Circulating and cell-bound antibodies increase coxsackievirus B4-induced production of IFN-α by peripheral blood mononuclear cells from patients with type 1 diabetes

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Increased levels of IFN-α have been found in patients with type 1 diabetes who have detectable levels of coxsackievirus B4 (CVB4) RNA in their blood. The IFN-α-inducing activity of CVB4 in vitro is weak but can be enhanced by human IgGs. Therefore, it was investigated in vitro whether a preferential IFN-α response of peripheral blood mononuclear cells (PBMCs) to CVB4 exists in patients with type 1 diabetes (n = 56) compared with healthy subjects (n = 20) and whether antibodies play a role. In patients, the levels of IFN-α obtained after stimulation by PBMCs with CVB4 were higher (P < 0.008), an individual IFN-α response by PBMCs to CVB4 was more frequent (P = 0.0004) and increased levels of IFN-α were observed in CVB4-infected whole blood cultures. The IFN-α-inducing activity of patients plasma and IgGs mixed with CVB4 and then added to PBMCs was high in comparison with healthy subjects (P < 0.001) and was inhibited by preincubating the cells with anti-FcγRII, anti-FcγRIII and anti-CAR (coxsackievirus and adenovirus receptor) antibodies. The strong IFN-α responsiveness of PBMCs to CVB4 suggested that IgGs bound to the cell surface might play a role. A short 56 °C incubation of PBMCs from patients responsive to CVB4 generated supernatants, which, when added to cells, exhibited IFN-α-enhancing activity in combination with CVB4, whereas those of controls did not. Specific antibodies for FcγRI, FcγRII and CAR inhibited this activity. These studies demonstrate that CVB4, through interactions with circulating and/or cell-bound IgGs, can strongly induce the production of IFN-α by PBMCs from patients with type 1 diabetes.

Introduction

IFN-α is a marker of ongoing virus infection (Green et al., 1982; Flowers & Scott, 1985). Recently we reported that increased levels of IFN-α in plasma were associated in 50% of cases with the presence of enterovirus sequences, particularly coxsackievirus B4 (CVB4), in the circulating blood of adults and children with type 1 diabetes (Chehadeh et al., 2000a). Moreover, IFN-α mRNA was detected in blood cells from patients with IFN-α in their plasma. These results suggest that IFN-α is produced during the course of CVB4 infection. In so far as CVB4 components were found in the circulating blood from patients, the synthesis of IFN-α in patients with type 1 diabetes could result from the interaction of CVB4 with peripheral blood mononuclear cells (PBMCs).

Enteroviruses like polioviruses and CVB are referred to as weak IFN-α inducers, as compared to strong IFN-α inducers like Sendai virus (SV) and herpes simplex virus type 1 (HSV-1)
(Pitkaranta & Hovi, 1993; Feldman et al., 1994). However, it has been shown that human polyclonal IgGs enhance the IFN-α-inducing capacity of poliovirus in vitro (Palmer et al., 2000). Furthermore, we have reported recently that CVB4-induced synthesis of IFN-α by PBMCs in vitro can be enhanced through interactions between CVB4, specific antibodies isolated from the plasma of healthy subjects, FcyR and a receptor for CVB called CAR (coxackievirus and adenovirus receptor) (Chehadeh et al., 2001; Bergelson et al., 1997). This suggests that antibodies can play a role in the IFN-α response to CVB4.

Infection by a CVB serotype induces production of serotype- and CVB group-specific antibodies (Frisk et al., 1989). Epidemiological studies have indicated that CVB infection is frequent in patients with type 1 diabetes (Yoon, 1990; Hyoty et al., 1995); therefore, these individuals may have a higher prevalence of CVB antibodies compared with appropriate controls (Szopa et al., 1993; Hyoty et al., 1995; Hiltunen et al., 1997). Thus, CVB-specific antibodies can be found in the circulating blood of diabetes patients and these antibodies may play a role in the interaction between CVB4 and PBMCs.

The mechanism of the increased expression of IFN-α in patients with type 1 diabetes is unknown. In the current study, we investigated in vitro whether a preferential IFN-α response by PBMCs to CVB4 exists in patients with type 1 diabetes compared with healthy subjects and whether antibodies can be involved.

