Human antibodies isolated from plasma by affinity chromatography increase the coxsackievirus B4-induced synthesis of interferon-α by human peripheral blood mononuclear cells in vitro

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Coxsackievirus B4 (CVB4) can be found in circulating blood of patients; however, the interaction of CVB4 with peripheral blood mononuclear cells (PBMCs) is poorly understood. CVB4 induced low levels of IFN-α synthesis in PBMCs from healthy donors. In contrast, preincubation of infectious CVB4 with plasma from these donors containing anti-CVB4 antibodies strongly enhanced the synthesis of IFN-α. IgG obtained from plasma by chromatography formed immune complexes with CVB4 and increased significantly the CVB4-induced production of IFN-α by PBMCs. These antibodies did not have a neutralizing effect on CVB4 infection of Hep-2 cells. The role of CVB and adenovirus receptor (CAR), FcγRII and FcγRIII in the increased synthesis of IFN-α induced by CVB4 preincubated with IgG was shown by inhibition with specific antibodies. The major interferon-α-producing cells in response to CVB4–IgG complexes were CD14+ cells and monocyte-enriched PBMCs. With the latter, detection of IFN-α by immunostaining was positive whereas in monocyte-depleted PBMCs it was not. This study shows that CVB4-induced synthesis of IFN-α by PBMCs can be enhanced by an antibody-dependent mechanism through interactions between the virus, non-neutralizing antivirus antibodies, FcγRII and III and CAR.

Introduction

Coxsackieviruses B1–B6 (CVB), members of the enterovirus group in the family Picornaviridae, are non-enveloped viruses with single-stranded plus-sense RNA contained in a capsid composed of 60 protomers, each consisting of one copy of the viral proteins VP1, VP2, VP3 and VP4 (Rueckert, 1996). A common receptor for CVB and adenoviruses, called CAR, has been identified (Bergelson et al., 1997; Tomko et al., 1997).

CVB are highly prevalent human pathogens associated with a variety of acute diseases (myocarditis, meningitis-encephalitis, Bornholm disease, herpangina…) (Melnick, 1996). Studies have indicated that CVB3 and CVB4 may have a causal role in chronic diseases such as chronic myocarditis and insulin-dependent type 1 diabetes (Kandolf et al., 1993; Graves et al., 1997).

Primary replication of enteroviruses takes place in tissues of the respiratory and gastrointestinal tracts. This may be followed by the occurrence of infectious virus in blood which leads to spread to secondary target organs (Melnick, 1996). A viraemia is often detected in hospitalized patients with enterovirus diseases (Prather et al., 1984). Increased levels of IFN-α in plasma have been associated with CVB infection, and IFN-α mRNA has been detected in the blood cells of individuals with enterovirus RNA, with high identity to CVB3 and CVB4 in most cases, in their blood (Dommergues et al., 1994; Chehadeh et al., 2000a).

A variety of viruses and virally infected cells are capable of inducing IFN-α production by human peripheral blood mononuclear cells (PBMCs) in short-term in vitro culture studies. Coxsackieviruses are frequently referred to as ‘weak’ IFN-α inducers compared with ‘strong’ IFN-α inducers like Sendai virus (SV) and herpes simplex virus type 1 (HSV-1) (Pitkaranta et al., 1988; Pitkaranta & Hovi, 1993; Feldman et al., 1994). In response to SV, the predominant IFN-α-producing cells have...
been reported to be monocytes (Saksela et al., 1984; Gobl et al., 1988), whereas in response to HSV-1, dendritic cell precursors produce IFN-α (Siegal et al., 1999). The mechanisms of CVB-induced synthesis of IFN-α have not been elucidated.

Infectious CVB4 and/or its components can be found in circulating blood of patients and consequently they may induce synthesis of IFN-α by IFN-α-producing blood cells. Recently, it has been reported that human polyclonal IgG enhances the in vitro IFN-α-inducing capacity of poliovirus, a member of the enterovirus group (Palmer et al., 2000). Humans are frequently exposed to sequential CVB challenges; therefore serotype and CVB group-specific antibodies can be found in the circulating blood of individuals meeting subsequent CVB infection (Frisk et al., 1989; Gauntt, 1997). In this study, the role of antibodies isolated from plasma of CVB4-seropositive subjects in the IFN-α-response of PBMCs to CVB4 has been investigated in vitro.

