DIABETES MELLITUS

Taber (2, p. D-20) provides 10 terms to explain the different entities known collectively as diabetes. The definition of diabetes mellitus, or true diabetes, follows.

> A disorder of carbohydrate metabolism characterized by hyperglycemia, and glycosuria, resulting from inadequate production or utilization of insulin.

Diabetes mellitus is further characterized into two general subcategories, depending on the patient's requirement for externally administered insulin to control hyperglycemia. These categories are juvenile-onset (type-1, or insulin-dependent) diabetes and adult-onset (type-2, or noninsulin-dependent) diabetes. This investigation is limited to a study of the juvenile-onset category of diabetes mellitus using an animal model.

Although diabetes mellitus is known to involve inadequate production or utilization of insulin, the initiation of the inadequacies of insulin activity is not well-understood. In 1927, Gunderson (3) proposed that viral infection could initiate the onset of diabetes. Although no virus had been identified as a causative agent of diabetes, he made the observation that an increased frequency of diabetes closely followed epidemics of mumps in children. More

recently, Craighead (4) reviewed findings which support viral infections as one possible cause of juvenile-onset diabetes. Mumps outbreaks have often been noted in close association to development of symptoms of diabetes. However, there is a lack of documented lesions in the islets of Langerhans in the few autopsy reports on individuals dying early in the course of infection with mumps virus. Other viruses common to childhood epidemic disease have also been implicated in juvenile-onset diabetes by close association in time with onset and/or by increased prevalence and higher titers of virus-specific antibodies. Relevant viruses known to cause pancreatitis in man include, in addition to mumps virus, rubella virus, coxsackievirus group B type 4, and cytomegalovirus (4).

To effectively study virus as a possible cause of diabetes mellitus, it is necessary to use an animal/virus model system. There are animal models for the study of virally induced diabetes that employ lymphocytic choriomeningitis virus (LCMV) (5), Venezuelan encephalitis virus (6), rubella virus (7), coxsackievirus B4 (8), foot-and-mouth disease virus (9), reovirus (10), and encephalomyocarditis (EMC) virus (11). Each is characterized by particular advantages and disadvantages. Thus, a model system was chosen that would best answer the questions posed by the study prospectus.

The mouse model using EMC virus was chosen for this study because EMC virus is a murine enterovirus (12), and is natural to the model host. It has also been isolated from stools of human beings and from several other genera of vertebrates, presumably occuring there as the result of natural infection (12). In addition, Yoon et al. (13) found neutralizing antibody titers to EMC virus in 12% of the juvenile diabetics they studied in contrast to only 6% of their control subjects. This virus/mouse model is also the most widely studied of such models.

It is obvious from the studies involving the EMC virus/mouse model that either the infectious virus particle itself or something in the cells in which the virus grows is one of the determining factors in whether or not the viral preparation will cause diabetes. In all previous studies, it was assumed that the virus particle itself was responsible for the clinical signs of diabetes. None of the investigators speculated that the extraneous material (from the cell culture in which the virus was propagated) had any bearing on the diabetogenicity of the virus preparation. The infecting virus particles have yet to be purified from the cell milieu in which the virus inoculum was grown, despite the fact that Giron et al. (14) found extreme differences in the diabetogenicity of EMC virus after single passages in different cell lines. Thus, it is important to determine if purified virus retains diabetogenic properties.

Interferon (IFN) has been implicated (14, 15, 16, 17, 18) in the mechanism of induction of the diabetic state when mice are infected with EMC virus. Since there have been only superficial investigations into the roll of IFN, more thorough investigations should be made into the type of IFN which is produced at specific times relative to diabetogenic infections by EMC virus. Results should then be compared to the type of IFN that is produced at similar times in non-diabetogenic infections by EMC virus. In addition, knowing which tissues are involved in the production of the IFN at those specific times could be enlightening.

Host factors such as immune competence (19, 20) are certainly involved in diabetogenicity following EMC virus infection; however, there are no investigations of the status of the cellular- or the humoral-immune system components during such diabetogenic infections.

All reported studies of EMC virus-induced diabetes have employed an artificial means, intraperitoneal (ip) injection, for introduction of the virus into

the host. However, a more relevant study of virus-induced diabetes might be possible if the virus were introduced by a natural route of infection.

The objectives of this study are 1) to determine if a natural route of infection can be reliably used to induce diabetes in mice and if so, to use that route of infection to continue with the other objectives of the study; 2) to determine if the virus particle is intrinsically diabetogenic or if it must be introduced along with supposedly extraneous materials present in the virus preparation; 3) to characterize host-defense activities in relationship to the EMC virus infection and development of diabetes; 4) to substantiate findings by others and extend the description of the characteristics of the EMC-D virus/mouse model of diabetes; and 5) to use reagent strips used for urinalysis in humans (Clinistix® and Diastix®) to determine their reliability as an indicator of diabetes in mice.

This study is important as a means to elucidate mechanisms involved in virus-induced diabetes in mice. If these mechanisms could be determined in animals, it would then be possible to intelligently design experiments to combat virally induced diabetes in humans.

CHAPTER II

REVIEW OF THE LITERATURE

Diabetes Mellitus. *Definition.* There are many definitions of diabetes, depending on the writer and the time that the definition was written. In Webster's New Collegiate Dictionary, copyright 1961 (21, p. 228), the definition is as follows:

> **di´a·be´tes** ...A disease attended with a persistent, excessive discharge of urine; specif., ***di´a·be´tes mel·li´tus*** ..., *or* ***sugar diabetes***, a grave form in which the body is unable to utilize properly the carbohydrates in the diet due to failure in the secretion of insulin by the pancreas. It is marked by sugar in the urine, excessive thirst and hunger, and progressive emaciation.

Currently, definitions by investigators more closely approximate that of Berhanu and Olefsky (22, p. 265):

> Diabetes mellitus is a complex disorder characterized by alterations in carbohydrate, lipid, and protein metabolism resulting from a deficiency of insulin or its cellular metabolic effects. As a function of time, these metabolic derangements lead to the development of chronic degenerative changes in the eyes, kidneys, nerves, and blood vessels, and often result in the well-known chronic clinical complications of diabetes including blindness, renal failure, peripheral and autonomic neuropathy, and occlusive peripheral vascular and coronary artery disease. Although diabetes mellitus has been recognized for several centuries, our concepts regarding its etiology and pathogenesis continue to undergo major changes.

The vivid description of the disease made by Aretaeus could almost serve as a definition and since it makes so many valid points regarding the disease, it is quoted here (23, flyleaf).

> Diabetes is a wonderful affection, not very frequent among men, being a melting down of the flesh and limbs into urine. Its cause is of a cold and humid nature, as in dropsy. The course is the common one, namely, the kidneys and bladder; for the patients never stop making water, but the flow is incessant, as if from the

> opening of aqueducts. The nature of the disease, then, is chronic, and it takes a long period to form; but the patient is short-lived, if the constitution of the disease be completely established; for the melting is rapid, the death speedy. Moreover, life is disgusting and painful; thirst unquenchable; excessive drinking, which, however, is disproportionate to the large quantity of urine, for more urine is passed; and one cannot stop them either from drinking or making water. Or if for a time they abstain from drinking, their mouths become parched and their bodies dry, the viscera seem as if scorched up; they are affected with nausea, restlessness, and a burning thirst; and at no distant term they expire.
>
> Hence, the disease appears to me to have got the name 'diabetes' as if from the Greek word διαβητησ (which signifies a siphon), because the fluid does not remain in the body, but uses the man's body as a ladder (διαβαθρη), whereby to leave it. They stand out for a certain time, but not very long, for they pass urine with pain, and the emaciation is dreadful; nor does any great portion of flesh pass out along with the urine.
>
> The cause of it may be, that some of the acute diseases may have terminated in this: and during the crisis the diseases may have left some malignity lurking in the part. It is not improbable, also, that something pernicious, derived from the other diseases which attack the bladder and kidneys, may sometimes prove the cause of this affection.
>
> Aretaeus the Cappadocian, A.D. 81-138

History. A disease, now termed diabetes, was described in the Ebers Papyrus in 1500 B.C. However, diabetes seems to be a "disease of civilization" since its prevalence increases as urbanization, working patterns, and diet change from primitive to civilized modes (24).

The name ***diabetes*** was applied to the disease by Aretaeus the Cappadocian, A.D. 81-138 (23, 24). The Greek word for honey, *mellitus,* was added when a sweet substance was noted in the urine of patients with diabetes (24). Butterfield (25) provided a succinct history of developments in the study of diabetes. He related (25, p. 78) that in 1798 Rollo (26) described diabetes as "a morbid condition of the stomach forming or developing from vegetable substances, saccharine matter which is quickly separated as a foreign body by the kidneys." It was not until 1851 that Bouchardat (27)

attributed diabetes mellitus to disease of the pancreas, although he wrongfully ascribed it to a failure of the external secretion of the pancreas. Von Mering and Minkowski (28) found that pancreatectomy in dogs caused permanent diabetes of a severe nature. They also reported that ligation of the duct led to atrophy of the pancreas but did not cause diabetes. They recognized that the pancreas has a function that is essential for the utilization of sugar by the organism. They evidently did not connect the cells (now called islets of Langerhans) previously reported by Langerhans (29) with their newly discovered function of the pancreas. Laguesse (30), however, suggested that the islets of Langerhans produce a hormone and Schäfer (31) related that hormone to carbohydrate metabolism. It was not until 1921 that Banting and Best (32, 33) extracted insulin from the pancreas of dogs and demonstrated its hypoglycemic activity. Sanger (34, 35) determined the structure of insulin in the mid-1950s.

According to Berhanu and Olefsky (22) it was thought, immediately following the reports of discovery of this pancreatic extract by Banting and Best (32, 33), that all diabetes mellitus resulted from insulin deficiency. Himsworth (36) was one of the first to recognize that diabetes is a heterogeneous disorder when he pointed out that diabetes could be differentiated into "insulin-sensitive" and "insulin-insensitive" types on the basis of blood-glucose response to insulin administered after an oral glucose load. He later suggested that insulin insensitivity, not insulin deficiency, is present in many diabetics who are nonketotic and of middle age. This view was strengthened when Yalow and Berson (37) reported a method (insulin radioimmunoassay) by which endogenous insulin could be measured in the plasma, and it was found that on the average higher levels of insulin occur in patients with the adult-onset form of the disease than in normal controls.

Epidemiology. The National Diabetes Data Group (NDDG) reported in 1985 (38) that approximately 5.8 million people in the U.S. had been diagnosed as being diabetic and an additional 4-5 million with diabetes remained undiagnosed. They reported 4 main types of diabetes 1) insulin-dependent diabetes mellitus (IDDM), which comprises 5-10% of all cases of diabetes in the U.S.; 2) noninsulin-dependent diabetes mellitus (NIDDM), which comprises most of the remaining cases; 3) gestational diabetes (GDM), which is a transient condition during pregnancy, occurring in 2-5% of all pregnancies; and 4) a type of diabetes that is secondary to or associated with the other conditions and makes up about 2% of the cases. In addition to these 4 types of diabetes, impaired glucose tolerance (IGT) affects those persons with glucose tolerance between normal and diabetic (38).

Approximately 500,000 persons each year are diagnosed as diabetic. The annual incidence is 12-14/100,000 children ages 0-16 years; by age 20, 0.3% of persons in the U.S. have IDDM. Two and thirty-five hundredths percent of the population of the U.S. is diagnosed as having NIDDM, and those 65 years and older with NIDDM make up 8.6% of the population (38).

Insulin-dependent diabetes mellitus tends to run in families but does not have a clear genetic etiology or a consistent mode of inheritance (39). The incidence of IDDM is similar in males and females and is 1.5 times higher in whites than in blacks, but incidence rates vary widely internationally. Individuals with particular human leucocyte antigen (HLA) types, especially DR3 and DR4, are at increased risk for development of IDDM. Additional risk factors may be advanced maternal age and the presence of islet cell antibodies. Siblings of diabetic children have a 7 to 18 times greater risk of developing IDDM than children of the general population. However, only about 1 child in 50 who is a brother or sister of an IDDM patient will develop

IDDM. IDDM is one of the major chronic diseases of children, having a risk similar to that of developing cancer in childhood but having a much greater risk than other well-known diseases (cystic fibrosis, leukemia, rheumatoid arthritis, muscular dystrophy, and Hodgkin's disease) (38).

Only limited data concerning the prevalence of IDDM in the U.S. are available but results obtained are consistent. One child in 600 of school age has IDDM. Very little is known concerning the prevalence of IDDM in adults (38).

Diabetes was the seventh leading underlying cause of death in the U.S. in 1982, leading to 34,583 deaths. An additional 95,000 deaths were listed with diabetes as a contributing cause (38). Mortality among white male and female IDDM patients was 5 to 11 times greater than in nondiabetics and the rates for blacks with IDDM were about two times higher than those for whites. Below age 20, the primary cause of death in IDDM was acute diabetic complications, but at older ages renal disease accounted for nearly half of the deaths. The frequency of renal disease deaths was 500 times higher and of cardiovascular disease deaths was 13 times higher in IDDM patients than in the general population (38).

Diagnosis. A number of prospective observational studies (40, 41, 42, 43, 44) have led to the tentative conclusion that the pathologic consequences of diabetes are most likely to occur when blood glucose levels are excessive over extended periods of time. Consequently, the World Health Organization (WHO) and the NDDG have recommended criteria for the diagnosis of diabetes based on plasma glucose levels which are believed to be predictive of the complications of diabetes. The NDDG and WHO do not consider diagnostic criteria that employ urine glucose or casual and postprandial glucose values to be adequate for the diagnosis of diabetes (38). They

recommend that diagnosis in a clinical setting be made by one of the following: 1) the presence of the classic symptoms of diabetes (polyuria, polydipsia, ketonuria, and rapid weight loss) together with gross and unequivocal elevation of plasma glucose, e.g., postprandial or random plasma glucose concentration > 200 mg/dl (11.1 mmol/L); 2) elevated fasting glucose concentration on more than one occasion as follows: venous plasma ≥140 mg/dl (7.8 mmol/L), venous whole blood ≥120 mg/dl (6.7 mmol/L), or capillary whole blood ≥120 mg/dl (6.7 mmol/L); or 3) when the fasting glucose concentration is less than that which is diagnostic of diabetes (see no. 2 above), an abnormal oral glucose tolerance test must meet the following criteria: venous plasma ≥200 mg/dl (11.1 mmol/L), or venous whole blood ≥ 180 mg/dl (10.0 mmol/L), or capillary whole blood ≥200 mg/dl (11.1 mmol/L). To meet these latter criteria, the NDDG requires both a 2-hour sample and some other sample taken between administration of the 75-gram glucose dose and the 2-hour sample, while the WHO requires only a 2-hour sample.

Interpretation of the results of a single glucose tolerance test should be done with care because there have been reports of a lack of reproducibility of this testing in normal subjects and in diabetic patients (45). McDonald et al. (45) found that the degree of carbohydrate intolerance can fluctuate in diabetics from a normal test at one time to marked intolerance in the same patient at another time. There have been occasional reports of total remission of severe diabetes (46, 47, 48), some of which may have been based on non-reproducible glucose tolerance tests.

Complications of diabetes. Diabetes is the leading cause of new cases of blindness in people of ages 20-74 years in the U.S. Other common complications are acidosis and coma, kidney disease, peripheral vascular disease, heart disease, hypertension, and cerebrovascular disease. It has

been reported that infections occur more often in diabetics than in nondiabetics but "many published inferences about infectious consequences of diabetes go beyond what available data permit." (49, p. XIX-1). Most types of infections are no more common in diabetics than in nondiabetics. However, kidney and urinary-tract infections do appear at a relatively high rate in diabetics; and diabetes is a pre-existing condition in a significant proportion of patients with certain fungal infections, as well as in some unusual clinical entities such as invasive external otitis (38).

Economic aspects of diabetes. It is difficult to estimate the full economic impact of diabetes because it is so closely related to other acute and chronic conditions that may also be the reason for medical expenses. The lowest estimates of costs directly attributable to diabetes in the U.S. are $13.8 billion annually, 3.6% of total health costs. Medical care expenses specifically for diabetes account for $7.4 billion, while $6.3 billion is due to indirect costs due to disability and premature death among diabetics. All costs incurred by diabetics for medical care are estimated to be about 2-times the above (38).

Animal Models of Virus-Induced Diabetes Mellitus. Several animal models for the study of virus-induced diabetes mellitus have been described. Some of these models have not been well-developed and have not received attention by many investigators. Such models include lymphocytic choriomeningitis virus in mice (5), foot-and-mouth disease virus in cattle (9), rubella virus in rabbits (7), reovirus in infant mice (10), and a model described by Rayfield et al. (6) of Venezuelan equine encephalitis virus in hamsters.

Another model of virus-associated diabetes mellitus is the coxsackievirus (CV) type B infection in mice (8). This model is especially interesting in light of the fact that some investigators (50, 51) found

correlations between antibodies to CV type B4 and the onset of insulin-dependent diabetes in humans. They also found a seasonal incidence of insulin-dependent diabetes mellitus with significant positive correlation with the annual prevalence data for this virus, but not for other types of viral infection. Other workers (52, 53, 54, 55, 56) have not been able to confirm those findings. Interestingly, however, Yoon et al. (57) isolated CV type B4 from the postmortem pancreas of a boy who had been admitted to a hospital in diabetic ketoacidosis. The boy was admitted within three days of onset of symptoms of a flu-like illness. The isolated virus caused diabetic signs when injected into mice. Serum samples from the boy had a titer of neutralizing antibody to the virus of less than 4 on the second hospital day and 32 on the 8th hospital day, the day of his death. This is the best evidence to date that a virus is involved in the initiation of diabetes in man.

Some workers have not been able to obtain hyperglycemic glucose levels in mice injected with CV (58, 59). Yoon et al. (60) found that repeated passage of CV type B4 in mouse pancreatic beta cells led to the isolation of a diabetogenic variant of this virus, which could then be used to induce diabetes signs in several inbred strains of mice. In this and another study (61) they found that repeated passage of CV or EMC virus in secondary mouse embryo cells did not increase diabetogenic capabilities.

The EMC virus/mouse model for juvenile-onset diabetes mellitus has been the most studied animal model for diabetes. Although a great deal has been published regarding this model system there are many questions to be answered before results obtained from the model can be properly interpreted. Some of these questions will be addressed in the remainder of this chapter.

Effects of Virus Strain on EMC Virus-Caused Diabetes. The virus strain is important in determining whether or not an infection will result in

the diabetic syndrome. The first report regarding the diabetogenic nature of EMC virus (11) specified that the diabetogenic activity is seen only in a variant selected by harvesting the virus from repeated passages in the myocardium of infected mice. Craighead and McLane (11) named it the M variant (from myocardium). Many other investigators have since used the M variant to successfully induce diabetes in various mouse strains; however, some mouse strains have consistently been resistant to the diabetogenic effects of the virus.

Yoon et al. (15) demonstrated that the stock of M variant EMC virus is composed of at least two separate strains, one a diabetogenic variant and the other a nondiabetogenic variant. The variants were separated by plaque purification. The range of ability of the different isolates to cause diabetes was found to be continuous between 100% and 0%. This could mean that there were many variants in the original virus stock, each with a different degree of ability to cause diabetes. Alternatively, it could mean that each plaque isolate is either a pure isolate of a single variant (either diabetogenic or nondiabetogenic) or a particular mix of the two variants which gives the range of variation seen. Two plaque isolates were selected, one of which gave 0% diabetes when injected into mice (the B variant) and one which induced diabetes in 100% of the injected mice (the D variant). When the B and D variants were mixed together at B:D ratios of 1, 9, and 99 and injected into susceptible mice, diabetes developed in 60%, 11%, and 0% of the mice, respectively (15). The investigators expressed confidence in the stability of the properties of these two variants since, unlike the M variant stock, repeated passage in secondary mouse embryo cells did not cause the D variant to lose the capacity to produce diabetes in mice.

It now seems evident that the diabetogenic nature of the D variant depends upon the type of cells in which it is grown. Giron et al. (14) found that

the D variant continues to be diabetogenic when grown in L-929 (L) cells, a mouse-derived line, while a single passage of the virus in baby hamster kidney (BHK-21) cells completely eliminates the diabetogenic ability of the virus. The diabetogenic ability of the D variant is fully restored by a single passage in a human cancer cell line (HeLa), but not by a single passage in L cells. Therefore, Giron et al. (14) suggested that the D variant, like the M variant from which it was isolated, either consists of more than one distinct virus or is relatively unstable and some of its properties are easily altered during replication in certain cell types.

