Spontaneous regeneration of pancreatic β-cells in EMC-D virus-induced diabetic mice and reversion from diabetic to normal state

R. R. Bhonde

National Centre for Cell Science, 'Jopasana', 85/1, Paud Road, Kothrud, Pune 411 029, India

With an aim of developing a model of experimental diabetes by which spontaneous recovery and regeneration processes can be investigated, EMC-D virus-induced diabetic mice were used as a system to study regeneration *in vivo*. Ninety per cent of the EMC-D virus-inoculated mice developed diabetes within a week after inoculation as judged by the glucose levels in blood and urine. Fifty-five per cent of the diabetic mice died within a fortnight after virus inoculation. Thirty per cent of the total infected mice slowly recovered from the diabetic state as evidenced by diminishing glycosuria, hyperglycaemia and near normal histological architecture. The results indicate reversion from clinical diabetes to normal status. Thus EMC-D virus-induced diabetes in SJL/J mice provides a new model to investigate mechanism and factors involved in regeneration of pancreatic β-cells *in vivo*.

INSULIN-dependent (Type 1) diabetes mellitus is an autoimmune disease caused by the selective destruction of islet β-cells, and the arrest of their regeneration potential may lead to a total disappearance of the cells. At the onset of diabetes about 80–90% of the β-cells are destroyed, resulting in severe hyperglycaemia[1]. Recently, it has been shown that viable and insulin-producing β-cells are present in human pancreatic islets at the onset of Type 1 diabetes[2]. It is not known how the β-cells can be triggered to proliferate in response to a moderate loss, providing that the residual β-cell mass is sufficient to maintain the glucose homeostasis under the condition where the glucose tolerance is already impaired[3]. Few cases of spontaneous and long lasting recovery from severe insulin-dependent diabetes mellitus in human and from drug-induced diabetes in animals are known[4,5]. It is not known whether β-cells in human beings regenerate. Since it is difficult to estimate regenerating β-cells in human cases of diabetes, it was thought worthwhile to use animal models of diabetes for this purpose. In the murine model of virus induced, insulin-dependent diabetes mellitus (IDDM), it has been shown that D-variant of EMC virus infects and lyses pancreatic β-cells[6]. The severity of diabetes is related to the extent of β-cell damage[7]. The earlier work on EMC-D induced diabetes in mice pointed out survival of diabetic mice for several days after virus infection, exhibiting abnormal glucose tolerance[6,8–10]. However, detailed clinical and histological studies of the surviving population were not carried out. Hence, the present study was designed to ascertain whether EMC-D virus-induced diabetic mice exhibit regeneration potential and eventual reversal of diabetes.

The D variant of EMC virus (plaque purified from mouse heart passaged M variant) was used in all the experiments. The source and preparation of EMC-D virus are described earlier[6]. Virus pools were prepared from L929 cells, and the virus titre was determined by plaque assay on L929 cells[11].

Male SJL/J mice were obtained from the Jackson Laboratory, Bar Harbor, Maine and housed in the animal facility in the Health Sciences Centre, University of Calgary, Calgary, Alberta, Canada. Except when noted, 5-week-old mice were used. A total of 60 mice were divided into 2 groups, control group of 20 mice and experimental group of 40 mice respectively. EMC-D virus was injected intraperitoneally into the experimental group (with 0.2 ml/mouse containing 100–500 p.f.u.). The control group was inoculated with 0.2 ml of phosphate buffered saline (pH 7.2).

Beginning 3 days after infection with virus, glucose levels in the urine were measured every alternate day with Diastix (Ames Division, Miles Laboratories Ltd., Etobicoke, Ontario, Canada). Blood glucose levels were measured in blood from the retro-orbital venous plexus by use of glucose oxidase assay with *o*-dianisidine dihydrochloride as the indicator dye[6]. Nonfasting glucose levels in the blood were measured on 5, 10, 15, 20, 25, 35 and 45 days after infection. The mean nonfasting glucose level of 20 uninoculated SJL/J male mice was 158 ± 19 mg/dl. In these experiments, any mouse with nonfasting glucose level greater than 215 mg/dl (3 standard deviations (SD) above the mean) was scored as diabetic.