**Methods**

**Viruses.** CVB4 (JVB strain, provided by J. W. Almond, Aventis Pasteur, Marcy-L’Etoile, France) were grown in Hep-2 cells (Bio-Whittaker) in Eagle’s MEM (Gibco BRL) supplemented with 10% heat-inactivated foetal calf serum (Gibco BRL) and 1% l-glutamine (Eurobio). Supernatants were collected 3 days post-infection and then clarified at 1000 r.p.m. for 10 min. Virus titres were determined by plaque formation assaying for CVB4 on Hep-2 cells and aliquots of virus preparations were then stored at −80 °C.

SV was provided by D. Garcin (Department of Genetics and Microbiology, University of Geneva, Switzerland). The virus was cultivated as previously described (Garcin et al., 1995). Briefly, SV was propagated in eggs and purified by first clarifying chorioallantoic fluid containing SV by centrifugation for 10 min at 1500 g at 4 °C, and then pelleting the SV at 15 000 g for 16 h. Virus titres were assayed by plaque formation on HeLa cells, and the virus preparation was stored frozen at −80 °C until use.

HSV (laboratory strain) was cultivated in a Vero cell line (ATCC) in MEM supplemented with 5% FCS and 1% l-glutamine. After 48 h incubation at 37 °C in an atmosphere of 5% humidified CO2, supernatants were collected and clarified at 1000 r.p.m. for 10 min. HSV-1 titres were assayed by plaque formation on Vero cells and virus preparations were stored at −80 °C.

**Blood donors.** Blood samples were obtained from 20 healthy subjects with anti-coxsackievirus B antibodies detected by neutralization assay in their blood (titre 1/2 to 1/32): 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 no suspected immunological, infectious or metabolic disease.

Blood samples were also obtained from 6 children (4 males and 2 females; median age 6 months, range 6–12) with negative detection of anti-CVB antibodies by neutralization assay.

**IFN-α induction.** Venous blood was collected in sterile 7.5 ml tubes (Becton Dickinson) containing 20 IU/ml heparin (Heparin Choay, Sanofi). PBMCs were separated over a Ficoll-paque solution (Diatrizoate Ficoll, Eurobio) (Boyum, 1976). The mononuclear cells were collected, washed three times with RPMI 1640, adjusted to a concentration of 2 × 10⁸ 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 a m.o.i. of 1 and plasma or various preparations obtained by using affinity chromatography of plasma was added to wells, followed by incubation for 48 h at 37 °C in a 5% CO₂ atmosphere. Then, culture supernatant was harvested, cleared of cells 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.

**Cell separation.** (1) A monocyte isolation technique based on Optiprep density-gradient medium was described previously (Graziani-Bowering et al., 1997). Briefly, PBMCs were isolated from whole blood as described above. Four ml of Optiprep (Sigma) was mixed with 10 ml of PBMCs before overlaying with 7.5 ml of a 1:076 g/ml lymphocyte-specific density layer. The latter was overlaid with 20 ml of 1:068 g/ml solution and 0.5 ml of HEPES-buffered saline (Sigma). Centrifugation was carried out at 600 g for 25 min at room temperature. Fractions of the resulting phases were collected. The first fraction corresponded to mononuclear cells at the 1:068 g/ml layer; a second fraction included the lymphocyte-trapping layer at the interface of the 1:078 g/ml and 1:068 g/ml layers. Each fraction was washed once with PBS and twice 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. The cells at 1:068 g/ml layer were 90–95% CD14⁺ monocytes as determined using FITC monoclonal anti-CD14 conjugated antibody (data not shown).

(2) PBMCs were isolated from whole blood as described above, and washed with PBS (E. Merck) – 2% FCS. Specific immunomagnetic beads (Dynal Dynabeads M-450 CD14) were used for depletion of CD14⁺ cells from PBMCs, according to the manufacturer’s instructions. 20 × 10⁶ PBMC/ml were incubated with 20 × 10⁴ beads/ml for 60 min at 4 °C. The proportion of CD14⁺ cells in the CD14-negative population was < 5%. CD14⁺-positive and -negative populations were washed and resuspended at 2 × 10⁶ cells/ml in RPMI supplemented with 10% FCS and 1% l-glutamine.