Recently Kruppenbacher et al. (62) reported that they were able to take the EMC-D variant supplied by Yoon and isolate a non-diabetogenic variant by further plaque purification. The D variant could have been in a continual mutation mode away from the diabetogenic variant, or the D variant as isolated by Yoon and his colleagues could have been an impure virus stock.

Physical and chemical differences of EMC virus variants. The only differences noted so far between EMC-D and EMC-B virus variants are 1) their diabetogenic abilities (15, 63), 2) their relative ability to induce production of interferon (IFN) in cell culture or in mice (although both variants are equally sensitive to inhibition by IFN) (15), 3) the plaque size in L cells (64), and 4) at least one oligonucleotide in the fingerprinting pattern of the T1-digested RNAs from the two variants (65, 63). Hybridization and thermal elution profiles of the RNAs from the two variants failed to demonstrate any differences, but the fingerprint of the T1-digested RNA of the B variant was missing at least one oligonucleotide, 20 to 25 nucleotides long, which was present in the digest of the D-variant RNA. Four of the structural proteins (alpha, beta, gamma, and delta) of the two variants migrate identically on SDS-PAGE, with a pattern similar to that already known for EMC virus capsid proteins. No differences in

the sensitivity to different pH values, salt concentrations, ultraviolet irradiation or temperature, as measured by infectivity of the viruses, could be found (65).

Host Regulated Factors Involved in Resistance or Susceptibility to EMC Virus-Induced Diabetes Mellitus. *Effect of genetics*. The resistance of certain mouse strains to induction of diabetes by infection with EMC virus was early proposed to be due to the genetic traits of the resistant mice (66). The inherited trait for resistance was said by Yoon and Notkins (67) to be due to resistance of the beta cells of the pancreas to both infection by, and production of, EMC virus. They found that the beta cells isolated from resistant strains of mice and grown *in vitro* had lower infection percentages and produced a less infectious virus than did the beta cells from sensitive strains of mice. In contrast, EMC virus replicated equally well in primary embryo or kidney cell cultures from susceptible or resistant strains of mice. They concluded that the development of EMC virus-induced diabetes is related to genetically determined host differences which affect the ability of the virus to infect beta cells. The same group of investigators (68) demonstrated that EMC virus selectively binds *in vitro* to the beta cells derived from a susceptible strain of mice as compared to the beta cells from a resistant strain of mice. They concluded that genetically inherited viral receptors cause the differences seen in susceptibility among mouse strains.

Ross et al. (69) and Yoon et al. (70) had shown in earlier studies that when mice were infected with EMC-M virus, some strains of mice were susceptible and other strains were resistant to EMC virus-induced diabetes. When susceptible mice were crossed with resistant mice, the F_1 progeny were resistant to the diabetogenic effects of the virus. However, more than 20% of the F_2 offspring developed diabetes when infected by the virus. The backcross of the F_1 progeny to the resistant parents gave offspring which were

also resistant. In contrast, when the F_1 progeny were backcrossed to the susceptible parents, approximately one-half of the offspring were susceptible (71). The authors concluded that these data are consistent with theories of Mendelian inheritance, and that susceptibility to EMC virus-induced diabetes is controlled by a single locus.

Dafoe et al. (72) performed an experiment in which male F_1 hybrids from a cross between resistant and susceptible strains of mice were induced to diabetes signs by chemical means. The diabetic hybrids then received pancreatic transplants from neonates of either the resistant or the susceptible parental strains of mice. Encephalomyocarditis virus was injected into these mice, into sex- and age-matched untreated F_1 hybrids, and into each parental strain. The parental strains were either susceptible or resistant to the diabetogenic effects of EMC infection, as expected. The untreated F_1 hybrids were intermediate in susceptibility, while the F_1 hybrids with transplants were also intermediate in susceptibility, regardless of source of the pancreatic transplant. These results indicate that host factors are more important than are inherited pancreatic factors in determining susceptibility to EMC-induced diabetes.

Effect of hormones and age. Testosterone is one host factor which appears to be important in the determination of mouse susceptibility to virus-induced diabetes. Morrow et al. (73) found that intact male DBA/2 mice (sham operated) and male castrates treated with testosterone and infected with the M variant of EMC virus had pancreatic insulin concentrations that dropped precipitously to 10% of control values with concomitant hyperglycemia. Untreated castrates failed to develop hyperglycemia, although the insulin content of the pancreas dropped somewhat in these infected animals. Equal amounts of viral antigen were demonstrated in beta cells of untreated

castrates and those of the other mice.

Testosterone is not a determining factor in diabetogenic susceptibility in all strains of mice, according to Giron and Patterson (74). They found that testosterone-treated ICR Swiss female mice, normally resistant to EMC virus-induced diabetes, were just as susceptible to diabetes induction as male mice when infected with the D variant of EMC virus. In contrast, testosterone-treated DBA/2J female mice were not susceptible to the virus. Other steroid hormones (estrone, cortisone, and progesterone) were not effective in increasing the susceptibility of ICR Swiss female mice to EMC virus diabetogenicity.

Giron et al. (75) have also found that age of the recipient mice largely determines their susceptibility to the diabetogenic effects of EMC-D virus. ICR Swiss mice younger than 7 weeks developed lethal encephalitis when infected with the D variant of EMC virus, while those older than 7 weeks developed diabetes. Testosterone injections enhanced the susceptibility of the young mice to the diabetogenic effects of the virus. On the other hand, testosterone had no effect on the susceptibility of 9-week-old mice to EMC-D virus. It is not clear at this time why the EMC-D virus loses the ability to infect the central nervous system of older mice.

Effect of interferon. Yoon et al. (15) speculated that differences in IFN stimulating ability of the two EMC virus variants could account for the difference in diabetogenic potential. The B variant stimulates normal levels of IFN while the D variant stimulates little or no IFN production either in cell culture or in mice. On the other hand, Giron et al. (14) found that the ability of the virus to induce interferon in cell culture had no bearing on whether or not the virus pool would retain its diabetogenic characteristics. Recently, Yoon et al. (16) repeated their claim that the IFN system is an important determinant in

the outcome of EMC virus-induced diabetes in mice. They reported that repeated administration of IFN or an IFN inducer reduced EMC-D virus-induced diabetes in mice, while a single treatment with either IFN or its inducer had no effect. They also found that treating mice with antibody to murine IFN allowed development of mild signs of diabetes in those mice injected with the B variant of EMC virus.

The host strain of mouse influences whether or not IFN, or a variety of IFN inducers, inhibit the diabetogenic effect of EMC-D virus. The diabetogenic effect of EMC-D virus in SWR/J mice was blocked by either β–IFN or a variety of IFN inducers, but the effect was not blocked in ICR Swiss mice by the same treatments (17). The authors implied that in ICR Swiss mice, β–IFN and the IFN produced in response to the inducers poly I:C, pyran, endotoxin, or tilorone either do not reach the beta cells of the pancreas, or the beta cells are not sensitive to the protective action of IFN. The same group of investigators (18) found that when β–(actually a mix of β– and α–) or γ–IFN was administered to adult male ICR Swiss mice four days after infection with EMC-D virus, the frequency and severity of diabetes signs were increased. In addition, the diabetic state was induced in the resistant C57BL/6 strain of mice under the same conditions. The IFN preparations were given after the acute stage of infection, but prior to the onset of insulitis (mononuclear cellular infiltration of pancreatic islets). They concluded that under certain conditions, the diabetic state is exacerbated and the normal course of EMC-D virus infection in mice is altered by IFN.

Effect of immune system capabilities. The cellular immune system of mice must be functional in order for EMC virus to cause diabetes in infected animals. However, the humoral immune system functionality seems to be of little consequence (20). Buschard et al. (19) found that the EMC-M strain of

virus was unable to induce diabetes in athymic (nu/nu) mice, but heterozygous littermates (+/nu) and normal mice of the background strain C57Bl/6 developed diabetes mellitus.

Immunosuppression of DBA/2 mice with the cyclophosphamide derivative Asta 5122 significantly increased the mean value of blood glucose levels compared to the positive controls, even though the percentage of diabetic animals (88%) was the same in both groups. In contrast, X-irradiation (500 R) caused a significant inhibition of the appearance of diabetes in the EMC-M virus infected mice. Neither chemical immunosuppression without viral infection nor X-irradiation alone caused diabetes in the mice. The irradiated mice infected with virus did not have insulitis, but the chemically treated mice with virus had a high incidence of insulitis (20).

In the study of Buschard et al. (19), EMC virus was isolated from all inoculated animals. They concluded, "it is the response of the thymus-dependent lymphocyte system evoked by the virus rather than the virus itself that leads to damage to the insulin producing cell" (19, p. 299). The same group (76) extended their investigations and found that the M strain of EMC virus also did not cause diabetes in athymic nude mice of BALB/c origin. In contrast, 36% of normal mice developed diabetes when infected with EMC-D virus and 93% of the infected normal mice, but none of the nude mice, developed paresis of one or more legs. Virus could be isolated in abundant amounts from the pancreas and heart of all virus-inoculated mice, including the non-diabetic nude mice. Although the thymus-dependent immune system seemed important in this model, the primary mechanism for affecting the islet beta cells was not a massive lymphocytic infiltration in the islets.

Virus replication in various tissues. Gould et al. (64) reported differences in the replication of the D and B variants of EMC virus in organs of

male ICR Swiss mice. The D variant replicated in the spleen, pancreas, heart, lung, and intestines of the mice but EMC-B virus replication was limited to the spleen and pancreas. The EMC-B virus strain interfered with the replication of EMC-D virus in each of the tissues examined. Insulin levels were initially increased by both viruses, but by 4 days postinfection, insulin levels were either normal or undetectable in the EMC-D virus-infected mice and were dramatically elevated in those mice infected with the B variant. The concentration of the D variant was much greater in the spleen than in the pancreas of mice with normal glucose tolerance test (GTT) results but the relative concentrations of virus were reversed in the 2 tissues of mice with abnormal GTT results. The D variant replicated, but to a very limited extent, in the heart, lung, and intestinal tissues. Even though the B variant did not replicate in these tissues, it still managed to block the multiplication of EMC-D virus. ICR Swiss mice were not protected against EMC-D virus-induced diabetes by IFN injections; therefore, IFN activity was not the mechanism of protection when EMC-B virus was used in these mice.

Immunohistochemical Techniques. *Immunoperoxidase staining.* The enzyme horseradish peroxidase (HRP), of molecular weight 40,000, is employed in the immunoperoxidase technique as a marker for identification of antibodies or antigens at both the cellular and subcellular levels. Interaction of horseradish peroxidase with certain substrates produces insoluble reaction products that can be demonstrated by both light and electron microscopy (77). Avrameas and Uriel (78) and Nakane and Pierce (79) were pioneer investigators of this method. They proposed independently that enzymes be used in the place of fluorochromes as labels for antibodies.

Disadvantages of immunofluorescence that immunoperoxidase staining overcomes include quick fading of fluorescence preparations, the

requirement of photography to provide permanent results, limited resolution of the fluorescence microscope, nonadaptability of fluorescence to electron microscopy, inability to use counterstains, and the inability to use paraffin- or resin-embedded sections to perform retrospective studies on stored blocks from past years.

Disadvantages of immunoperoxidase staining include 1) the demonstration of relatively minute positively staining areas is limited by the light microscope's resolution, 2) endogenous peroxidase, which causes nonspecific staining, may not be completely eliminated from the tissue under investigation, and 3) products resulting from the enzyme reaction may diffuse away from the area where antigen is localized (77).

Mukai and Rosai (80) applied immunoperoxidase techniques to histochemical staining for diagnosis in surgical pathology. This technique has greatly improved the diagnostic capabilities of the surgical pathologist.

Peroxidase-antiperoxidase technique. Sternberger et al. (81) developed a peroxidase-antiperoxidase (PAP) method that allowed use of unlabeled antibody, thus overcoming a disadvantage of the original peroxidase-labeled primary antibody technique in which it was necessary to label each antibody specificity and individually. The same PAP complex may be used for many different unlabeled antibody specificities.

Avidin-biotin-peroxidase complex technique. The avidin-biotin-peroxidase complex (ABC) technique, introduced by Hsu et al. (82, 83), is a major advance. This method allows use of highly diluted antibody preparations previously only used in radioimmunoassay techniques. The ABC procedure employs primary antibody without label, secondary antibody labeled with biotin, a low molecular weight (244 daltons) vitamin, and a preformed avidin-biotinylated horseradish peroxidase complex. Avidin (from

egg white) is a glycoprotein of molecular weight 68,000. It has an extremely high affinity ($Kd=10^{-15}$ M^{-1}) for biotin which leads to an essentially irreversible union, in contrast to reversible antigen-antibody reactions. To stain by this method, the tissue section is incubated with a primary antibody specific for the antigen in question and then biotinylated secondary antibody is applied. This is followed by exposure to the ABC complex which binds very tightly to the biotin of the secondary antibody. Visualization is accomplished by incubation in a solution of peroxidase substrate to form an insoluble pigment at the site of the peroxidase enzyme. Avidin has 4 separate binding sites for biotin. When it is incubated, in excess, with biotin-peroxidase molecules, a large lattice-like complex develops which is still capable of binding to other biotin molecules. The structure of the complex is unknown, but is believed to consist of numerous biotinylated horseradish peroxidase molecules cross-linked by avidin to give a three-dimensional structure (84).

The ABC staining method is capable of providing multiple molecules of the peroxidase enzyme at the site of antigen-antibody reaction, in contrast to the PAP method in which an average of only 2.4 molecules of peroxidase can be attached to each molecule of tissue antigen (77). Hsu et al. (82, 83) found their ABC method to be 8-40 times more sensitive than the PAP technique. This method is also more universal than the PAP technique in that it eliminates the necessity of preparing peroxidase-anti-peroxidase complexes in different species. There is also no need for excess linking antibody, as in the PAP method, because biotin coupled to the secondary antibody serves as the link and only a minimum amount is necessary because of the high affinity of the biotin-avidin reaction (77).

Many biological molecules, including enzymes and antibodies, can be biotinylated without significant loss of biological activity (85).

> Unlike directly conjugated enzyme-labelled second antibodies, biotinylated second antibody is, essentially, identical in size to the immunoglobulin molecule and is therefore able to penetrate tissue sections much more readily than its more bulky counterpart. This property can prove important in strongly fixed sections and it reduces the need for protease treatment of sections. (85, p. 34)

Likewise avidin can be conjugated with a wide variety of labels without inactivation of activity. Thus, the biotin-avidin system provides a great promise of numerous potential applications. However, there are two major problems that arise because of the chemical structure and properties of avidin derived from egg-white. 1) Avidin is highly positively charged at neutral pH because of its isoelectric point, which is approximately ten (86). Staining done at a pH near neutrality leads to nonspecific binding of the avidin to negatively charged molecules (87) such as nucleic acids or phospholipids, resulting in high background labeling. 2) Avidin is a glycoprotein and will thus react with biological molecules such as lectins through its carbohydrate moiety (88).

Streptavidin, a protein of molecular weight 60,000, isolated from *Streptomyces avidinii* (89), has four high-affinity ($Kd=10^{-15}\ M^{-1}$) binding sites and may be conjugated with a variety of label molecules in a manner that retains activity. The properties of streptavidin are very similar to those of avidin. However, in contrast to egg-white avidin, streptavidin has an isoelectric point near neutral pH, contains no carbohydrate moieties, and has a molecular weight that is 8000 daltons less than avidin (85). In contrast to avidin, streptavidin exhibits little non-specific binding to mast cells, kidney, liver, or brain tissue at physiological pH (89, 90).

Controls. It is absolutely essential that proper controls be included with each staining procedure (77). There are two necessary controls: 1) A positive control slide which is fixed and embedded in an identical manner to the test slides and which is known to contain the antigen in question. This slide is

necessary to determine that the reagents are performing properly. 2) A negative control slide, with the same characteristics as the positive control slide, is essential to show nonspecific staining reactions. This slide has primary antibody omitted and nonimmune serum substituted during the staining procedure. This control slide permits examination for endogenous peroxidase activity as well as nonspecific binding of secondary antibody.

Substrates. One of the earliest substrates utilized in immunoperoxidase staining was 3,5'-diaminobenzidine (DAB). The enzyme produces an insoluble brown pigment when DAB is the substrate. There is a modification of the DAB peroxidase staining to produce a jet blue-black pigment [personal communication of Hsu in (77)]. These pigments are insoluble in water as well as in organic dehydrating and mounting media. Thus, when DAB is used, the slides can be permanently mounted and referred to over long periods of time since the pigment also does not fade. This substrate has the disadvantage of being a potential carcinogen (77). A substrate utilized to avoid the carcinogenic potential of DAB as well as to provide a contrasting staining color is 3-amino-9-ethyl-carbazole (AEC). It provides a red to brownish-red reaction product which is soluble in organic solvents. Thus, aqueous mounting media must be used to mount tissue sections when this substrate is used. Some AEC sections fade during storage over a period of several months (91, 92).

CHAPTER III

A NATURAL ROUTE OF INFECTION STUDIED IN THE MOUSE MODEL OF DIABETES INDUCED BY EMC VIRUS

Introduction. Previously published studies on the induction of diabetes by EMC virus in mice have used an artificial route, intraperitoneal (ip) injection, of the virus. While this means of introducing the virus is certainly effective in producing diabetic signs in the mice, it may yield results which are somewhat different than those obtained following a natural route of infection.

This study was carried out in order to determine if a natural route of infection, either per os (po) or intranasal (i.n.), by EMC virus would reliably produce diabetes in mice. The i.n. route was effective in establishing diabetes in mice. It allowed a comparison of diabetic signs obtained following infection by the i.n. route to those seen after ip injection of the virus.

Materials and Methods. *Mice.* Male and female adult mice of the SJL/J strain, a highly inbred strain of white mouse, obtained from The Jackson Laboratory (Bar Harbor, ME) were used to begin a small breeding colony in our laboratory. This strain of mice was used by Yoon et al. (15) to demonstrate different strains of EMC in a stock of M variant EMC virus. Mice of various ages were used to determine if there is a relationship between mouse age and the diabetogenic potential of the virus. The mice were housed in shoe box style cages, fed Wayne Lab Blox and given tap water ad libitum. Ground corn cobs were used as bedding.

Cells. L-cells, a continuous culture of mouse connective tissue cells, were obtained from the American Type Culture Collection (ATCC), Rockville,

Maryland, as NCTC clone 929 cells and were maintained in growth medium which was different from the original medium in which they had been developed. For this reason, they are no longer considered to be the NCTC clone 929 cells and are thus called L-cells. See the following quote from the ATCC Catalogue of Strains II, Fourth Ed., 1983, page 17 (93), regarding the description of this clone of cells. "If the cells are maintained in the original medium, they may be considered NCTC clone 929 cells; if grown in other media, they may exhibit different properties and should be considered to be derivatives or sublines" (93, p.17). The continuous culture of BHK-21, Syrian hamster, *Mesocricetus auratus*, kidney cells, was purchased from ATCC.

Medium. Eagle's Minimum Essential Medium (EMEM) with Earle's salts and non-essential amino acids (GIBCO, Grand Island, NY), containing 5% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT), 0.1% $NaHCO_3$, and without antibiotics was used as growth medium for L-cells. The growth medium for BHK-21 cells differed only in that the concentration of FBS was increased to 9%. EMEM containing 2% FBS, 0.18% $NaHCO_3$, and 50 µg of gentamicin/ml was used as diluent when virus or other material was being titered and is referred to as test medium in this study.

Virus. Two different EMC-D virus preparations, one labeled "EMC-D, 4-29-82, 100% diabetes" and the other labeled "EMC-D, P-3, 7/80", were kindly provided by Dr. J. Yoon of the National Institutes of Health. The one dated 4-29-82 will be referred to as the '82 virus pool and the other preparation will be referred to as the '80 virus pool. The '82 virus pool was passaged in L-cells in our laboratory for use in all other experiments. The cells were infected with the virus, which was allowed to adsorb for 1 hr, test medium was added, and the cells were incubated at 37° C. When the viral cytopathogenic effect (CPE) was graded 4 (100% of the cells showing CPE) the cells, with medium and

virus, were frozen at -80°C. Later, the cells were allowed to thaw at room temperature, with occasional shaking of the ice sheet over the surface on which the cells had been growing, in an attempt to suspend all cell material. The resulting suspension was homogenized for 30 seconds at the full speed setting of a Sorvall® Omni-Mixer (DuPont Co., Newtown, CT) while the stainless steel cup was in an ice bath. The homogenate was dispensed to freezer vials, labeled, and placed at -80°C.

