# Virus-induced Diabetes Mellitus

## No Evidence for Immune Mechanisms in the Destruction of β-Cells by the D-Variant of Encephalomyocarditis Virus

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**SUMMARY**

**A possible contribution of the immune system to the pathogenesis of virus-induced diabetes mellitus was investigated using the D-variant of encephalomyocarditis (EMC-D) virus. Studies on the $F_1$ and backcross progeny of susceptible and resistant strains of mice gave no suggestion of a linkage between susceptibility and the major histocompatibility locus. Immunosuppression by antilymphocyte serum did not prevent the induction of EMC-D-induced diabetes. Athymic nude mice infected with EMC-D virus showed a nearly identical diabetogenic response as compared with heterozygous littermates. Passive transfer of lymphocytes from mice made diabetic with EMC-D virus into normal mice failed to produce diabetes. From these and other studies, we conclude that the development of EMC-D-induced diabetes is due to the direct destruction of β-cells by the virus and that the contribution of the immune response to the pathogenesis of this disease is, at the most, minor. DIABETES 1985; 34:922–25.**

In the murine model of virus-induced, insulin-dependent diabetes mellitus (IDDM), the D-variant of EMC virus infects and lyses pancreatic β-cells.[1] Over a period of 2–3 days, infected mice become hypoinsulinemic and hyperglycemic.[1] The severity of these symptoms is related to the extent of β-cell damage,[2] which in turn is dependent on the strain and sex of the mouse.[3] Despite the fact that the onset of the disease is very rapid, several reports[4–6] have suggested that immune-mediated mechanisms are involved in EMC virus-induced diabetes.

We have reinvestigated the role of immune functions in the pathogenesis of EMC virus-induced diabetes by looking at the susceptibility of athymic nude mice, thymectomized mice, immunosuppressed mice, and the segregation of H-2 haplotypes in crosses of resistant and susceptible mice. We find no evidence of immune involvement in the induction of diabetes mellitus by the D-variant of EMC virus.

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Received for publication 13 November 1984 and in revised form 1 March 1985.

### MATERIALS AND METHODS

**Mice.** Male athymic (nu/nu) CD-1 mice and phenotypically normal heterozygous (+/nu) CD-1 mice were kindly provided by Dr. S. I. Shin, Albert Einstein College of Medicine, Bronx, New York. Male SJL/J, C57BL/6J, $F_1$, and backcrosses of these strains as well as DBA/2J mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). Male athymic (nu/nu) NIH/Swiss mice and phenotypically normal heterozygous (+/nu) littermates were obtained from the breeding colony at the National Institutes of Health (Bethesda, Maryland). Mice were maintained on mouse ration (Ralston-Purina, Purina Chow Division, Richmond, Indiana) containing 5% fat and 23.5% protein. Five- to six-week-old mice were used for all virus inoculations.

**Virus.** The D-variant of EMC virus (plaque-purified from mouse heart-passaged M-variant) was used in all experiments and mice were inoculated intraperitoneally (i.p.).[1] Virus titer was determined by plaque assay on secondary mouse embryo cells as described previously.[7]

**Glucose and insulin assays.** Plasma glucose levels were measured in blood from the retro-orbital venous plexus by use of a glucose-oxidase assay with *o*-dianisidine as the indicator dye.[7] Sixty-minute glucose tolerance tests were performed and glucose indices were calculated as previously described.[8] Pancreatic immunoreactive insulin (IRI) was measured in a radioimmunoassay.[9]

**H-2 typing of mice.** All animals were marked for identification on arrival, and coat and eye color were recorded. At the completion of the glucose index protocol, animals were bled from the retro-orbital plexus and erythrocytes were collected and washed in isotonic saline citrate. H-2 haplotypes as displayed on the erythrocytes were determined in hemagglutination assays using standard reference alloantisera (i.e., sera

D-2 and D-28, kindly provided by the National Institute of Allergy and Infectious Diseases) as previously described.[10]

**Antilymphocyte serum.** Glucose indices were determined on infected and uninfected mice treated with antilymphocyte serum (ALS) to see if immunosuppression had any effect on virus-induced diabetes. Each mouse received 0.2 ml of ALS, obtained from Microbiological Associates, on different days before and after infection with EMC-D virus. The immunosuppressive effect of the ALS treatment was determined by counting peripheral blood lymphocytes. When tested 3–5 days after administration, the ALS-treated group showed approximately one-tenth the number of lymphocytes ($4.9 \times 10^6$/ml) as compared with untreated controls ($4.1 \times 10^7$/ml). The same batch of antilymphocyte serum was previously shown to be effective in preventing reovirus-induced autoimmunity.[11]