**Immunooassay for IFN-α.** The concentration of IFN-α was determined by a specific and sensitive dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) based on the direct sandwich technique using a mixture of two murine monoclonal antibodies (MAbs) to human IFN-α, LT27:273 and LT27:293, which bind more than 90% of natural IFN-α subtypes coated onto microtiter plate wells (LKB Wallac), and a europium-labelled murine anti-human IFN-α, as previously described (Cederblad et al., 1998). Samples (100 µl) diluted with an equal volume of dilution buffer containing an irrelevant IgG1 mouse monoclonal (B1:2) at 50 µg/ml were then added to the plate. After 2 h, plates were washed three times, and 0.2 ml of the europium-conjugated antibody at 1:800 dilution was added per well in dilution buffer. After 1 h, the plates were washed six times and 0.2 ml of Enhancement solution (LKB Wallac) was added per well to promote dissociation of Eu³⁺ cations from the labelled antibody into solution, where they form fluorescent chelates with components of the Enhancement solution. After 20 min, the fluorescence in the wells was measured in a time-resolved fluorimeter (1230 Arcus fluorimeter, LKB Wallac). The National Institutes of Health leucocyte reference interferon G-23-902-530 was used as standard.

**Affinity chromatography.** (1) Protein A-Sepharose CL-4B. Plasma samples (2 ml) collected from five healthy subjects were diluted twofold with PBS at pH 7.0, filtered (0.45 µm) and passed at flow rate of 0.1 ml/min over a 1.6 cm × 20 cm column of Protein A-Sepharose CL-4B (Pharmacia), equilibrated with PBS buffer. The column was then washed extensively with the same buffer until the A280 of the effluent was zero, and eluted at a flow rate of 1 ml/min with elution buffer containing 50 mM glycine (Sigma) and 150 mM NaCl (E. Merck), pH 3.0. Fractions

containing the eluted immunoglobulins were pooled and concentrated on a Biomax-30 membrane in the Centricron Plus-20 system (Millipore). To preserve the activity of acid-labile IgG, the eluted fractions were neutralized by adding 50–100 µl of 1 M Tris–HCl, pH 9 (Sigma). IgG concentrations were estimated using an assumed extinction coefficient of 1.43 for a 1 mg/ml solution at 280 nm.

(2) CVB4-Sepharose CL-4B. Sepharose CL-4B was activated with divinylsulphone (DVS; Sigma) as described previously (Lihme et al., 1986). 2 × 10^9 p.f.u./ml CVB4 obtained by ultracentrifugation for 1 h at 500 000 g was resuspended in 25.5 g/l NaHCO₃ (40 ml) (E. Merck) and mixed with DVS-activated Sepharose CL-4B (20 ml) which had been washed with NaHCO₃. After gentle mixing for 16 h at room temperature, the free reaction sites were saturated by incubation for 3 h with glycine buffer, pH 9.0, containing 80.5 g/l glycine and 25.5 g/l NaHCO₃. The slurry was then washed five times with distilled water and packed into a 1.0 cm × 20 cm column in Tris–saline buffer, pH 7.5, containing 12.1 g/l Trizma base (Sigma) and 2.3-4 g/l NaCl. Pooled immunoglobulin fractions obtained by Protein A affinity chromatography were diluted in 5 ml of Tris–saline buffer and passed over the column at flow rate of 0.1 ml/min. The column was then washed with the same buffer and the flow-through fraction containing the unadsorbed antibodies was recovered. Elution of specific anti-CVB4 antibodies was performed at a flow rate of 1 ml/min with elution buffer at pH 3.0. The fractions were pooled, concentrated and neutralized as described above. IgG concentrations in each fraction were estimated using an assumed extinction coefficient of 1.43 for a 1 mg/ml solution at 280 nm.

■ Plaque neutralization assay. To assess the presence of anti-CVB4 neutralizing antibodies in plasma and affinity-chromatography fractions, twofold serial dilutions of each fraction in MEM supplemented with 2% FCS and 1% t-glutamine were incubated with 100 p.f.u. of CVB4 in 96-well microtitre plates for 2 h at 37 °C. Hep-2 cells in suspension were then added at approximately 1 × 10⁴ cells per well and the plates were re-incubated for 3 days at 37 °C in a humidified incubator with 5% CO₂. Results were expressed as the inverse final dilution (titre) of sample that totally inhibited the viral cytopathic effect.

■ Immune complexes detection by complement fixation test. Plasma or plasma fractions were serially diluted twofold in calcium–magnesium–Veronal buffer at pH 7.2 (bioMerieux) and incubated with 10² p.f.u./ml CVB4 for 1 h at 37 °C. Fifty µl of CVB4–antibody mixture and 25 µl of guinea pig complement at 1/48 (Dade Behring) were dispensed into a microtitration plate and incubated at 4 °C overnight. The indicator system consisted of 25 µl of antibody-coated red cells added to wells and incubated at 37 °C for 10–30 min. The indicator system was prepared by incubating 50% sheep red cells (bioMerieux) with rabbit anti-sheep haemolysin at 1/1600 (bioMerieux) for 30 min at 37 °C. Sedimented red cells forming a button indicate a positive reaction.