**Methods**

**Patients and healthy subjects.** Blood samples were obtained from 56 patients with type 1 diabetes: 12 newly diagnosed and 13 previously diagnosed children (median age 13 years, range 3–17 years) and 20 newly diagnosed and 11 previously diagnosed adults (median age 37 years, range 20–69 years). Of the 56 patients, 30 had metabolic decompensation (diabetic ketoacidosis or diabetic ketoacidosis) and the remaining 26 had no metabolic decompensation. All patients included in the study were treated with insulin. Informed consent was obtained from the adults and the parents of the children; the committee on research ethics approved the study protocol.

Blood samples were obtained from 20 healthy subjects: 10 adults (6 males and 4 females; median age 28 years, range 23–45) and 10 children (5 males and 5 females; median age 12 years, range 7–16), who had not any suspected immunological, infectious or metabolic disease. These subjects were hospitalized or were outpatients at the hospital.

**Viruses.** The JBV strain of CVB4, kindly provided by J. W. Almond (Aventis Pasteur, Marcy-L’Etoile, France), was grown in Hep-2 cells (BioWhittaker) in Eagle’s minimum essential medium (MEM) (Gibco BRL) supplemented with 10% heat-inactivated foetal calf serum (FCS) (Eurobio) and 1% L-glutamine (Eurobio). Supernatants were collected 3 days post-infection (p.i.) and then clarified at 3000 r.p.m. for 10 min. Virus titres were determined by plaque-formation assay on Hep-2 cells and expressed as p.f.u./ml. Aliquots of virus preparations were stored frozen at −80 °C.

SV was kindly provided by D. Garcin (Department of Genetics and Microbiology, University of Geneva, Switzerland). HSV-1 (a laboratory strain) was cultivated in Vero cells (ATCC) in MEM supplemented with 5% FCS and 1% L-glutamine.

**IFN-α induction**

**Whole blood cultures.** Venous blood was collected in sterile 7.5 ml tubes (Becton Dickinson) containing 20 IU/ml heparin (Heparin Choya). Samples (25 μl) of heparinized whole blood were dispensed into each of triplicate wells of a 96-well tissue culture plate containing 225 μl RPMI 1640 (Gibco BRL) or 225 μl RPMI 1640 containing 10% FCS. The blood cultures were incubated in a humidified incubator at 37 °C with 5% CO₂.

PBMCs. PBMCs from heparinized blood were separated over a Ficoll–paque solution (Diatrizoate Ficoll, Eurobio). Mononuclear cells were collected, washed three times with RPMI 1640, adjusted to a concentration of 2 × 10⁶ cells/ml in RPMI supplemented with 10% FCS and 1% L-glutamine and then distributed as 0.1 ml aliquots into 96-well tissue culture plates. Thereafter, 0.1 ml of medium containing virus at an m.o.i. of 1 or medium containing virus at an m.o.i. of 1 and plasma at different dilutions were added to wells. Cultures were then incubated for 48 h at 37 °C in a 5% CO₂ atmosphere.

After this incubation period, whole blood and PBMC cultures were irradiated with UV light for 1 h to inactivate residual viruses. Supernatants of culture were then harvested, clarified by centrifugation for 10 min at 1000 r.p.m. and stored at −20 °C until assayed for the presence of IFN-α. Cultures not treated with viruses served as controls.

**Immunooassay for IFN-α.** The concentration of IFN-α was determined by a specific and sensitive dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA), as described previously (Chehadeh et al., 2000b). Antibodies were kindly provided by G. Alm, Biomedical Center, Uppsala, Sweden. The assay detection limit was 0.5 IU IFN-α/ml.

**Bioassay for IFN-α.** Samples of whole blood and PBMC cultures were assayed for antiviral activity by protection of MDBK cells (BioWhittaker) against vesicular stomatitis virus-induced cytopathic effects, as described previously (Chehadeh et al., 2000b). The assay detection limit was 2 IU IFN-α/ml. The antiviral activity of supernatant samples was neutralized with a rabbit antiserum to IFN-α (Biosource).

