Virus Induced Diabetes and the Immune System

I. Suggestion that Appearance of Diabetes Depends on Immune Reactions*

F. K. Jansen¹, H. Müntefering², and W. A. K. Schmidt³

¹ Diabetes Research Institute, ² Pathological Institute, and ³ Institute for Medical Microbiology and Virology of the University, Düsseldorf

Summary. The participation of immune reactions in the EMC virus induced diabetes of the mouse was studied by immunosuppression with 500 R sublethal X-irradiation or 120 mg/kg Asta 5122, a cyclophosphamide derivative. Average glucose levels after X-irradiation and infection remained normal, while virus infected, otherwise untreated mice, had significantly higher mean glucose levels, indicating that immune reactions are necessary for the development of virus induced diabetes. Immune suppression by the cyclophosphamide derivative led, in contrast, to a significantly increased mean glucose level and increased insulinitis in comparison with the controls only infected. This indicates an important role of the cellular immune reaction, insulinitis, in the destruction of the islets.

Key words: Encephalomyocarditis virus, diabetes mellitus, virus induced experimental diabetes, immune reaction, X-irradiation, immunosuppression, cyclophosphamide, blood glucose, insulinitis.

In the pathogenesis of juvenile diabetes immune reactions may play a role for the following reasons:

- a) autoantibodies directed against the islets of Langerhans were found in a high percentage of juvenile diabetics [1, 2];
- b) at the onset of severe juvenile diabetes lymphocytic infiltrations were found in the islets of Langerhans [3];
- c) juvenile diabetes correlates with some HL-A antigens depending on the histocompatibility com-

plex, which is known to code also for immune reactions [4, 5].

On the other hand evidence has been established that juvenile diabetes may be initiated by a virus. The mouse model of a virus-induced, insulin-dependent diabetes [6], accompanied by lymphocytic infiltration of the islets of Langerhans [7], and some epidemiological studies [8] support the virus hypothesis.

It is also possible that both mechanisms, the virus infection and the autoimmune reaction, may play a role in the sense that the virus infection initiates an immune reaction leading to a lymphocytic infiltration, "insulinitis", with consequent islet destruction. This hypothesis is supported by the finding that mouse strains of different H-2 types in the acute stage of the infection have almost identical necrosis in the majority of B-cells and intact A-cells, but only certain strains (i. e. DBA₂) develop "insulinitis" and diabetes [9]. In the lymphocytic choriomeningitis of the mouse an immune reaction initiated by the virus infection leads to the acute phase of the inflammation and death in adult mice. Therefore the hypothesis that diabetic symptoms provoked by the EMC virus in DBA₂ mice might be due to immune reactions was examined.

Materials and Methods

Mice and Virus

DBA₂ male mice (SPF) from Bomholtsgård, Ry, Denmark were used at the age of 8 and 12 weeks. The EMC virus (M-variant) was obtained from Dr. Craighead and was reproduced by 3 passages on heart tissue [7]. Mice were infected subcutaneously with 0.5 ml heart extract in a dilution of 10⁻² in

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¹ Present address: Centre de Recherches Clin-Midy, Montpellier, France

Earles medium. The virus titre in the end dilution was $10^{3.6}$ TCID₅₀/0.25 ml. The virus antibodies were determined by haemagglutination inhibition (HI) [10]. Blood glucose levels were determined on non-fasting mice, as fasting was not tolerated very well after infection. Blood samples were taken from the orbit before infection and at weekly intervals thereafter. Glucose was measured on a Technicon auto-analyser using an o-toluidine-method [11].

Immunosuppression

X-irradiation was performed with the help of Dr. Eichmann from the Institut für Genetik in Köln. With the help of a standard curve, time was calculated to get 500 R per surface occupied by one mouse.