**Lymphocyte preparation and transplantation.** Male SJL/J mice were infected with $1 \times 10^5$ PFU of EMC-D virus. After 7 days, blood glucose levels were measured. Highly diabetic animals (>450 mg/dl) were selected as donors. Lymphocytes were harvested at day 7 from both the spleens and peripheral blood of the donors. Spleens were pressed through a wire-mesh screen and stroma was removed by low-speed centrifugation. Red blood cells were removed by treatment with ammonium chloride. The remaining lymphocytes were washed three times with PBS. Cells were counted and the concentration was adjusted to $5 \times 10^7$ viable cells/ml with PBS. Lymphocytes from peripheral blood were isolated by use of a Ficoll-Paque gradient (Pharmacia Fine Chemicals, Piscataway, New Jersey). The collected lymphocytes were washed three times with PBS, the cell concentration was adjusted to $5 \times 10^7$ viable cells/ml with PBS, and 1 ml of the suspension was injected i.p. into each of the recipients. To avoid the possibility that the lymphocyte preparations from mice that had been infected with EMC virus might still contain infectious virus, the spleen cell preparation was incubated at 20°C for 1 h with an equal volume of a high-titered mouse antiserum against EMC virus.

**Thymectomy.** Within 24 h of birth, DBA/2J mice were cooled on ice for anesthesia. The skin and sternal notch were incised and the thymus was removed by gentle suction using a sharp glass Pasteur pipette.[12] Surviving mice were infected with $5 \times 10^5$ PFU of EMC-D virus at 6 wk of age. Beginning 1 wk later, blood glucose levels were determined and the glucose index was calculated for each mouse.

## RESULTS

**Virus-induced diabetes and H-2 haplotypes.** In humans, insulin-dependent diabetes mellitus (IDDM) is strongly associated with certain HLA haplotypes.[13,14] The major histocompatibility locus of the mouse (H-2) contains the genes that are the murine counterparts of the HLA system and can influence immune function. We have previously reported[15] that, in crosses of susceptible SWR/J and resistant C57BL/6J mice, susceptibility to diabetes induced by the M-variant of EMC virus is inherited as an autosomal recessive gene. By analogy to human IDDM, susceptibility of mice to virus-induced diabetes might be linked to the major histocompatibility locus. With the availability of the more diabetogenic D-variant, we have tested the association of H-2 haplotypes with susceptibility to virus-induced diabetes in crosses of susceptible SJL/J (homozygous for the s-haplotype) and resistant C57BL/6J (homozygous for the b-haplotype) mice. To determine if resistance or susceptibility were associated with expression of either of the parental H-2 haplotypes, we H-2 typed animals from each of the crosses. If an association existed, then in the backcrosses one would expect diabetic animals to show a high frequency of one haplotype and resistant animals to show a high frequency of the other haplotype. As seen in Table 1, after i.p. infection with the D-variant, virtually all SJL/J mice developed diabetes, while none of the C57BL/6J mice developed diabetes. A small fraction of the $F_1$ animals (11%) developed mild diabetes. In the backcross of $F_1$ mice to SJL/J, a broad distribution of glucose indices was found, and slightly over 50% of the animals became diabetic. In the backcross of $F_1$ to C57BL/6J mice, only 4–5% of the animals became diabetic. When the results of the H-2 typing were tabulated (Table 1), no association or linkage of H-2 haplotype and susceptibility was seen. In the backcross to SJL/J mice, progeny with the s/s genotype (51% diabetic) were as susceptible as progeny with the b/s genotype (53% diabetic), and in the backcross to C57BL/6J, b/b and b/s offspring were equally susceptible (4% and 5% diabetic, respectively).

**Induction of diabetes in nude and thymectomized mice.** If islet cell destruction in virus-induced diabetes were T-cell mediated, then athymic nude mice should show less severe diabetes than normal mice. Table 2 shows no significant difference in the frequency or severity (as measured by the glucose index) of diabetes in CD-1 homozygous nude mice as compared with the heterozygous normal (+/nu) mice. Measurements of immunoreactive insulin in the pancreas revealed a similar decrease in the pancreatic insulin of both CD-1 nu/+ and nu/nu mice. In fact, the nude mice showed a slightly greater decrease in insulin levels and a slightly greater increase in blood glucose levels than their heterozygous littermates. Moreover, histopathologic evaluation of sections of pancreata from infected CD-1 nu/nu and nu/+ mice showed no discernible difference in the extent of islet cell pathology (not shown). Similarly, EMC-D-infected homozygous (nu/nu) and heterozygous (+/nu) NIH/Swiss mice showed approximately the same elevation in blood glucose