■ Immune complexes quantification. A Quidel Circulating Immune Complex (CIC) C1q EIA kit (Ingen, Rungis, France) was used, according to the manufacturer’s instructions, for quantitative detection of specific immune complexes (IC) formed in vitro by incubating either plasma or plasma fractions obtained by CVB4 affinity chromatography, with 10² p.f.u./ml CVB4 for 1 h at 37 °C. The concentration of IC present in the test specimen was determined by reference to a standard curve. Results were expressed as µg of heat-aggregated human gamma globulin equivalents per ml (µg Eq/ml). Values greater than 4 µg Eq/ml were considered positive for significant levels of IC. C1q-coated and control microwells from the kits were used for IC depletion experiments.

■ Immunofluorescence (IF). CVB4-infected PBMCs, CVB4/antibody-infected PBMCs and mock-infected PBMCs were carefully washed in RPMI at 8 h post-infection. The cells were then treated with trypsin–Versene (Eurobio) for 10 min at 37 °C, removed from the wells by pipetting and washed twice before 10⁴ cells were cytocentrifuged onto clean glass slides. The slides were air-dried and fixed in a solution of 4% paraformaldehyde (PFA; Sigma) for 20 min at 4 °C. The slides were washed twice in PBS and permeabilized with cold acetone–methanol (1:2, v/v) for 10 min at 20 °C. After washing twice in PBS, the slides were incubated with 5% normal rabbit serum for 1 h at room temperature in a humidified chamber, washed twice in PBS, and incubated for 1 h at room temperature with monoclonal antibodies to human IFN-α (LT27:273 and LT27:293) at 1 µg/µl. After three washes in PBS, the slides were stained with a 1:100 dilution of FITC-conjugated anti-mouse IgG antibodies (Sigma), counterstained with Evans blue (Sigma), and washed three times in PBS. The slides were then mounted with Permafluor (Coulter), and positive cells were counted in a fluorescence microscope (Leitz Diaplan).

■ RNA extraction. Native RNA was extracted from cells at 8 h post-infection by the acid guanidinium thiocyanate–phenol–chloroform extraction procedure using a commercial system (RNAgent Total RNA Isolation System; Promega). Extracted RNA was then dissolved in 50 µl of DEPC-treated water (Sigma) and used in the RT–PCR assays.

■ Oligonucleotide primers. Human IFN-α primers were purchased from Clontech: upstream primer, 5′ TAATGCGGAACTTCCCACAGGCTCAAAG-3′; downstream primer, 5′ ACAACCTCCAGCAACAGGCTG TATTTT 3′. The amplified PCR fragment size is 303 bp. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were synthesized by Eurogentec (Seraing, Belgium): upstream primer, 5′ GTCTTCAACCATGGAGAGATGTCAC 3′; downstream primer, 5′ CCAAAGTTGTCATGAGACC 3′. The amplified PCR fragment size is 206 bp.

■ One step RT–PCR for IFN-α RNA detection. cDNA synthesis and cDNA amplification were performed in a single tube using the Enhanced Avian RT–PCR Kit (Sigma) according to the manufacturer’s instructions. The procedure has been described in a previous report from us (Chehadeh et al., 2000b). One step RT–PCR was performed in a total volume of 50 µl using 1 µM of each primer and 0.1 µg of extracted RNA and the reaction was carried out using a Perkin Elmer Applied GeneAmp PCR System 2400.

For each RNA sample, GAPDH mRNA was retrotranscribed in cDNA, amplified by RT–PCR and used as a positive control to demonstrate the absence of RT–PCR inhibitors. A negative control (no RNA) was also included in each PCR. The absence of introns within IFN-α genes precludes the distinction between amplification products resulting from reverse-transcribed IFN-α mRNA and those arising from residual genomic DNA. Therefore, the absence of contaminating genomic DNA was verified for each RNA sample by carrying out the reverse transcriptase. Only samples without contaminating genomic DNA have been considered.

■ Detection of PCR products. The amplified RT–PCR products were analysed on 2% agarose gel containing 0.5 µg/ml ethidium bromide (Sigma) and visualized using the Gel Doc 2000 system (Bio-Rad). Image processing and analysis operations of DNA bands were performed using Quantity One software (Bio-Rad). A 100 bp DNA Ladder (Gibco BRL) was used as a molecular mass marker. The relative quantity of RT–PCR IFN-α and GAPDH products were compared by serial end-point dilution. The results were expressed as the absorbance ratio of IFN-α and GAPDH amplions.