Vesicular stomatitis virus (VSV), Indiana strain (ATCC, Rockville, MD), was passaged in MA104 cells in a manner similar to that described for EMC-D virus.

Titration of virus in vitro. Two methods, cell culture infectious dose-50% endpoint ($CCID_{50}$) and plaque assay, were used in these experiments for *in vitro* virus titrations. These titrations were accomplished in the same cells (L or BHK-21) utilized to conduct other assays in which the virus was used as a challenge.

The $CCID_{50}$ endpoint was determined by titration in cells grown in 96-well, flat-bottomed, tissue culture plates (A/S Nunc, Denmark or Corning Glass Works, Corning, NY) (96-well microplates). Cells suspended in growth medium were seeded in these sterile plates at 2×10^5 cells/ml, 0.2 ml/well. The plates were wrapped in Saran Wrap™ (The Dow Chemical Co., Indianapolis, IN) and incubated overnight at 37°C to allow the cells to form confluent monolayers. Virus was diluted by serial 10-fold dilutions in polystyrene, 12 x 75 mm, sterile, snap-cap tubes (Becton Dickenson and Co., Lincoln Park, NJ or ElKay Products , Inc., Shrewsbury, MA) containing test medium as diluent. The growth medium was decanted from the plates and the diluted virus was placed in specified wells within the plate (0.1 ml/well, 4 replicate wells/dilution). The plate was wrapped with fresh Saran Wrap™ and

returned to the 37°C incubator. The CPE resulting from virus growth within the cells was graded by microscopic examination after 3 days of incubation. The 50% endpoint of the titration was determined by the method of Reed and Muench (94), considering wells with any degree of CPE within the contained cells to be infected, and those wells lacking CPE within the cells to be uninfected.

For plaque assay of virus, L-cells or BHK-21 cells were seeded in 6-well tissue culture plates (A/S Nunc, Denmark) at 2 x 10^5 cells/ml, 3 ml /dish. The dishes were placed at 37°C in a humidified atmosphere of 5% CO_2, 95% air, and the cells were allowed to grow to confluence (2-3 days). The growth medium was aspirated from the cells and 0.1 ml of a specified virus dilution (in test medium) was placed in the center of the cell sheet in each of 2 wells. The virus was allowed to adsorb for one hour at 37°C with rocking every 15 minutes to keep the cells moist. The cells were then overlaid with 3 ml of a mixture of equal volumes of doubly concentrated test medium and 2% Sea Plaque® agarose (FMC Corp., Marine Colloids Div., Rockland, ME) prepared in glass distilled water. The medium and agarose were tempered to 37°C before mixing. The agarose was allowed to solidify at room temperature and the dishes were placed at 37°C. When CPE was evident in the cell sheets (1-2 days), 1 ml of sterile neutral red in normal saline (one part in 5000 parts) was placed in each dish and allowed to penetrate the agarose at 37°C for a minimum of 2 hours. The excess neutral red was aspirated and the dishes returned to the incubator in the dark. The plaques could usually be counted 3-4 hours later.

Mouse interferon activity in L-cells and in BHK-21 cells. Interferon (IFN) production was induced in L-cells by exposing 7-day old cell sheets, in tissue culture flasks, to a mixture of 0.1 mg/ml DEAE Dextran (Pharmacia, Uppsala,

Sweden) and 0.01 mg/ml polyinosinic-polycytidylic acid, sodium salt (poly I:C) (Sigma Chemical Co., St. Louis, MO) in test medium lacking serum. The mixture was left on the cells at 37°C for 1 hr and then decanted. The cell sheets were rinsed 2 times with modified Puck's balanced salt solution. Test medium supplemented with 0.2% Primatone® RL, a peptic digest of animal tissue (Humko Sheffield Chem., Memphis, TN), was added to the flasks. The cells were incubated in this medium at 37°C for 42 hr to allow production of IFN. Medium was collected from the flasks of L-cells, centrifuged to remove cells and cell debris, dispensed to vials, and frozen at -80°C for future assay.

The IFN activity of this preparation was titered by diluting it in test medium and assaying it in both L-cells and BHK-21 cells. The assay was carried out by placing ten-fold dilutions of the IFN (from undiluted to 10^{-6}) on 18-hour monolayers of cells in 96-well microplates from which the medium had been decanted. Each dilution of IFN was placed in 4 replicate wells of the plate at 0.1 ml/well. The IFN dilutions (or diluent medium without IFN) were incubated on the cells at 37°C for 42 hr before they were decanted from the cells, and 0.1 ml of challenge virus was added to each well. Both VSV and EMC-D virus, each diluted to the same concentration of 100 $CCID_{50}$/0.1 ml, were used as challenge virus to titer this IFN preparation in the two cell lines. When the virus control cells (those exposed to medium lacking IFN) were 100% destroyed by the virus, all of the wells in each plate were examined microscopically and graded for the extent of development of viral CPE. The dilution of sample which provided a 50% endpoint of viral CPE was calculated and the inverse of this endpoint was defined as the IFN titer (units/0.1 ml) of the sample (95).

Tests on mouse urine. Clinistix®, Diastix®, and Bili-Labstix® Reagent Strips for Urinalysis (Ames Division, Miles Laboratories, Elkhart, IN) were used

for analysis of urine samples. Both Clinistix® and Diastix® are plastic strips with single reagent areas designed to react with glucose. The reagent area of each type of strip contains 2 enzymes, glucose oxidase, which catalyzes the formation of gluconic acid and hydrogen peroxide from the oxidation of glucose, and peroxidase, which catalyzes the reaction of hydrogen peroxide with a chromogen. The Clinistix® reagent area contains the chromogen orthotolidine, with which hydrogen peroxide produces colors ranging from purple through blue. A potassium iodide chromogen in the Diastix® reagent area is oxidized by hydrogen peroxide and peroxidase, to colors ranging from green to brown, according to the concentration of peroxide present. Bili-Labstix® Reagent Strips are plastic strips with 6 separate reagent areas fixed to the strip. One of the reagent areas is the same as the Diastix® reagent area and is used to quantitate glucose in the urine. The other reagent areas are individually designed to detect blood, bilirubin, ketone, protein, and the pH of the urine. Detailed instructions provided with each of these products were followed carefully in the testing procedure.

Preparation of mouse plasma samples. Pasteur pipets and 1 ml polystyrene centrifuge tubes (Allied Fisher Scientific, Pittsburgh, PA) were heparinized by dissolving 4 g of sodium fluoride (Sigma chemical Co., St. Louis, MO) in 100 ml of water to which 0.5 g (~170 units/mg) of heparin, sodium salt, (Sigma) was added and allowed to dissolve. The heparin-sodium fluoride solution was drawn into and expelled from the Pasteur pipets which were then dried in an incubator. One drop of the heparin-sodium fluoride solution was added to each centrifuge tube and the tubes were dried in an incubator before use.

Selected mice were individually anesthetized with ether, the brachial artery clipped, and the blood collected in a heparinized Pasteur pipet and

transferred to a heparinized centrifuge tube. These samples were centrifuged. Plasma was collected from each sample, placed in a labeled freezer vial, and stored (-80°C) for future tests.

Determination of plasma glucose levels. Glucose concentration was determined by an enzymatic colorimetric method proposed by Keston (96) and modified by Raabo and Terkildsen (97). The method is based on the coupled enzymatic reactions of glucose oxidase and peroxidase, as in the urine test strips; however, in this case, the chromogen is o-dianisidine. Oxidized o-dianisidine is brown and this color intensity, measured at 425-475 nm, is proportional to the glucose concentration in the sample tested. The procedure outlined in Sigma Technical Bulletin #510, provided with the kit, was modified as to volumes of sample, enzyme mixture, and color reagent solution to facilitate utilization of small amounts of sample in a 96-well flat-bottomed tissue culture plate. The results were measured at 450 nm in a microplate reader (Bio-Tek Instruments, Winooski, VT).

Titration of neutralizing antibodies in plasma. Each plasma sample was diluted in test medium by preparing a single 10-fold dilution (on occasion a 20- or 50-fold dilution was made if the sample volume was insufficient for a 10-fold dilution). This dilution tube was heated at 56°C for 30 minutes and then serial 2-fold dilutions were made from the initial dilution. Equal volumes (0.5 ml) of each diluted plasma sample and a virus preparation containing 100 $CCID_{50}$ of EMC-D virus/0.1 ml, were mixed together and incubated at 37°C for 1 hr in a water bath. Virus control mixtures (0.5 ml of diluent, without mouse plasma, and 0.5 ml of the virus preparation) were similarly incubated. One-tenth ml aliquots of each mixture were placed in each of 3 wells of a 96-well microplate containing confluent monolayers of BHK-21 cells. Diluent medium (0.1 ml) without virus or mouse plasma, was placed in each well designated

as cell control wells. The cells were incubated at 37°C until all virus control wells had a CPE grade of 4 and the cell control wells were all still in very good condition (CPE grade of 0). The neutralizing antibody titer of each serum preparation was determined by calculating the 50% endpoint by the method of Reed and Muench (94). Wells with any degree of CPE were considered to be unprotected and those without CPE were considered to be totally protected by the antibodies in the plasma.

Mouse experiments in which infection was by the ip route. The '82 virus pool was diluted in test medium to obtain approximately 1 x 10^6 pfu/0.1 ml. The '80 virus pool was used undiluted to obtain the approximate same concentration of virus. Neither of these viral preparations had been passaged in our laboratory prior to use in this experiment. Seven mice received an ip injection of 0.1 ml of the '82 virus preparation, 7 mice received an equal volume of the '80 virus preparation, and 7 mice received 0.1 ml of diluent without virus by the same route. Twelve male and 9 female mice were used in the initial experiment. Clinistix® test strips were used each day to test all urine samples for glucose spillage (glycosuria). Glycosuria was first evident on day 4 post virus inoculation (p.i.), so 3 mice from each group (control and 2 virus preparations) were sacrificed for plasma glucose and antibody determinations. The remainder of the mice were sacrificed on day 7 p.i. for like determinations, except that one of the '80 virus-infected mice died on day 7 p.i.

The '82 virus preparation was used to infect L-cells. The virus pool obtained from this first L-cell passage was assayed by ip injection of mice to determine if cell passage in our laboratory would diminish the diabetogenic potential of the virus. All experimental materials and methods were similar to those used in the first experiment except for the following: 1) the virus pool

was different, as noted above; 2) concentrations of 10, 10^2, 10^3, 10^4, 10^5, and 10^6 pfu of virus/mouse, given by the ip route, were used to determine the potency of the virus for diabetogenicity in mice; 3) one mouse from each group, given different concentrations of virus, was sacrificed on days 4, 7, 14, and 21 to determine levels of plasma glucose and neutralizing antibody; 4) several female mice, negative for glycosuria on days 4, 7, and 14, were saved until day 21 to determine if additional female mice would develop glycosuria when given a longer time period after the virus infection; and 5) Diastix® test strips were also used to measure glycosuria on the day of sacrifice if the mouse was positive by Clinistix® on that day.

Selection of a natural route of infection. The L-cell passaged virus was introduced to male SJL/J mice by po, and i.n. routes of infection, with the ip route of infection used as a control. Five mice were selected for each group, but only 4 mice received virus by the i.n. route because one mouse was killed by the anesthetic. Five control mice each received 0.1 ml of sterile diluent medium by the ip route. The ip injections were given by use of a one ml syringe with a 26 gauge needle attached. The po inoculations were administered with a 20G-1.5" perfektum® (Popper and Sons, Inc., New Hyde Park, NY) intubation needle attached to a one ml syringe. The i.n. virus delivery was accomplished by use of a micro-pipetter and micro-pipet tips after the mice had been lightly anesthetized with ether. The mice exposed to virus by the i.n. route received 50 µl of virus which was 5×10^4 pfu/mouse, instead of the 1×10^5 pfu/mouse received by mice exposed to virus by the ip and po routes. On day 6 p.i., all living mice were exsanguinated by clipping the brachial artery and collecting blood while the mouse was anesthetized. Plasma samples were collected, labeled, and frozen for later testing. Urine samples were tested daily for glucose by Clinistix® and by use of Bili-Labstix®

on the day of sacrifice.

Titration of the diabetogenicity of EMC-D virus in mice inoculated by the i.n. route. Male SJL/J mice were inoculated i.n. with virus concentrations ranging in 10-fold increments from 4 pfu/mouse to 4 x 10^5 pfu/mouse. The virus was given in 20 µl aliquots because in the initial experiment, some of the mice failed to inhale all of the 50 µl inoculum that was presented. Five mice were used for each concentration of virus. Five control mice were each given 20 µl of the sterile virus diluent by the i.n. route. Glycosuria was determined daily by use of Clinistix® test strips. Diastix® test strips were used daily for each mouse previously shown to be positive by Clinistix®. On day 7 p.i., all mice were exsanguinated to obtain plasma samples that were frozen for later assay.

Statistical analysis. Statistical analysis on the number of positive results/total samples was done by chi square analysis. The Student's t-test was used to analyze significance of means obtained from glucose measurements.

Results. *Activity of mouse IFN in L-cells and BHK-21 cells.* The IFN induced by treatment of L-cells with poly I:C was devoid of protective activity against either VSV or EMC-D virus in BHK-21 cells, but had good activity when assayed against either virus in L-cells. Results of the titration are shown in Table III-I.

Experiments by the ip infection route. Parameters used as diabetic signs in the mice were glycosuria and plasma glucose levels greater than 3 standard deviations above the mean plasma glucose concentration in the uninfected controls.

Results of Clinistix® testing of the urine of mice injected with EMC-D virus

TABLE III-I. TITRATION OF MOUSE INTERFERON (IFN) IN BHK-21 CELLS AND L-CELLS.

IFN dilution	Percent of cells with virus CPE vs VSV in: BHK-21 Cells	L-cells	vs EMC-D Virus in: BHK-21 cells	L-cells
Undilute	100	0	100	0
10^{-1}	100	0	100	0
10^{-2}	100	0	100	0
10^{-3}	100	9	100	81
10^{-4}	100	94	100	100
No IFN	100	100	100	100

which had not yet been passed in tissue culture in our laboratory are summarized in Table III-II. These data represent tests on most of the mice at each day indicated; however, since some mice withheld urine at one or more sample times, all mice in the experiment were not represented by data at each time period.

All mice with glycosuria on the day of their sacrifice also had plasma glucose concentrations greater than 243 mg/100 ml, which was 3 standard deviations above the mean glucose level of the 7 control mice. The mean of the plasma glucose concentrations of the 4 mice with diabetes that were sacrificed on day 4 (all males) was 331 mg/100 ml, with a range of 273-381 and a S.D. of 46. The 5 mice with diabetes that were sacrificed on day 7 (3 males and 2 females) had a mean plasma glucose concentration of 377 mg/100 ml, with a range of 274-500 and a S.D. of 100.

The 4 female mice which provided a urine sample on day 4 were negative for glycosuria as were all male and female mice sampled previous to day 4. Two of 4 female mice tested on day 5 were positive by Clinistix®. They

TABLE III-II. GLYCOSURIA IN MICE INJECTED BY THE ip ROUTE WITH 2 EMC-D VIRUS POOLS[a].

Source of sample	Days after virus inoculation							
	0	1	2	3	4	5	6	7
Control mice	0/6[b]	0/6		0/4	0/6[c]	0/3	0/3	0/4[d]
Infected mice	0/12	0/9		0/5	7/12	6/8	6/8	5/7
Infected males	0/7	0/5		0/3	7/8	4/4	4/4	3/3
Infected females	0/5	0/4		0/2	0/4	2/4	2/4	2/4
All receiving '82 virus	0/7	0/4		0/3	3/6[e]	3/4	3/4	3/4[f]
All receiving '80 virus	0/5	0/5		0/2	4/6[e]	3/4	3/4	2/3[g]

[a] Both virus pools were provided by Dr. Yoon and had not been passed in our lab prior to use in this experiment.

[b] Number positive/number of samples provided.

[c] Two males and 1 female were sacrificed.

[d] Two males and 2 females were sacrificed.

[e] Two positive males and 1 negative female were sacrificed.

[f] Two positive males and 2 females (1 positive) were sacrificed.

[g] One positive male and 2 females (1 positive) were sacrificed.

both remained positive until day 7, when the experiment ended. None of the other female mice showed signs of diabetes by either parameter, although all mice injected with virus exhibited evidence of infection by development of neutralizing antibodies to the virus. The 6 mice which were sacrificed on day 4 had a mean titer of 322 units of neutralizing antibody/0.1 ml of plasma (range: 200-480; S.D.=119). The 7 infected mice which were sacrificed on day 7 had a mean titer of 1706 units of neutralizing antibody/0.1 ml (range: 480-4470; S.D.=1334). None of the control mice sacrificed on day 4 or day 7 p.i., had detectable antibody (10 units/0.1 ml was the lower limit of detection).

Four male mice infected with virus (3 which received the '80 virus preparation and 1 infected with the '82 virus pool) developed paralysis of one, or both, hind legs. Three of these mice were sacrificed on day 4 p.i., and the remaining mouse was dead on day 7 p.i.

The '82 EMC-D virus passed in L-cells also caused diabetes in mice. All infected male mice became diabetic, even when as little as 10 pfu of the virus was administered ip to the animals. All males except 1 were positive for diabetes by Clinistix®, Diastix®, and plasma glucose. The one exception had a plasma glucose concentration well within the control range, although both indicators of glycosuria were positive. The total number of infected mice with glycosuria varied considerably (Table III-III). All male mice injected with virus were glycosuric by day 5 and remained so throughout the experiment. In contrast, only 9 of 14 females became glycosuric at any time in the experiment, 1 as late as day 7. Two of the female mice had cured of glycosuria before they were sacrificed. Therefore, the variation was due to the numbers of female mice that developed, or recovered from, glycosuria. All female mice that developed glycosuria did so by day 7 p.i.

TABLE III-III. GLYCOSURIA IN MICE INJECTED BY THE ip ROUTE WITH VIRUS PASSED IN L-CELLS

Source of sample	Number of days after virus inoculation				
	0	4	7	14	21
Control mice	0/4[a]	0/4	0/3	0/2	0[b]
Mice infected with 10 pfu	0/4	0/4	2/3	1/2	0/1
Mice infected with 10^2 pfu	0/4	2/3	3/3	1/2	0/1
Mice infected with 10^3 pfu	0/4	2/2	2/2	1/2	0/1
Mice infected with 10^4 pfu	0/4	2/3	2/3	1/2	0/1
Mice infected with 10^5 pfu	0/4	3/3	3/3	2/2	1/1
Mice infected with 10^6 pfu	0/4	2/3	2/3	0/1[c]	0/1
All infected mice	0/24	11/18	14/17	6/11	1/6
Infected male mice	0/10	5/6[d]	7/7[e]	3/3[f]	0[g]
Infected female mice	0/14	6/12[h]	7/10[i]	3/8[c,k]	1/6[m]

[a] Number positive/number of samples obtained.

[b] Mouse died on day 18.

[c] Mouse died on day 13.

[d] Two positive male mice sacrificed.

[e] Five positive male mice sacrificed.

[f] Three positive male mice sacrificed.

[g] All males previously sacrificed.

[h] Four females sacrificed (3 positive and 1 negative).

[i] One positive female sacrificed.

[k] Two positive females sacrificed.

[m] Six females sacrificed (1 positive and 5 negative).

Results of Clinistix® testing of the urine of the mice did not always agree with results of plasma glucose testing. Most discrepancies (3 of 4) occurredin female mice (Table III-IV). Results of tests on mice sacrificed on day 21 were not included because plasma samples were mistakenly discarded.

