Monoclonal antibody-mediated cytotoxicity against rat Beta cells detected in vitro does not cause Beta-cell destruction in vivo

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Summary. Two monoclonal Beta-cell surface antibodies M10H6 und K14D10 were obtained by fusion of spleen cells of Balb/c mice with the myeloma cell line P₃0. The monoclonal antibody M10H6 was induced by immunization with rat insulinoma cells finally boosted with disintegrated rat islets, whereas the K14D10 was generated after immunization with porcine proinsulin. Both monoclonals belong to the IgG_{2a} isotype and were screened with insulin-producing rat insulinoma cells by an indirect immunofluorescence test as well as by a cellular enzyme linked immunosorbent assay. In addition to the cell surface binding on living Beta cells the monoclonals react with islets on cryostat sections of rat pancreas. The anti-islet cytotoxic potential of these monoclonals was measured by ⁵¹Chromium-release in the presence of complement or Fc-receptor bearing leucocytes using ⁵¹Chromium-labelled rat islet cells as target. Both antibody secret-

ing hybridomas were propagated in syngeneic mice resulting in high levels of islet cell surface antibodies in ascites and sera from the recipient. High anti-islet cytotoxicity was mediated by ascites fluid, but no mouse developed hyperglycaemia. Furthermore, the repeated injections of the monoclonals into rats did not exert a diabetogenic action and failed to reduce the pancreatic insulin content although the attraction of the K14D10 to the pancreatic islets in vivo could be demonstrated. We conclude that islet cell surface antibody-mediated Beta-cell lysis in vitro may not be relevant to Beta-cell destruction in vivo.

Key words: Monoclonal islet cell surface antibodies (mc-ICSA), anti-islet cell toxicity, application in vivo, pancreatic insulin content, rat.

There is now abundant evidence that Type 1 (insulin-dependent) diabetes mellitus results from the destruction of pancreatic Beta cells by mononuclear cells of the immune system. Even in the preclinical phase anti-islet cell cytoplasmic antibodies (ICA) can be detected [1]. Recently glutamic acid decarboxylase has been discovered to be the prominent Beta-cell specific cytoplasmic 64 kD autoantigen in Type 1 diabetes [2]. Islet cell surface antibodies (ICSA) preferentially bind to Beta cells [3] and mediate anti-islet toxicity in vitro [4], but their role in the destruction of Beta cells in vivo remains unclear. The production of monoclonal ICSA (mc-ICSA) and their propagation by growing hybridomas in syngeneic mice offers the possibility of investigating the significance of ICSA for Beta-cell destruction.

In the present study mc-ICSA were generated by different immunization procedures; their islet cell toxic potential by activation of complement or Fc-receptor bearing leucocytes was investigated in vitro using rat islet cells or islets as targets and in vivo after application into rats, as well as in hybridoma bearing mice with high levels

of ICSA in their circulation. In addition to the binding of the monoclonals as ICSA on living rat islet and insulinoma cells, their reactivity in vitro and in vivo as ICA was detected on cryostat sections of rat pancreas after in vitro incubation and 24 h after in vivo application into rats.

Materials and methods

Animals and cells

Balb/c mice were supplied by the Institute of Biomodels (Berlin, FRG) and were allowed to acclimatize for at least one week before use in experimental work. NEDH rats were from our breeding colony, originally supplied by Dr. A. Lernmark, Hagedorn Research Laboratory (Gentofte, Denmark). Cultured rat insulinoma cells (RIN) are derived from this rat strain [5]. The RIN cell line was cultured in standard medium RPMI 1640 containing 7.5 % heat-inactivated fetal calf serum supplemented with 2 mmol/l glutamine, 1 mmol/l pyruvate, 145 mg/l streptomycin, 50 mg/l gentamycin and passaged every week by detachment in the presence of 0.02 % EDTA and 0.05 % trypsin. Normal rat and mouse islets and islet cells were isolated after collagenase digestion of pancreata from 10–14-day-old animals by standard procedure as detailed previously [6].