■ Antibody-dependent assays. PBMCs or monocytes were distributed into 96-well tissue culture plates as described above. Virus
antibody complexes were made by incubation of plasma fractions at various dilutions with CVB4 for 1 h at 37 °C. CVB4 or CVB4–antibody complexes were added to cells at an m.o.i. of 1 before incubation for 2 h at 37 °C. The cells were then washed three times and cultured in RPMI 1640 containing 10% FCS (48 h at 37 °C, 5% CO₂ atmosphere. After incubation, culture supernatants were harvested, cleared and stored until assayed for the presence of IFN-α.

■ Antibodies. Monoclonal IgG1 neutralizing anti-human FcγRI (CD64), clone 10.1 (Biotest, Buc, France), monoclonal IgG2a neutralizing anti-human FcγRII (CD32), clone 2E1 and monoclonal IgG1 neutralizing anti-human FcγRIII (CD16), clone 3G8 antibodies were purchased from Coulter. Monoclonal IgG1 anti-human CAR (coxsackievirus and adeno-virus receptor) antibody (RmcB) was kindly provided by J. Bergelson (Division of Infectious Disease, Children’s Hospital of Philadelphia, Philadelphia, PA, USA). Anti-D human monoclonal IgG1 and IgG3 immune sera were provided by ‘Etablissement de Transfusion Sanguine’ (Lille, France) and used as irrelevant human IgG antibodies. Murine IgG1 and IgG2a (Coulter) were used as control antibodies.

■ Statistical analysis. Data are summarized as means ± SD. The significance of differences in IFN-α levels was determined by the Mann–Whitney U-test.

Results

Plasma enhances CVB4-induced synthesis of IFN-α

In preliminary experiments, high levels of CVB4-induced IFN-α were produced by PBMCs when virus was preincubated with plasma of healthy subjects at different dilutions (1:10⁴ to 1:10⁵) for 1 h and then added to PBMC cultures. The optimal dilution of plasma differed between subjects and ranged from 1:10⁴ to 1:10⁵ (data not shown). The mean levels of CVB4-induced IFN-α in culture supernatants of PBMCs incubated with plasma at a dilution of 1:10⁵ were significantly higher than those in absence of plasma (CVB4: 135 ± 80 IU/ml vs 3 ± 3 IU/ml, P < 0.0001, n = 20) (Fig. 1a). FITC staining of IFN-α in CVB4-stimulated PBMCs showed no IFN-α-positive cells, whereas in (CVB4 + plasma)-stimulated cells, 5 ± 1% of cells were IFN-α-positive at 8 h post-infection. However, IFN-α mRNA was detected by RT–PCR in extracts of CVB4- and (CVB4 + plasma)-stimulated PBMC cultures at 8 h post-infection, but the absorbance ratio for IFN-α amplicons and GAPDH amplicons obtained from (CVB4 + plasma)-stimulated PBMC cultures was much higher than that from CVB4-stimulated PBMC cultures (9 ± 3 vs 0.3 ± 0.1). In contrast, IFN-α mRNA was not detected in extracts of control PBMCs (data not shown).

IgG isolated from plasma enhance CVB4-induced synthesis of IFN-α

Plasma samples from five healthy subjects were passed over a Protein A–Sepharose CL-4B column. The resulting eluted fractions containing the IgG antibodies (IgG1, IgG2 and IgG4) and the flow-through fractions (IgG1, -2, -4-free plasma) were preincubated with CVB4 for 1 h at 37 °C before being added to PBMC cultures. High levels of IFN-α were obtained in the presence of eluted fractions (20 µg/ml IgG), whereas no IFN-α production was detected in the presence of flow-through fractions (Fig. 1b).

The enhancing effect of eluted fractions 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 (Fig. 2a). Irrelevant control antibodies did not inhibit the IgG-mediated enhancement of CVB4-induced IFN-α production. When PBMCs were preincubated with anti-FcγRI, anti-FcγRII or anti-FcγRIII antibodies before adding SV or HSV1, the levels of IFN-α were not reduced (data not shown).