The immunosuppressive drug, Asta 5122, a gift from the Astawerke, was applied P in a daily dose of 0.5 ml of a 1 mg/ml solution the 6 days following infection (total = 120 mg/kg) [12]. This chemical, 3-(2-chloroethyl) -2-(2-mesyloxyethylamino) -tetrahydro-2H-1, 3, 2, -oxazaphosphorine-2-oxide (Asta 5122) is a derivative of Cyclophosphamide, known for its immunosuppressive action or, under certain conditions, for the immunostimulation of T-lymphocytes [13–15].

Histopathology

The pancreata were removed immediately after killing the animals, fixed for a period of 20 hours in Bouin's solution and embedded in paraplast. Evaluation was carried out with 6 μ thick sections, which were stained by Haematoxylin-Eosin. Alpha- and beta-granules were demonstrated by indirect immunofluorescence.

Statistics

The results of all groups were expressed as means + standard error of means (SEM). The distribution of the individual values for all groups was studied by calculation of their normality, in order to justify the use of the t-test. Calculation of β_1 and β_2 was done by the classical method of Pearson. In a normal distribution β_1 tends to 0 and β_2 to 3. This had been verified for the experimental values (Table 1–2).

Results

a) Age Dependence

Young mice (8 weeks) were more resistant to the diabetogenic effects of the virus than older mice. The latter were therefore used in subsequent experi-

ments. With increasing mean glucose values the standard deviation increased as well, showing heterogeneity of blood glucose levels in infected mice. 14 days after infection 55% of the older mice exceeded the level of 257 mg/100 ml glucose, the upper normal limit (= mean level + 3 times the standard deviation of blood glucose in normal animals).

b) X-Irradiation

A group of 130 DBA₂ mice with a mean weight of 22.2 g was divided into 4 subgroups: 20 mice received the virus only (positive controls), 50 mice X-irradiation plus virus, 50 mice X-irradiation only and 10 mice no treatment (negative controls). The average nonfasting glucose level of mice before the experiment was 163 ± 4 mg/100 ml. On day 6 after inoculation the positive control group, receiving virus only, had reached a value of 286 ± 27 mg/100 ml in the 95% surviving animals and on day 14 a mean value of 376 ± 43 mg/100 ml in the 80% surviving mice. The negative control group with no treatment and the group receiving X-irradiation only had very similar glucose levels with a low standard deviation, but the mean values increased slightly with time.

The experimental group treated with X-irradiation (500 R) plus virus had a mean glucose level almost identical to the negative control group, 169 ± 16 mg/100 ml in the 82% surviving animals on day 6, which is significantly decreased ($p < 0.001$) in comparison with the positive controls. Thereafter the high mortality rate led to only 10% of animals surviving by day 14 ($n = 5$), with an average glucose concentration identical to the negative control group (214 ± 7 mg/100 ml). The high standard deviation on day 6 demonstrates the presence of some high and some subnormal glucose levels in this group. In the positive control group 42% of the animals were diabetic (i. e. exceeding 3 times the standard deviation of normal animals), but only 17% of the mice with virus plus X-irradiation. Histologically no insulitis was found in any of the animals examined, but necrosis was found in the B-cells without infiltrations in 14/17 mice and in the exocrine pancreas in 12/17. This is very uncommon in animals without immune depression (Table 3).

Virus could be cultured from pancreatic tissues in all animals, but the antibody titre against the EMC virus remained negative.

c) Chemical Immunosuppression

Mice (200) were randomly divided into 4 groups: 40 mice received the virus only (positive control), 100

Table 1. Immunosuppression by X-irradiation. Nonfasting levels of blood glucose at different times after treatment with: virus alone, virus plus 500 R X-irradiation, X-irradiation alone or no treatment