**Protein A-affinity chromatography.** IgGs were obtained from the plasma of healthy subjects or patients using a protein A–Sepharose CL-4B column, as described previously (Chehadeh et al., 2001).

**Antibody-dependent assays.** PBMCs were distributed into 96-well tissue culture plates as described above. Virus–antibody complexes were made by incubating plasma or IgGs at various dilutions with CVB4 for 1 h at 37 °C. The optimal dilution of plasma differed between subjects and ranged from 1:100 to 1:1000, as described previously (Chehadeh et al., 2001). CVB4 or CVB4–antibody complexes were added to cells at an m.o.i. of 1 before incubation for 2 h at 37 °C. In certain experiments, PBMCs were incubated with IgGs for 1 h and, after washing, were incubated with CVB4 at an m.o.i. of 1 for 90 min. The cells were then washed three times and cultured in RPMI 1640 containing 10% FCS for 48 h at 37 °C in a 5% CO₂ atmosphere. After the incubation period, supernatants were harvested, clarified and stored until assayed for the presence of IFN-α.

**Isolation of cytophilic antibodies.** PBMCs (5 × 10⁶/ml) were incubated for 30 min in a 56 °C water bath. Cells were removed by centrifugation at 300 g for 10 min. The cell-free supernatants generated (eluted fraction by PBMCs) were used in assays of CVB4-induced production of IFN-α by PBMCs to detect the presence of cytophilic antibody dissociated from the cell surface during the 56 °C incubation.
**Antibodies.** Monoclonal IgG1 neutralizing anti-human FcγRI (CD64), clone 10.1 (Biotest), monoclonal IgG2a neutralizing anti-human FcγRII (CD32), clone 2E1, and monoclonal IgG1 neutralizing anti-human FcγRIII (CD16), clone 3G8, antibodies were purchased from Coulter Immunotech. Monoclonal IgG1 anti-human CAR antibody (RmEβ) was kindly provided by J. Bergelson (Division of Infectious Disease, Children’s Hospital of Philadelphia, Philadelphia, Pennsylvania, USA). Anti-D human monoclonal IgG1 and IgG3 immune sera were kindly provided by Etablissement de Transfusion Sanguine (Lille, France) and used as irrelevant human IgG antibodies. Murine IgG1 and IgG2a (Coulter Immunotech) were used as control antibodies.

**Assays of CVB antibodies**

Plaque neutralization assay. The presence of anti-CVB4 neutralizing antibodies (NAS) in plasma were routinely assessed according to the method described elsewhere (Chehadeh et al., 2000a). Results were expressed as the inverse of the final dilution (titre) of the sample that totally inhibited the cytopathic effect of CVB4 in Hep-2 cells.

**ELISA.** The Serion ELISA Classic kit (Institut Virion-Serion) was used for quantitative detection of specific human anti-CVB IgG antibodies, according to the manufacturer’s instructions. Results were expressed in IU/ml.

**Statistical analysis.** Data are summarized as mean ± SD. The significance of the differences in IFN-α levels was determined by the Mann–Whitney U-test. The χ²-test was used when appropriate.

**Results**

**CVB4-induced production of IFN-α in vitro**

In healthy subjects, the mean level of CVB4-induced IFN-α in the supernatant of PBMC cultures was significantly lower than the one in the supernatant of whole blood cultures (2·0 ± 2·76 versus 46 ± 15 IU/ml; P < 0·001, n = 20) (Fig. 1a). In patients with type 1 diabetes, the range of IFN-α levels was large; high individual IFN-α levels were observed. The mean level of IFN-α in the supernatant of PBMC cultures was significantly higher than the one in healthy subjects (85 ± 134 IU/ml; P = 0·008, n = 56). An individual IFN-α response by PBMCs of ≥ 32 IU/ml (mean ± 3 SD of healthy subjects = 11 IU/ml) was obtained in 24 of 56 patients (43%) and 0 of 20 healthy subjects (0%) (P = 0·0004, χ²-testing). The mean level of IFN-α in the supernatant of whole blood cultures from patients was higher than the one in healthy subjects but the difference was not significant (109 ± 158 IU/ml; P = 0·07, n = 56 versus healthy subjects). An individual IFN-α level in whole blood cultures of ≥ 128 IU/ml (mean ± 3 SD of healthy subjects = 91 IU/ml) was obtained in 17 of 56 patients (30%) and 0 of 20 healthy subjects (0%) (P < 0·02, χ²-testing). In patients, high levels of IFN-α in CVB4-infected whole blood cultures (up to 1024 IU/ml) were associated with low levels of IFN-α in CVB4-infected PBMC cultures (0 IU/ml). In contrast with CVB4, when PBMCs and whole blood were infected with SV or HSV-1, the mean levels of IFN-α in patients were not significantly higher than those in healthy subjects (SV-infected PBMCs: 903 ± 1069 versus 716 ± 328 IU/ml, respectively, P = 0·45; HSV-1-infected PBMCs: 830 ± 525 versus 691 ± 300 IU/ml, respectively, P = 0·26) (data not shown).

