GENETIC INFLUENCES AFFECTING THE OCCURRENCE OF A DIABETES MELLITUS-LIKE DISEASE IN MICE INFECTED WITH THE ENCEPHALOMYOCARDITIS VIRUS*

By JOHN E. CRAIGHEAD and DUNCAN A. HIGGINS

(From the Department of Pathology, University of Vermont, College of Medicine, Burlington, Vermont 05401)

(Received for publication 5 November 1973)

Substantive clinical evidence supports the view that genetic factors play a role in the causation of diabetes mellitus in man (1–4). Despite considerable study the pattern of inheritance of the predisposition to the disease remains to be defined. The recent studies of Pyke and his associates (5, 6) indicate that environmental influences may be of critical pathogenetic importance, particularly in the juvenile form of the disease.

Epidemiological observations by Gamble et al. (7) and Gamble and Taylor (8) suggest that at least some cases of human diabetes mellitus may have a viral etiology. Assuming the validity of the conclusions of these workers, one might ask whether or not their findings are inconsistent with the genetic hypotheses. The present investigation was undertaken to explore this question using an experimental animal model of viral-induced diabetes mellitus.

In 1968 we described a diabetes mellitus-like disease in adult mice infected with a variant of the encephalomyocarditis (EMC)[1] virus (9). It was found that the onset of the metabolic abnormalities was associated with virus replication in the pancreas and damage to beta cells in the islets of Langerhans. Detailed virologic, biochemical, and pathologic studies of this animal model have been reported in several recent publications (10–13).

In the present investigation we show that genetic factors affect the occurrence of the viral-induced diabetes mellitus-like disease in mice, apparently by influencing the severity of the insular lesion.

Materials and Methods

Virus.—The M variant of the EMC virus was prepared as a suspension of mouse heart tissue after a series of 21 animal passages (14). The virus pool used throughout these studies was diluted and stored as aliquots at −70°C so that animals were inoculated by the subcutaneous route with approximately 25 plaque-forming units in 0.1 ml. Titrations of the virus inocula, and the tissues obtained at autopsy from infected mice were carried out in mono-

* This work was supported by U. S. Public Health Service Grant No. AI09118 from the National Institute of Allergy and Infectious Diseases.

[1] *Abbreviation used in this paper:* EMC, encephalomyocarditis.

layer 35-mm plate cultures of L cells using the plaque technique. Procedures for the preparation of animal tissues for virological study, and the details of our neutralization test procedures have been described in previous publications (10, 14).

Animals.—Adult mice of the DBA/2 and C3H strains were supplied by the Jackson Laboratories, Bar Harbor, Maine and Microbiological Associates, Inc., Bethesda, Md. The animals were housed in individual plastic shoebox size containers using a chipped, dry corn cob bedding. Food and water were administered ad libitum. Breeding mice were provided Purina breeder chow (Ralston Purina Company, Inc., St. Louis, Mo.) whereas adults were given Purina maintenance chow. The animals were maintained on a 12 h light–12 h darkness schedule.

Biochemical Studies.—Glucose determinations were carried out by a microglucose oxidase method on blood obtained from the retro-orbital sinus of animals either in the nonfasting state or after food had been withheld for a period of 18 h. Tests for glucose tolerance were done using blood obtained from alternate eyes at 0, 60, 120, and 300 min after the intraperitoneal inoculation of 50 mg of glucose in 0.5 ml buffered salt solution. Glycosuria was assessed by the glucose oxidase method using chromagen indicator impregnated paper strips (TES-TAPE M-73-Lilly, Eli Lilly and Co., Indianapolis, Ind.).

Experimental Design.—Assays for blood glucose concentrations were made on one or more occasions at weekly intervals before the animals were inoculated with virus. Glycosuria was assessed routinely on a daily basis for the 1st wk of an experiment and three times per week for the next 2 wk. The weight of the animals was determined at weekly intervals.

Young adult animals were mated to provide the F_1 and the backcross generations. To maintain consistency, 12-wk old animals which had not served as parents were used for the virologic and biochemical studies summarized in the tables and figures below. Parental animals were also infected and studied by an identical protocol, after breeding had been accomplished. Although these mice were usually about 16 wk of age, results were comparable to those obtained with the somewhat younger virgin animals.