Natural routes of infection. In an experiment to determine if a natural route of infection could be used to obtain diabetic signs in SJL/J male mice, two natural routes, i.n. and po, were compared to the commonly used ip route. As evident in Table III-V, none of the control mice nor the mice which received virus by the po route showed signs of diabetes on any of the sample days. One of the mice given virus by the po route died on day 3 p.i., and one which received virus by the i.n. route was dead on day 6. One mouse infected by the ip route (the one with the lowest plasma glucose level) was near death immediately before sacrifice on day 6. Three of 4 mice infected by the ip route began showing glycosuria as early as day 4 p.i. and all mice in this group had developed high glycosuria, as well as diabetic plasma glucose levels, at the time of sacrifice. The mice infected by the i.n. route began spilling glucose one day later than those infected ip, with the earliest onset on day 5 p.i. However, on day 6 p.i., all of these mice exhibited high levels of plasma and urine glucose similar to those found in the ip-infected mice. In both situations (ip and i.n.), the increased positives/total were statistically significant. None of the control mice, nor those exposed to virus by the po route, developed neutralizing antibody to the virus. However, all mice exposed to the virus by either the i.n. or the ip routes developed neutralizing antibody titers greater than 200 units/0.1 ml by day 6 p.i. The magnitude of the antibody titers, as related to the concentration of plasma glucose present on the day of sacrifice, is recorded in Fig. III-1, along with similar results from other experiments. Little correlation could be made between concentration of plasma glucose and

TABLE III-IV. COMPARISON OF 3 PARAMETERS USED TO DETERMINE THE DIABETIC STATE IN MICE INJECTED BY THE ip ROUTE WITH VIRUS PASSED IN L-CELLS.

Source of sample	Day of sacrifice post-virus inoculation								
	4			7			14		
	Cl[a]	Di[b]	PG[c]	Cl	Di	PG	Cl	Di	PG
Female, 10 pfu	0*	0*	196*						
Female, 10^2 pfu	D	5	477						
Female, 10^3 pfu	D	1	188*						
Female, 10^4 pfu	L	0*	302						
Male, 10^5 pfu	D	5	514						
Male, 10^6 pfu	D	2	392						
Female, 10 pfu				D	n.s.[d]	154*			
Male, 10^2 pfu				D	1	186*			
Male, 10^3 pfu				D	5	372			
Male, 10^4 pfu				D	5	562			
Male, 10^5 pfu				D	5	426			
Male, 10^6 pfu				D	5	412			
Male, 10 pfu							D	5	611
Female, 10^2 pfu							L	0*	176*
Male, 10^3 pfu							D	5	767
Male, 10^4 pfu							D	5	722
Female, 10^5 pfu							D	5	385

[a] Clinistix® results: 0=negative; L=light; M=medium; D=dark.

[b] Diastix® results: 0=negative; 1=100; 2=250; 3=500; 4=1000; 5=≥2000 mg/100 ml.

[c] Plasma glucose concentration (mg/100 ml).

[d] No sample obtained.

* Non-diabetic result.

TABLE III-V. DIABETOGENICITY OF EMC-D VIRUS ADMINISTERED BY DIFFERENT ROUTES IN MALE SJL/J MICE.

Virus inoculation route	Days After Virus Inoculation: 3 Clinistix Pos./ total	4 Clinistix Pos./ total	5 Clinistix Pos./ total	6 Diastix Pos./ total	6 Plasma glucose Pos.[a]/ total	6 Plasma glucose Titer[b]	6 Neutral. antibody titer[c]
None	0/3	0/3	0/4	0/5	0/5	151	0
po	0/3	0/3	0/4	0/4	0/5	150	0
i.n.	0/4	0/3	2/3	3/3	3/3	452	3840
ip	0/4	3/4	2/3	5/5	5/5	522	2582

[a] ≥3 S.D. above the mean plasma glucose conc. of control mice sacrificed on day 6.

[b] mg/100 ml (mean).

[c] units/0.1 ml (mean).

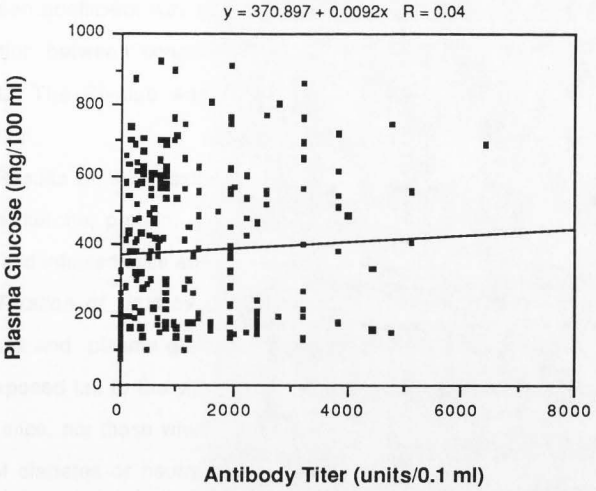

FIG. III-1. Scatter graph of the relationship between plasma glucose concentration and antibody titer in mice inoculated with EMC-D virus.

antibody titer. The correlation coefficient (R) calculated for the straight line of best fit to the data points was 0.04. A table of critical values for the Pearson correlation coefficient was used to test the validity of the hypothesis that a correlation between concentration of plasma glucose and antibody titers existed. The P-value was found to be > 0.05, indicating no statistical correlation.

Results of Bili-Labstix® tests of the urine other than glucose (blood, bilirubin, ketone, protein, and pH) were not consistently different between control and infected mice and were not done in other experiments.

Titration of virus by the i.n. route of infection. Results of tests for glycosuria and plasma glucose, as well as neutralizing antibody titers of all mice exposed i.n. to the virus, are summarized in Table III-VI. None of the control mice, nor those which received either 4 or 40 pfu of virus, developed signs of diabetes or neutralizing antibodies. Only one of five mice which received 400 pfu of virus was diabetic during the experiment. This mouse exhibited glycosuria for the first time on day 6, was positive for diabetes by glycosuria and plasma glucose, and was the only mouse in this group positive for neutralizing antibody on day 7. Two of 5 mice which received 4×10^3 pfu of virus were not diabetic by any parameter. However, one of these mice had a high titer of neutralizing antibody while the other mouse had no neutralizing antibody. The other 3 mice in this group developed high levels of urine glucose, plasma glucose, and neutralizing antibody. All of the mice inoculated with 4×10^4 pfu of virus showed glycosuria at some time during the experiment. Two of the mice were negative for glucose in the urine by Diastix® on the day of sacrifice, although one of these mice earlier (day 5) had a Diastix® reading of 500 mg/100 ml. This reading dropped to 250 mg/100 ml on day 6 and was negative on day 7, even though the Clinistix® reading was

TABLE III-VI. TITRATION OF INTRANASALLY ADMINISTERED EMC-D VIRUS IN MALE SJL/J MICE.

Virus conc. (pfu/mouse)	Days after virus inoculation: 3 Clinistix Pos./ total	4 Clinistix Pos./ total	5 Clinistix Pos./ total	6 Clinistix Pos. total	7 Diastix Pos./ total	7 Plasma glucose Pos.[a]/ total	7 Plasma glucose Titer[b]	7 Neutralizing antibody titer[c]
4	0/4	0/5	0/4	0/3	0/5	0/5	168	0
4x10	0/5	0/3	0/5	0/3	0/5	0/5	196	0
$4x10^2$	0/5	0/4	1/4	1/4	1/5	1/5	249	512
$4x10^3$	0/4	1/4	3/5	1/3	3/5	3/5	373	1278
$4x10^4$	0/5	1/4	4/5	4/5	3/5	3/5	374	3460
$4x10^5$	0/5	1/4	5/5	5/5	5/5	5/5	384	1711

[a] ≥3 S.D. above the mean plasma glucose conc. of control mice sacrificed on day 7.

[b] mg/100 ml (mean).

[c] units/0.1 ml (mean).

medium for glucose on the day of sacrifice. The other Diastix®-negative mouse had a positive Clinistix® reading (dark) for the first time on the day of sacrifice. Neither of the two Diastix®-negative mice had high plasma glucose, but the other 3 mice were diabetic by plasma glucose as well as by Diastix® on day 7. All of the mice in the group exhibited high neutralizing antibody titers in the plasma taken on day 7. The earliest times of glycosuria in this group of mice was day 4 (one mouse). Three others began spillage on day 5, and the last mouse showed no glycosuria until day 7. All of the mice in the group receiving 4×10^5 pfu of virus were diabetic by both parameters tested. One of these mice began spilling glucose in the urine on day 4, and the other 4 began spilling on day 5. All of these mice also developed neutralizing antibody specific to the virus.

Discussion. The comparative titration of mouse IFN in L-cells and BHK-21 cells was carried out in order to identify a cell line which would not respond to mouse IFN. A cell line of this type was necessary to insure that neutralizing antibody titrations could be performed without interference by mouse IFN, which was present in some of the plasma samples to be tested. BHK-21 cells served this purpose well. They were very sensitive to infection by EMC-D virus and pretreatment with mouse IFN did not alter this sensitivity. Thus, the "species specificity" of mouse IFN is valid in this case; i.e. mouse IFN is inactive in cells from the hamster (BHK-21 cells).

There was no apparent difference in the diabetogenic potential of the 2 different pools of virus received from Dr. Yoon when studied prior to cell-culture passage in our laboratory. However, the '80 virus pool caused a few more mice to develop central nervous system infections than did the '82 virus pool. For this reason, the '82 virus pool was chosen as seed for cell culture

passage in our laboratory to prepare virus for additional experiments.

The preparations of EMC-D virus supplied by Dr. Yoon, as well as the virus pools prepared in our laboratory in L-cells, were diabetogenic in SJL/J mice to the same extent as that previously published by others (14, 15, 18, 62). In agreement with previous investigators (74), it was found that this virus induced diabetes in a higher percentage of male mice than it did in female mice when they were injected with identical amounts of the virus. This was apparently due to a hormonal effect, according to the work of Morrow, et al. (73), Giron and Patterson (74), and Giron, et al. (75). Male mice began spilling glucose in the urine one day earlier than the female mice, as seen in Table III-II. In the experiment summarized in Table III-III, 4 of the male mice were glycosuric on day 3, while none of the female mice spilled glucose on that day (data not shown). Others have not reported a difference in time of development of diabetes among male and female mice.

Only four of the published reports (11, 62, 73, 76) dealing with this mouse model of diabetes referred to the measurement of glucose spilled in the urine. These reports only briefly mentioned that the investigators had tested urine for glucose. No comparisons were made between results of the urine- and blood-glucose tests. None of the investigators consistently utilized this measurement as an indicator of development of diabetes in the mice. It seemed logical that methods developed for, and widely used by, humans for demonstration of diabetic symptoms, could also be used to demonstrate diabetic signs in mice. Since mice tend to urinate as they are being handled, it was decided to investigate the use of commercially prepared indicators of glucose in the urine, namely Clinistix® and Diastix® reagent strips. Bili-Labstix® were also used in one experiment to determine if other abnormal conditions would develop in the urine in the short period of time necessary for

the virus to cause diabetes in mice. Lack of abnormal results, other than glycosuria, unique to the diabetic mice, as well as the difficulty of collecting sufficient urine at the proper time to accomplish all of the tests on each reagent strip, precluded use of Bili-Labstix® strips in additional experiments.

The experiments presented here demonstrate that normal SJL/J mice do not spill sufficient glucose in the urine to be detected by reagent strips designed to detect glycosuria. A total of 93 control mice were tested for glycosuria on a daily basis over periods of time from 4 days to 64 days, and none had a positive test. Thus, it was assumed that a positive finding with either Clinistix® or Diastix® test strips indicated that the tested animal has had some part(s) of its normal glucose control mechanism altered toward production of diabetic signs.

In the experiment in which the '80 and '82 EMC-D pools were examined, all of the mice which spilled glucose in the urine, as determined by Clinistix® measurement, also had plasma glucose levels which were considered to be in the diabetic range. These results suggested that Clinistix® testing is in complete agreement with the more cumbersome method of measuring glucose levels in the plasma of the mouse. However, in other experiments, there were some positive Clinistix® tests of urine from mice which had plasma glucose concentrations in the normal control range. These results seem contradictory because one would not expect spillage of glucose into the urine until after blood glucose levels were above the "renal threshold concentration" at which tubular reabsorption of glucose no longer matched the filtered load (98). Certainly, one would have expected spillage of glucose into the urine once the "threshold concentration" was reached. In cases of such contradictory results, virus infection may have altered the active transport mechanism of the renal proximal tubular cells so that glucose was spilled into

the urine while blood glucose concentration remained within a normal range. Alternatively, plasma glucose might have increased to a level sufficient to cause a threshold concentration in the proximal tubule for a period of time, thus allowing urine in the bladder to contain glucose. However, by the time the mouse was sacrificed, the plasma glucose level was back to normal, while the urine stored in the bladder still contained measurable glucose.

Pitts (99) described a clinical condition known as intercapillary glomerulo-sclerosis in which glycosuria could be absent despite high plasma glucose concentrations. This condition is due to a reduced glomerular filtration rate caused by deposition of a mucopolysaccharide-protein complex in the glomerular capillary. If the passive glomerular filtration rate is so easily affected, the more complicated, active transport mechanism of tubular reabsorption could be inhibited greatly by something as pervasive as viral infection.

The Diastix® test strips were not as sensitive in these experiments as the Clinistix® strips, but they are designed to be quantitative. Therefore, a Diastix® reading would be expected to more closely agree with the results of plasma glucose measurements. Either an initial threshold level of glucose or a transient threshold level could allow non-reabsorbed glucose to be diluted sufficiently in the bladder to give negative, or low, Diastix® readings, even though Clinistix® tests were positive. Only a glucose imbalance which had increased over a period of time would be sufficient to give high Diastix® readings. All test mice had plasma glucose levels greater than 3 S.D. above the mean plasma glucose level of control mice when the Diastix® reading of their urine was 250 mg/100 ml or above.

Lack of sufficient virus to cause a generalized infection was not the reason that some of the mice failed to develop diabetic signs. All mice

injected with virus by the ip route developed virus-specific antibody in the plasma, even as early as 4 days after the virus had been injected, and when only 10 pfu of virus was given to each mouse. Not all of these mice became diabetic.

Experiments by the i.n. route of infection demonstrated that all mice which failed to develop neutralizing antibody also failed to show diabetic signs. One mouse, although given the high dose of 4000 pfu of virus, failed to develop either circulating antibody or diabetes. Four of the 5 mice given 400 pfu of virus were similarly negative in both categories. Thus, either some specific site must be infected by the virus, or some specific set of infection/resistance parameters must be achieved, in order to trigger development of diabetic signs. During the development of the particular set of circumstances necessary for triggering diabetic signs, the virus must achieve an infection that is sufficiently generalized to initiate antibody production. However, the magnitude of virus neutralizing antibody production has a low correlation with the concentration of glucose in the plasma, indicating that the neutralizing antibody alone is not responsible for high glucose levels.

It was clear from the results of these experiments that mice can be in a transient state of glucose imbalance to the extent that they spill glucose into the urine for a period of time, and then return to normal conditions. Female mice appeared better able than male mice to resist and/or overcome the diabetogenic effect of this virus. However, a few male mice, at least those exposed to a low to intermediate dosage of the virus, were sometimes also able to overcome the diabetogenic effect of EMC-D virus. In these cases, the males also had transient glycosuria, and seemed to return to normal glucose balance. The mechanisms by which some individuals were able to successfully resist the diabetogenicity of this virus is not known. One purpose

of this study was to provide an improved model to be used in the determination of these resistance mechanisms.

The i.n. route of infection is an excellent natural route of infection for this virus/mouse model of diabetes development. When this route of infection is used, the diabetic signs generally begin to appear a day later than when infection is by the ip route. However, when a high titer ($\geq 10^5$ pfu) of virus is used to infect males, the ultimate percentage of mice induced to develop diabetic signs is essentially the same whether the i.n. or the ip route of infection is used. On the other hand, the po route of infection was completely ineffective for induction of diabetic signs in the mice. In fact, the mice were totally resistant to generalized infection as determined by a complete lack of development of neutralizing antibody when a high concentration of the virus (10^5 pfu/mouse) was introduced by this route.

It was surprising that the po route of introduction of EMC virus was ineffective in establishing an infection in the mice. This virus is classified as a mouse enterovirus and is resistant to low pH, although somewhat conflicting reports as to the extent of this resistance have been published (12, 100, 101). Because of its classification as an enterovirus, EMC virus would be expected to be infectious by the po route. In fact, according to one author (102, p.565), "Occasionally, mice can be infected with virus-contaminated drinking water.", and, "Contact transmission among cotton rats and infection by feeding in the case of albino mice have been demonstrated" (102, p.567). Others have reported that several species of monkeys have been infected by the po route (12). Craighead et al. (103) have infected swine with EMC virus by the oral route. It is possible that selection of the diabetogenic strain of the virus also eliminates the virus strain which is resistant to low pH. However, this possibility, as well as the possibility that the wild-type virus is not infective by

the po route of infection, has not been tested.

The diabetogenicity of the virus is more dose responsive when given by the i.n. route than when given by the ip route. This could be due to the fact that virus introduced by the artificial route of infection circumvents some of the mechanisms of natural resistance to infection encountered in the natural route of infection. This conclusion is supported by study of neutralizing antibody data obtained following i.n. exposure to different concentrations of EMC-D virus. All of the mice that received less than 400 pfu of virus were able to eliminate the virus before it was able to stimulate antibody production, i.e. before a generalized infection was established. Four of the 5 mice that received 400 pfu of virus were able to eliminate the virus prior to establishment of sufficient infection to induce circulating antibody. Even when virus was given at a concentration of 4×10^3 pfu/mouse, 1 of the 5 mice escaped infection sufficient to produce detectable antibodies in the plasma. Conversely, when virus was given by the ip route, all mice developed neutralizing antibodies, even when as little as 10 pfu of virus was given.

Titration of the diabetogenicity of the virus in male mice revealed that low doses of virus, similar to those which caused diabetic signs when given by the ip route, were ineffective when introduced by the i.n. route. However, with increasing concentrations of virus, the percentage of diabetic mice increased to 100%. With the i.n. infection, diabetogenicity is related to the dosage of virus administered. Thus, adjustments of the virus concentration should allow one to more fully study the differences in host resistance occurring in mice that are or are not susceptible to the diabetogenic aspects of the virus. This should be possible, even if the virus strain and dosage are identical in each case. Conversely, when the virus causes diabetic signs in 100% of the mice, control mice for study of resistance factors involved in permitting or prohibiting the

diabetic state must either be non-infected mice or mice infected by a non-diabetogenic virus. Thus, the i.n. route of infection by intermediate concentrations of this virus may enable one to differentiate the quirks in resistance mechanisms that allow the virus to establish a diabetic condition in the mouse.

Conclusions. Mouse IFN was not active in BHK-21 cells. Therefore, the neutralizing antibody titers determined in these cells were not affected by the presence of IFN in the mouse plasma.

Measurement of glucose in the urine of mice by use of Clinistix® and Diastix® reagent strips provided a reliable method to determine if the mice were diabetic or not. The method was easily and quickly accomplished without harm or pain to the mice. The only deficiency in the method was that sometimes individual mice failed to provide a sample of urine for testing.

A natural route of infection, the intranasal route, was found to be reliable in allowing the EMC-D virus to cause a diabetogenic infection in male SJL/J mice. The i.n. route of infection, unlike the ip route, provided a wide range of dose response for the diabetogenicity of EMC-D virus. When mice were given either 4 or 40 pfu of virus i.n., they remained free of diabetes and also did not develop neutralizing antibody. Twenty percent of the mice developed diabetes when given 4×10^2 pfu of the virus. When 4×10^3 pfu of virus was given i.n., 60% of the mice developed diabetes. Half of those without diabetes at this concentration of virus, also lacked antibody specific for the virus. All of the male SJL/J mice given $\geq 4 \times 10^4$ pfu of EMC-D virus i.n. developed diabetes. As little as 10 pfu of this virus, given to male SJL/J mice by the ip route, caused 100% of the mice to become diabetic. All mice which developed diabetes, regardless of the route of infection, also produced

neutralizing antibody to the virus.

When virus was given by the po route, no signs of infection were observed. The mice did not develop diabetes nor did they produce neutralizing antibody.

Some mice became temporarily diabetic by the EMC-D virus infection and then spontaneously recovered. The mechanism by which this recovery occurred is unknown.

CHAPTER IV

EFFECTS OF PURIFICATION ON THE DIABETOGENICITY OF EMC-D VIRUS

Introduction. Craighead and McLane (11) published the first report of a strain of encephalomyocarditis (EMC) virus causing diabetic signs in mice. These investigators named the diabetogenic strain of EMC virus, which they had isolated from the heart tissue of infected mice, the M-variant; M standing for myocardium. More recently, Yoon et al. (15) were able to purify several strains of EMC virus from the M-variant by plaquing techniques. The purified strains seemed to differ only by their relative ability to cause diabetes in a particular strain of mouse. Some of the plaque isolates had no ability to cause diabetes in the mice, some had the ability to cause 100% of infected mice to develop diabetes, and other isolates were intermediate in their diabetogenic ability.