**High IFN-α-inducing activity of plasma from patients with type 1 diabetes combined with CVB4**

When plasma samples from patients with type 1 diabetes were preincubated with CVB4 and then added to the patient’s own PBMCs, the levels of IFN-α were higher than those obtained in the absence of plasma (P < 0·001) (Fig. 1b). Moreover, the mean level was higher than the one obtained with plasma and PBMCs from healthy subjects (472 ± 348 IU/ml, n = 56, versus 134 ± 72 IU/ml, n = 20; P < 0·001). Fig. 2(a) shows that the levels of IFN-α were higher when plasma from patients, instead of plasma from healthy subjects, were added to PBMCs from healthy subjects (P < 0·001), whereas the levels of IFN-α were lower when plasma from healthy subjects, instead of plasma from diabetes patients, were added to PBMCs from diabetes patients (P < 0·001).

To assess whether IgGs were involved in the enhancement of CVB4-induced IFN-α production in these experiments, plasma from controls or patients were passed over a protein
PBMCs isolated from the blood of three healthy subjects were preincubated with anti-Fc antibodies to Fc.

Table 1. Antibodies to FcγR inhibit the production of IFN-α by PBMCs infected with CVB4 preincubated with IgGs

PBMCs isolated from the blood of three healthy subjects were preincubated with anti-FcγR or control antibodies for 1 h before adding CVB4 mixed with IgGs obtained from the plasma of healthy subjects (n = 3) or diabetic patients (n = 3). IFN-α levels in culture supernatants at 48 h p.i. were measured by DELFIA. Results are expressed as mean ± SD.

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<tr>
<th>IFN-α (IU/ml) produced by PBMCs infected with CVB4 + IgG from</th>
<th>Preincubation of PBMCs with</th>
<th>Medium</th>
<th>IgG1</th>
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<th>Anti-FcγRI antibody</th>
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A-affinity chromatography column. The eluted fractions containing IgG antibodies and the flow-through fraction (free from IgG1, -2 and -4) were preincubated at 20 µg/ml with CVB4 for 1 h at 37 °C before being added to PBMC cultures. As seen in Fig. 2(b), eluted fractions enhanced CVB4-induced IFN-α production, whereas flow-through fractions did not. The enhancing activity obtained with IgGs from patients was significantly higher than the one obtained with IgGs from controls (P < 0.001). In contrast, irrelevant human IgGs had no IFN-α-enhancing activity.

The enhancing effect of IgGs from controls and patients on CVB4-induced IFN-α production in PBMC cultures was suppressed significantly when PBMCs were preincubated with 2 µg/ml of either anti-human FcγRII IgG2a antibodies or anti-human FcγRIII IgG1 antibodies but not with anti-human FcγRI IgG1 antibodies (Table 1). Irrelevant control antibodies did not inhibit IgG-mediated enhancement of CVB4-induced IFN-α production. To examine the role of CAR on the efficiency of IgG-mediated IFN-α production, PBMCs were preincubated for 1 h with anti-human CAR antibodies before challenging with CVB4 preincubated with IgGs from healthy subjects or patients. IFN-α production was inhibited in the presence of anti-CAR antibodies [80 ± 12 (n = 3) and 93 ± 5% (n = 3) inhibition in experiments with IgGs from donors and patients, respectively], whereas control isotypes did not inhibit IgG-mediated enhancement of CVB4-induced IFN-α production (data not shown). When PBMCs were preincubated with anti-FcγRI, anti-FcγRII or anti-CAR antibodies before adding SV or HSV-1, the levels of IFN-α were not reduced (data not shown), which demonstrated that these antibodies did not give a negative signal to IFN-α-producing cells that blocked IFN-α synthesis.