Measured variables

The pancreatic insulin and glucagon content of rats and mice was estimated by radioimmunoassay after homogenization in phosphoric acid/ethanol and overnight storage at 4°C [7, 8]. Blood samples were taken from the retro-orbital sinus or by heart catheterisation under ether anaesthesia after aspiration of the intraperitoneal ascites fluid. Plasma glucose was assayed by the glucose oxidase method in a Beckman glucose analyzer (Beckman Instruments, Fullerton, Calif., USA). The ICSEA level in the ascites and serum of mice bearing mc-ICSEA-producing hybridomas and in serum of rats after injection of monoclonal ICSEA was detected by indirect immunofluorescence technique using RIN cells [9].

Hybridoma generation and antibody screening

Immune response was induced by five i. p. injections of 50 µg porcine proinsulin into a female 8-week-old Balb/c mouse. For the first injection proinsulin solution was emulsified in complete Freund's adjuvant followed by four further i. p. injections of proinsulin emulsified in incomplete Freund's adjuvant given on days 14, 28, 55 and 86. Four days before fusion the mouse was boosted with 100 µg proinsulin on day 105. Another mouse received 100 µl complete Freund's adjuvant on day 0 followed by i. p. injections of 5×10^6 RIN cells on days 1, 14, 42, 54. The last boosters were performed with 350 ultrasonically disintegrated neonatal rat islets on days 185, 186, 187 in 154 mmol/l NaCl followed by fusion on day 188 according to the method of Lane et al. [10] and as detailed previously [11]. After 2 weeks during which the hybrid cells were grown semiconfluently, the medium was removed and assayed for antibodies reacting with the surface of living rat insulinoma cells tested by indirect immunofluorescence as described previously [12–13] or by ELISA using desiccated RIN cells (5×10^4 per sample) as target [14]. Antibody positive microcultures were cloned and recloned by limiting dilution and grown in culture medium or as ascites tumours in Balb/c mice primed with pristane 10 days before i. p. injection of 2×10^6 hybridoma cells into each mouse [15]. The immunoglobulin concentration in hybridoma supernatants and in the ascites fluids as well as the isotypes were determined by ELISA standard procedures using goat anti-mouse immunoglobulin class and subclass-specific antibodies [13, 14].

In addition, the reaction of certain mc-ICSEA was detected on cryostat sections of rat and mouse pancreas using the APAAP (alkaline phosphatase anti-alkaline phosphatase) method [16]. Consistent with the RIN cell donor strain, in this assay pancreata of NEDH rats were used as targets [5]. Every incubation step of the assay was performed for 30 min at room temperature. The cryostat sections were incubated with culture supernatants containing mc-ICSEA followed by incubation with rabbit anti-mouse immunoglobulin (Dakopatts, Glostrup, Denmark) diluted 1:20 and with APAAP (Dakopatts) diluted 1:25. The bound antibodies were visualized using the chromogen substrate naphthol-AS-bi-phosphate. Cross-reaction of mc-ICSEA with insulin and glucagon was examined by radioimmunoassay [17].

Beta-cell binding of mc-ICSEA

Purified rat islet Beta and non-Beta cells were prepared by autofluorescence-activated cell sorting according to a previously described procedure [18]. The isolated cells (6×10^4 in 200 µl) were incubated with the mc-ICSEA and irrelevant monoclonals for 60 min. The cells were washed twice and resuspended in rhodamine-labelled swine anti-mouse immunoglobulin antiserum, incubated for 30 min, washed and finally fixed in 4% (volume/volume) formaldehyde. The cell preparations were analysed by fluorescence-activated cell sorting (FACS) in a Facstar (Sunnyvale, Calif., USA) equipped with a 2025-05 Argonlaser (Spectra Physics, Mountain View, Calif., USA) to quantify the percentage of fluorescent cells [19].

Investigation of in vivo effects of mc-ICSEA

To examine the binding of the mc-ICSEA in the islets after in vivo application, NEDH rats received a single i. p. injection of 1 ml ascites containing 30 mg of the monoclonal K14D10, M10H6 or an irrelevant monoclonal of identical isotype, respectively. The rats were killed 24 h later, serum was saved for detection of circulating mc-ICSEA and the pancreas was immediately frozen in liquid nitrogen. The in vivo binding of the mc-ICSEA on the islets was examined with the APAAP method described previously by direct incubation of the pancreatic cryosections with rabbit anti-mouse immunoglobulin and APAAP. Furthermore, the possible cytotoxic effects in vivo on Beta cells were examined in the NEDH rats after six i. p. injections of 1 ml ascites per rat every second day. The animals were killed 5 days after the last application of mc-ICSEA. For examination of specific Beta-cell destruction, pancreatic insulin and glucagon content were quantified by radioimmunoassay [8] for each animal loaded with mc-ICSEA.