Yoon's group expressed confidence that their D variant (EMC-D), diabetogenic in 100% of the male mice infected by it, was stable in this property, since they had passaged the virus variant repeatedly in secondary mouse embryo cells without diminishing its diabetogenic ability. However, Giron et al. (14) suggested that the D variant consists of either more than one distinct virus strain, or that it is a relatively unstable strain, the properties of which are easily altered during replication in various cell types. They came to this conclusion after observing that EMC-D virus displayed different degrees of diabetogenicity according to the type of cells from which it was harvested.

The altered diabetogenicity observed by Giron et al. could have been

due to cell material in the crude virus preparations used in the experiments. Perhaps it is not the virus alone, but a combination of certain materials from the cell culture along with the virus, that is responsible for producing the diabetic state. Alternatively, materials from certain cell cultures may mask the diabetogenic ability of the virus. At this time, it is only known that certain strains of EMC virus appear to vary in their ability to cause diabetes in mice according to the type of cells in which the virus was prepared.

The purpose of this investigation was to determine if purification of EMC-D virus would alter its diabetogenic characteristics.

Materials and Methods. *Virus.* EMC-D virus was prepared by a single passage in L-cells as outlined in the materials and methods section of Chapter III. A few small aliquots of the crude virus preparation were dispensed to vials and frozen for later comparative assays. The remainder of the virus pool was purified as outlined in the next section.

Virus purification. Sixty-seven ml of ice-cold Freon 113 was added to 250 ml of the homogenized virus pool in a 400 ml stainless steel Sorvall® Omni-Mixer cup (DuPont Co., Newtown, CT), and the mixture was homogenized for one min at 8,000-10,000 rpm in an ice bath. The homogenate was transferred to Nalgene® #3119-0050 polyallomer 50 ml screw cap tubes (American Scientific Products, Sunnyvale, CA) which had been sterilized in the autoclave. The tubes were placed in the SS-34 rotor of the RC-2-B centrifuge (Ivan Sorvall, Inc., Norwalk, CT) and centrifuged at 2,000 rpm for 10 min. at 0°C.

The clear red supernatant liquid was carefully decanted from each centrifuge tube and collected in a sterile 1,000 ml Erlenmeyer flask. This resulting virus pool was mixed and placed in an ice bath. A few 0.2 ml aliquots of the pool were dispensed to freezer vials and placed at -80°C for

later assay.

The pH of the Freon extracted pool was 7.8. After adding 3.5 ml of 10% acetic acid in an ice bath, the pH was 4.8. Very fine, white, hair-like particles began to appear in the bright yellow liquid after about 5 min. After approximately 30 min., some of the liquid was poured into 2 sterile Nalgene polyallomer tubes and these were centrifuged in the SS-34 rotor of the Sorvall RC-2-B centrifuge at 4,000 rpm for 10 min. at 4°C. The supernatant liquid was decanted into a sterile tissue culture flask (Corning Glass Works, Corning, NY) and the above centrifugation step was repeated after more of the acidified virus preparation was decanted into the tubes. These two steps were repeated until all of the virus preparation had been centrifuged. After the final centrifugation, a small white button could be seen in one of the centrifuge tubes before the supernatant liquid was decanted. Both centrifuge tubes were washed with 0.5 ml of acidic medium (pH 4.5-5.0) in an attempt to resuspend the small pellets, and then washed 5 times with 1 ml volumes of sterile PBS. All washings were collected in a sterile stainless steel micro Omni-Mixer cup and homogenized at full speed for 1 min. in an ice bath. Samples of this acid precipitate (A.P.) virus homogenate were stored at -80°C for future assay.

One ml of sterile-filtered CsCl solution (895.3 mg/ml) was pipetted into each of 6 centrifuge tubes (Beckman® #331370, cellulose nitrate, Beckman Instruments, Fullerton, CA). One ml of sterile PBS was placed in tube #6 and 1.0 ml of the A.P. virus homogenate was placed in each of the other 5 tubes. Approximately 11 ml of Nujol® Laxative Extra Heavy Mineral Oil (Plough, Inc., Memphis, TN) was placed in each of the 6 tubes. The tubes were centrifuged in the Beckman® L8-70 ultracentrifuge (Beckman Instruments) at 14°C, 25,000 rpm, slow acceleration, brake off, for 16.5 hours with tubes in their respectively numbered buckets of the SW41 rotor. After centrifugation, fractions were

obtained by collecting single drops from a hole punctured in the bottom of each tube. The fractions were collected from tube #6 first, and then from each of the other tubes in the order #1 through #5. A single drop was collected in each of the 1.0 ml non-sterile polystyrene centrifuge tubes (Fisher Scientific, Pittsburgh, PA) used to collect from tube #6. The same amount was collected in each of the sterile Fisher #05-407-5 centrifuge tubes used to collect from tube #1. One ml polystyrene centrifuge tubes were used to collect single drops from tube #2. The single drops collected from each of tubes #3 through #5 were pooled in sterile Fisher #05-407-5 centrifuge tubes.

Refractive indices of each sample collected from tube #6 were measured by use of a Bausch and Lomb refractometer (Fisher Scientific). A Haake type F constant temperature water bath (American Scientific Products, Sunnyvale, CA) was used to maintain a uniform 25°C temperature on the refractometer glass plate. The density of each sample was calculated from the refractive indices by use of the formula of Bruner and Vinograd (104): $\rho^{25°C} = 10.2402\ \eta_D^{25°C} - 12.6483$ for densities between 1.00 and 1.37 g/cm^3, where $\rho^{25°C}$ = density at 25°C and $\eta_D^{25°C}$ = refractive index measurement at 25°C. The refractive index was corrected for the PBS in the CsCl samples before the density was calculated.

Tubes containing fractions collected from centrifuge tubes #1 through #5 were capped and placed at -80°C for storage until assays could be performed.

Protein assay. Protein in each sample was assayed by the Bio-Rad Protein Assay procedure (Bio-Rad Laboratories, Richmond, CA). Bovine plasma gamma globulin was used as a protein standard (Bio-Rad Protein Assay Standard I).

Medium. The same types of cell culture medium as those described in

the materials and methods section of Chapter III were used in these experiments.

Cells. See the materials and methods section of Chapter III.

Virus assay. Virus was assayed by the plaque assay method outlined in Chapter III, materials and methods section.

Mice. Mice were used as described in the materials and methods section of Chapter III.

Neutralizing antibody assay. Sera of mice were assayed for neutralizing antibody as explained in the materials and methods section of Chapter III.

Experiments comparing diabetogenicity of crude and purified virus pools. Male mice received either 4×10^3, 4×10^4, or 4×10^5 pfu of crude or of purified virus by the intranasal (i.n.) route while they were lightly anesthetized by ether. In the first experiment, female mice were included to determine if they would respond to purified virus differently than the male mice. The females received only the 4×10^4 pfu dosage from each virus pool by the i.n. route of inoculation. All mice in this experiment were sacrificed 7 days after receiving virus in order to determine plasma glucose and neutralizing antibody levels.

A second experiment was run to determine if additional mice would develop diabetes when allowed a longer period of time for development of the disease. Both crude and purified virus preparations were used for infection to determine if one would be more diabetogenic than the other. The rate of death and number of diabetes cures were also observed for differences between crude and purified virus. Mouse sacrifice was delayed from the 7 days of the first experiment to either 9 or 21 days to allow more time for development of diabetes. Up to 10 mice from each group that had glycosuria

on day 9 p.i., or that had demonstrated a remission of glycosuria by day 9, were selected for sacrifice on that day. The other mice in each group were retained for observation over the full 21 days. A 10-fold higher dosage of virus was given to females in this experiment to determine if increased virus would cause more females to become diabetic.

The mice of both experiments were monitored daily for glycosuria by use of Clinistix® (Ames Div., Miles Laboratories, Inc., Elkhart, IN). After they were positive by this test, Diastix® (Ames) test strips were used in order to obtain quantitative results.

Bleeding and plasma sample collection. Bleeding and plasma sample collection techniques were the same as those in the materials and methods section of Chapter III. The plasma samples were frozen (-80°C) and analyzed later for glucose and neutralizing antibody, as outlined in the materials and methods section of Chapter III.

Results. *Virus purification.* The infectious virus recovered in the pooled fractions from density gradient tubes 3, 4, and 5 is shown in Fig. IV-1a. The virus recovered in each fraction from density gradient tube 2 is shown in Fig. IV-1b along with the density of corresponding fractions recovered from density gradient tube 6.

At each step of the purification procedure, the percentage recovery of plaque forming units of virus was compared to the percentage recovery of total protein to obtain a purification index (105). These results for sample #25 of pooled fractions from density gradient tubes 3, 4, and 5 are shown in Table IV-I. Other samples from the virus peak shown in Fig. IV-1a provided similar results. The virus preparations labeled "purified" in these experiments were approximately 67-fold more pure than the crude virus preparation.

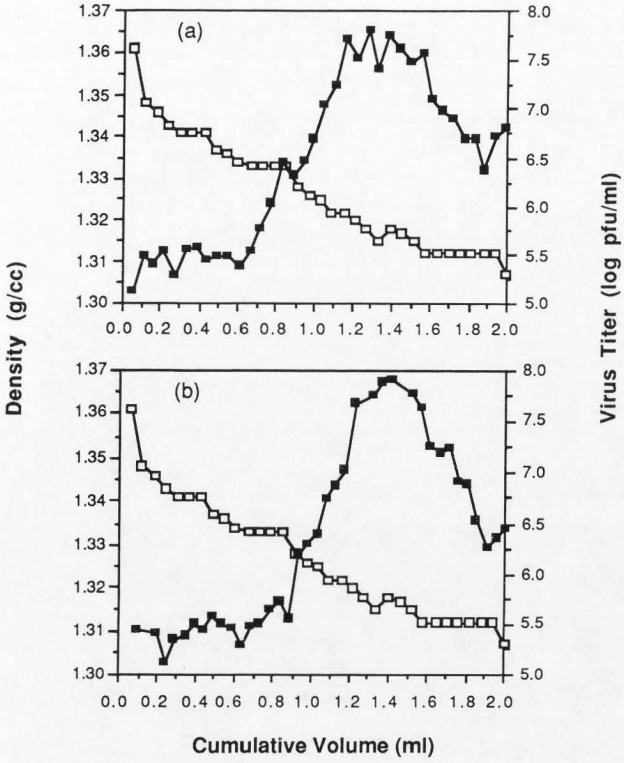

FIG. IV-1. Density gradient purification of EMC-D virus. Open squares are density results of fractions without virus (centrifuge tube #6). Closed squares are virus titers obtained from fractions (a) pooled from centrifuge tubes #3-5; and (b) from centrifuge tube #2.

TABLE IV-I. EMC-D VIRUS PURIFICATION.

Sample	Total volume (ml)	Virus titer (pfu/ml)	Total virus (pfu)	Virus recovery (% of initial)	Protein conc. (μg/ml)	Total protein (μg)	Protein recovery (% of initial)	Purification index[a]
Crude pool	258	5.4×10^7	1.4×10^{10}	100	1,790	4.6×10^5	100	1
Freon supernate	323	8.4×10^7	2.7×10^{10}	192.8	1,040	3.4×10^5	73.9	2.6
Acid precipitate	5.5	2.7×10^7	1.5×10^8	1.1	332.8	1.8×10^3	0.4	2.8
Density gradient[b]	0.06	5.7×10^7	3.4×10^6	0.02	24.8	1.4	0.0003	66.7

[a] Purification index = percentage recovery of virus ÷ percentage recovery of protein.

[b] Density gradient sample #25 from 3-5 pooled tubes was assayed.

Diabetogenicity of crude and purified virus preparations. The number of male mice that developed diabetic signs by day 7 post virus inoculation (p.i.) was directly proportional to the dosage of virus given whether the virus was the crude or the purified preparation (Table IV-II). Both parameters for diabetic signs (glycosuria and plasma glucose) were in agreement in samples from male mice on the day of sacrifice. Two of 5 female mice given 4×10^4 pfu of purified virus were diabetic by plasma glucose, but only one had glycosuria by Diastix®. The females given the same dosage of crude virus failed to develop any signs of diabetes. All of the female mice which were tested developed neutralizing antibodies. Only the lowest dosage of virus failed to infect the male mice sufficiently to cause every mouse to produce antibodies. At this lowest virus dosage, only those male mice infected sufficiently to produce circulating antibodies became diabetic.

Glycosuria, plasma glucose, and neutralizing antibody data gathered from mice sacrificed 9 or 21 days post virus inoculation are summarized in Table IV-III. Glycosuria data from day 7 are included for comparison with data from the first experiment. Standard deviations for the mean values of plasma glucose and neutralizing antibody concentrations are shown in Table A-I of the Appendix. Neutralizing antibody data are not combined since the antibody titers would be expected to be, and were significantly higher in those mice sacrificed 21 days after infection than in those sacrificed 9 days p.i.

A time-line with events of change, either cure from previous glycosuria or death, indicated on the days they were observed is depicted in Fig. IV-2. The type of virus pool and the concentration of virus used in the mice involved in each event is represented by the location and the type of symbol, respectively.

Four mice out of 63 inoculated with crude virus died during the

TABLE IV-II. TITRATION OF CRUDE vs PURIFIED EMC-D VIRUS POOLS. MICE WERE SACRIFICED 9 DAYS POST VIRUS INOCULATION.

Titer of virus inoculum (pfu/mouse)	Glycosuria (pos./total) Day 7 Crude	Day 7 Purified	Day 9 Crude	Day 9 Purified	Plasma glucose (day 9) Crude Pos.[a]/total	Crude Mean[b]	Purified Pos./total	Purified Mean	Neutral. antibody[c] (mean ± S.D.) Crude	Purified
4×10^3	7/7	9/10	8/10	9/10	9/10	518±165	9/10	541±180	704±466	555±393
4×10^4	9/10	10/10	10/10	10/10	10/10	593±167	9/9	517±120	597±283	443±309
4×10^5	10/10	9/9	10/10	9/10	10/10	567±95	9/10	498±167	354±185	482±209
4×10^5 female	10/10	5/5	10/10	6/6	7/10	358±125	6/6	466±106	458±248	627±508

[a] ≥3 S.D. above the mean plasma glucose concentration of control mice.

[b] mg/100 ml (± S.D.)

[c] Units/0.1 ml of plasma.

TABLE IV-III. TITRATION OF CRUDE VS PURIFIED EMC-D VIRUS POOLS.
MICE WERE SACRIFICED 9 OR 21 DAYS POST VIRUS INOCULATION.

Titer of virus Inoculum (pfu/mouse)	Data from mice sacrificed on (day)	Glycosuria (pos./total)						Plasma glucose				Neutralizing antibody[c]	
		Day 7		Day 9		Day 21		Crude		Purified			
		Crude	Purified	Crude	Purified	Crude	Purified	Pos.[a]/ total	Mean[b]	Pos./ total	Mean	Crude	Purified
4×10^3	9	7/7**	9/10**	8/10**	9/10**			9/10**	518**	9/10**	541**	704	556
	21	0/5	2/3**	0/5	2/3**	0/5	2/5*	0/5	182	2/5*	308*	1120	1313
	Combined	7/12**	11/13**	8/15**	11/13**			9/15**	406**	11/15**	463**		
4×10^4	9	9/10**	10/10**	10/10**	10/10**			10/10**	593**	9/9[d]**	517**	597	443
	21	2/3**	4/4**	1/3	4/4**	1/3	3/4**	2/3**	371	3/4**	459**	2933	1651
	Combined	11/13**	14/14**	11/13**	14/14**			12/13**	542**	12/13**	499**		
4×10^5	9	10/10**	9/9**	10/10**	9/10**			10/10**	567**	9/10**	498**	354	482
	21	4/4**	5/5**	4/4**	2/4*	3/4**	1/3	3/4**	512**	1/3	392	3203	1731
	Combined	14/14**	14/14**	14/14**	11/14**			13/14**	551**	10/13**	473**		
4×10^5 female	9	10/10**	5/5**	10/10**	6/6**			7/10**	358**	6/6**	466**	458	627
	21	2/6*	0/5	1/4	0/5	0/7	0/12	0/6[d]	170	0/11[d]	161	1613	1878
	Combined	12/15**	5/10**	11/14**	6/11**			7/16**	287**	6/17**	269**		
0	9	0/19		0/19				0/19	174			0	
	21	0/10		0/10		0/10		0/9[d]	166			0	
	Combined	0/29		0/29				0/28	171			0	

[a] ≥3 S.D. above the mean plasma glucose concentration of control mice.

[b] mg/100 ml.

[c] Units/0.1 ml of plasma.

[d] Sample not available from one mouse.

* $P<0.05$; ** $P<0.01$; compared to controls.

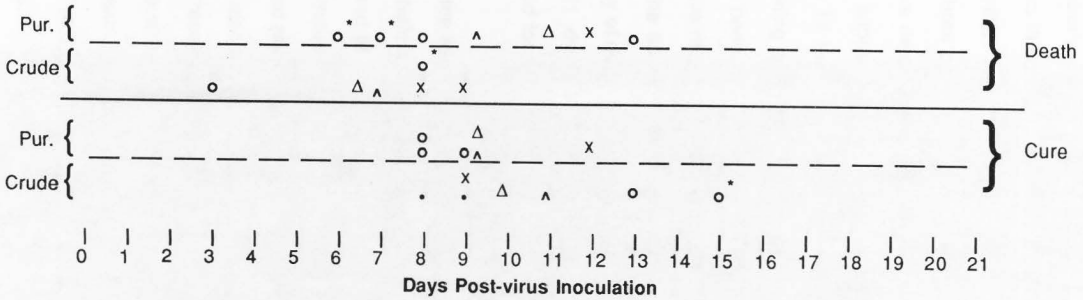

Fig. IV-2. Time-line diagram of cures or deaths in mice inoculated with crude or purified preparations of EMC-D virus. •, 4 x 10^3 pfu; x, 4 x 10^4 pfu; o, 4 x 10^5 pfu; *, female; ∧, mean day of change--all mice; Δ, mean day of change--male mice only.

experiment. The mean day of death for these mice was 7.0 ± 2.7. Although the cause of death was not determined, it did not seem to be due to central nervous system infection because the mice that died did not show obvious signs of paralysis. A general parenteral infection by the virus would seem more likely. Three of the mice were noted to have a bright red and swollen penis just before death. One of the mice had a bloody penis. In general, the mice suddenly appeared very ill, and died within 1 or 2 days. Of the 59 mice (42 males and 17 females) that lived to provide a blood sample, 3 failed to produce circulating antibody specific for the virus. All 3 of these mice were males that had been inoculated with 4×10^3 pfu of crude virus. One of these antibody-negative mice was sacrificed on day 9 and the other 2 on day 21 p.i.

Five of the 65 mice inoculated with purified virus died prior to day 21, with a mean day of death of 9.2 ± 3.1. All of the 59 mice that provided enough blood to test (1 diabetes-negative female sacrificed on day 21 provided insufficient blood for the antibody assay) had ≥80 units of antibody /0.1 ml of plasma.

None of the mice that died during the experiment developed glycosuria which remitted before death. However, 2 of the male mice that died (days 8 and 12) appeared to be progressing toward remission just prior to death, in that the concentration of glucose in their urine samples was decreasing. Both of these mice had received purified virus. Two other male mice that died (days 9 and 13) were also positive for glycosuria at the time of their deaths. One of these mice had received 4×10^4 pfu of crude virus and the other had received 4×10^5 pfu of purified virus. The 3 mice that died prior to day 8 had never developed glycosuria, and the 3 that died after day 8 were all glycosuric at the time of death. The 3 mice that died on day 8 included 2 without glycosuria and 1 with glycosuria.

Five of the mice given crude virus spontaneously recovered from glycosuria before they were sacrificed. The mean day of cure for these mice was 10.8 ± 3.0. Four mice which were given purified EMC-D virus recovered with the mean day of cure being 9.2 ± 1.9.

Discussion. The greatest purification ratio for the EMC-D virus was obtained within the peak area of infectious virus in the density gradient centrifugation. The virus within that peak was approximately 67 times more pure than was the crude pool.