Insulin was extracted from pancreas of mice sacrificed on 5, 20 and 45 days after infection by acid ethanol method[12] and the insulin content of the pancreatic extract was measured by RIA[13] and expressed as µg/g of pancreas. Briefly, pancreas were disintegrated by ultrasonic procedure at 4°C in 0.5 ml acid–alcohol solution (75% ethanol and 1.5% 10 N HCl v/v). The homogenates were kept at -20 °C until insulin was assayed by RIA.

At 5, 20 and 45 days after virus inoculation, several mice from control and experimental group were sacrificed and the pancreas were fixed in Bouin's fixative. Paraffin-embedded sections were stained with haematoxylin and eosin and examined to score presence or absence of insulitis and normal islet morphology.

Data obtained are summarized in Tables 1–3. Table 1 shows the glucose levels of blood and urine at various days after virus inoculation and the incidence of diabetes in the 2 groups. Animals in the control group did not show signs of development of diabetes throughout the

period of observation as indicated by absence of glucose in urine and blood glucose levels < 200 mg/dl at all the time points noted. However, in EMC-D virus inoculated group 36 out of 40 mice (90%) became diabetic within a week after virus inoculation, as revealed by the presence of glycosuria and hyperglycaemia (blood glucose > 450 mg/dl) which persisted for about 20–25 days post inoculation. A large number of mice in the experimental group died (20 out 36) within 2 weeks post-inoculation. In the surviving mice, 75% (12 out of 16) of the mice exhibited signs of reversal of diabetes as revealed by decreasing levels of glucose in the urine and blood. The process of reversal of clinical diabetes began in the 3rd week post-inoculation and was completed after 6 weeks, as evidenced by the absence of glucose in urine and establishment of normoglycaemia (Table 1). In the remaining 25% of the population amongst the surviving mice in the experimental group, blood and urine glucose did not disappear, indicating continued diabetic status.

Table 1. Changes in blood glucose (mg/dl) and urine glucose in control (C) and EMC-D virus infected (EMC-D) mice at different post-inoculation days

Days after EMC-D inoculation	Group	Blood glucose (mg/dl)	Urine glucose (+ ve/– ve)	No. of mice
5	C	180 ± 11	– ve	20
	EMC-D	470 ± 18	++++	36
10	C	140 ± 17	– ve	10
	EMC-D	468 ± 16	++++	26
15	C	200 ± 12	– ve	10
	EMC-D	430 ± 7	++++	16
20	C	180 ± 15	– ve	10
	EMC-D	472 ± 10	++++	4
	EMC-D (R)	264 ± 13	++	12
25	C	140 ± 17	– ve	10
	EMC-D	380 ± 6	+++	4
	EMC-D (R)	210 ± 9	– ve	12
35	C	110 ± 11	– ve	10
	EMC-D	400 ± 12	++++	4
	EMC-D (R)	186 ± 9	– ve	12
45	C	140 ± 11	– ve	10
	EMC-D	390 ± 8	+++	4
	EMC-D (R)	182 ± 15	– ve	12

EMC-D (R) = Regenerating mice; ++++ = Strongly positive; – ve = Absence of sugar.

The reversal of clinical diabetes in the experimental group was further documented by the increase in total weight and insulin content of the pancreas (Tables 2 and 3). It is clear from Table 2 that on day 20 post-inoculation the weight of the pancreas in uninoculated control group (159.0 ± 10.57 mg) is comparable to that of regenerating mice (170 ± 4.0 mg) whereas it is significantly lower in diabetic mice (71.5 ± 7.26 mg). Similarly, there was a significant difference in the insulin content of the pancreas in the control and experimental group of mice on day 5 post-inoculation (Table 3) whereas on day 45 post-inoculation insulin content of pancreas was comparable in both the groups (Table 3), indicating regeneration of islets and documenting reversal of diabetes as revealed by clinical picture. The histopathological studies were carried out on pancreas of control and experimental group at day 5 and day 45 post-inoculation. Figure 1 shows normal histological architecture, islet morphology in control group, whereas Figure 2 shows loss of islet morphology, β-cell lysis and insulitis in experimental group on day 5 post-inoculation. However, histological picture of experi-

Table 2. Changes in wet weight of the entire pancreas in control and EMC-D virus inoculated diabetic and regenerating mice at different post-inoculation days

Days	Control group (mg)	EMC-D virus inoculated group	
		Diabetic (mg)	Regenerating (mg)
5	62.6 ± 4.59	45.3 ± 4.62	
20	159.0 ± 10.57	71.5 ± 7.26	170 ± 40

8 to 10 mice of each group were used. Results are mean ± SD ($P = 0.0003$).