RESULTS

Studies with DBA/2 and C3H Mice.—The body weights and blood glucose concentrations of uninfected, 12-wk old, DBA/2 and C3H male mice are shown in Table I. The response of representative animals of these two strains to a carbohydrate challenge was similar but not entirely comparable inasmuch as DBA/2 mice exhibited a relatively "flat" curve in response to several different dosages of glucose (Fig. 1). Histologic examination of the pancreatic tissue revealed no consistent differences in the morphology of the islets of Langerhans in the two strains, and granulation of beta cells appeared similar in aldehyde fuchsin stained tissue sections.

Both DBA/2 and C3H mice developed systemic infections after inoculation

TABLE I

Some Characteristics of 12-Wk old, Male DBA/2 and C3H Mice

	DBA/2	C3H
Weight (g)	23 ± 2	26 ± 2
Blood glucose (mg/100 ml)		
Fasting	74 ± 13	84 ± 25
Nonfasting	113 ± 24	124 ± 28

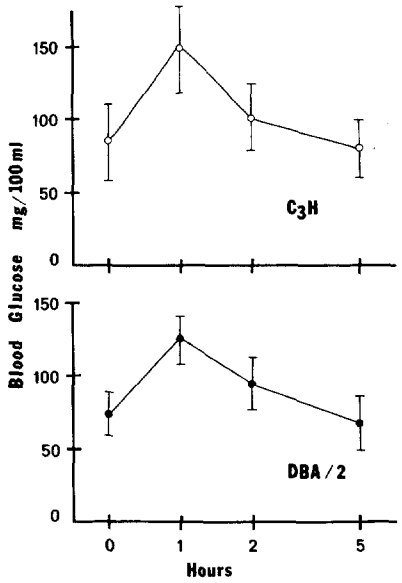

FIG. 1. Glucose tolerance of uninfected 6-wk old, male DBA/2 and C3H mice. Animals were inoculated at 0 h intraperitoneally with 50 mg glucose in 0.5 ml. Each point represents the mean of determinations on four or more animals.

with EMC virus (Table II). The blood serum yielded virus by 48 h and roughly comparable concentrations usually were detected in the heart, pancreas, and lacrimal glands at that time. Although viremia persisted in animals of both strains for an additional two days, increasing amounts of virus were recovered from the heart and pancreas. Throughout the course of the infection, the viral titers of the pancreatic tissue from the DBA/2 and C3H mice were similar although the virus appeared to persist somewhat longer in the pancreas of animals of the latter strain. Interestingly enough, greater amounts of virus were recovered from the heart and brain tissue of C3H mice.

Animals of the two strains died sporadically as a consequence of infection 7–20 days after inoculation (Table III). The mice exhibited vague signs of lethargy, inappetence, and ruffled fur but failed to develop specific neurological signs. Histologic studies on fatally-affected animals of both strains documented the presence of diffuse myocardial necrosis, a feature characteristic of the M variant (15).

Fig. 2 summarizes the results of blood glucose determinations on nonfasting mice at intervals during the 2-wk period after virus inoculation. Elevated concentrations of blood glucose became apparent in DBA/2 mice by the 5th day. Subsequently, the majority of the animals of this strain became hyperglycemic, whereas the amounts of glucose in the blood of C3H mice remained in the normal range. The results of a second experiment in which the animals were bled

TABLE II

*Virus Recovered from Organs of 12-wk old, Male DBA/2 and C3H Mice at Intervals after Inoculation of M Variant of EMC Virus**

Day	Serum		Heart		Pancreas		Brain	
	DBA/2	C3H	DBA/2	C3H	DBA/2	C3H	DBA/2	C3H
1	0	0	0	0	0	1.4×10^1	0	0
2	1.3×10^2‡	1.6×10^3	3.2×10^1	4.0×10^2	2.5×10^3	8.0×10^3	0	2.0×10^1
3	8.0×10^2	1.1×10^3	4.0×10^3	2.4×10^4	6.0×10^5	8.0×10^4	4.0×10^1	8.0×10^3
4	1.0×10^1	3.0×10^2	1.8×10^4	1.0×10^5	6.0×10^4	8.0×10^4	7.2×10^2	3.2×10^3
5	0	0	3.2×10^4	1.2×10^5	2.5×10^4	1.3×10^4	1.2×10^4	2.8×10^4
7	0	trace	8.0×10^2	1.8×10^5	2.5×10^3	1.7×10^3	3.2×10^1	6.0×10^4
9	0	trace	0	7.2×10^4	0	3.3×10^2	0	2.0×10^4

* Inoculated subcutaneously with ca. 25 plaque-forming units.