Role of cytophilic IgGs in CVB4-induced production of IFN-α by PBMCs from patients with type 1 diabetes

We observed that PBMCs from patients with type 1 diabetes produced up to 512 IU/ml IFN-α in response to CVB4, whereas those from healthy subjects were low re-
As seen in Fig. 3(b), a significant IFN-α-inducing activity of CVB4 can be enhanced by IgGs, as shown in our experiments. We investigated whether antibodies bound to PBMCs from healthy subjects played a role in the IFN-α response by PBMCs to CVB4.

Preincubation of PBMCs from healthy subjects with IgGs, after washing and before adding CVB4, resulted in the production of IFN-α (Fig. 3a). These results suggested that CVB4-specific antibodies bound to the surface of cells. To dissociate and recover antibodies from PBMCs, the PBMCs were preincubated in the absence or presence of 20 μg/ml IgG for 1 h, then they were washed and mock-infected or infected with CVB4. IgGs were obtained from the plasma of healthy subjects by using protein A-affinity chromatography. The experiments were repeated with IgGs from 10 different donors. Mean ± SD are presented. (b) IFN-α levels in cultures of PBMCs preincubated with supernatants generated from heated PBMCs. PBMCs from healthy subjects were preincubated for 1 h in the absence or presence of cell-free supernatant of PBMCs from healthy subjects either treated or not with IgGs, heated for 30 min in a 56 °C water bath. Then, the cells were washed three times and mock-infected or infected with CVB4 at an M.o.i. of 1 for 90 min. Mean ± SD of four independent experiments are presented. (a, b) The IFN-α levels in culture supernatants at 48 h p.i. were measured by DELFIA.

Fig. 3. CVB4-induced production of IFN-α in cultures by PBMCs. (a) IFN-α levels in cultures of PBMCs preincubated with IgGs before adding CVB4. The cells were preincubated in the absence or presence of 20 μg/ml IgG for 1 h, then they were washed and mock-infected or infected with CVB4. IgGs were obtained from the plasma of healthy subjects by using protein A-affinity chromatography. The experiments were repeated with IgGs from 10 different donors. Mean ± SD are presented. (b) IFN-α levels in cultures of PBMCs preincubated with supernatants generated from heated PBMCs. PBMCs from healthy subjects were preincubated for 1 h in the absence or presence of cell-free supernatant of PBMCs from healthy subjects either treated or not with IgGs, heated for 30 min in a 56 °C water bath. Then, the cells were washed three times and mock-infected or infected with CVB4 for 1 h. Supernatants were generated from heated PBMCs from healthy subjects either treated or not with IgGs, heated for 30 min in a 56 °C water bath. These supernatants were incubated for 1 h with PBMCs from healthy subjects, then the cells were washed three times and mock-infected or infected with CVB4 at an M.o.i. of 1 for 90 min. IFN-α levels in culture supernatants at 48 h p.i. were measured by DELFIA. Mean ± SD of four independent experiments are presented. (a, b) The IFN-α levels in culture supernatants at 48 h p.i. were measured by DELFIA.