EMC Virus	X-Irradiat. 500 R	N	Pretreatment Glucose level ^a mg/100 ml Mean + SEM	Day 6		Normality		Signif. p	% diabetic values ^(F)	Day 13	
				% Surviving	Glucose level mg/100 ml Mean + SEM	β_1	β_2			% Surviving	Glucose level mg/100 ml Mean + SEM
+	-	20		95%	286±27	0.85	2.67	< 0.001	42%	80%	376±43
+	+	50	163±4	82%	169±16	2.19	4.99		17%	10%	214± 7
-	+	49		100%	166± 5	0.00	3.32	> 0.1	0%	94%	223± 4
-	-	10		100%	169±11	n.d.	n.d.	> 0.1	0%	80%	239±13

^a pretreatment glucose levels were only measured on a randomized sample of 50 out of the 129 mice^(F) Values $\geq$ mean value + 3 SD**Table 2.** Chemical immunosuppression with ASTA 5122. Nonfasting levels of blood glucose at different times after treatment with: virus alone, virus plus ASTA 5122 (6 × 0.5 mg), Asta 5122 alone or no treatment

EMC Virus	5122 Asta 6 × 0.5 mg	N	Pretreatment Glucose level ^a mg/100 ml Mean + SEM	Day 7		Normality		Signif. p	% Diabetic values ^(F)	Day 15	
				% Surviving	Glucose level mg/100 mg Mean + SEM	β_1	β_2			% Surviving	Glucose level mg/100 ml Mean + SEM
+	-	40		100%	367±21	0.15	1.89	< 0.01	88%	90%	392±25
+	+	100	144±3	40%	482±28	0.62	2.75		88%	0%	- -
-	+	50		96%	123±4	0.56	2.98	> 0.1	0%	90%	134± 4
-	-	10		100%	148±5	n.d.	n.d.		0%	80%	151± 5

^a pretreatment glucose levels were only measured on a randomised sample of 50 out of the 200 inbred SPF mice^(F) values $\geq$ mean value + 3 SD**Table 3.** Histology and virology after immunosuppression. Virological and histological findings of infected DBA 2 mice without immunosuppression, after 500 R X-irradiation or after 6 × 0.5 mg Asta 5122 chemical immunosuppression

Treatment		Examination		Virology		Histology		
Virus	Immune suppression	Day	N	Virus in pancreas	HI-anti- body titer of serum	Insulitis	B-Cell necrosis without infiltration	Exocrine necrosis
+	-	6	20	20/20	1/>2000	20/20	0/20	0/20
+	X-irradiation	6-8	17	17/17	neg.	0/17	14/17	12/17
+	Asta 5122	7-8	20	15/16	not done	20/20	2/20	7/20

mice the virus plus Asta 5122 in the dose of 0.5 mg/mouse (20 mg/kg) for 6 days after the virus infection; 50 mice were given Asta 5122 alone for the same period and 10 mice were without treatment (negative controls). The mean glucose level of 144 ± 3 mg/100 ml resembled that in the former experiment and did not change on days 6 and 13 in the negative controls without treatment or in the group with Asta 5122 only. The positive control group became more rapidly diabetic than in the former experiment (367 mg/100 ml on day 6 and 392 mg/100 ml on day 14). The experimental group with virus

plus Asta 5122 had a higher mortality than the corresponding group after X-irradiation as only 40% survived on day 7 and no animal on day 14. The average glucose level on day 7 (482 ± 28 mg/100 ml), however, is significantly ($p < 0.01$) increased in comparison with the group receiving virus only, though the number of diabetic animals (88%) is identical in both groups. Most of the animals (16/20) showed a severe insulitis, while necrosis without infiltration was only seen in 2/20 mice. Necrosis of the exocrine pancreas was much more frequent (7/20). Virus was demonstrated by culture of

pancreatic tissue in 15/16 animals. Virus antibodies were not determined because of a lack of serum (Table 3).