⁵¹Cr release assay for assessment of antibody-mediated cytotoxicity (ADCC and C'AMC) in vitro

The detection of antibody-dependent cellular cytotoxicity (ADCC) against rat islets in vitro was performed as detailed previously [20]. Briefly, 100 collagenase-isolated islets obtained from 6-8-day-old rats were ⁵¹Cr-labelled by incubation in 200 µl tissue culture medium RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum and with 75 µCi Na₂⁵¹CrO₄ (Institut für Kernforschung, Rossendorf, FRG) at 37°C for 1 h. After washing five times by centrifugation, batches of five islets were incubated for 6 h at 37°C with 3×10^5 living mononuclear effector cells from a newly-diagnosed diabetic patient, in 100 µl RPMI 1640 containing 50% hybridoma supernatant or diluted ascites fluid (1:20) containing islet cell reactive or irrelevant monoclonals. For determination of complement-dependent antibody-mediated cytotoxicity (C'AMC) ⁵¹Cr-labelled neonatal rat islet cells were incubated with mc-ICSEA containing culture supernatant or diluted ascites and 1:5 diluted rabbit complement as described previously [21]. The percentage of specific ⁵¹Cr-release was calculated:

$$\text{specific } ^{51}\text{Cr} - \text{release} = \frac{\text{cpm}_{\text{exp.release}} - \text{cpm}_{\text{spont.release}}}{\text{cpm}_{\text{max.release}}} \times 100$$

The mc-ICSEA were considered to be islet-cell toxic if the ⁵¹Cr-release was greater than $\bar{x} + 2 \text{ SD}$ as measured with irrelevant monoclonals. The spontaneous ⁵¹Cr-release of islets incubated with medium alone amounted to $8.77 \pm 0.51\%$ ($\bar{x} \pm \text{SEM}$ of 21 separate islet cell preparations). In addition, the monoclonals were tested for antilymphocyte C'AMC activity. In these experiments the ⁵¹Cr-release of splenic lymphocytes was estimated using the same protocol as described above.

Statistical analysis

Data are shown as means $\pm$ SEM. Significant differences ($p < 0.01$) were determined with the Student's one-tailed *t*-test.

Results

The binding of monoclonal antibodies to RIN cells and islet sections is shown in Figure 1. Two stable hybridomas secreting monoclonal antibodies of the IgG_{2A} isotype primary screened by CELISA were detected as cell surface antibodies by indirect fluorescence technique using the insulin-producing RIN cells as target (Fig. 1a and b). Both monoclonals were confirmed as surface antibodies with a strong binding on normal living rat and mouse islet cells (ICSEA).

Fig. 1a-f. Immunostaining of the surface of viable rat insulinoma cells by incubation with the monoclonal antibody M10H6 (a) and K14D10 (b) using the indirect immunofluorescence technique (magnification $\times 1500$). Reactivity of the same antibodies M10H6 (c) and K14D10 (d) in vitro on islets of pancreatic cryosections of NEDH rats. The binding of K14D10 (e) in the islets of NEDH rats in vivo was detected on pancreatic cryosections 24 h after application of 1 ml K14D10 ascites immunostained by the use of alkaline phosphatase anti-alkaline phosphatase method (c-f; magnification $\times 250$). In spite of identical conditions, no in vivo islet binding was detectable for the monoclonal M10H6 (f)

Fig. 2. Facstar-analysis of the monoclonal K14D10 binding to islet Beta and non-Beta cells purified from the rat pancreas. Each cell is plotted according to forward scatter intensity and rhodamine-associated fluorescence intensity. The left panel represents the Beta-cell surface binding. More than 14% of the Beta cells are recovered in the higher window. The right panel represents that less than 0.8% of the non-Beta cells are bound by K14D10 and recovered in the higher window, reflecting the Beta-cell specificity of this monoclonal relative to islet cells