‡ Arithmetic mean titer of blood serum or tissue from two animals.

TABLE III

Glycosuria and Mortality in 12-wk old, Male DBA/2 and C3H Mice and the F_1 and Backcross Generations after Inoculation with EMC Virus

Genotype	No. tested	Glycosuria*		Dead*	
		No.	%	No.	%
DBA/2	45	37	82	13	29
C3H	45	1	2	8	18
DBA/2 × C3H	55	15	27	42	76
F_1♀ × DBA/2♂	92	56	61	45	49
F_1♂ × C3H♀	32	2	6	17	53

* 2–21 days after inoculation.

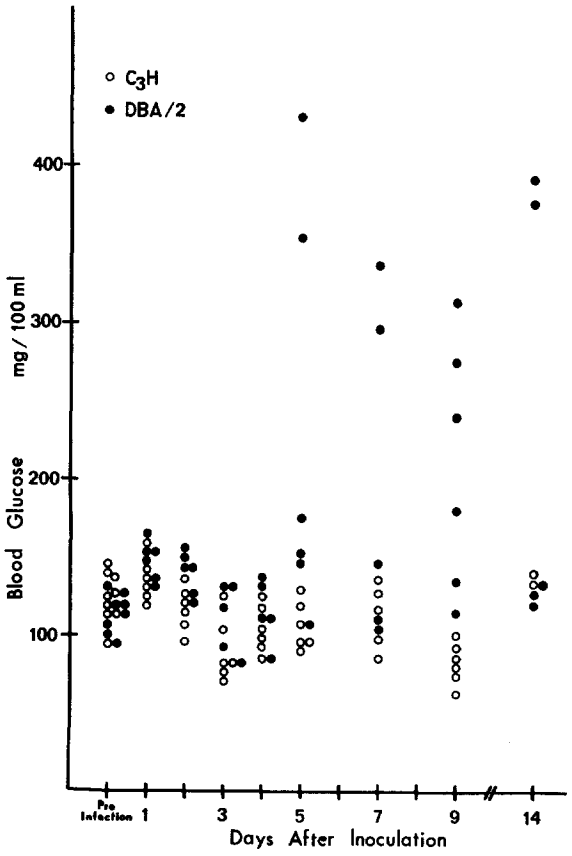

FIG. 2. Concentrations of blood glucose in nonfasting 12-wk old, male DBA/2 and C3H mice before and at intervals after the inoculation of EMC virus. Each point represents the result of a determination on an individual animal.

during convalescence from the infection are recorded in Fig. 3. A wide range of blood glucose concentrations was found in animals of the DBA/2 strain, although the majority were frankly hyperglycemic. In contrast, preinfection and convalescent concentrations of blood glucose in mice of the C3H strain were similar.

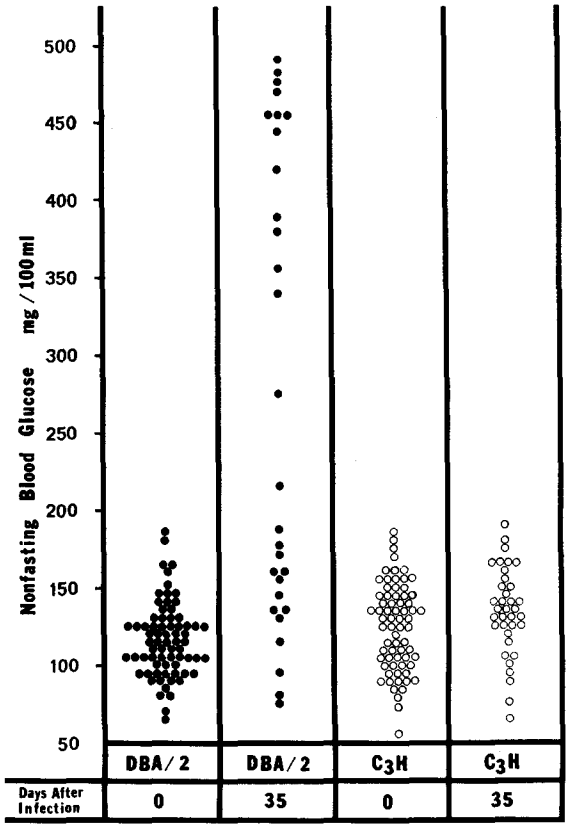

FIG. 3. Concentrations of blood glucose in nonfasting 12-wk old, male DBA/2 and C3H mice before and 35 days after the inoculation of EMC virus. Each point reprsents the result of a determination of an individual animal.