IgG fractions isolated from healthy subjects with anti-CVB neutralizing antibodies had an enhancing activity on CVB4-induced IFN-α production at high dilutions (1:10⁴–1:10³) but not at lower dilutions (1:2–1:10). On the contrary, human plasma obtained from six children (age range 6–12 months),
CVB4-antibody complexes induce IFN-α production

CVB4-antibody complexes induce IFN-α production

Fig. 2. Role of IgG in CVB4-induced IFN-α production by PBMC. (a) Role of FcγRI and FcγRIII in IFN-α production by PBMCs exposed to CVB4 preincubated with eluted fractions (IgG) obtained from plasma passed over a Protein A chromatography column. PBMCs from three subjects were incubated for 1 h with anti-FcγRI, -II or -III neutralizing antibodies or control antibodies before adding virus preincubated with IgG. Culture supernatants were harvested 48 h post-infection. (b) Role of specific IgG in IFN-α production by PBMCs exposed to CVB4 preincubated with plasma IgG obtained from individuals with anti-CVB neutralizing antibodies (CVB-immune individuals) and plasma IgG obtained from individuals without anti-CVB neutralizing antibodies (CVB-non immune individuals). Culture supernatants were harvested 48 h post-infection.

which did not contain anti-CVB neutralizing antibodies directed against the six serotypes of CVB (CVB1–6), showed no enhancing activity on synthesis of IFN-α (Fig. 2b). The role of specific anti-CVB IgG antibodies in IFN-α induction was also suggested when no IFN-α production could be detected in supernatants of PBMC cultures stimulated with CVB4 that were preincubated with irrelevant human IgG at different concentrations (data not shown).

**Antibodies without neutralizing activity bind CVB4 and enhance CVB4-induced IFN-α production**

To isolate specific anti-CVB IgG antibodies from plasma of healthy subjects, IgG eluted fractions obtained by Protein A affinity chromatography were passed over a DVS-activated Sepharose CL-4B column coupled to CVB4. Adsorbed IgG antibodies were eluted at pH 3.0. From a typical preparation, IgG concentrations in the flow-through and eluted fractions were 6 and 0.08 mg respectively. By using a plaque neutralization assay, we detected high titres of anti-CVB4 neutralizing antibodies in eluted fractions but not in flow-through fractions (Fig. 3a). However, by using the complement fixation test (CFT), CVB4–antibody complexes were detected, as a result of the binding of complement, when CVB4 was preincubated with flow-through or eluted fractions (Fig. 3b). CVB4–IgG complexes were also able to bind to C1q coated onto microwells, allowing quantification of IC (Fig. 3b). The IC concentrations were IgG-dose dependent (data not shown).

Preincubation of CVB4 with eluted fractions containing anti-CVB4 neutralizing antibodies at different concentrations before adding to PBMC cultures resulted in no detection of IFN-α production, whereas high levels of IFN-α production by PBMCs were detected when CVB4 was preincubated with flow-through fractions at 20 µg/ml (Table 1). IFN-α concentrations were higher than those obtained when CVB4 was preincubated with IgG obtained from plasma by using Protein A chromatography at the same IgG concentration (20 µg/ml).
Table 1. Enhancing effect of antibodies on CVB4-induced IFN-α production by PBMCs

IFN-α levels in PBMC cultures infected with CVB4 preincubated in the absence or presence of different preparations containing IgG: plasma (1/100 dilution), flow-through and eluted fractions of Protein A chromatography of plasma; flow-through and eluted fractions of CVB4 chromatography of plasma IgG obtained by using Protein A chromatography. For each experiment, plasma and PBMCs were obtained from the same donor. Final IgG concentration for each fraction was 20 µg/ml. Experiments were repeated with five different donors. The mean ± SD is presented.

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<th>Protein A chromatography</th>
<th>CVB4 chromatography</th>
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<td></td>
<td>Medium</td>
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<td>IFN-α (IU/ml)</td>
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Fig. 4. Role of CAR in production of IFN-α by PBMCs infected with CVB4 preincubated with enhancing IgG. PBMCs were incubated for 1 h with anti-CAR neutralizing antibodies or control antibodies before adding CVB4 preincubated with enhancing IgG obtained from CVB4-chromatography of plasma IgG. Culture supernatants were harvested 48 h post-infection. Means ± SD of five independent experiments are presented.

Next, CVB4/enhancing IgG mixtures were put into C1q-coated microwells to remove immune complexes (IC) before being added to PBMCs. The IC concentrations in CVB4/IgG mixtures after incubation in control or C1q-coated microwells were 37 ± 5 µgEq/ml and 2 ± 1 µgEq/ml respectively (n = 5). The IC depletion resulted in a decrease of IFN-α levels in PBMC cultures from 166 ± 65 IU/ml to 23 ± 12 IU/ml (n = 5) (data not shown).

CAR plays a role in antibody-mediated enhancement of CVB4-induced IFN-α production

To examine the effect of CAR on the efficiency of IgG-mediated IFN-α production, PBMCs were preincubated for 1 h with anti-human CAR antibodies or control antibodies before challenging with CVB4–IgG complexes. As shown in Fig. 4, IFN-α production was significantly decreased in the presence of anti-CAR antibodies, whereas control antibodies did not inhibit the IgG-mediated enhancement of CVB4-induced IFN-α production. When PBMCs were preincubated with anti-CAR antibodies before adding SV or HSV-1, the levels of IFN-α were not reduced (data not shown).

