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Letter to the Editor

## Impaired glucose tolerance in rat islet isograft recipients after cytomegalovirus infection

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To the Editor

The long-term survival rates of human islet allografts are unsatisfactorily low (1). Despite improved immunosuppressive protocols, only approximately 10% of the recipients are still normoglycemic and insulin independent at 5 years after transplantation. For a long time, the role of cytomegalovirus (CMV) in islet graft failure was neglected, as its effect could not be reliably determined because of the overshadowing effects of other deleterious factors, such as recurring autoimmunity, alloimmunity, or a combination of these (1). However, recently, CMV has been recognized, both in experimental animals and in humans, as a possible risk factor contributing to islet graft failure (2).

In our efforts to identify the mechanisms by which CMV contributes to failure, we demonstrated that CMV could directly infect human and rat beta cells *in vitro*

(3, 4). *In vivo*, in rat islet allografts in rats, we showed that rat CMV (RCMV) accelerates failure of the grafts (5). Also, the routinely applied antiviral treatment with ganciclovir has been shown not to be effective in blocking the negative effects of RCMV (5). Antiviral treatment blocks replication of the virus, but cannot prevent adverse effects on the cells such as elevated immunogenicity (5).

We found evidence that the accelerated allograft failure could be explained by CMV-induced enhancement of allogeneic CD8+ and NK cells responses (5). A question that could not be addressed in our previous studies was whether RCMV also has direct effects on the grafts *in vivo* (5). Therefore, we undertook this study, in which we determined the direct effect of RCMV on the functional survival of islets by performing infections in isografts, thus, in the absence of

confounding allogeneic immune responses. This study was done with low doses of RCMV, to be able to determine whether RCMV might influence graft function even in the absence of clinical viremia and characteristic clinical symptoms.

To this end, diabetic Albino Oxford rats, induced with streptozotocin (via the tail vein with 75 mg/kg of Zonasar; Upjohn Co., Kalamazoo, Michigan, USA), were transplanted with a syngeneic islet graft. The baseline glucose levels before streptozotocin injection were always between 5 and 5.5 mM glucose and before islet transplantation between 19 and 30 mM glucose. No statistically significant differences were found in these values between the different groups. The endocrine graft volume was 5  $\mu$ L, which induces normoglycemia in 100% of the non-CMV infected recipients (5). Islets were transplanted under the kidney capsule. Transplantation was considered successful when non-fasting blood glucose concentrations reached levels <10 mmol/L. At the time of sacrifice we took biopsies from the naïve pancreas to exclude pancreas regeneration, as previously described (6). To mimic a primary RCMV infection in a negative recipient, the islet graft recipients received an RCMV infection ( $n = 7$ ) (Maastricht strain) of  $2 \times 10^5$  plaque-forming units at day 1 after implantation. Control animals ( $n = 6$ ) received the homogenate without the virus; this is called a mock infection. Three weeks after transplantation, all successful graft recipients received a permanent silicone catheter in the right jugular vein, for blood sampling and infusion of glucose in unanesthetized, undisturbed animals. RCMV-specific (nested) polymerase chain reaction (PCR) analysis was performed as described previously (5). Results are presented as the mean  $\pm$  standard error of mean. Statistical significance was calculated using the Mann–Whitney *U*-test. *P*-values <0.05 were considered to be statistically significant.

In RCMV-infected animals it took  $10.8 \pm 3.1$  days before normoglycemia was established after islet transplantation. In the mock-treated controls this was faster, i.e.,  $7.8 \pm 1.7$  days. This difference did not reach statistical significance. Normoglycemia was maintained in all control recipients for the study period of 90 days. In the RCMV-infected animals, this was 83% instead of 100%, illustrating that in isografts an effect of RCMV on the success rate of the grafts can be measured.