Most of the data was statistically significant when infected mice were compared to uninfected control mice, as seen in the P values given in Tables IV-II and IV-III. When crude virus-infected mouse data was compared to data from mice infected with purified virus, there was no statistically significant data ($P \leq 0.05$). The number of male mice which developed diabetic signs was not statistically different ($P > 0.05$) according to the purity of the virus. These differences also varied according to virus dosage and according to experiment performed, but not to an extent to make them statistically significant. Female mice were less sensitive to the diabetogenicity of the virus. Their lack of diabetic signs was not due to an ability to prevent a generalized infection by the virus, since each of the female mice tested for circulating antibodies had titers $\geq$100 units/0.1 ml. When the virus inoculum was increased 10-fold, the percentage of female mice which developed diabetes was nearer to that of the male mice. In fact, in the case of higher virus inoculum, the only statistically significant difference (at $P \leq 0.05$) between the number of males and females that developed diabetes was when purified virus was used and numbers of mice with glycosuria at 7 days p.i. were compared.

The crude and purified virus preparations were almost equally infectious as judged by the number of mice that developed neutralizing

antibodies. It is interesting that all of the mice that failed to develop detectable circulating antibodies specific to the virus also did not develop diabetic signs; however, the development of circulating antibody alone was not sufficient to cause diabetic signs. Some of the mice with very high antibody titers, as high as 1920 units/0.1 ml, also had negative tests for glycosuria and their plasma glucose concentrations were very near, or even below, the mean concentration found in control mice.

The extended time between infection and sacrifice did not increase the percentage of male mice which became diabetic, regardless of the virus pool used for infection. None of the mice became diabetic between day 9 and day 21 p.i. Only one of the female mice apparently became glycosuric between 7 and 9 days p.i., and that could have been an artifact due to a history of no urine samples prior to day 9. Some mice recovered from the diabetic state after day 7 p.i. The reasons for recovery of some mice when others remained strongly diabetic is not known.

No significant differences in the numbers of mice reversing to normal from glycosuria could be seen when either crude or purified preparations of virus were used. Equal numbers of animals died or went into remission during the experiment. Most of the animals which reversed from glycosuria or died, did so within 9 days of introduction of the virus; however, some changes were seen as long as 15 days after virus inoculation (Fig. IV-2).

The relative frequencies of male mice which died was somewhat dose dependent in that none given 4×10^3 pfu of virus died, but equal numbers died when given either 4×10^4 or 4×10^5 pfu. Unexpectedly, the male mice given the highest dose of virus were also the ones that had the highest cure rate (14.8%). Those given 4×10^4 or 4×10^3 pfu of virus had lower cure rates (7.4% and 6.7%, respectively). The differences in recovery rates were not

statistically significant. The mice were able to cure themselves in a way that is not understood.

The death rate of mice given 4 x 10^5 pfu of virus was slightly higher (4/35) when purified virus was used to infect than when crude virus was given (2/33). The cure rate for mice given 4 x 10^5 pfu of purified virus was also higher (3/30) than when crude virus was given (2/31). These differences were not statistically significant.

Conclusions. Additional mice were not found to become diabetic during the extended time between infection and sacrifice. In fact, just the opposite occurred. Some mice that had been diabetic by glycosuria early in the experiment spontaneously recovered and returned to normal during the extended time period. These mice evidently retained some functioning beta cells that were able to regenerate sufficiently to produce adequate levels of insulin. It is possible that even more cures would have occurred if many of the glycosuric mice had not been sacrificed on day 9 p.i. There was no significant difference in remissions from diabetes when the use of crude virus was compared to that of purified virus.

No extraneous (non-infectious viral or cellular) material was shown to contribute to, or oppose, the diabetogenic capabilities of EMC-D virus. There were no statistically significant differences in the numbers of mice that died, showed remission from glycosuria, developed diabetes, or developed antibody to the virus when either crude or purified virus was used for infection. Titration of each type of virus pool (crude or purified) yielded approximately equal numbers of mice in each of the categories above, when equal concentrations of virus were used from each type of pool. Thus, it seems that the infectious EMC-D virus particle is intrinsically diabetogenic. However, purification of the virus sufficient to remove all of the cell material from the

preparation would be necessary to absolutely prove that cellular material does not play a part in the diabetogenicity of EMC-D virus.

CHAPTER V

HOST DEFENSE ACTIVITIES IN RELATIONSHIP TO DIABETES INDUCED BY EMC-D VIRUS

Introduction. Previous investigators have demonstrated a variety of different characteristics that affect the outcome of EMC virus infection of mice, especially relating to whether or not diabetes is induced in the animal. It is impossible to determine the characteristics that are necessary for the infection to lead to diabetes because of the many conflicting results obtained by these investigators. However, host defense mechanisms appear to be one group of characteristics that is consistently important in the outcome of this infection. This study was designed to obtain additional information regarding what is occurring to selected host defense mechanisms at the time virus infection is inducing the diabetic state in mice.

Many strains of mice have been shown to be resistant to the diabetogenicity of EMC virus. Boucher et al. (66) recognized a genetic trait of resistance and Yoon and Notkins (67) attributed the inherited trait to a resistance of the beta cells of the pancreas to infection by EMC virus. However, experiments by Dafoe et al. (72) demonstrated that host factors other than inherited pancreatic resistant factors are most important in determining susceptibility to the diabetogenicity of EMC virus.

Testosterone has been shown to be very important in causing mice to become susceptible to the diabetogenicity of EMC virus, at least in certain strains of mice (73). Giron and Patterson (74) found that females of one strain of mouse (DBA/2J) remained resistant to EMC-D-induced diabetes when

treated with testosterone even though ICR Swiss female mice became susceptible when treated with testosterone.

Giron et al. (75) also found that ICR Swiss mice must be older than 7 weeks for EMC-D virus to induce diabetes. In mice younger than 7 weeks, the virus induced a lethal encephalitis instead of diabetes.

Yoon et al. (15) speculated that differences in IFN-stimulating ability of their B and D variants of EMC virus could account for the differences in diabetogenicity exhibited by the two variants. They found that the D variant, which caused near 100% of the mice injected to develop diabetes, stimulated little, or no, IFN production in either cell culture or mice. The B variant, with near 0% diabetogenicity, had the opposite IFN-stimulatory ability, with normal levels being stimulated in both cell culture and mice.

IFN or its inducers have different effects on the diabetogenicity of EMC-D virus, according to results reported by others (17, 18). While IFN-ß, or a variety of IFN inducers, blocked the diabetogenic effect of the virus in SWR/J mice, no effect was seen in ICR Swiss mice when the same treatments were used according to a report by Giron's group (17). The same group (18) extended the studies and reported that if IFN-ß or IFN-γ were given at a specific time in the infection, it exacerbated the diabetogenic nature of the virus.

Earlier investigators (19, 20, 76) reported that the cellular immune system must be functional for EMC strain M virus to cause diabetes. They also found that diabetes is caused even in the absence of a functional humoral immune system.

Each of these prior findings emphasizes that the diabetogenicity of EMC virus is certainly affected by the resistance mechanisms of the mouse. Exactly how these mechanisms influence the course of development of the

diabetic syndrome has not been elucidated. The research reported here was undertaken with the goal of discovering what happens to some of the resistance mechanisms of the mouse during induction of the diabetic state by EMC-D virus infection.

Materials and Methods. *Mice*. Male SJL/J mice were used in these experiments. They were obtained and maintained as outlined in Chapter III.

Medium, cells, and virus. All were explained in Chapter III.

Titration of animal serum for neutralizing antibody. These titrations were done in a manner similar to that outlined for mouse plasma samples in Chapter III.

Titration of interferon. Samples were titered in a manner similar to that outlined in Chapter III for the titration of poly I:C-induced IFN with the following exceptions: 1) Only L-cells were used for the titrations. 2) Three wells, instead of four, were used for each dilution. and 3) The plates were incubated for only 22 hr at 37°C before the samples were aspirated from the cells and 0.1 ml of virus (VSV only) was placed in each well.

Plaque assay of density gradient samples. Plaque assays to determine the concentrations of EMC-D virus present in density gradient samples were carried out as described in the materials and methods section of Chapter III.

Comparison of diabetic signs with signs of EMC infection at various intervals. Male SJL/J mice were inoculated by the i.n. route with the L-cell-passaged virus at a concentration of 4×10^5 pfu/mouse or with an equal volume (20 µl) of sterile virus diluent for control mice. At various intervals from 0 to 125 hr after virus exposure, mice were sacrificed by bleeding and tissue samples were taken; some samples were frozen and some were fixed in buffered 10% formalin, for future analysis. Analyses were made of the urine samples taken at the time of sacrifice by use of Clinistix®; Diastix® were used

after the Clinistix® tests were positive. Two mice exposed to virus and one control mouse were sacrificed at each interval.

Titration of infectious virus from mouse tissues. Tissues stored at -80° were weighed, thawed and homogenized in sufficient test medium to prepare a 10% (w/v) suspension. In some cases, especially with lung and thymus tissues, only a 5% suspension was made because there would have been insufficient volume for the homogenizer to function if a 10% suspension had been made.

The tissue homogenates and plasma samples were diluted in test medium by serial 10-fold dilutions. One-tenth ml of each dilution was placed in each of triplicate wells of a 96-well plate containing monolayers of L-cells. The plates were wrapped with Saran Wrap™ (The Dow Chemical Co., Indianapolis, IN), incubated at 37° C for 3 days, and the CPE was read by use of an inverted microscope. Virus titers were calculated by the 50% endpoint method of Reed and Muench (94) and expressed as cell culture infectious dose, 50% endpoint ($CCID_{50}$)/0.1 ml.

Guinea pig serum collection. Guinea pigs were bled by cardiac puncture after they had been anesthetized by intramuscular (im) injections of VETALAR® (Ketamine HCl Injection, NF) Veterinary--equivalent to 100 mg/ml Ketamine (Parke Davis and Co., Detroit, MI) plus Acepromazine (1:10). Collected blood samples were placed in 15 ml sterile Corning centrifuge tubes (Corning Glass Works, Corning, NY) and allowed to clot. Each clot was rimmed with a wood applicator stick and the tube was centrifuged to separate the serum, which was collected and frozen for future assay.

Preparation of primary antibody specific for EMC-D virus. Virus was purified by CsCl density gradient as outlined in Chapter IV with the following changes: 1) Four and one-half ml of CsCl was used in each of 4 tubes. 2)

Four and one-half ml of sterile PBS was added to 2 of the tubes and 4.5 ml of the virus to each of the other 2 tubes (tubes 1 & 4 of the ultracentrifuge). 3) The tubes were centrifuged at 35,000 rpm for 38.5 hr. 4) Both virus-containing tubes had a visible, ragged-looking heavy band approximately half way down in the gradients of each tube. This precipitate, which looked as though it were protein, was collected mainly in fractions numbered 9 & 10. However, the precipitate was seen to form a very narrow "stream" down the center of each tube as fractions were beginning to be collected. The visible "stream" reached the bottom of the tube at approximately the time that fractions 4 and 5 were being collected.

As the fractions were being collected from tube #1 and the precipitate reached the collection needle, it plugged the needle until sufficient pressure was applied to suddenly break the plug loose. The drops flowed so rapidly from the tube that two fractions (20 drops) were collected in fraction tube #9. The very rapid flow caused some visible mixing action at the very bottom of the density gradient tube.

The fractions obtained from the density gradient tubes were titered by plaque assay to determine which samples contained the highest concentrations of infectious virus. The samples with peak virus titers (#1-8, #1-9, #4-8, and #4-9) were selected for dialysis. The first number represents the density gradient centrifuge tube number from which fractions were collected. The second number represents the fraction number collected from that centrifuge tube, i.e. #1-8 is fraction number 8 collected from centrifuge tube number 1. These samples were diluted 1:10 in sterile 5 mM phosphate buffered saline, pH 7.3 (PBS-1) and dialyzed against PBS-1. All dialyses occurred at 4°C with stirring. The first dialysis was for 2 hr in 500 ml of PBS-1, the second for 4 hr in 500 ml of fresh PBS-1, and the final dialysis was for 19

hr in 1,000 ml of fresh PBS-1. The samples were removed from the dialysis tubing and placed in freezer vials for storage at -80°C. All samples were clear except #1-9, which had a white precipitate.

Portions of sample #4-9 were diluted 1:3 in sterile PBS-1 and in 10% buffered formalin. The sample diluted in PBS-1 was placed at 49°C for 15 hr for heat inactivation (4-9-Δ) and the sample in formalin (4-9-formalin) was placed at 4°C for an equal time period to allow inactivation by formalin action. Sample #1-9 was diluted 1:16 in sterile PBS-1 and placed at 49°C for 15 hr for heat inactivation (1-9-Δ). Samples of 1-9-Δ, 4-9-Δ, and 4-9-formalin were placed on L-cells to determine if complete inactivation had occurred. Neither of the heated samples was completely inactivated, so they were additionally heated at 55°C for 20 hr, which completely inactivated the virus infectivity in L-cells. The formalin-treated sample was completely inactive in L-cells.

Three female Hartley guinea pigs were used. One of the guinea pigs received 0.3 ml of an equal volume mixture of 1-9-formalin and Freund's complete adjuvant (FCA) (Calbiochem-Behring Corp., LaJolla, CA) in each rear flank by the im route. The mixture was made by forcefully injecting the FCA into the virus preparation, which was already in a serum vial. The mixture was pulled into the syringe and forcefully injected back into the vial 2 times. The resulting milky-white suspension was used for injection. The two heat-inactivated virus samples were mixed in equal volumes with FCA, as outlined above. These suspensions were injected im into each of the other two guinea pigs. The next day, one of the latter 2 guinea pigs was found dead and 2 days later, the second guinea pig that had received heat-inactivated virus died.

Two male Hartley guinea pigs were bled. One was injected im with a sample of heat-inactivated virus, without adjuvant, while the other received a formalin-inactivated virus sample im without adjuvant. The guinea pigs were

bled and given booster shots on somewhat different schedules, as shown in Table V-I.

Sample #1-9 from the density gradient centrifugation was emulsified with an equal volume of FCA by repeatedly (approximately 20 total syringe pushes) forcing the mixture between 2 syringes (5 ml) connected by 16 gauge x 1.5" needles and silicone tubing. When a drop of the emulsion on water held a bead, it was considered sufficiently emulsified (106). This emulsion was immediately injected into the gluteal muscles of a New Zealand White male rabbit. Approximately 2.6 ml of the emulsion was divided into 4 injection sites, 2 on each side of the rabbit. The virus was not inactivated in any deliberate manner prior to injection. The rabbit was bled and boosted with virus injections as summarized in Table V-II.

Serum collection from rabbit. The outside of the chosen ear was clipped closely by use of electric clippers. Xylene was rubbed on the central ear artery and the artery was vigorously rubbed from the base of the ear toward the tip with a dry cotton ball. A sterile 20 gauge x 1" injection needle was inserted into the upper end of the artery with the needle pointed toward the head of the rabbit. Blood was allowed to flow from the needle hub into a sterile Venoject® AutoSep™ tube with inert separator gel and clot activator (Terumo Medical Corp., Elkton, MD). Immediately after the blood was collected, a sterile cotton swab was pressed tightly over the hole left when the needle was withdrawn, and held until a clot stopped the bleeding. The ear was then thoroughly washed with warm water and baby shampoo. After thoroughly rinsing and drying the ear, Sulfa Urea Ointment (Veterinary Laboratories, Inc., Lenexa, KS) was applied and rubbed into the skin of the ear. Alternate ears were used with each successive bleeding when this was possible. The blood was allowed to clot at room temperature and then

TABLE V-I. GUINEA PIG INJECTION AND BLEEDING SCHEDULES AND ANTIBODY TITERS.

Pig no.	Action	Number of days from initial antigen inoculation						
1	Bleed	0						
	Inject[a]	0[b]	dead on day 4					
2	Bleed	0						
	Inject	0[c]	dead on day 1					
3	Bleed	-1	26	34	60	117	158	188[d]
	Titer[e]	0	1.0	1.0		1.0	3.4	4.2
	Inject	0[f]	26[g]	51[g]	111[h]	152[h]	180[i]	
4	Bleed	-1	33	42	71			
	Titer	0			1.3			
	Inject	0[k]	34[k]	58[m]	79[m]	dead on day 83		
5	Bleed	-8	13	34	78	92	163[d]	
	Titer	0		0	0	0	0	
	Inject	0[n]	26[n]	72[m]	86[p]			

[a] All injections by im route unless otherwise stated in footnote.

[b] 4-9-Δ + Freund's Complete Adjuvant.

[c] 1-9-Δ + Freund's Complete Adjuvant.

[d] Sacrificed

[e] Log_{10} neutralizing antibody titer (0 indicates no antibody detected).

[f] 4-9-formalin + Freund's Complete Adjuvant.

[g] 4-9-formalin no adjuvant.

[h] 4-9, live virus, no adjuvant.

[i] 4-8, live virus, no adjuvant.

[k] 4-9-Δ, no adjuvant.

[m] EMC-D, crude pool, $10^{5.9}$ pfu given by i.n. route.

[n] 1-9-formalin, no adjuvant.

[p] Both EMC-D, crude pool by i.n. route and 1-9-formalin, no adjuvant.

TABLE V-II. RABBIT INJECTION AND BLEEDING SCHEDULES AND NEUTRALIZING ANTIBODY TITER.

Action	Number of days from initial antigen inoculation																		
Bleed	-4	3	10	17	24	31	38	59	63	416	418	420	422	427	431	437	452	456	466
Titer[a]	n.d.[b]	1.53	4.31	3.63	3.54	3.91	3.86	3.85	3.57	3.00	3.08		4.15	4.30	4.30	4.15	4.15	4.30	4.15
Inject	0[c]				21[d]	31[e]		59[f]		416[g]							452[h]		

[a] Neutralizing antibody titer (log_{10} units/0.1 ml).

[b] None detected.

[c] 1-9 (live), (0.5 ml + 1.0 ml PBS, emulsified with 1.5 ml Freund's complete adjuvant), 2.6 ml by im injection.

[d] 4-9 (live), 0.5 ml undilute by iv injection.

[e] 4-9 (live), 0.25 ml undilute by iv injection.

[f] 1-9 (live), 0.1 ml undilute by sc injection.

[g] EMC-D, Pur. #1, 9-27-85 (live), 1.0 ml of a 1:10 dilution by iv injection.

[h] EMC-D, Pur. #1, 9-27-85 (live), 1.0 ml of a 1:10 dilution by iv injection.

centrifuged at the highest speed setting of an International clinical centrifuge (International Equipment Co., Needham Heights, MA) for 5 minutes. The serum was collected from the top of the plug and dispensed to freezer vials which were placed at -80°C or stored at refrigerator temperature for later assay of antibody in the serum.

Separation of IgG from IgM and other serum components. ZetaChrom™ 60 D1 Amine Disks (AMF Laboratory Products, 400 Research Parkway, Meriden, CT) were utilized as outlined in the product use brochure supplied with the disks. In brief, the process was as follows. The sample of serum was diluted 1:10 in "Buffer A" (0.015M NaH_2PO_4, pH 6.3, conductivity 1.0 milli mho/cm). The ZetaChrom 60 disk was equilibrated by washing it with 75 ml of "Buffer B" (0.1M NaH_2PO_4, pH 6.8, conductivity 9.0 milli mho/cm) and then passing 75 ml of Buffer A through the disk. The sample was applied to the disk by use of a syringe with the flow rate adjusted to 5-10 ml/min. The effluent from the disk was collected as purified IgG. The disk was washed with an additional 28 ml of Buffer A to remove all of the IgG. Bound proteins, including IgM, were eluted by passing 50 ml of Buffer B rapidly through the disk. From the time the sample was applied until the entire 50 ml of Buffer B was pushed though the disk, 4.0 ml fractions were collected as disk effluent. The O.D. (at 280 nm) of each fraction was read on a Perkin-Elmer, Coleman 124 double beam spectrophotometer (Hitachi Ltd., Tokyo, Japan), using 1 cm cuvettes. Each sample was titered for neutralizing antibody.

Other primary antibody preparations. Anti-porcine insulin prepared in the guinea pig was obtained from Miles Scientific Division, Miles Laboratories, Inc., Naperville, IL. Rabbit anti-mouse IFN (alpha) antiserum was purchased from Chemicon International, Inc., Los Angeles, CA. Anti-mouse IFN (beta) globulin raised in a rabbit was purchased from Lee BioMolecular Research

Laboratories, Inc., San Diego, CA. Rabbit anti-mouse IFN (gamma) was obtained from Enzo Biochem, Inc., New York, NY.