Fig. 3. Assessment of complement-dependent antibody-mediated cytotoxicity using ^{51}Cr -labelled neonatal viable rat islet cells and rabbit complement. The specific ^{51}Cr -release was measured in the presence of various ascites dilutions of the monoclonal islet cell surface antibodies M10H6 (▨) and K14D10 (■) and of an irrelevant monoclonal (□) of the identical isotype IgG_{2A}. Data given as $\bar{x} \pm \text{SEM}$, $n = 3$

As shown by binding analysis in FACS on Beta and non-Beta islet cells both monoclonals preferentially recognised Beta cells. A typical antibody binding pattern of the monoclonal K14D10 on pre-sorted living rat islet cells is demonstrated in Figure 2.

Additionally, both monoclonal ICSA gave a typical ICA pattern on NEDH rat pancreatic cryosections (Fig. 1 c and d). The monoclonals did not cross react with insulin and glucagon when examined by radioimmunoassay.

By growing both antibody-producing hybridomas as ascites tumours in syngeneic mice, a high level of the monoclonals at a range of 20 to 50 mg/ml serum was also detectable in the circulation. However, the mice did not develop hyperglycaemia, and neither the pancreatic insulin/glucagon ratio nor the insulin content was diminished.

The ascites as well as the serum of the tumour bearing mice displayed a strong anti-rat islet cell toxicity in vitro, both in the presence of complement (C'AMC) (Fig. 3) or Fc-receptor bearing effector cells (ADCC) (Fig. 4). Anti-islet cytotoxicity was also mediated by mc-ICSA if rabbit serum was replaced by rat serum as complement source. Neither monoclonal mediated a cytotoxicity against lymphocytes (^{51}Cr -release $1.4 \pm 0.2\%$; $n = 6$).

Both mc-ICSA were repeatedly injected into NEDH rats to evaluate their anti-Beta-cell toxicity in vivo; however, all animals remained normoglycaemic (plasma glucose 7.1 ± 0.7 mmol/l; $n = 5$) and their pancreatic insulin content corresponded to those of rats receiving ir-

Fig. 4. Assessment of antibody-dependent cellular cytotoxicity using ^{51}Cr -labelled neonatal rat islets and mononuclear effector cells from a newly-diagnosed Type 1 (insulin-dependent) diabetic patient. The specific ^{51}Cr -release was measured in the presence of the monoclonal islet cell surface antibodies M10H6 (▨) and K14D10 (■) and of an irrelevant monoclonal of the identical isotype IgG_{2A} (□) as well as in the absence of a monoclonal (▤). Data given as $\bar{x} \pm \text{SEM}$ of three separate islet preparations

Fig. 5. Dependence of the percentage of rat insulinoma cell number with surface fluorescence on dilution of the NEDH rat serum of recipients received 24 h before 1 ml ascites fluid of the islet cell surface antibodies K14D10 ■ or M10H6 □ and of an irrelevant monoclonal △. Immunostaining of rat insulinoma cells was detected by indirect immunofluorescence technique

relevant antibodies (27.4 ± 1.7 pmol/mg pancreas vs 26.3 ± 1.5 pmol/mg pancreas; $n = 5$ in each group).

After injection of 1 ml ascites of M10H6 or K14D10 into rats only K14D10 showed a binding on the islets as demonstrated by the cryosections of pancreas, whereas the mc-ICSA M10H6 with the same binding pattern in vitro was not detectable (Fig. 1 e and f) even though a high level of M10H6 was detected in the circulation of recipient animals (Fig. 5).

Discussion

Whether the circulating islet cell reactive autoantibodies detectable before the clinical onset of Type 1 diabetes are involved in Beta-cell destruction is not yet clear. However, if any, only the Beta-cell surface reacting antibodies (ICSA) should be able to mediate Beta-cell damage by activation of the complement (C'AMC) or the Fc-receptor bearing cells (ADCC).