Role of FcγRII and CAR in the CVB4-induced production of IFN-α. PBMCs from healthy subjects were preincubated with supernatants from PBMCs infected with CVB4 for 1 h. PBMCs were isolated from blood samples of healthy subjects (n = 3); patients with type 1 diabetes whose PBMCs produced IFN-α in response to CVB4 (responsive, n = 3); patients with type 1 diabetes whose PBMCs did not produce IFN-α in response to CVB4 (nonresponsive, n = 3). Supernatants were generated from heated PBMCs from healthy subjects either treated or not with IgGs, heated for 30 min in a 56 °C water bath. Then, the cells were washed three times and mock-infected or infected with CVB4 for 1 h. Supernatants were generated from heated PBMCs from healthy subjects either treated or not with IgGs, heated for 30 min in a 56 °C water bath. These supernatants were incubated for 1 h with PBMCs from healthy subjects, then the cells were washed three times and mock-infected or infected with CVB4 at an M.o.i. of 1 for 90 min. IFN-α levels in culture supernatants at 48 h p.i. were measured by DELFIA. Mean ± SD of three independent experiments are presented. (b) Role of FcγRI and CAR in the CVB4-induced production of IFN-α. PBMCs from responsive (n = 3) and nonresponsive (n = 3) healthy subjects were preincubated with supernatants from PBMCs infected with CVB4 for 1 h. PBMCs were isolated from blood samples of healthy subjects (n = 3); patients with type 1 diabetes whose PBMCs produced IFN-α in response to CVB4 (responsive, n = 3); patients with type 1 diabetes whose PBMCs did not produce IFN-α in response to CVB4 (nonresponsive, n = 3). Supernatants were generated from heated PBMCs from healthy subjects either treated or not with IgGs, heated for 30 min in a 56 °C water bath. These supernatants were incubated for 1 h with PBMCs from healthy subjects, then the cells were washed three times and mock-infected or infected with CVB4 at an M.o.i. of 1 for 90 min. IFN-α levels in culture supernatants at 48 h p.i. were measured by DELFIA. Mean ± SD of three independent experiments are presented. (b) Role of FcγRI and CAR in the CVB4-induced production of IFN-α. PBMCs from healthy subjects (n = 3) and patients with type 1 diabetes (n = 3) were preincubated with supernatants from PBMCs infected with CVB4 for 1 h. PBMCs were isolated from blood samples of healthy subjects (n = 3); patients with type 1 diabetes whose PBMCs produced IFN-α in response to CVB4 (responsive, n = 3); patients with type 1 diabetes whose PBMCs did not produce IFN-α in response to CVB4 (nonresponsive, n = 3). Supernatants were generated from heated PBMCs from healthy subjects either treated or not with IgGs, heated for 30 min in a 56 °C water bath. These supernatants were incubated for 1 h with PBMCs from healthy subjects, then the cells were washed three times and mock-infected or infected with CVB4 at an M.o.i. of 1 for 90 min. IFN-α levels in culture supernatants at 48 h p.i. were measured by DELFIA. Mean ± SD of three independent experiments are presented.
supernatants of heated PBMCs from patients producing IFN-α in the presence of CVB4 (256, 64 and 64 IU/ml). Anti-FcγRI, anti-FcγRII and anti-CAR but not anti-FcγRIII inhibited the IFN-α-inducing activity of these supernatants (Fig. 4b).

**Relationship between CVB4-induced production of IFN-α by PBMCs and the level of anti-CVB antibody**

The production of IFN-α by PBMCs in response to CVB4 was compared with the levels of anti-CVB4 NAs in healthy subjects and patients with type 1 diabetes (Fig. 5). Higher levels of NAs were found in patients compared with controls. For each individual, the titre of antibody was plotted with regard to the level of IFN-α. There was no correlation between IFN-α induced by CVB4, or CVB4 preincubated with plasma, and the titres of NAs. In certain patients, the detection of NAs was negative or the titres were very low, whereas the levels of IFN-α were high; therefore, the levels of CVB antibodies were determined using another method. However, using ELISA, the levels of anti-CVB IgG antibodies were concordant with the CVB-neutralization test results and there was still no correlation between the level of antibodies detected by ELISA and the level of IFN-α (data not shown).

**Discussion**

The current study demonstrates that CVB4 can strongly induce the production of IFN-α by PBMCs from patients with type 1 diabetes in the absence or presence of plasma, whereas the levels of CVB4-induced synthesis of IFN-α by PBMCs from healthy controls are low or moderate in the same conditions.

There are several noteworthy considerations for the systems used in the study. The measurement of IFN-α-producing capacity by the whole blood method has been reported previously by us and other teams (Hober *et al.*, 1998; Uno *et al.*, 1996). The production of IFN-α per mononuclear cell was higher in whole blood than in PBMC cultures from healthy controls and patients with type 1 diabetes, although the cell number in whole blood cultures was about three times as low as that in PBMC cultures (data not shown). The discordant IFN-α levels in whole blood and PBMC cultures infected with CVB4, in particular the high values in whole blood cultures from patients, prompted us to study the role of plasma in CVB4-induced production of IFN-α.