Cumulative data on the occurrence of glycosuria in mice of the two strains during the 3-wk period after virus inoculation are summarized in Table IV. As might be expected, glycosuria became apparent 5 days after inoculation and maximal numbers of animals were glycosuric by the 8th day.

The results of serum neutralization tests for antibodies directed against EMC virus are shown in Fig. 4. DBA/2 mice responded to a systematic infection by raising substantially higher titers of antibody than did animals of the C3H

TABLE IV

Glycosuria by 12-wk old, Male DBA/2 Mice at Intervals after Inoculation with EMC Virus

Day	No. of animals		Percent positive*
	Living	Glycosuria	
1	33	0	0
4	33	1	3
5	33	6	18
6	32	17	53
7	31	18	58
8	31	19	61
9	31	19	61
10	29	12	41
11	27	9	33
13	23	5	22
15	23	8	35

* Four mice died after exhibiting glycosuria. Thus, 70% of the inoculated mice had detectable glucose in their urine during the course of the experiment.

strain. These observations are of particular interest in view of the evidence suggesting that the tissue titers of virus in mice of the latter strain were somewhat higher during the acute stages of infection (Table II).

Histologically, the pancreatic tissue of virus-infected C3H mice revealed only subtle alterations of the islets of Langerhans. Necrosis of beta cells was observed rarely and mononuclear cell infiltrates were not present. Aldehyde fuchsin stains showed that the majority of the beta cells were heavily granulated. (Fig. 5 *a–d*). In contrast, striking lesions were found in the insular tissue of DBA/2 mice. The overall architecture of the islets was distorted and beta cells were shrunken and focally necrotic. In addition, many islets exhibited mononuclear cell infiltrates. Beta cells consistently were degranulated in tissue sections stained by the aldehyde fuchsin method (Fig. 6 *a–d*).

Studies with Progeny F_1 and Backcross Generations.—Glycosuria developed in 27% of the male F_1 progeny of a DBA/2 × C3H cross (Table III). In contrast to results with infected DBA/2 parental animals, glucose was detected in the urine of the majority of the members of the F_1 generation for only brief periods of time (median duration 1 day, range 1–8 days). These results were complicated by the high mortality of the animals of the F_1 generation consequent to infection. Thus, 15% of the mice succumbed between the 5th and 7th days of the experiment, having failed to exhibit glycosuria before death. It seems likely that a greater proportion of the animals might have exhibited glycosuria had they survived after virus inoculation. As can be seen in Fig. 4, the immunologic response of members of the F_1 generation was similar to the DBA/2 parents.

The results of studies on the progeny of backcrosses of the F_1 generation with the parental strains are also summarized in Table III. The prevalence of

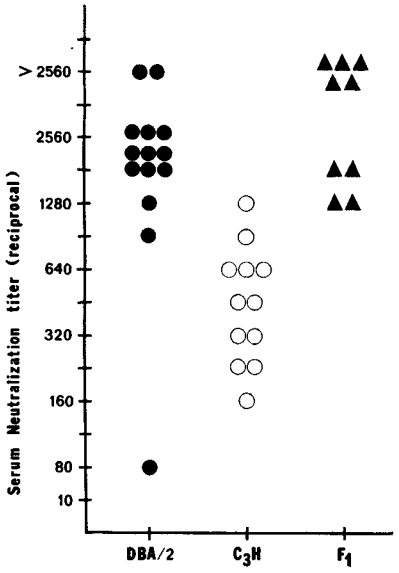

FIG. 4. Serum neutralization antibody titers in surviving mice of the DBA/2 and C3H strains and the F_1 generations, 17 days after inoculation with EMC virus. Heated (56°C, 30 min) dilute serum was mixed with ca. 100 PFU of the r^+ variant of EMC virus and 0.1-ml aliquots were introduced into each of two plate cultures of L cells. 50% end points were determined at 4 days.

glycosuria in these mice was similar to the parent strains although again interpretation of the data was complicated by the frequent occurrence of death during the acute stages of infection.