In this paper both the islet cell binding properties and the cytotoxic activity of two monoclonal ICSA (mc-ICSA) have been studied in vitro and in vivo. The mc-ICSA M10H6 was induced by immunization with RIN cells and after boosting with disintegrated rat islets, whereas, surprisingly, the K14D10 was obtained by an immortalized splenocyte from a mouse immunized by proinsulin. It is assumed that this antibody was induced secondarily after proinsulin antibody formation.

Independent of the different regimes used to establish primary induction the two monoclonals, M10H6 and K14D10 showed striking surface binding to both the pri-

mary rat islet cells and the insulin-producing RIN cells, which confirms similar results observed with other mc-ICSA [13, 22–25]. The insulinoma cells obviously share many antigens of normal pancreatic islet cells and are suitable for detecting not only mc-ICSA but also polyclonal ICSA in Type 1 diabetic sera [9, 26, 27]. Neither of the two monoclonal ICSA are reactive with insulin and glucagon as tested by radioimmunoassay and both react preferentially with Beta cells as demonstrated by FACS analysis. There was no binding of the pre-sorted viable non-Beta cells by the monoclonal K14D10 compared to the Beta cells.

Interestingly, both surface antibodies react as ICA on rat pancreatic cryosections, which also means the monoclonal antibodies K14D10 and M10H6 recognize antigens which occur in the cytoplasm as well as on the surface of Beta cells. This dual reactivity as ICSA and ICA has been described previously by Eisenbarth et al. [28] for the mc-ICSA A₂B₅, whereas most mc-ICSA failed to label the islets of pancreatic cryostat sections [23, 24]. Probably cell surface antigens are less abundant on pancreatic cryostat sections.

After injection of both mc-ICSA as well as an irrelevant monoclonal into NEDH rats only K14D10 was detectable in the islets of the cryosectioned pancreas although the antibody level of M10H6 in the circulation was higher than that of K14D10. This suggests that both mc-ICSA recognize different antigens as supported by significantly reduced (50%) surface binding of M10H6 to trypsin-pretreated RIN cells whereas trypsin failed to affect RIN-cell binding of K14D10. Since the monoclonals belong to the same isotype of IgG_{2A} it is suggested that an artifact due to different accumulation in the islets' capillaries should be excluded. Independent of the different in vivo accumulation of both the mc-ICSA in the islets there was no significant insulin loss seen after six i.p. injections of 1 ml ascites per rat on every second day.

However, both mc-ICSA specifically mediated in vitro anti-rat islet cell toxicity in the presence of complement or Fc-receptor bearing effector cells. But no splenocytotoxicity was mediated by the mc-ICSA, supporting the Beta-cell specific binding as detected by FACS analysis for islet cells. The lack of anti-islet toxicity of K14D10 in vivo despite high antibody level in the circulation, and striking islet cytotoxicity of these rat sera in vitro as in ascites supports the concept that ICSA-mediated activation of complement or of Fc-receptor-bearing cells alone is not sufficient to destroy the Beta cells. Alternatively these activations do not take place in vivo. Our results confirm data obtained by Pontesilli et al. [29] with an IgM monoclonal ICSA generated from a diabetic non-obese diabetic mouse. Although C'AMC against RIN cells was readily detected in the serum of the mice injected with the mc-ICSA, the mc-ICSA did not exert a diabetogenic action, even if injected into diabetes-prone mice [29]. The homologous species restriction of the complement-mediated lysis and the cytolytic inefficiency of murine serum can account for the lack of a diabetogenic action of mc-ICSA in the mice [30, 31].

Our results support the view that ICSA-mediated cytotoxicity to Beta cells plays only a minor role for Beta-cell destruction in the context of the cellular autoimmune

mechanism causing Type 1 diabetes. This is shown by the ability of activated lymphocytes but not serum to transfer the diabetic syndrome of BB rats and non-obese diabetic mice [32, 33]. Nevertheless, the monoclonal antibodies reactive with Beta-cell antigens which are competitively inhibited in their antigen binding by antibody-positive serum from newly-diagnosed Type 1 diabetic patients remain an important tool for identifying and preparing antigens which are recognized by diabetes relevant autoantibodies. Some of these antigens may also be recognized by the immune effector cells which cause the Beta-cell destruction.

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