Monocytes are involved in antibody-mediated enhancement of CVB4-induced IFN-α production

To identify which cells were responsible for IgG enhancement of CVB4-induced IFN-α production, a monocyte-enriched PBMC population was segregated from a monocyte-depleted PBMC population as described in Methods. CVB4 preincubated with enhancing antibodies induced high levels of IFN-α production in monocyte-enriched cultures, whereas no IFN-α production was detected in monocyte-depleted cultures (Fig. 5a). A similar pattern of results was obtained with SV. In contrast, with HSV-1, IFN-α levels were low in supernatants of monocyte-enriched cultures and high in supernatants of monocyte-depleted cultures. Then, CD14+ and CD14− cells were selected from PBMCs by using immunomagnetic beads before being stimulated with CVB4 preincubated with enhancing IgG. High IFN-α levels were detected in CD14+ cell cultures, whereas IFN-α was not detected in CD14− cell cultures (Fig. 5b). Monocyte-enriched cultures and monocyte-depleted cultures were stained for IFN-α in an IF test. When monocyte-enriched cultures were infected with CVB4, the proportion of IFN-α-positive cells was < 0.1%. Preincubation of CVB4 with enhancing IgG (20 µg/ml IgG) increased the proportion of IFN-α-positive cells to 10%. In contrast, there were no IFN-α-positive cells when monocyte-depleted cultures were infected with CVB4 preincubated in the absence or presence of antibodies (Fig. 5c).

Discussion

The current study demonstrates that in the presence of plasma, CVB4 can strongly induce the synthesis of IFN-α, as demonstrated by high levels of mRNA and IFN-α protein. IFN-
CVB4-antibody complexes induce IFN-α production

Fig. 5. Role of monocytes in antibody-dependent enhancement of CVB4-induced IFN-α production by PBMCs. (a) IFN-α levels in monocyte-enriched and monocyte-depleted PBMCs infected with CVB4 (m.o.i. 1) preincubated with enhancing IgG, SV or HSV-1 (m.o.i.1). Culture supernatants were harvested at 48 h post-infection. Monocytes were isolated by using a density gradient method. (b) IFN-α levels in CD14+ cell-enriched cultures obtained by using immunomagnetic specific anti-CD14 beads and CD14+ cell depleted cultures. The cells were infected with CVB4 preincubated with enhancing IgG. (c) IFN-α immunostaining at 8 h post-infection of monocyte-enriched and monocyte-depleted cultures infected with CVB4 or CVB4 preincubated with enhancing IgG. Results, expressed as a percentage of IFN-α-positive cells, are means ± SD of three independent experiments.

x mRNA was detected in extracts of CVB- and (CVB—IgG)-stimulated PBMCs, whereas IFN-α protein was detected only in (CVB—IgG)-infected cells; in contrast, in mock-infected cells, IFN-α mRNA and immunoreactive IFN-α were not detected. IFN-α may be released and consumed locally at the site where the reaction occurred, which may explain, under the limits of our assay, the negative or low level of IFN-α in the supernatant of CVB4-treated PBMCs.

IgG contained in plasma was responsible for the enhancement of CVB4-induced synthesis of IFN-α as demonstrated by the role of an eluted fraction containing IgG obtained from plasma passed over a Protein A-Sepharose column. It can be assumed that IgG played a role in the form of immune complexes with CVB4 for the following reasons. (1) The enhancing effect of virus/eluted fraction mixtures was suppressed by anti-FcγRII and FcγRIII antibodies, which bind IgG effectively only in the form of immune complexes, but not by anti-FcγRI antibodies, which bind Fcγ monovally with high affinity (Gessner et al., 1998). (2) Immune complexes formed between enhancing IgG antibodies and CVB4 were able to bind complement and protect sheep red cells from lysis. The adsorption of immune complexes on C1q-coated micro-well plates, blocked the enhancing activity of IgG, provided further evidence of the role of specific immune complexes in the enhancement process. (3) IgG fractions isolated from subjects with anti-CVB4 antibodies, but not those obtained from individuals without anti-CVB4 antibodies, enhanced CVB4-induced IFN-α synthesis. Insofar as the Protein A–Sepharose CL-4B column used to isolate the IgG fraction from human plasma binds only IgG1, IgG2 and IgG4, and that FcγRII and FcγRIII bind IgG1 and IgG3 in the form of immune complexes (Gessner et al., 1998), it can be suggested that virion–IgG1 complexes were involved in the enhancement process.