DISCUSSION

Coxsackie viruses Group B, types 4 and 5, have been associated epidemiologically with abrupt onset, insulin-dependent diabetes mellitus in man (7, 8). If indeed these, or other common viruses, play a causative role in the disease, factors unique to certain "wild" virus strains, or peculiar to the individual host, must be important pathogenetic considerations since so few of the many humans who are naturally infected during life develop the disease (16). Although genetic determinants are believed to affect the occurrence of diabetes mellitus (1–4), their importance and mechanism of action as well as the mode of inheritance of the predisposition have not been defined. Inasmuch as genetic factors affect the susceptibility of animals to viruses (17–21), it might plausibly be asked whether or not heritable influences are reflected in the expression of a viral infection which may be etiologically responsible for diabetes in man.

EMC, a picornavirus biologically similar to the group B coxsackie viruses, produces a disease in mice which exhibits many features of human juvenile-type

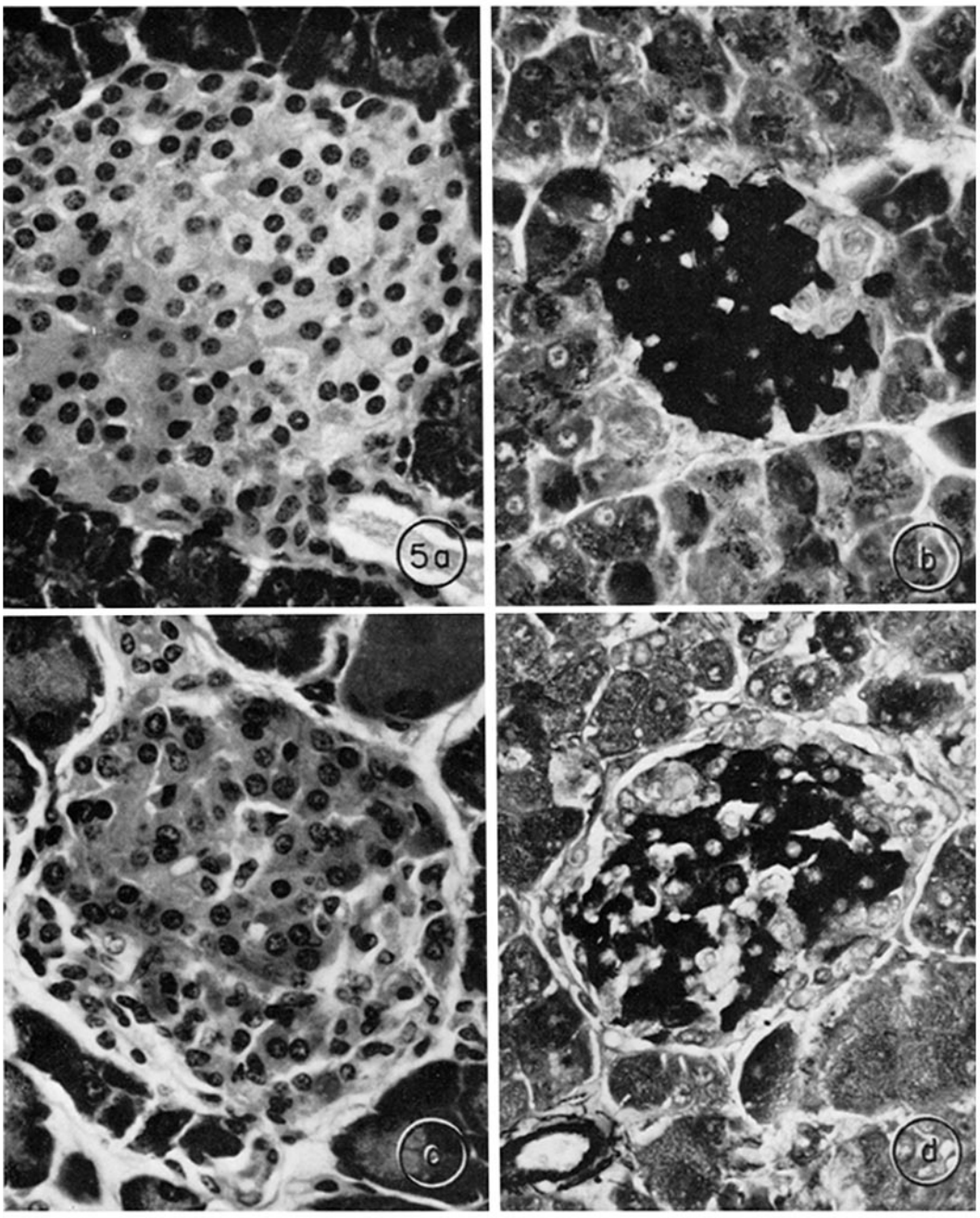

FIG. 5. Islet of Langerhans in the pancreases of 12-wk old, male C3H mice. (*a* and *b*), uninfected controls; (*c* and *d*), 6 days after virus inoculation. Note the slightly distorted architecture of the islet and the modest decrease in beta granules. Compare the features of the insular tissue with Fig. 6 *a* and *c*, hematoxylin and eosin stains, and Fig. 6 *b* and *d*, aldehyde fuchsin stain. × 3,400.