Table 3. Changes in insulin content (µg/g) of the pancreas in control and EMC-D virus inoculated diabetic and regenerating mice at different post-infection days

Days	Control group (mg)	EMC-D virus inoculated group	
		Diabetic (mg)	Regenerating (mg)
5	75.4 ± 12.45	20.5 ± 7.88	
20	77.8 ± 5.71	25.1 ± 6.44	33.2 ± 8.22
45	88.7 ± 3.75	15.3 ± 7.87	78.6 ± 7.65

8 to 10 mice were used in each group. Results are mean ± SD ($P = < 0.0001$).

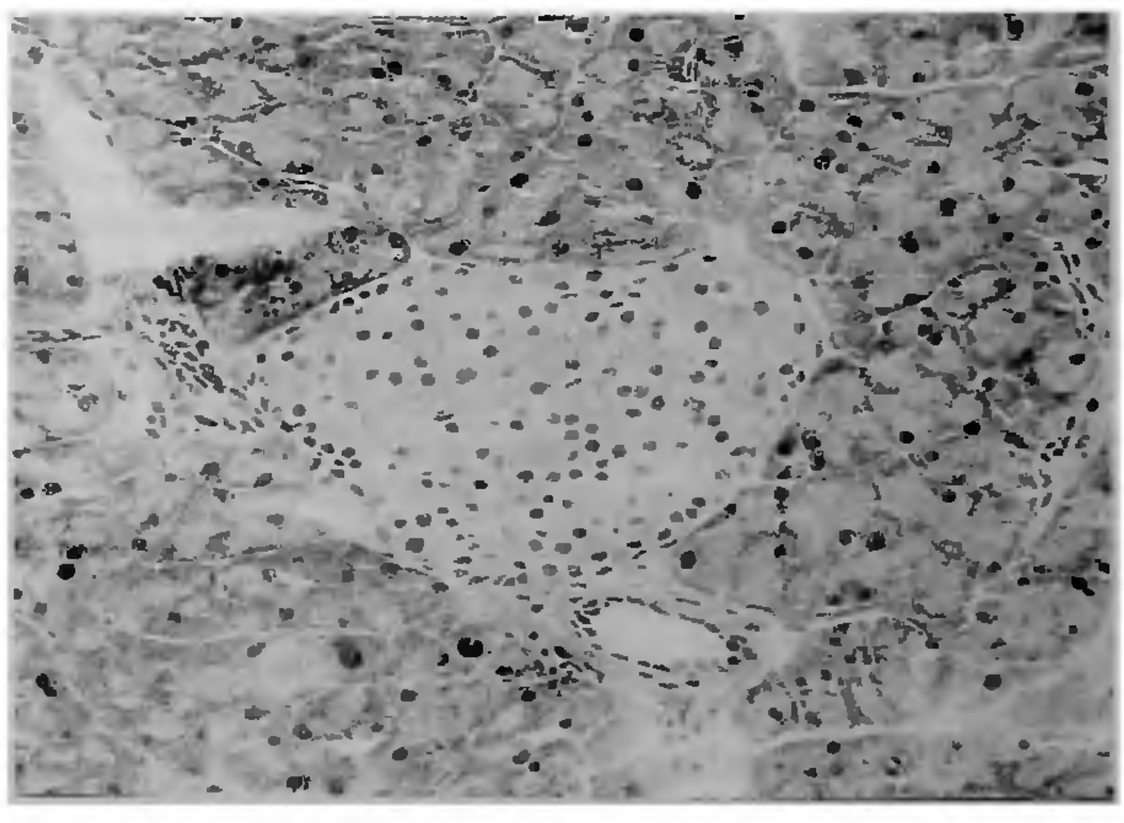

Figure 1. H&E stained section of pancreas from control mouse, showing normal islet surrounded by acinar cells (× 320).

mental group pancreas on day 45 post-inoculation was comparable to that of control group of pancreas on day 45 except for smaller islets in the former (Figure 3).