Neutralizing anti-CVB4 antibodies were contained in eluted fractions, whereas enhancing antibodies were recovered in flow-through fractions of affinity chromatography with DVS-activated Sepharose CL-4B coupled to CVB4. We cannot exclude the possibility that enhancing antibodies were not detected in the eluted fraction because of the presence of high levels of neutralizing antibodies. However, these results suggest that IgG involved in the enhancement of CVB4-induced IFN-α synthesis and in the neutralization of CVB4 may be different; otherwise, both types of antibodies would have been recovered in the eluted fraction. The attachment of CVB4 to activated Sepharose may result in conformational changes in the virus structure which prevented binding of enhancing antibodies. It can be explained by previous reports suggesting that portions of enterovirus capsid proteins can be reversibly exposed to the surface (Roivainen et al., 1993). Together, our data suggest that neutralizing and enhancing antibodies recognize different epitopes at the virus surface. Natural infections of humans by a CVB serotype can induce production of both serotype- and CVB group-specific antibodies (Pattison, 1983; Frisk et al., 1989), which can explain the fact that eluted anti-CVB4 neutralizing antibodies could also neutralize, to a lesser degree, CVB3 (data not shown). We cannot exclude the
possibility that the enhancing activity seen in the flow-through fraction is due to the presence of anti-CVB enhancing antibodies which cross-react with different CVB serotypes. This is in line with studies showing that specific enhancing antisera to purified haemagglutinin or neuraminidase of influenza A viruses cross-react with different influenza A subtypes (Tamura et al., 1991). Further investigations are needed to explore these hypotheses.

The presence of both low-affinity FcγR and CAR molecules on the target cells was required for IgG-dependent enhancement of CVB4-induced IFN-α synthesis; otherwise, blocking of FcγRII or CAR independently could not inhibit IFN-α synthesis. It has been reported that FcγRIII colocalizes with decay-accelerating factor (DAF, CD55), which is a receptor for CVB3 (Bhatia et al., 1998). Whether FcγRII, FcγRIII and CAR colocalize at the surface of IFN-α-producing cells remains to be determined. The strong reduction of IFN-α levels in culture obtained by preincubating PBMCs with anti-FcγRI or anti-FcγRIII antibodies or anti-CAR antibodies in IgG-enhanced IFN-α synthesis suggests a role of interactions between CVB4–IgG complexes, FcγR and CAR which result in induction of IFN-α. It cannot be ruled out that CVB4–IgG complexes act as cross-linking agents on FcγRII, FcγRIII and CAR altogether to trigger a combined signal for IFN-α. Further studies are needed to clarify whether these proteins act together at the same time or sequentially. Our results are similar to those reported recently by Palmer et al. (2000) showing that FcγRII plays a role in the induction of IFN-α in PBMCs by poliovirus–antibody complexes (Palmer et al., 2000). However, there are some differences with the present study since in their experiments FcγRIII did not play a role. These differences could be related either to the techniques and/or to the virus used.

Production of IFN-α by monocytes and dendritic cell precursors in response to in vitro stimulation with SV and HSV-1 has been well characterized (Saksela et al., 1984; Gobl et al., 1988; Siegal et al., 1999). Therefore, the pattern of results obtained with CVB4 compared with SV and HSV-1 in our experiments, together with the detection of IFN-α-positive cells by IF staining in monocyte-enriched cultures and not in monocyte-depleted cultures and the production of IFN-α by CD14⁺ cells, suggest that the major interferon-producing cells in response to CVB4–IgG complexes were CD14⁺ monocytes.

The mechanism of CVB4/IgG-induced IFN-α synthesis in PBMCs is unknown. It has been suggested that the induction of IFN-α by poliovirus–antibody complexes depends on CD32-mediated phagocytosis of RNA-containing particles (Palmer et al., 2000). Further investigations are necessary to determine whether the synthesis of IFN-α in our experiments reflects an enhanced entry of CVB genome into monocytes through an antibody-dependent mechanism.

These data show that antibodies devoid of neutralizing activity can play a role in the interaction between CVB and PBMC by enhancing the synthesis of IFN-α, which may increase the innate antiviral defence of the host against CVB. Whether such an effect of antibodies also operates in vivo is still unclear. Studies are in progress in our laboratory to investigate whether antibodies can be involved in the previously reported production of IFN-α in individuals with CVB RNA in their peripheral blood (Chehadeh et al., 2000a).

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