The production of IFN-α by PBMCs from healthy controls and patients with type 1 diabetes, obtained with a mixture of CVB4 and plasma, depended on the antibodies contained in the plasma, as evidenced by: (1) the eluted fractions containing IgGs obtained from plasma passed over a protein A-Sepharose column; and (2) the suppression of the enhancing effect of virus/eluted fraction by anti-FcγRII and anti-FcγRIII antibodies. The activity of plasma and IgGs obtained in our experiments suggested that the level of IFN-α-enhancing antibodies was higher in patients with type 1 diabetes than in healthy subjects. It may be assumed that IgGs from diabetics formed immune complexes with CVB4, since the adsorption of CVB4 complexed to patients IgGs on C1q-coated microwell plates, according to the procedure described previously (Chehadeh *et al.*, 2001), inhibited the IFN-α-enhancing activity of IgGs (data not shown). Furthermore, our experiments with neutralizing anti-CAR antibodies suggested that CAR, a specific receptor for CVB4, in addition to FcγRI and FcγRIII, played a role in the induction of IFN-α by CVB4–antibody complexes in PBMCs from patients and healthy controls. This result showed that IFN-α production was not just a consequence of cell activation by binding of CVB4–IgG complexes but also depended on binding of CVB4 to a specific cell surface receptor. Our results agree with those of Palmer *et al.* (2000), suggesting that FcγRII plays a role in the poliovirus–antibody complex-induced production of IFN-α by PBMCs. However, in the present study, FcγRIII played a role, whereas in their experiments, it did not; the difference could be related to the virus used and/or to the nature of IFN-α-producing cells. Indeed, previous works from our laboratory showed that the major IFN-α-producing cells in response to CVB4–IgG complexes were CD14+ monocytes (Chehadeh *et al.*, 2001), whereas Palmer and colleagues suggested that poliovirus–antibody complexes...
activated the synthesis of IFN-α by natural IFN-α-producing cells characterized as type 2 dendritic cell precursor (Siegal et al., 1999; Palmer et al., 2000). In an extension of the present work we will attempt to determine whether monocytes are involved in the production of IFN-α observed in vivo and in vitro in response to CVB4 infection in individuals with type 1 diabetes.

We demonstrated that PBMCs armed in vitro with cytophilic antibodies produced IFN-α in the presence of CVB4. Moreover, we provide evidence of the presence of antibodies on the surface of freshly isolated PBMCs from patients producing IFN-α in response to CVB4. The role of antibodies was evidenced by the following experiments: (1) a 30 min incubation at 56 °C was used to recover cell surface-associated antibodies, as described previously (Tyler et al., 1989); (2) the supernatants generated in this manner contained significant levels of IFN-α-enhancing activity in combination with CVB4, as measured by IFN-α assays. A role for IgGs in CVB4-induced production of IFN-α in these experiments was indicated by the ability of monoclonal antibodies against FcγRI and FcγRII to significantly inhibit the production of IFN-α. The anti-FcγRII monoclonal antibody (clone 2E1) used in this study binds well to FcγRII-expressing cells but is marginally reactive with cells expressing FcγRIIb (Van de Winkel & Anderson, 1995). Thus, the effect of the anti-FcγRII monoclonal antibody in our experiments do not result from a possible activation of FcγRIIb, which negatively regulates intracellular signalling passing through immunoreceptor tyrosine-based activation motifs and their cytoplasmic ligands like FcγRI-mediated activation of IFN-α (Daeron, 1997). IFN-α production was inhibited by anti-FcγRII but not by anti-FcγRIII antibodies, suggesting that FcγRIII, which, like FcγRII, binds immune complexes, is not involved in IFN-α production nor is it expressed on the IFN-α-producing cells in experiments with PBMCs armed with cytophilic antibodies. The presence of both FcγRI (I and II) and CAR molecules on cells was required for CVB4-induced IFN-α synthesis by PBMCs armed with cytophilic antibodies; otherwise, blocking of FcγR or CAR independently could not inhibit IFN-α synthesis. The results of our experiments suggest that IgGs bound FcγRI and FcγRII, which is in agreement with the fact that these receptors bind monomeric IgGs. In contrast with FcγRI, FcγRII binds monomeric IgGs weakly but reacts mostly with IgG complexes (Sautès, 1997; Carayannopoulos & Capra, 1993). However, when isolated from blood, FcγR-bearing cells are saturated by serum IgGs. In our system, one possible explanation for the effect of antibodies preincubated with freshly isolated PBMCs already saturated by IgGs is the following: IgGs added to cells bind to cell surface molecules (glycosyltransferases and/or lectin-like molecules) (Carayannopoulos & Capra, 1993), then, when CVB4 is added, immune complexes formed compete with monomeric IgGs for receptors and displace bound monomeric IgGs. Indeed, conformational changes in the Fc region by virus–antibody complex formation facilitates interactions with the Fc receptor (Frey & Einsfelder, 1984); therefore, immune complexes are several times more effective than monomeric IgGs at competing for receptors.