It is shown in this study that a single injection of EMC-D virus can induce diabetes in SJL/J male mice within a week after virus inoculation by reducing the β-cell mass and insulin content of the pancreas. However, the remaining β-cell volume seems to be sufficient to regain normoglycaemia in at least 30% of the animals (12 out of 40). Taking into consideration the course and outcome of diabetes in the experimental group over a period of time it is seen that there are definite signs of reversal of diabetes as evidenced by the absence of glycosuria, achieving normoglycaemia and insulin content of the pancreas comparable to those of control group. The experimental data indicates reversion of EMC-D virus-induced diabetes. Such type of reversion from diabetic to nondiabetic status has not been reported previously in the case of EMC virus-induced diabetes. The reversal of diabetes obtained in the present study appears to be a case of spontaneous regeneration of the residual β-cell mass that was left intact after EMC-D virus infection. Thus, EMC-D virus-induced diabetes in SJL/J mice provides a new model to study the process of regeneration in pancreatic β-cells and reversal of diabetes. Such a type of spontaneous regeneration has been reported in streptozotocin (STZ)-induced diabetes in mice[5], which provides another model to investigate mechanisms and promoting factors of such restorative processes. However, the present model differs from STZ-induced diabetic model[5] in many respects. Firstly, our model involves adult mice as opposed to neonatal mice in STZ model. Secondly, the time of onset and the time of recovery are much earlier compared to the STZ model. The only limitation is the high mortality rate in our model (55%) as opposed to STZ model. It is remarkable that the mice were able to recover in spite of severe hyperglycaemia, considering the fact that high blood glucose levels *per se* may have toxic effects on pancreatic β-cells[8]. This model also differs from other models of pancreatic regeneration such as partial pancreatectomy[9] wherein there is 60–90% loss of whole pancreas, which results into regeneration of entire pancreatic tissue as opposed to selective loss and regeneration of only islet tissue as observed in the present study. The EMC-D induced diabetes model also differs from autoimmune models of diabetes such as biobreeding rat[10] and nonobese diabetic mouse[11], wherein total destruction of β-cells occurs due to immune cytolytic process, thereby reducing the amount of residual β-cell mass and eventually reducing the chances of regeneration and recovery. It seems plausible to control the degree of damage of β-cells in the present model by reducing the dose of virus injected or by suppressing virus replication[12]. Although the present study neither permits elucidation of mechanisms involved in the process of regeneration nor indicates origin of newly developed islets, the results obtained reflect an intrinsic limited capacity or potential of β-cell regeneration. Probably the recovery process might be caused by factors released from damaged β-cells, a mechanism observed in many other tissues after cell damage[13]. Since the number of functionally intact islets is of decisive importance for the development, course and outcome of diabetes, there is very little hope of recovery and regeneration of β-cells in insulin-dependent (Type 1) diabetes which is a consequence of progressive β-cell destruction[14,15]. However, the recent report[2] on human pancreatic islet function at the onset of Type 1 diabetes clearly indicates the presence of insulin producing viable cells, suggesting a new