Discussion

Participation of immune reactions in virus induced diabetes can be demonstrated by immunosuppression. This procedure, however, simultaneously decreases the hosts defence, thus favouring virus multiplication. In consequence there was a high mortality in mice treated with the EMC-virus (M variant) plus immunosuppression by X-irradiation or chemicals. Only 10% of the animals after X-irradiation, and no animal after chemical immunosuppression plus virus, survived on day 13 or 15, respectively. On day 6 there was still a sufficient percentage of surviving mice: 82% ($n = 41$) for the X-irradiated and 40% ($n = 40$) for the chemically suppressed group for comparative purposes. Mice with 500 R X-irradiation or immunosuppressive chemicals alone had no higher mortality than the negative controls without treatment. On day 6 the immunosuppression by X-irradiation significantly inhibited ($p < 0.001$) the appearance of diabetes in the EMC-virus infected DBA₂ mice. The mean glucose value was the same as that of untreated controls or the group with X-irradiation alone. However the standard deviation of ± 105 mg/100 ml indicates that a few animals had high glucose levels and a few others subnormal low concentrations. The subnormal values are probably from animals no longer able to eat. When the diabetic animals in both groups were checked, only 17% (7/41) were found in the X-irradiated group, but 42% (8/19) in the positive controls. Preliminary histopathological examination suggests a complete lack of insulinitis in all irradiated mice, but islet cell necrosis without lymphocytic infiltration indicated that the virus was present.

The additional lack of antibodies against the EMC virus showed that immunosuppression at the level of T- and B-cells was complete at that period and the necrosis of the islets was presumably due to the toxic effect of the virus. As the necrosis was not sufficient to induce diabetes, an additional reaction of the immune system seems to be necessary for its appearance.

The chemical immunosuppression with the cyclophosphamide derivative Asta 5122 significantly ($p < 0.01$) increased the mean value of blood glucose levels compared to the positive controls, though the percentage of diabetic animals (88%) was the same in both groups. Chemical immunosuppression without virus did not lead to diabetes. In

connection with the high blood glucose levels the histological examination showed a very high incidence of severe insulinitis.

The antibody titres against EMC virus could not be measured, but, according to the literature, a decrease of antibody production should be expected [12]. Therefore there was a cellular immune response with probably no serological immune response. The preferential depression of the antibody producing B-lymphocytes under certain conditions of treatment with cyclophosphamide was described in 1972 [13] and then confirmed by two other groups [14–15]. The appearance of the lymphocytic infiltration in the islet correlates with a high level of blood glucose. This is quite evident in the chemically immunosuppressed group.

There are other possible interpretations of the data. Thus the 18% mortality in the virus plus irradiation group could represent animals with severe diabetes. Inclusion of values for such animals could have greatly increased the mean glucose value. The same criticism could apply to the chemical immunosuppression group with a 60% mortality by day 6. However, the histopathological findings suggest that the high mortality is not due to diabetes but to an increased myocardial necrosis as there was less destruction of the islet of Langerhans after X-irradiation compared to positive controls.

Immunosuppression may lead to virus multiplication, which may itself exacerbate hyperglycaemia by increases of glucagon and corticosterone secretion. This explanation does not take into account that X-irradiation inhibited high glucose levels despite increased virus multiplication.

The subnormal glucose levels of some animals in the X-irradiated group may have been due to an inability to eat. However, this possibility would be as likely in the more severely affected group with chemical immunosuppression which, nevertheless, had the highest glucose levels of all groups. It cannot alone explain the decreased glucose levels after virus plus X-irradiation.

X-irradiation is known to affect T- and B-lymphocytes simultaneously. On the other hand cyclophosphamide, under certain conditions, enhances the activation of T-lymphocytes [13, 14, 15]. Therefore the increased insulinitis, after drug induced immunosuppression, suggests that a cellular immune response might destroy those B-cells of the islets which escaped the virus attack.

These experiments can only be considered as pilot experiments. They have to be repeated in a way that prevents the high mortality and demonstrates immunological specificity.

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Dr. F. K. Jansen
Service d'Immunologie
Centre de Recherches Clin Midy
rue du Professeur Blayac
F-34082 Montpellier Cedex
France