The IFN-α response mediated by the supernatants of heated PBMCs from patients producing IFN-α in the presence of CVB4 was reduced compared with the responses of these PBMCs to CVB4 (13 ± 4 versus 128 ± 64 IU/ml). This may be due to the relatively low concentrations of enhancing antibodies in the preparations. Moreover, the effects of supernatants of heated PBMCs from patients can be underestimated. Indeed, they were tested with cells from healthy controls; whether these cells can bind IgGs as well as cells from patients is not known.

When PBMCs from patients with type 1 diabetes were exposed to CVB4 in the absence or presence of their own IgGs, the levels of IFN-α were higher than those obtained with cells and IgGs from healthy subjects. The high IFN-α response to CVB4 in diabetic patients was not related to age of onset of diabetes, duration of diabetes or metabolic decompensation (data not shown), which indicates intrinsic hyperresponsiveness to CVB4 in type 1 diabetes. It has been reported that there is an hyper-IFN-α responsiveness by PBMCs to IFN-α inducers like polyinosinic:polycytidilic acid [poly(I:C)] in type 1 diabetes (Toms et al., 1991); however, in the current work we found no difference between patients and healthy subjects in response to IFN-α inducers like HSV-1 and SV. The difference observed in our study of CVB4 response in patients and healthy subjects can be explained by a history of CVB infections responsible for the presence of higher levels of IFN-α-enhancing anti-CVB antibodies in patients than in healthy subjects. Nevertheless, there was no correlation between the titres of anti-CVB antibodies detected in patients by neutralization assay or by ELISA and the production of IFN-α by their PBMCs. These results are in agreement with our previous studies showing that IFN-α-enhancing antibodies directed towards CVB4 detected in healthy controls were different from NAs against that virus (Chehadeh et al., 2001) and a priori suggest that IFN-α-enhancing antibodies do not bind H antigen, which is incorporated into the immunoenzymatic assay used in our experiments for detecting anti-CVB antibodies. Together these data are consistent with the existence of anti-CVB antibodies in plasma from patients with type 1 diabetes not detected by methods usually available.

The preferential IFN-α response by PBMCs from patients with type 1 diabetes to CVB4 supports the hypothesis that CVB4 can play a role in the development of type 1 diabetes, although the mechanism has not been elucidated. Further studies are needed to know whether the observed IFN-α hyperactivity towards CVB4 is an event prior to (predisposing) or post clinical onset of type 1 diabetes.

In conclusion, the current studies demonstrate that CVB4 can strongly induce the in vitro production of IFN-α by PBMCs from patients with type 1 diabetes in comparison with healthy subjects. The IFN-α-inducing activity of CVB4 is strongly
enhanced by IgGs from patients with type 1 diabetes in comparison with IgGs from healthy subjects. Moreover, it has been shown that the CVB4-induced production of IFN-α by PBMCs from patients can result from the presence of IFN-α-enhancing-specific antibodies on the cell surface. Studies are needed to characterize the antibodies involved in the CVB4-induced production of IFN-α and to define their role in the pathogenesis of CVB4 infection. Future works will be directed along this line in our laboratory.

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