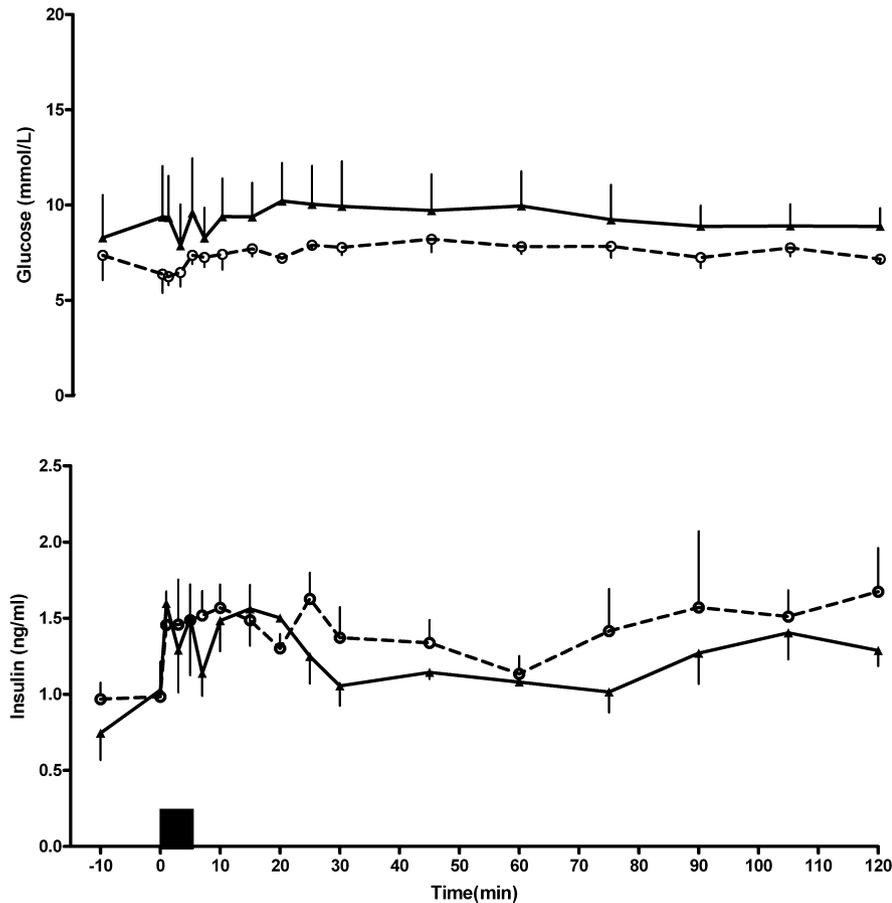
The metabolic capacity of successful grafts was studied at day 28 after implantation by performing a meal test. At this time point, 4 animals were sacrificed. In the salivary glands we found no expression of RCMV

by PCR (5), confirming that the infection is subclinical and does not lead to viremia. RCMV-specific R44 protein was demonstrated in the grafts by immunohistochemistry, as described (5). We inspected the grafts for RCMV R44 protein, but, as also shown in our previous study, infected cells were rare or absent (5). Only a small portion of the cells are infected, but are responsible for dysfunction, as we showed for human and rat islets (3, 4).

As demonstrated in Figure 1, RCMV-infected recipients were less glucose tolerant than the control animals, as illustrated by the higher glucose levels and the lower insulin responses during the meal test (areas under the curve calculations showed that *P* was always <0.05). To determine whether this was a result of a decreased beta cell mass or should be attributed to a disturbed potentiation of the response, we also performed an intravenous glucose tolerance test by administering a glucose bolus infusion of 200 mg of glucose ( $n = 6$ ). This intravenous glucose tolerance test did not demonstrate any differences in glucose or insulin levels, illustrating that it was not the beta cell mass but rather a disturbed potentiation of the response that should be held responsible for the reduced responses during the meal test (7, 8). In a previous study, we showed that only a few cells in the graft are infected but responsible for enhancement of the immunogenicity of the graft as a whole (3, 4). So we show that not only enhancement of allogeneic CD8+ and NK cell responses is involved in islet grafts failure (5) but also more direct effects on the grafts, as allograft responses were excluded in the present experimental set up. Possibly, RCMV-specific T and NK cells are involved. These cells are also likely a portion of the NK cells we observed to be enhanced in our previous study (5).

As we found only a few beta cells to be infected by RCMV in the present and previous studies (3, 4), we believe we can conclude that the increased immunogenicity and cytokine expression in the beta cells (3, 4), after RCMV infection, should be held responsible for the disturbed engraftment and reduced responses during the meal test.

Up to now, many clinicians neglect CMV as a threat for islet graft function when viremia is absent. Viremia and CMV disease is assumed to be a mandatory hallmark to consider CMV as a factor influencing graft function (9). We were not able to detect viremia in the CMV group. Furthermore, the group did not develop clinical CMV. Despite these facts, we still found many undesired effects of CMV, such as a disturbed glucose tolerance as shown in this report, and accelerated graft failure with allografts (5). Our argument is supported



**Fig. 1.** Success rates and metabolic capacity (upper and lower graphs) of rat cytomegalovirus-infected and control islet isograft recipients. The meal tests were performed after a 2-h fast. Meal tests were performed by offering the animals 2 g of normal rat chow (53% carbohydrates, 20% protein, 5% fat, and 22% other constituents) mixed with 2 mL of water. The animals were trained to consume the meal within 5 min. Results are presented as the mean  $\pm$  standard error of mean. Statistical significance was calculated using the Mann-Whitney *U*-test. *P*-values <0.05 (\*) were considered to be statistically significant.

by Hayashi and Fujisaki (10) in mice, who demonstrated impaired insulin secretion by native pancreatic beta cells after CMV infection of fully immunocompetent mice. Our data also corroborated the findings of Eckhard et al. (11), who also found evidence that, in the absence of CMV disease, human islet allograft function may be impaired. Only studying effects in patients with viremia may therefore lead to an underestimation of the effects of CMV on impaired graft function.

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**Author contributions:** M.J.S. and B.J.deH. contributed to performance of the experiments. A.deH., M.M.F.,

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