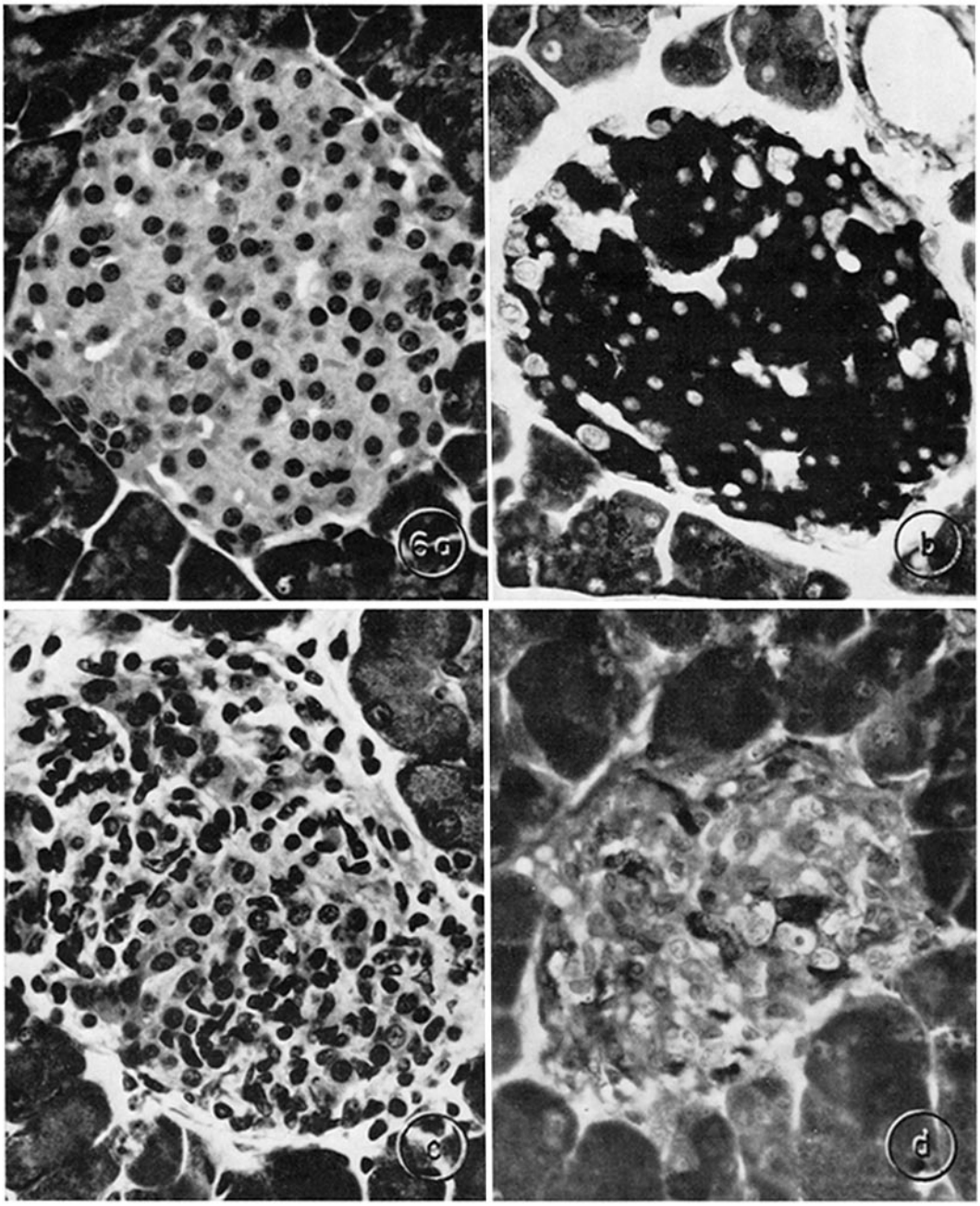

FIG. 6. Islets of Langerhans in the pancreases of 12-wk old, male DBA/2 mice. (*a* and *b*), uninfected controls; (*c* and *d*), 6 days after virus inoculation. The islet is disrupted and beta cells appear necrotic. Note the mononuclear cell infiltrate and the prominent degranulation. (*a* and *c*), hematoxylin and eosin stains; (*b* and *d*), aldehyde fuchsin stain. × 3,400.