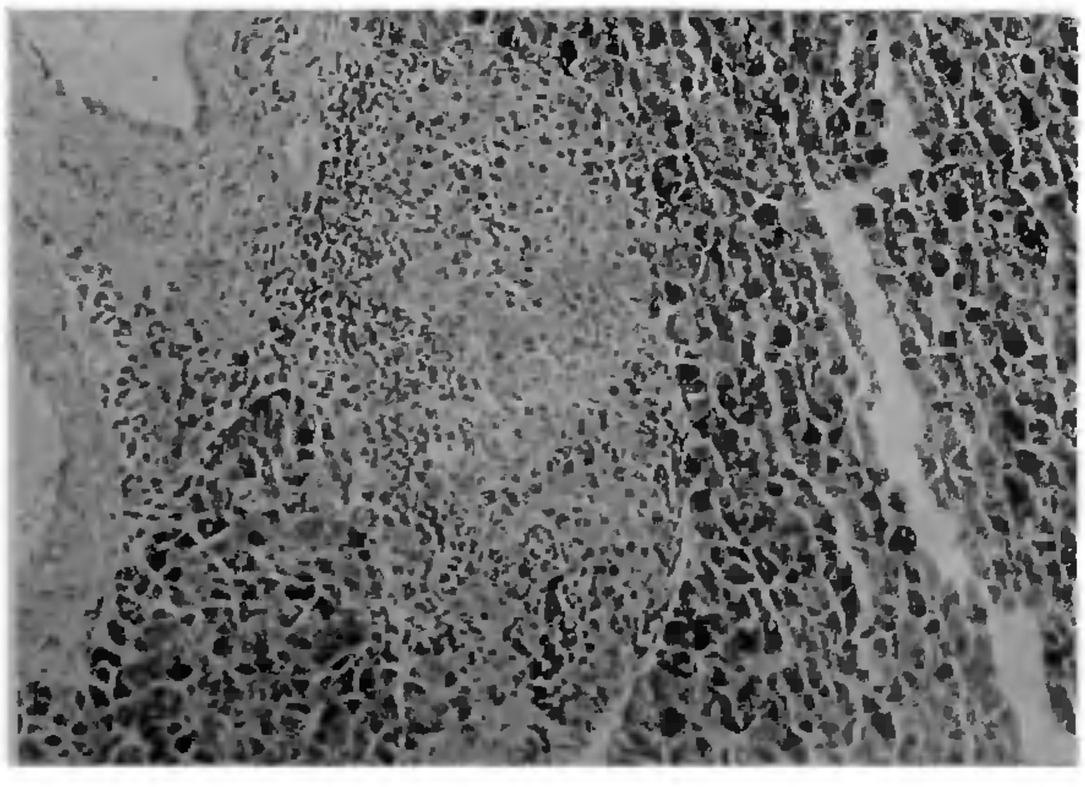

Figure 2. H&E stained section of mouse pancreas, 5 days after EMC-D virus infection showing extensive inflammatory infiltrate with mononuclear cells in the islets of Langerhans (× 320).