Immunohistochemical staining. Tissue sections, 7 µm thick, were prepared by the Animal, Dairy, and Veterinary Science diagnostic group, using mouse tissues that had been placed in 10% buffered formalin solution after they were freshly excised from the mice. The tissues were stored in this solution until they were embedded and sectioned. The sections were placed on glass slides without gelatin or other added material to fix the section to the slide. It is important that certain adherence factors, especially gelatin, be avoided since these can cause a strong false positive background staining (Vectastain® Product Insert).

The Vectastain® ABC immunoperoxidase staining procedure (Vectastain ABC Kits, Vector Laboratories, Burlingame, CA) was generally followed but some major adjustments were made to avoid the non-specific staining initially experienced. The following procedure was found to be satisfactory. The sections were deparafinized by soaking in 3 separate baths of Histosol (National Diagnostics, Somerville, NJ) for 2 minutes in each. Hydration of the tissues was accomplished by 2 separate baths of absolute ethanol (EtOH) for 30 seconds each, then 2 baths of 95% EtOH (30 seconds in the first and 10 dips in the second), 10 dips in a 50% EtOH bath, 10 dips in a tap water bath and 5 minutes in a distilled water bath. Blocking serum (1.8% normal serum from a species of animal in which the secondary antibody was made) was flooded over the tissue sections and the slides were placed in a humidified chamber to incubate for 20 minutes at room temperature (all steps took place at room temperature). Excess serum was blotted from the slides and primary antibody used to flood the tissue. The slides were again placed in the chamber for 30 minutes. The sections were rinsed for 10 minutes in

10mM phosphate-buffered saline, pH 7.6 (PBS-2) and the slides were incubated for 30 minutes with diluted biotinylated antibody solution. The slides were again rinsed for 10 minutes in fresh PBS-2 and incubated for 30 minutes with Vectastain® ABC reagent. The slides were rinsed for 10 minutes in fresh PBS-2 and exposed for 10 minutes to the peroxidase substrate solution (320 ml of 50mM Tris buffer, pH 7.6; 9.3 g NaCl; 160 mg 3,3'-diaminobenzidine tetrahydrochloride [DAB]; 1.6 ml 8% $NiCl_2$; all filtered through Whatman #2 paper, then immediately before use 43 µl of 30% H_2O_2 was added, with mixing). The slides were rinsed in tap water for 5 minutes and placed in methyl green/pyronin counterstain for 1.5 minutes. The tissues were rinsed and dehydrated before cover slips were mounted. Rinsing and dehydration steps were accomplished by ten dips in each of distilled water, acetone (2 baths), acetone/Histosol (50:50), and Histosol. The slides were placed in a fresh Histosol bath until cover slips were mounted with Permount (Fisher Scientific, Pittsburgh, PA).

Histogen® immunohistology kits (BioGenex Laboratories, Dublin, CA) were used for some of the staining. In this procedure, the tissue sections were deparafinized by soaking in 2 separate baths of Histosol for 3 minutes each. The tissues were hydrated by 2 separate baths of absolute EtOH, 3 minutes each, followed by 2 baths of 95% EtOH, 3 minutes each, and finally 5 minutes in 10 mM potassium phosphate buffered saline, pH 7.6 (PBS-3). Blocking serum was placed on the tissues for 20 minutes at room temperature (all incubations took place at room temperature). Excess serum was removed and primary antibody was placed on the slides for 30 minutes. The slides were rinsed well in PBS-3 and link antibody (streptavidin-labeled antibody specific to the IgG of the species in which the primary antibody was raised) was allowed to react for 20 minutes. The slides were rinsed again in PBS-3 and

labeling reagent (biotinylated peroxidase) was flooded over the slides for 20 minutes. The slides were rinsed well with PBS-3 and freshly prepared substrate solution was placed on the slides for 5-40 minutes. The slides were rinsed with glass distilled water, counterstained with hematoxylin, and cover slips were mounted with aqueous mounting medium.

Results. *Diabetic signs compared to virus yield and resistance parameters at various time periods.* Mice infected i.n. with EMC-D virus were killed at varying times post inoculation (p.i.) and various disease signs were assayed. Three parameters of infection and resistance, virus yield, IFN induction, and neutralizing antibody production, are depicted graphically in Fig. V-1 as they developed at various times in the plasma of infected mice. Infectious virus was not detectable ($< 10^{0.7}$ $CCID_{50}$/0.1 ml) in the plasma until 11 hr p.i., but achieved a peak titer of $10^{5.4}$ $CCID_{50}$/0.1 ml at 54 hr, and declined to non-detectable levels by 125 hr p.i. The plasma IFN activity was undetectable until 29 hr, then reached a maximal level by 35 hr, declined slightly in titer between 35 and 96 hr, and dropped to undetectable levels at 125 hr p.i. Neutralizing antibody began to show detectable levels in the plasma as early as 72 hr after virus inoculation and continued to increase in titer through the end of the experiment, 125 hr p.i.

Diabetic signs are represented by plasma glucose levels in Fig. V-2. The concentrations rose sharply from the normal level (below 200 mg/100 ml) to well above normal between 96 and 125 hr following i.n. introduction of the virus. Glycosuria was seen in one of the virus-infected mice at 96 hr p.i. This mouse was not sacrificed to obtain plasma glucose measurements until 125 hr p.i.

Results of virus, IFN, glucose, and neutralizing antibody in blood plasma are combined from 2 experiments. One of the experiments had

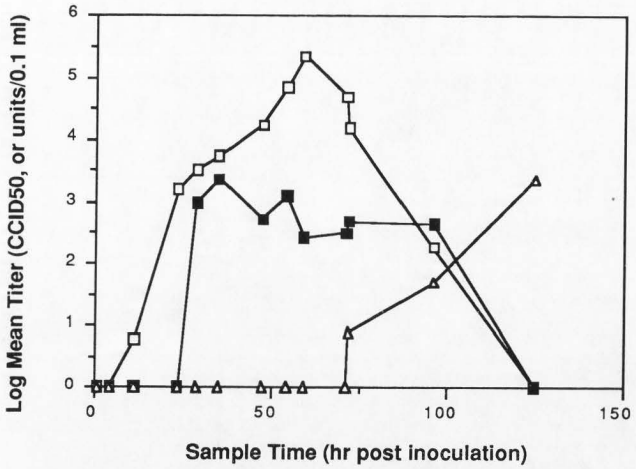

FIG. V-1. Comparison of virus, IFN, and neutralizing antibody concentrations in the plasma of mice at various times after inoculation of EMC-D virus by the i.n. route. Male SJL/J mice were inoculated with virus and two mice were sacrificed at each of various time periods to collect plasma for later assay for virus (CCID50/0.1 ml), □; IFN (units/0.1 ml), ■ ; and neutralizing antibody (units/0.1 ml), Δ. Single control mice (receiving virus diluent only) sacrificed at each time period were uniformly negative for tested items in the plasma.

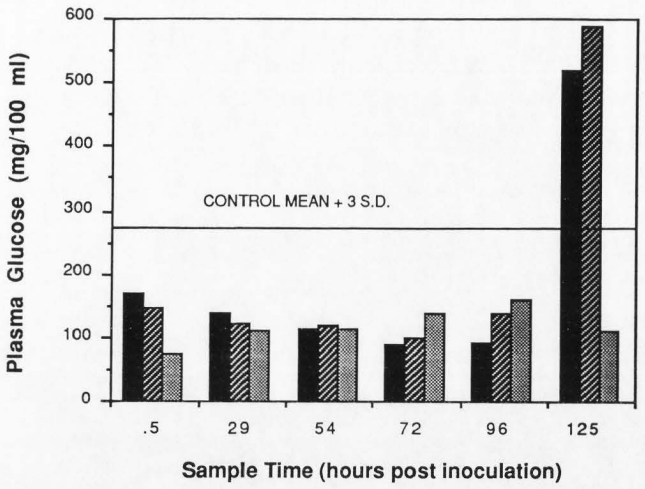

FIG. V-2. Plasma glucose concentration in mice at various times after i.n. inoculation with EMC-D virus or with sterile virus diluent. Individual mice inoculated with virus, ■ and ▨ ; individual mice inoculated with virus diluent ▩.

sample times ranging from 0 to 71 hr post-virus inoculation and solid tissues were taken for analysis of virus as well as for immunohistochemical staining. The other experiment had sample times ranging from 0 to 125 hr post-virus inoculation and solid tissues were taken only for immunohistochemical staining.

Although the virus concentration in the plasma of the mice was reduced somewhat by 71 hr p.i., the concentration in other tissues seemed generally to be increasing at this time, as seen in Fig. V-3. Only the thymus tissue, in addition to the plasma, seemed to have reached a peak of virus concentration prior to 71 hr p.i.

Primary antibody specific for EMC-D virus. Results of virus titrations of density gradient samples of the virus preparation partially purified for antibody stimulation are shown in Fig. V-4. The peak virus titer occurred in the sample with a density of approximately 1.36. Neutralizing antibody titers in the sera collected from the guinea pigs are shown in Table V-I. One of the guinea pigs developed a peak antibody titer of $10^{4.2}$ units/0.1 ml of serum. Table V-II gives neutralizing antibody titers of serum samples obtained from the rabbit. The highest titer reached was $10^{4.31}$ units/0.1 ml of serum. Results of IgG separation from other serum components and titrations of neutralizing antibody in the serum samples of the rabbit are shown in Fig. V-5. The highest titer of neutralizing antibody was found in the IgG peak. However, the neutralizing antibody found in the IgM peak was approximately half as concentrated as that seen in the IgG peak fraction.

Immunohistochemical staining with anti-insulin primary antibody. Islet cells stained very prominently in pancreas tissue of control animals and of virus-infected mice until 54 hours post-inoculation. At this time and after, the staining became spotty, Fig. V-6, or disappeared altogether in tissue sections

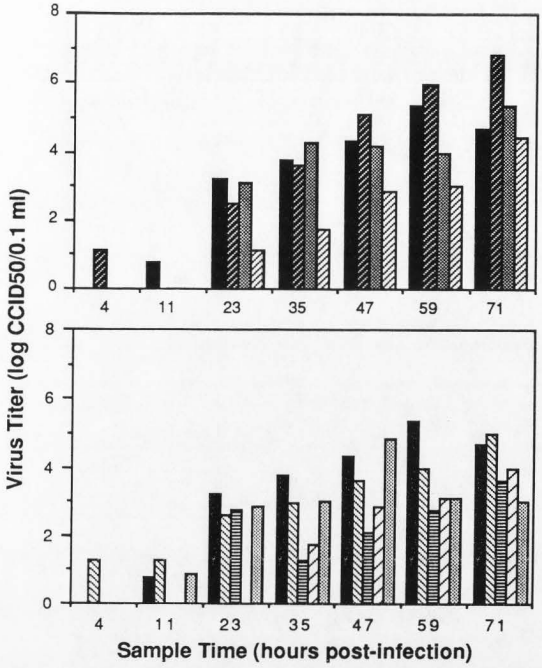

FIG. V-3. EMC-D virus yields from tissue samples taken at various times after mice were inoculated by the i.n. route. Male SJL/J mice were inoculated with virus and sacrificed at various times after virus was given. The tissues were frozen until they could be homogenized as a 10% (w/v) suspension and the homogenate titered for infectious virus in cell culture. Symbols represent titers obtained from the following tissues: ■, plasma; ▨, pancreas; ▩, spleen; ▨, kidney; ▧, liver; ▤, lung; ▨, heart; and ▩, thymus.

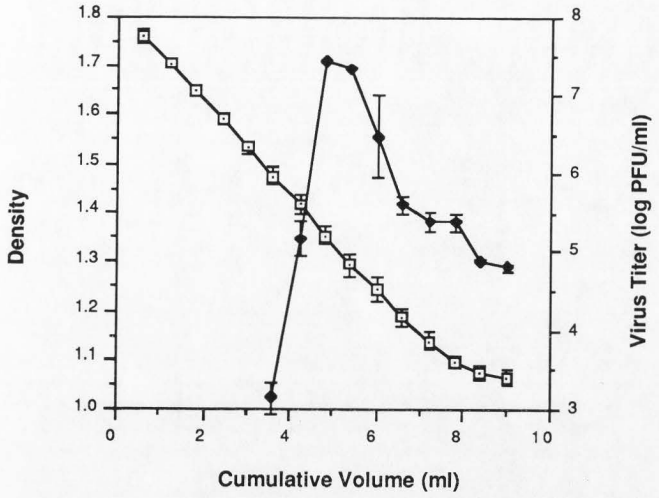

FIG. V-4. Virus titers of density gradient samples. Open squares are mean densities of samples from 2 density gradient control tubes. Closed diamonds are geometric mean virus titers of samples from 2 density gradient tubes with virus. Error bars represent the standard deviations of the sample means.

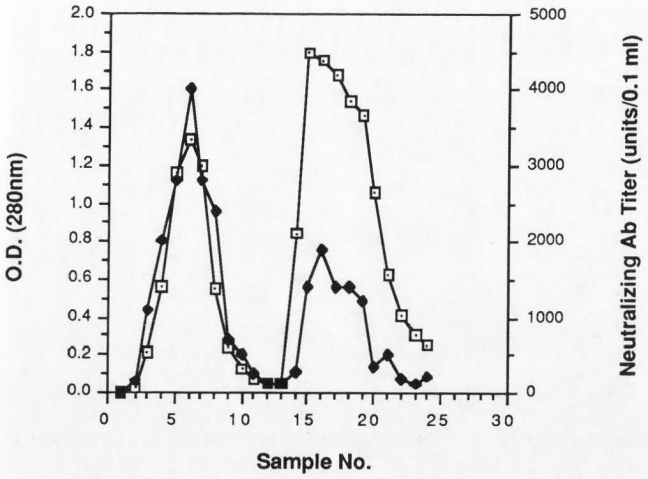

FIG. V-5. IgG separation and neutralizing antibody titer of rabbit anti-EMC-D serum. Open squares are the O.D. Filled diamonds are titers of neutralizing antibody. The peak to the left is IgG and the remaining peak includes all other serum components.

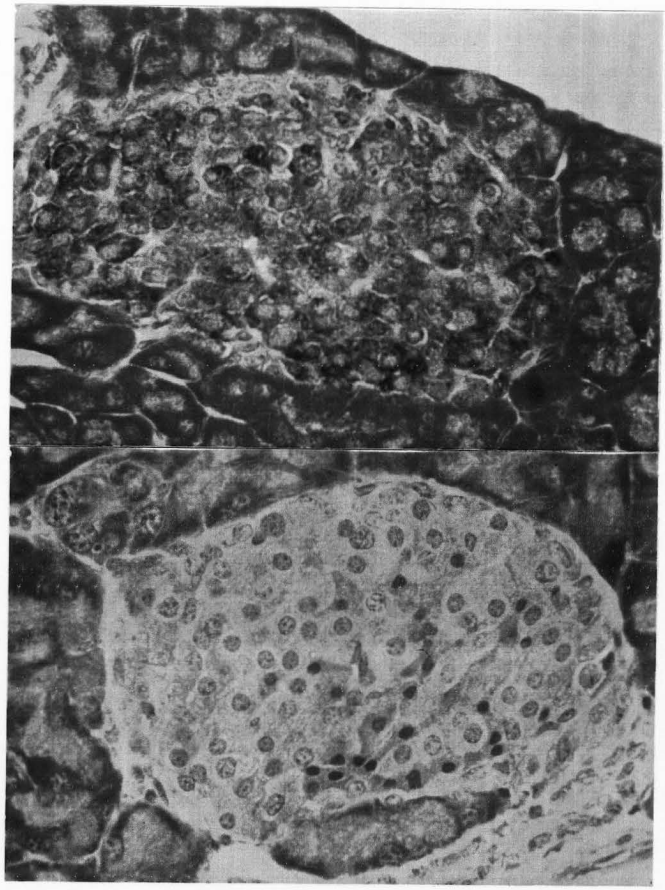

FIG. V-6. Immunohistochemical staining of insulin (dark brown) in pancreatic tissue. (A) Section from an infected mouse at 54 hr p.i. Stained with Vectastain® ABC kit, guinea pig anti-porcine insulin used as primary antiserum. (B) Section from a control mouse; stained as above except that primary antiserum was omitted, as a negative control.

from virus infected animals. Sections of pancreas from control animals had islets that stained strongly throughout the 125 hr of the experiment. Pancreas tissue of mice infected with EMC-D virus and sacrificed 125 hr p.i. either completely lacked recognizable islets or the few islets present were devoid of stain.

Immunohistochemical staining with anti-EMC-D virus primary antibody. No specific staining of any tissues could be demonstrated with the guinea pig antiserum used as primary antibody, nor could any be demonstrated when the rabbit antiserum was used. However, when rabbit IgG isolated from the anti-EMC-D virus antiserum was used as primary antiserum, specific staining of spleen tissue could be seen which began at 11 hr and showed staining through 71 hr p.i. No other tissue was specifically stained when the rabbit IgG was used as primary antibody. Uninfected control mice had no staining of any tissue.

Staining with antibody specific for the three different types of mouse IFN. Immunohistochemical staining for IFN-β in the tissue sections seemed specific since sections treated with negative control serum instead of primary antiserum completely lacked stain in all tissues. However, when glandular tissue (pancreatic acini or tubular glands of the small intestine) was exposed to the primary antibody and stained, some nuclei were seemingly non-specifically stained. Even control mice had tissues which stained well. The virus infected mice appeared to have darker staining tissues than tissues from control mice. The antiserum had a fairly low titer so that only 1:20 or 1:40 dilutions could be used.

Tissues exposed to negative control serum also did not stain when staining was done for IFN-α in the tissue sections. The only cells that stained in lung tissue when anti-IFN-α was used as primary antibody were smooth

muscle cells surrounding some of the larger airways. Tissues from uninfected control mice as well as virus infected mice had areas of plasma which stained quite well. The anti-IFN-α serum caused positive staining when diluted as much as 1:100 or 1:500. If diluted 1:1,000 or more the endpoint was surpassed.

Staining patterns for IFN-γ was surprising in that thymus and spleen tissues from control mice stained a little more intensely than the same types of tissue from virus infected mice. Other tissues had uniform intensity of staining between tissues of control or virus infected mice. The staining seen in kidney tissue is represented in Fig. V-7. The anti-IFN-γ antiserum could be diluted 1:1,000 or 1:5,000 to obtain intense staining.

Discussion. Experiments to determine the time of appearance of diabetic signs in mice relative to the signs of EMC virus infection showed that plasma glucose was not elevated until after infectious virus in the plasma was eliminated and neutralizing antibody was rapidly increasing in concentration. In this i.n. infection with a dose of virus designed to cause 100% of the mice to develop diabetes, the circulating IFN response pattern was very similar to that described by Yoon et al. (15). Their data indicated that the IFN titer peaks later and is sustained over a longer period than that seen in mice infected with the non-diabetogenic EMC-B virus.

Of the 8 tissue types examined (blood, pancreas, spleen, kidney, liver, lung, heart, and thymus), only blood and thymus reached peak virus titers in the 71 hr period studied. Titers in the thymus peaked at 47 hr and blood titers peaked at 59 hr. Since the virus was introduced by the i.n. route, it was surprising that virus appeared in pancreas, liver, blood, and thymus tissues before it was detected in lung tissue. Virus was not measurable in lung tissue until 23 hr p.i., whereas virus was detected in pancreas and liver as early as 4

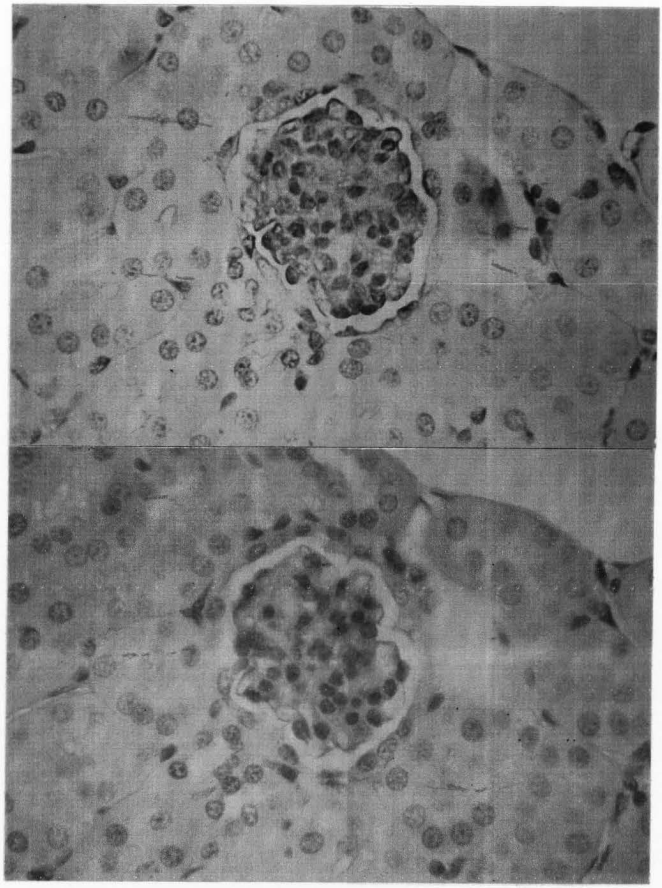

FIG. V-7. Immunohistochemical staining of mouse IFN-γ in kidney tissue. (A) Section from a control mouse with specific staining (red) associated with the glomerulus. Stained with Histogen® kit, rabbit anti-mouse IFN-γ used as primary antiserum. (B) Serial section from same mouse; stained as above except that primary antiserum was omitted, as a negative control.

hr p.i. and in blood and thymus at 11 hr p.i. Spleen and kidney tissue had detectable virus as early as lung tissue. Heart tissue was the last tissue type (at 35 hr p.i.) to begin yielding detectable virus. This was also a surprise since the virus had initially been cultivated repeatedly in mouse heart tissue to obtain the M-strain of EMC virus. These results indicate that free virus transport in circulating plasma is abating well before diabetic signs begin to appear.