TABLE 1
Relationship of diabetes and H-2 haplotypes in crosses of SJL/J and C57BL/6J mice*

| Strain | H-2 genotype† | Number of animals tested | Number of diabetic animals‡ | Diabetes (%) |
|---|---|---|---|---|
| SJL/J | s/s | 77 | 75 | 97 |
| C57BL/6J | b/b | 50 | 0 | 0 |
| $F_1$§ | b/s | 45 | 5 | 11 |
| $F_1$ × SJL/J | s/s | 65 | 33 | 51 |
| | b/s | 55 | 29 | 53 |
| $F_1$ × C57BL/6J | b/b | 52 | 2 | 4 |
| | b/s | 37 | 2 | 5 |

*Male mice were inoculated i.p. with $1 \times 10^5$ PFU of EMC-D virus. Control mice were inoculated with an equal volume of Eagle's minimal essential medium with 5% fetal bovine serum (the vehicle used for the virus).
†H-2 genotypes were determined by hemagglutination as described in MATERIALS AND METHODS.
‡An infected animal was considered diabetic if its glucose index exceeded the mean of uninfected controls by 3 SD.
§Both $SJB6_{F_1}$ and $B6SJ_{F_1}$ were tested.

TABLE 2
Induction of diabetes in nude and thymectomized mice

| Strain* | Virus infected | Glucose index (mg/dl) | IRI† (μg/g pancreas) | Diabetes‡ (%) |
|---|---|---|---|---|
| CD-1 nu/nu | + | 339 ± 109 | 21 ± 5 | 86 |
| | − | 135 ± 14 | 101 ± 12 | 0 |
| CD-1 +/nu | + | 249 ± 90 | 40 ± 41 | 71 |
| | − | 142 ± 17 | 119 ± 13 | 0 |
| NIH/Swiss nu/nu | + | 211 ± 90 | ND§ | 45 |
| | − | 132 ± 10 | ND | 0 |
| NIH/Swiss +/nu | + | 200 ± 72 | ND | 33 |
| | − | 125 ± 8 | ND | 0 |
| DBA/2J (thymectomized) | + | 372 ± 81 | ND | 90 |
| DBA/2J (nonthymectomized) | + | 376 ± 90 | ND | 95 |
| DBA/2J (thymectomized) | − | 141 ± 13 | ND | 0 |

*CD-1 mice were infected with $1 \times 10^4$ PFU, and NIH/Swiss and DBA/2J mice were infected with $5 \times 10^5$ PFU of the D-variant of EMC virus. Each group contained between 7 and 11 mice. Only male mice were used.
†Immunoreactive insulin.
‡Mice with a calculated glucose index >5 SD above the mean of uninfected controls were considered diabetic.
§ND, not determined.

levels. The less severe diabetes in NIH/Swiss as compared with CD-1 mice is due to the well-known strain difference in susceptibility.[1,3]

To see whether thymic-dependent, cell-mediated immune destruction of pancreatic β-cells might contribute to EMC virus-induced diabetes, DBA/2J mice were thymectomized 24 h after birth. Surviving animals were infected with EMC-D virus at 6 wk of age, and beginning 7 days later blood glucose levels were measured. As seen in Table 2, 90% of the thymectomized and 95% of the nonthymectomized controls developed diabetes. The results from both nude mice and thymectomized mice suggest that EMC virus-induced β-cell destruction is independent of T-cell-mediated immune responses.

**Immunosuppression.** Immunosuppressive drugs such as ALS inhibit the development of hyperglycemia in the autoimmune-prone BB rat[16] and in reovirus-induced diabetes.[11] To see whether EMC virus-induced hyperglycemia could be inhibited by immunosuppression, mice were divided into groups and treated with ALS on different days before and after EMC infection. The frequency of virus-induced diabetes and the extent of the hyperglycemia were not statistically different in the treated and untreated animals (Table 3).

**Passive transfer of lymphocytes.** If virus-induced diabetes mellitus were due to a cell-mediated immune mechanism, transfer of lymphocytes from diabetic mice into normal mice might produce hyperglycemia in the recipients. As described in MATERIALS AND METHODS, lymphocytes from diabetic (7 days after infection) and nondiabetic animals were given to normal recipient mice, and glucose tolerance tests were performed 5, 7, 13, 18, and 25 days later. None of the recipients showed abnormally elevated glucose levels. In other experiments, mice irradiated with 500 R failed to develop diabetes after transfer of spleen cells from virus-infected diabetic donors. In still other experiments, pooled sera from diabetic mice failed to induce diabetes when given to normal recipients.