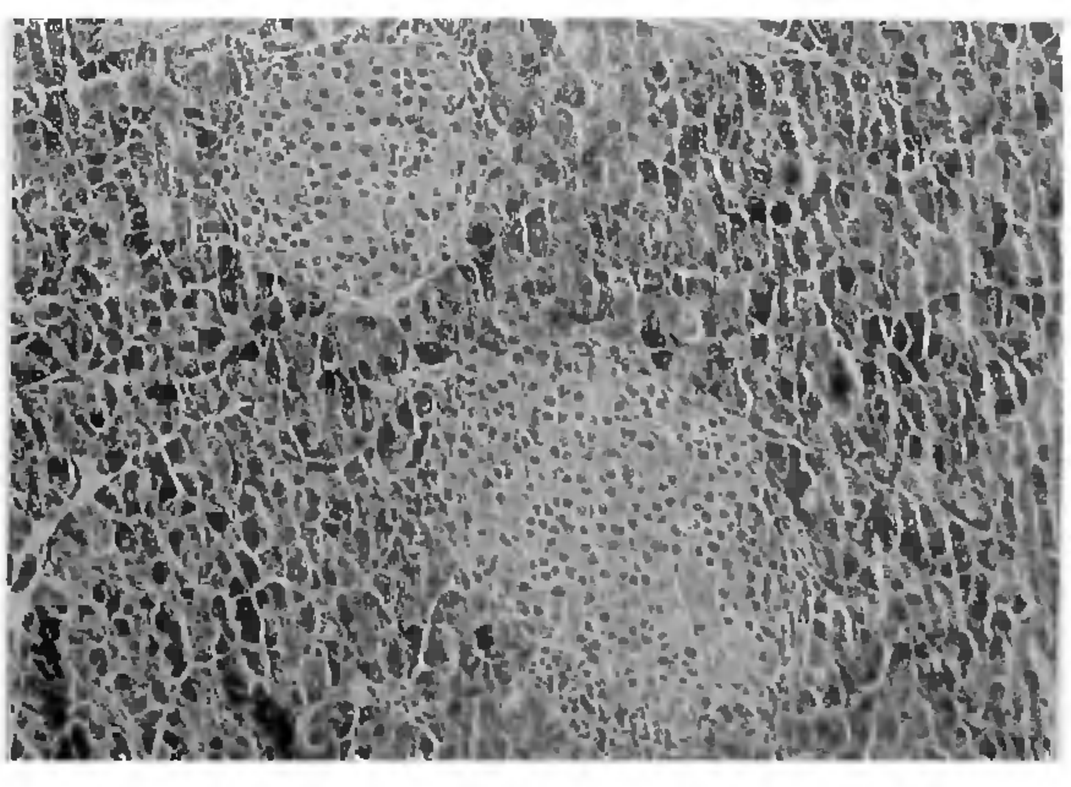

Figure 3. H&E stained section from regenerated mouse pancreas on day 45 exhibiting normal islet morphology comparable to that of the control group (× 320).

dimension in the treatment of Type 1 diabetes. In this context, the results of the present study are of importance as they reflect on the intrinsic capability of residual islet cell mass to restore transiently after EMC-D induced decrease in β-cell number.

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Ascorbic acid counteracts the prooxidant effect of alloxan in erythrocytes *in vitro*

Poonam Yadav*, S. Sarkar, Nisha Rathore, Susan John, Manisha Kale and D. Bhatnagar†**

Department of Biochemistry, D.A. University, Khandwa Road, Indore 452 001, India

*Department of Biochemistry, **Centre for Reproductive Biology and Molecular Endocrinology, Indian Institute of Science, Bangalore 560 012, India

Alloxan-induced experimental diabetes has been reported due to the production of toxic O_2^- and $OH^\cdot$ radicals and H_2O_2. Alloxan induced lipid peroxidation (LPO) in rat erythrocytes in the absence of ascorbic acid (AA). Superoxide dismutase (SOD) activity decreased while catalase (CAT) activity increased in erythrocytes with alloxan treatment without AA. Alloxan treatment in presence of AA showed no significant changes in LPO, SOD and CAT activities in erythrocytes, indicating neutralization of alloxan induced free radical production. Treatment with glucose in presence of AA showed no significant changes in LPO and in SOD and CAT activities in erythrocytes. Erythrocytes incubated with alloxan and glucose without AA showed increased LPO and decreased activity of SOD and CAT. However, LPO decreased and enzyme activities were comparable to control when treatment with alloxan and glucose was followed in presence of AA.

ALLOXAN is known to cause cytotoxicity to β-cells of pancreas by producing highly reactive free radical species[1]. The action of alloxan is inhibited by superoxide dismutase (SOD) and the enzyme has been shown to have therapeutic value in alloxan-induced diabetes[2]. These and other studies showed the involvement of free radical species such as O_2^- and $OH^\cdot$ radicals in the action of alloxan. In the present study, rat erythrocytes exposed to alloxan have been used as an *in vitro* model to show the action of alloxan. As the cyclic reaction involving alloxan and its reduced product dialuric acid spontaneously produce O_2^- and $OH^\cdot$ radicals and H_2O_2 (refs 2, 3), it was intended to show whether alloxan causes changes in antioxidant enzymes in the erythrocytes. Further, whether addition of free radical scavengers such as ascorbic acid (AA) inhibits the action of alloxan. Ascorbic acid was used to investigate whether it protects erythrocytes from the prooxidant effect of alloxan. Glucose was added in physiological amounts to erythrocytes to compensate for any glucose loss during incubation and to observe the effects of alloxan in presence of glucose. In the present study, the action of alloxan *in vitro* and the protective action of AA on erythrocyte lipid peroxidation (LPO) and antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) were determined. The report indicates that alloxan induced LPO and changes the activities of SOD and CAT, and the action is mitigated by AA in the rat erythrocyte system.

Male Wistar rats weighing 150–180 g were housed in polypropylene cages under standard conditions with free access to drinking water and basal diet. The animals

†For correspondence.