diabetes (9–13). Accordingly, it seemed appropriate to employ this model using inbred strains of mice in an effort to define the role of genetic factors which might affect the occurrence of the disease in animals. The studies reported here show that heredity is important. However, the basis for the differences in response between mouse strains is defined incompletely and the pattern of inheritance of the predisposition is not clear.

The DBA/2 and C3H mice employed in this study were selected for detailed investigation after a preliminary survey showed that commonly available strains of inbred mice differ in their response to the diabetogenic effect of EMC virus. In our studies, uninfected DBA/2 and C3H animals were similar in their metabolic response to a carbohydrate challenge and histologic examination of the pancreas failed to demonstrate differences in the morphology of the islets of Langerhans. Animals of these two strains developed systemic infections with EMC and the organs supported the replication of roughly comparable amounts of virus. Additional work showed that fibroblast cultures prepared from DBA/2 and C3H fetuses were equally susceptible to the virus and produced identical quantities of infectious virus.[2] Thus, animals of both strains appeared to be metabolically competent and their cells were readily infected with EMC. The major differences between the mouse strains which have been detected thus far are the viral cytopathology of the insular tissue with the resulting metabolic effects, and the immunologic response to infection as documented by quantitative neutralization tests.

The pathogenesis of the insular lesion in EMC-infected mice is incompletely understood. Although viral antigen is present in the islets of mice during the acute stages of infection (13), the number of beta cells which undergo necrosis is variable and many degranulate but survive (22). Moreover, the extent of the lesion is affected by gonadal and adrenal cortical hormones and metabolic stresses such as hyperphagia and obesity (23). Thus, in the two mouse strains studied in this series of experiments, subtle metabolic influences which only indirectly affect carbohydrate metabolism may differ and be reflected in the severity of the insular lesion. Alternatively, the viral susceptibility of the cells of the two mouse strains may be dissimilar on a genetic basis. These questions require further investigation and are beyond the scope of the present report.

The data concerned with the prevalence of diabetes in parental and progeny strains of animals are inexact inasmuch as a fully satisfactory determinant of subtle metabolic disease and beta cell insufficiency has not been found as yet. Tests for glycosuria, hyperglycemia, and carbohydrate intolerance in the mouse are relatively insensitive and subject to error. Moreover, they may not consistently detect the transient episodes of beta cell dysfunction which are known to occur in some infected animals. Despite these shortcomings, we believe the data are sufficiently precise to permit a preliminary assessment of strain differ-

[2] Craighead, J. E. Unpublished data.

ences and the mode of inheritance of the diabetic predisposition. When this is done it is clear that the genetic pattern of transmission is not simple Mendelian but suggestive of effects mediated by multiple genes (17). More refined genetic and biochemical experiments will be required to establish the validity of this conclusion.

SUMMARY

The M variant of encephalomyocarditis virus produces a diabetes mellitus-like disease in DBA/2 mice but not in animals of the C3H strain. Fewer than one-third of infected F_1 (DBA/2 × C3H) progeny exhibit the disease, whereas the prevalence in backcrosses (F_1 × DBA/2, F_1 × C3H) is comparable to the parental inbred strain. Thus, the mode of inheritance of the diabetic predisposition appears to be polygenic. DBA/2 animals develop striking inflammatory and necrotizing lesions of the islets of Langerhans; in contrast, alterations of the insular tissue in the C3H mice are minimal. Although metabolic abnormalities appear to be consequent to lesions of beta cells, the factors influencing the severity of these insular changes are incompletely understood.

Mr. Herman West provided valuable technical assistance.