## DISCUSSION

Several investigators have suggested that immune mechanisms may be involved in EMC-induced diabetes. Jansen

TABLE 3
Effect of ALS on virus-induced diabetes

| Infection | ALS treatment (days)* | | | | | | | Mean glucose index ± SD | Diabetes† (%) |
|---|---|---|---|---|---|---|---|---|---|
| | −5 | −3 | −1 | 0 | +1 | +2 | +3 | | |
| + | | | | | | | | 446 ± 75 | 100 |
| + | + | | | | | | | 465 ± 76 | 100 |
| + | + | | | | + | | | 372 ± 78 | 90 |
| + | + | | + | | | | | 436 ± 73 | 100 |
| + | + | | | | + | + | | 421 ± 107 | 100 |
| + | | + | | | + | | | 462 ± 30 | 100 |
| + | | + | | | + | + | | 423 ± 50 | 100 |
| + | | | + | | | | + | 416 ± 70 | 100 |
| + | | | + | + | + | | | 372 ± 57 | 100 |
| − | | | + | + | + | | | 151 ± 17 | 0 |
| − | | | | | | | | 162 ± 15 | 0 |

*Antilymphocyte serum (0.2 ml per mouse) was injected on each day indicated. Mice were infected with $1 \times 10^5$ PFU of EMC-D virus on day 0. Five- to six-week-old male SJL/J mice were used and each group contained between 8 and 20 mice.
†Mice with a calculated glucose index >5 SD above the mean of uninfected controls were considered diabetic.

reported that 500 R x-irradiation protected mice from virus-induced IDDM.[4] Buschard et al.,[5,6] in experiments with two strains of nude mice, reported that these mice did not develop virus-induced diabetes, whereas their heterozygous littermates became diabetic. Mortality was high in some of the experiments, and both groups of investigators infected mice with the M-variant of EMC virus, which, in the strains of mice that they used, did not produce severe diabetes.

Our studies with the D-variant of EMC virus do not support an immune mechanism. We have tested athymic nude mice, thymectomized mice, and chemically immunosuppressed mice and have found them no less susceptible to EMC virus-induced diabetes than normal animals. In crosses of susceptible and resistant mice, the inheritance of susceptibility did not show an association with H-2 haplotype. In short, we could not find evidence of immune involvement. Similarly, Vialettes and colleagues recently failed to find evidence for a thymic-dependent, cell-mediated immune response in EMC-M-induced diabetes.[17]

The different findings among laboratories[4–6,17–19] may be due, in part, to the exquisite sensitivity of EMC virus to interferon. Subtle differences in the interferon response of different strains of mice or differences in the induction of interferon by various treatments (e.g., immunosuppression) could influence the development of virus-induced diabetes. Moreover, the EMC-M virus pool, used by several of the laboratories,[4–6,17–19] is a mixture of the diabetogenic D-variant and the nondiabetogenic B-variant.[1] The B-variant, in contrast to the D-variant, is a good interferon inducer and protects β-cells from infection by the D-variant. The ratio of these two variants determines the severity of the resulting diabetes.[1] In our experiments, we attempted to avoid this problem by using the plaque-purified D-variant.

Studies on the pathogenesis of EMC virus-induced diabetes mellitus also argue against an immune component. As do other picornaviruses, EMC virus rapidly infects and lyses host cells. In the case of EMC virus infection of pancreatic β-cells, evidence of this lysis can be seen within 24–48 h.[1] The virus destroys large numbers of β-cells and many of the surviving cells contain viral antigens in the cytoplasm, as determined by immunofluorescence.[1,8] In this early phase of the infection, there is little or no evidence of an inflammatory response.[1] There is also no indication that EMC virus modifies the host cell surface by inserting viral antigens or that the virus triggers an autoimmune response (i.e., no evidence of islet cell cytoplasmic antibody or islet cell surface antibody, unpublished data).

In other models of virus-induced disease, immunopathologic mechanisms are known to be important. Destruction of cells by the humoral and/or cellular immune system is well known in diseases such as lymphocytic choriomeningitis[19] and some models of chronic demyelinating diseases.[20] In fact, in mice, reovirus type 1 causes a polyendocrine disease characterized by a mild and transient type of diabetes that can be prevented by immunosuppression.[11] In the case of EMC-D-induced diabetes, at least in the strains of mice used, the contribution of the immune response to the pathogenesis of the disease appears, at the most, to be very minor.

**ACKNOWLEDGMENTS**

We wish to thank Dr. Kathleen Melez for performing the thymectomies, Tecolia Brown for technical assistance, and Eloise Mange for editorial assistance.

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