Only one of the mice had glucose in the urine by 96 hr p.i., and that mouse was spilling even more glucose the next day when it was sacrificed (1000 mg/100 ml at 96 hr and ≥2000 mg/100 ml at 125 hr). It is possible that some immunological mechanisms are playing a role in the development of diabetes in the mice, since the signs of diabetes appear at the time antibody production is building strongly and after the virus has been largely removed from the circulation. Additional studies must be done to determine what is taking place to cause the diabetic signs to appear at this time.

The staining for insulin in the tissues worked very well and seemed to be specific. However, it did not appear sensitive enough to stain insulin in the plasma that was contained in the tissue sections. It is possible that fixation with formalin masked the insulin antigens in the plasma but left sufficient insulin antigens unmasked in β-cells to enable good staining to take place in the cells.

At the time this investigation began, it was not possible to purchase antiserum directed against EMC virus. Since it was necessary to have a good primary antibody specific for EMC virus in order to discern those tissues producing virus antigen during the infection, production of antiserum in animals other than mice was necessary. Guinea pigs were the first choice, since Tyrrell (107) stated that they produced better antibody to rhinoviruses

(which are in the same family, picornavirus, as EMC virus) in general than did rabbits. It was thus reasoned that less antigen would be needed to illicit a good antibody response if guinea pigs were used. However, the results of attempts to produce antiserum that would cause specific staining in the immunohistochemical staining procedure were disappointing. Two of the inoculated guinea pigs died of unknown reasons and a postmortem examination (gross necropsy observations and histopathology) did not reveal the cause of death. Both animals appeared healthy the day before they were found dead in their cages. Other guinea pigs inoculated with virus produced neutralizing antibody in moderately high titers, but no specific staining could be obtained on the tissue sections.

It is possible that IgM antibody was responsible for much of the neutralization of the virus. Any virus-specific IgM antibody would probably interfere with the immunohistochemical staining because the secondary antibody used was specific for guinea pig IgG. Thus, if IgM antibody covered some, or all, of the viral antigens in the tissues, there would be fewer sites at which IgG antibody could attach. The secondary antibody necessary for specific staining would then have few, if any, sites for attachment.

Results of attempts to produce anti-EMC virus antiserum in the rabbit were more promising in that higher neutralizing antibody titers were obtained in this species. However, when these antiserum samples were used as the primary antibody in the immunohistochemical staining technique, a similar lack of specific staining was seen. It was decided to separate the IgG from the IgM of the rabbit serum, since it was easier to obtain large volumes of the rabbit serum and since this serum also had higher titers of neutralizing antibody than did the guinea pig serum samples. Once the IgG was separated, it was found to react specifically in the immunohistochemical

staining technique. The IgM must have been reacting with the virus antigens in the tissues to such an extent that IgG was not able to bind to these antigens. The secondary antibody was thus unable to attach and begin the network necessary to provide a visible marker at the site of virus antigen production.

It was an unexpected finding that spleen tissue was the only tissue which stained in a specific manner when anti-EMC-D virus antiserum was used. Infectious virus was isolated at respectable titers from all of the other tissues studied. In fact, infectious virus was recovered from pancreatic tissue at higher titers (on a per gram of tissue basis) than found in spleen tissue, except at 23 and 35 hr p.i. (Fig. V-3). Yoon et al. (15) had shown the presence of viral antigen in the islets of Langerhans of infected mice by direct fluorescence assay. They used mouse anti-EMC virus antibody labeled with fluorescein isothiocyanate (FITC) and frozen sections of mouse pancreas. No one has described staining sections other than pancreas tissue, and that tissue has only been stained by FITC-labeled anti-EMC virus antisera.

The reasons for failure to specifically stain the EMC-D virus antigen in all tissues are not known. However, it is known that fixation of the tissue can mask the antigens within the tissue. The antigens masked by formalin-fixation can often be partially rejuvenated by brief protease treatment (108, 109).

Treatment of tissue sections with trypsin did seem to restore specific staining to lung, liver, kidney, heart, and thymus tissues. However, pancreas tissue was always dislodged from the slide during the trypsin treatments. Lung, liver and heart tissues were dislodged 2 of 5, 2 of 5, and 3 of 5 times, respectively, when trypsin treatment was tried on these tissues. Therefore, trypsin treatment is not recommended when one is attempting to stain several different tissues at several time periods in order to obtain comparative results.

Immunohistochemical staining for IFN-β in the tissue sections seemed to be specific according to a lack of stain when negative control serum was used instead of primary antiserum, and according to the direct relationship between concentration of primary antiserum used and the resulting stain intensity. However, the staining of the nuclei of certain cells indicated that some non-specific staining was occurring, since IFN should be produced in the cytoplasm of the cell. This stain in the nucleus was possibly due to avidin binding to the nucleus, as had been seen on a much more pronounced scale when particular batches of distilled water were used to prepare rinse buffers for the Vectastain® procedure. If the nuclear staining was due to non-specific avidin binding, such binding should also have occurred when the negative control serum was used, and this did not happen.

At this time, it is not possible to rule out non-specific staining in the case of the stain seen over the nuclei of certain cells. On the other hand, a definite reason for such staining is not known. The staining seen in tissues from uninfected mice would also lead one to suspect non-specific staining. However, it could also indicate that sufficient IFN is locally produced to accomplish its normal regulatory functions, and the staining is sensitive enough to detect this low level of IFN.

Comparative staining of both virus-infected and control tissues was successfully done at only the 125 hr time period. In that comparison between 2 mice, all tissues taken from the infected mouse stained darker than those from the uninfected mouse. Tissues from virus-infected mice were also stained at the 29 hr and the 72 hr time periods. They showed no stronger staining than those taken at 125 hr p.i., even though the antiviral activity of circulating IFN taken at the 2 earlier time periods was 300- to 1,000-fold higher than at 125 hr p.i.

Staining of tissues from both virus-infected and control mice which could be directly compared was attained at only 47 hr p.i., when anti-IFN-α was used. The tissues from these 2 mice were stained by use of the Histogen® immunostaining kit. Pancreas, lung, spleen, and thymus were the only tissues which specifically stained. Most of the stain occurred in areas which appeared to be plasma. Smooth muscle cells around large airways of the lung tissues (both mice) had dark staining cytoplasm. There was no difference in stain intensity between control and virus-infected mouse tissues.

The negative control serum did not allow staining of any tissue, thus indicating that the IFN-γ staining patterns were specific, though somewhat unanticipated. Tissues from only 2 time periods were successfully stained with the anti-IFN-γ antiserum. The differences in intensity were not overwhelming. Therefore, general conclusions cannot be established without further work to determine if such differences are consistent through all time periods.

It is interesting to note that the differences occurred in tissues in which high concentrations of T-cells would be expected. It is possible that the virus infection could reduce the overall T-cell population or at least reduce one of the T-cell sub-species.

Conclusions. Although the two immunostaining kits examined for these studies are advertised for use on formalin-fixed, paraffin-embedded tissue sections, other fixatives may be best for some of the antigen/tissue combinations involved in these studies. In particular, B5 fixative is better for most antigens than is formalin, according to a personal communication from Jonathon N. Schumaker, histologist/technical services manager, Lipshaw Corp.

Immunostaining for EMC-D virus antigen was only successful in tissues other than spleen if the tissues were treated with trypsin. The trypsin uncovered some of the virus antigen. It also completely dislodged some of the tissue sections from the slides, even though the slides had been treated with Elmer's glue to gain better adherence.

Insulin was well stained in the islets of Langerhans. The islets were reduced in size and/or demonstrated a reduced presence of insulin in pancreas tissue taken from virus-infected mice beginning at 54 hr p.i. This was somewhat surprising since Yoon et al. (15) had shown infiltration of leukocytes in the islets of mice inoculated with virus 7 days earlier. No islet infiltration of leukocytes was seen in the 5 day period of these experiments.

Infectious virus yield was shown to increase in pancreas, spleen, kidney, liver, lung, and heart beyond the peak of infectious virus seen in the plasma. Infectious virus peaked in thymus tissue before it peaked in the plasma.

Circulating IFN titer began a precipitous decline to undetectable levels at the time that infected mice began to develop glycosuria. Whether this rapid decline in IFN titer had a causative relationship to development of glycosuria or not remains to be determined.

Observations of the relative time of appearance and change in titer of various measurable signs of infection, resistance, and diabetes development have been made. It is hoped that these preliminary observations will enable a more detailed examination of resistance parameters in mouse tissue by immunohistochemical methods. The more detailed examination should provide some evidence to determine why some mice infected with EMC-D virus become diabetic while others recover fully from the virus infection.

CHAPTER VI

SPECIAL OBSERVATIONS CONCERNING EMC-D VIRUS-INDUCED DIABETES IN MICE

Three significant observations were made during the outlined EMC-D virus-induced diabetes research for this dissertation which may appropriately be described separately from the remainder of the research. These observations are outlined in the following sections.

Glucose Excretion in the Saliva of EMC-D-Infected Mice. *Introduction.* While attempting to get "obstinate" mice to provide urine samples for glucose tests, it was observed that they were more likely to urinate when excited than when placid. The observation was also made that mice were prone to urinate while biting. In attempts to excite the mice that were not excreting urine, Clinistix® test strips were provided as a "chew stick" for mice to bite. On the occasions when mice with glycosuria would bite the stick in a way that transferred saliva to the indicator pad, a typical glucose reaction would occur. This observation was pursued and the results are described as follows.

Materials and methods. Clinistix® or Diastix® test strips and SJL/J mice were described in the materials and methods section of Chapter III.

Results and discussion. Both EMC-D virus infected and control mice were tested for this reaction. Diastix® test strips were used to obtain quantitative glucose readings. Results of the tests are shown in Table VI-I.

Saliva testing was usually positive for glucose when glycosuria was present. Only one test of saliva was negative when glycosuria was ≥2,000

TABLE VI-I. COMPARISON OF GLUCOSE IN SALIVA AND URINE BY CLINISTIX® OR DIASTIX® TESTS.

Group	No. mice	No. tests	No. positive
Not infected	26	35	0
Infected, no glycosuria	13[a]	15	0
Infected, with glycosuria	26[a]	52	50**

[a] One mouse tested through day 5 was negative for both saliva and urine glucose, but on day 6 and after, was positive for both. This same mouse is included in both counts.

** $P<0.001$

mg/100 ml. The other negative saliva test occurred on a day when the mouse provided no urine for testing. The day previous, the urine test on that mouse was 500 mg/100 ml and 3 days after the negative saliva test, his urine was negative for glucose.

Diastix® readings were usually lower when saliva was tested as compared to urine from the same mouse. When the urine reading was ≥2,000 mg/100 ml the saliva reading would often match the urine reading. Control mice and infected mice without glycosuria were always negative for glucose in the saliva.

The mechanism of glucose secretion in the saliva is not known. It is possible that other body secretions also contain glucose when plasma glucose levels are high, but this has not been studied.

Conclusions. Mice with high plasma glucose, sufficient to cause glycosuria, also secrete glucose in the saliva. Normal mice did not have measurable glucose in their saliva.

Effect on Diabetogenicity of EMC-D Virus by the Cell Type Used for Virus Propagation. *Introduction.* As noted in Chapter IV,

partially purified EMC-D virus was found to have the same diabetogenic properties in mice as crude EMC-D virus pools. An experiment was carried out to confirm the observation of Giron et al. (14) that a single passage of EMC-D virus in BHK-21 cells eliminated its diabetogenic properties. In contrast to the observation of Giron and his associates, it was found that the EMC-D virus passaged one time in BHK-21 cells was equally diabetogenic in SJL/J male mice as the EMC-D virus passaged in L-cells. This experiment is described in this section.

Materials and methods. A crude virus pool, prepared by passage of EMC-D virus a single time in L-cells, was diluted to provide 5 x 10 pfu, 5 x 10^2 pfu, or 5 x 10^3 pfu of virus/mouse when 0.1 ml was injected ip. A crude virus pool prepared by passage of the same EMC-D virus a single time in BHK-21 cells was diluted to provide 4 x 10^4 pfu of virus/mouse when 0.1 ml was injected ip. Five mice were injected with each concentration of virus and 4 mice were injected with 0.1 ml of diluent as controls. All mice were male SJL/J mice. Titration of both virus pools was done in L-cells as outlined in Chapter III. Giron et al. (14) also plaque titered their virus pools in L-cells. Their single-passage virus in BHK-21 cells was used in mice at approximately 1.2 x 10^4 pfu/mouse.

The mice were monitored for glycosuria as explained in Chapter III; however, the duration of the experiment was extended to 64 days. At 64 days, remaining mice were sacrificed and plasma was tested for glucose and neutralizing antibody titers as outlined in Chapter III.

Results and discussion. Results of the experiment are shown in Table VI-II. Obviously, the virus passaged once in BHK-21 cells was effective as a diabetogenic agent, with 100% of the mice developing diabetes. The earlier glycosuria seen in mice given BHK-21 passaged virus was probably due to

TABLE VI-II. EFFECT OF CELL PASSAGE ON THE DIABETOGENICITY OF EMC-D VIRUS IN MALE SJL/J MICE.

Virus conc. (pfu/ mouse)	Cell passage	Glycosuria[a]		Day 64 post virus inoculation		
		Pos./total	First day all samples were positive	Plasma glucose Pos.[b]/total	Titer[c]	Neutral. antibody titer[d]
5x10	L	5/5	6	1/4	≥338	148
$5x10^2$	L	5/5	8	4/5	≥601	229
$5x10^3$	L	5/5	6	1/1	≥800	70
$4x10^4$	BHK-21	5/5	4	4/4	≥785	125
0	-	0/4	-	0/4	128	<10

[a] Determined by Clinistix® or Diastix®, after Clinistix® was positive.

[b] ≥3 S.D. above the mean plasma glucose conc. of control mice. The same mice were also glycosuric.

[c] Mean of all sampled mice (mg/100 ml).

[d] Mean of all sampled mice (units/0.1 ml).

the higher dosage of virus given. The day of first appearance of glycosuria was dosage dependent in the titration of L-cell passaged virus also.

Giron's group did use a different strain of mouse (ICR Swiss) than was used in these experiments. They also used a different method, a combination of plasma glucose levels and glucose tolerance tests, to determine which mice were considered diabetic or normal. Neither of these differences would seem to account for the dramatic difference of results seen (0% diabetes with Giron's group as opposed to 100% diabetes in this study). The reasons for this difference are not known.

Conclusions. Passage of EMC-D virus in L- or BHK-21 cells did not affect its diabetogenicity. This observation is in contrast to a report by Giron et

al. (14). Results of this study indicate that the diabetogenic properties of EMC-D virus are reasonably stable upon cell passage.

Correlation of Spontaneous Recovery of Mice from EMC-D Virus-Induced Diabetes with Reduced Polydipsia and Polyphagia. *Introduction.* It had previously been noted (Chapter III) that most mice with EMC-D virus-induced glycosuria developed very wet bedding within a day after glycosuria was evident. Some mice had been observed to have normal bedding, with no noticeable sticky wetness present, even though they had high glycosuria. Mice with normal bedding, whether they had glycosuria or not, did not utilize as much water or food as the mice with continuously wet bedding. It was decided to measure the water and food intake of the mice at different intervals in the experiment to determine if a relationship existed between severity of diabetes and food or water consumption.

Materials and methods. The water bottle of each individually housed mouse was weighed each day from days 36 through 40 and again from days 57 through 60 to determine individual water consumption. Food remaining in the feeding trays of cage lids was weighed on each of the above days to determine food consumption. Individual mouse weights were recorded on the same days. The ratio of mean mouse weight per gram of food utilized or per gram of water utilized was calculated for each time period. The data for the time periods were also combined and the above ratios calculated for the combined data.

Results and discussion. When results of ratios of individual mouse weights/weight of food or water consumed were sorted in an ascending order, particular groupings of mice were observed. Those with strong glycosuria, and wet bedding, at the end of the experiment were grouped as low numbers

(high food or water intake). Those mice with normal bedding but which had experienced high glycosuria at some time in the experiment were grouped as significantly higher numbers. Mean values and standard deviations of the ratios for each group are listed in Table VI-III. There was an obvious difference in the data obtained from control mice and mice with wet, sticky bedding.

TABLE VI-III. RATIO OF MOUSE WEIGHT TO DAILY FOOD OR WATER CONSUMPTION.

Group	Mouse wt./wt. of food			Mouse wt./wt. of water		
	Early	Late	Combined	Early	Late	Combined
Diabetic	2.9**±0.5	2.8**±0.6	2.9**±0.5	0.5**±0.1	0.6**±0.2	0.6**±0.1
Curing or cured	5.8±0.6	6.4±0.7	6.1±0.7	2.6±0.9	3.1±1.0	2.7±0.9
Controls	6.5±0.2	6.9±0.2	6.7±0.1	2.9±0.2	4.4±0.6	3.4±0.4

** $P < 0.01$, when compared to Curing or cured group.

Two ranges of data were observed for infected mice. 1) A relatively continuous range of values from mice with high glycosuria at the end of the experiment. These values ranged from 2.0 to 3.7 for weight/food consumption ratio. The ratios for weight/water consumed ranged from 0.4 to 0.9. 2) A range of values for mice that were able to control tendencies toward polyphagia and polydipsia. These mice had recovered, or were in the process of recovery, from glycosuria. Extremes for this range were 5.2 to 7.2 for food consumption and 1.4 to 4.0 for water intake. This range of ratios overlapped that of the control, uninfected mice, which had extremes of 6.3 to 7.2 for food and 2.7 to 5.3 for water consumption.

Conclusions. Some of the mice induced to a diabetic state by EMC-D virus infection were able to control the polydipsia and polyphagia common to

diabetes. These mice recovered spontaneously from the virus-induced diabetes.

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APPENDIX

TABLE A-I. TITRATION OF CRUDE VS PURIFIED EMC-D VIRUS POOLS. STANDARD DEVIATIONS FOR PLASMA GLUCOSE AND NEUTRALIZING ANTIBODY.

Titer of virus inoculum (pfu/mouse)	Data from mice sacrificed on (day)	Plasma glucose[a] (Mean ± S.D.)		Neutralizing antibody[b] (mean ± S.D.)	
		Crude	Purified	Crude	Purified
4×10^3	9	518**±165	541**±180	704±466	556±393
	21	182±20	308*±180	1120±1213	1313±1036
	Combined	406**±211	463**±208		
4×10^4	9	593**±167	517**±120	597±283	443±309
	21	371±288	459**±182	2933±462	1651±701
	Combined	542**±210	499**±136		
4×10^5	9	567**±95	498**±167	354±185	482±209
	21	512**±255	392±357	3203±1730	1731±941
	Combined	551**±148	473**±211		
4×10^5 female	9	358**±125	466**±106	458±248	627±508
	21	170±28	161±25	1613±1381	1878±1284
	Combined	287**±136	269**±162		
0	9	174±37		0	
	21	166±26		0	
	Combined	171±33		0	

[a] mg/100 ml (± S.D.).

[b] Units/0.1 ml of plasma. Statistics were not run on this data.

* $P<0.05$; ** $P<0.01$; compared to controls.

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Brigham Young University, Provo, Utah, M.S., 1969, Microbiology.
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Publications and Presentations:

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