REFERENCES

1. Neel, J. V., S. S. Fajans, J. W. Conn, and R. T. Davidson. 1965. Diabetes mellitus in genetics and the expidemiology of chronic diseases. U. S. Government Printing Office, Washington, D.C. 105.
2. Simpson, N. E., Jan. 1969. Heritabilities of liability to diabetes when sex and age at onset are considered. *Ann. Hum. Genet.* **32**:283.
3. Gottlieb, M. S., and H. F. Root. 1968. Diabetes mellitus in twins. *Diabetes.* **17**:693.
4. Harvald, Bent. 1967. Genetic perspectives in diabetes mellitus. *Acta. Med. Scand. Suppl.* **476**:17.
5. Pyke, D. A., J. Cassar, J. Todd, and K. W. Taylor. 1970. Glucose tolerance and serum insulin in identical twins of diabetics. *Br. Med. J.* **4**:649.
6. Tattersall, R. B., and D. H. Pyke. 1972. Diabetes in identical twins. *Lancet.* **2**: 1120.
7. Gamble, D. R., M. L. Kinsley, M. G. Fitzgerald, R. Bolton, and K. W. Taylor. 1969. Viral antibodies in diabetes mellitus. *Br. Med. J.* **3**:627.
8. Gamble, D. R., and K. W. Taylor. 1973. Coxsackie B virus and diabetes. *Br. Med. J.* **1**:289.
9. Craighead, J. E., and M. F. McLane. 1968. Diabetes mellitus: Induction in mice by encephalomyocarditis virus. *Science (Wash. D.C.).* **162**:913.
10. Craighead, J. E., and J. Steinke. 1971. Diabetes mellitus-like syndrome in mice infected with encephalomyocarditis virus. *Am. J. Pathol.* **63**(1):119.
11. Wellmann, K. F., D. Amsterdam, P. Brancato, and B. W. Volk. 1972. Fine structure of pancreatic islets of mice infected with the M variant of the encephalomyocarditis virus. *Diabetologia.* **8**:349.
12. Müntefering, H., W. H. K. Schmidt, and W. Körber. 1971. Zur virusgenese des diabetes mellitus bei der weissen maus. *Dtsch. Med. Wochenschr.* **16**:693.

13. Boucher, D. W., and A. L. Notkins. 1973. Virus-induced diabetes mellitus. I. Hyperglycemia and hypoinsulinemia in mice infected with encephalomyocarditis virus. *J. Exp. Med.* **137**:1226.
14. Craighead, J. E. 1966. Pathogenicity of the M and E variants of the encephalomyocarditis (EMC) virus: II. Lesions of the pancreas, parotid and lacrimal glands. *Am. J. Pathol.* **48**:375.
15. Craighead, J. E. 1966. Pathogenicity of the M and E variants of the encephalomyocarditis (EMC) virus: I. Myocardiotropic and neurotropic properties. *Am. J. Pathol.* **48**:333.
16. Kogon, A., I. Spigland, T. E. Frothingham, L. Elveback, C. Williams, and C. Hall. 1969. The virus watch program: A continuing surveillance of viral infections in metropolitan New York families. VII. Observations on viral excretion, seroimmunity, intrafamilial spread and illness association in coxsackie and echovirus infections. *Am. J. Epidemiol.* **89**:51.
17. Fenner, F. 1972. Genetic aspects of viral diseases of animals. *In* Progress in Medical Genetics. A. G. Steinberg and A. G. Bearn, editors. Grune and Stratton, New York. 1–60.
18. Parry, H. B. 1962. Scrapie: a transmissible and hereditary disease of sheep. *Heredity.* **17**:75.
19. Gorham, J. R., R. W. Leader, G. A. Padgett, D. Burger, J. B. Henson. 1965. Slow, Latent and Temperate Virus Infections. U. S. Government Printing Office, Washington, D.C. U. S. Public Health Serv. Publ. 1378, NINDB Monograph, No. 2. 279.
20. Hicks, J. D., and F. M. Burnet. 1966. Renal lesions in the "autoimmune" mouse strains NZB and F_1 NZB × NZW. *J. Pathol. Bacteriol.* **91**:467.
21. Rowe, W. P. 1972. Studies of genetic transmission of murine leukemia virus by AKR mice. I. Crosses with Fv-1^n strains of mice. *J. Exp. Med.* **136**:1272.
22. Craighead, J. E., R. E. Kanich, and J. Kessler. 1972. Lesions of islets of Langerhans in encephalomyocarditis (EMC) virus-infected mice with diabetes mellitus-like disease. *Am. J. Pathol.* In press.
23. Craighead, J. E., Insulitis associated with viral infection. *In* Immunity and Autoimmunity in Diabetes Mellitus. P. A. Bastenie, editor. Exerpta Medica, Amsterdam.