

Inhibition of Diabetes in NOD Mice by Human Pregnancy Factor

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ABSTRACT: Clinical symptoms of Th1 mediated autoimmune diseases regress in many patients during pregnancy. A prominent feature of pregnancy is the presence of human chorionic gonadotrophin hormone (hCG) in blood and urine. In this report we tested the effect of clinical grade hCG (c-hCG) on the development of diabetes, a Th1 mediated autoimmune disease, in nonobese diabetic (NOD) mice. We show that treatment of NOD mice with c-hCG before the onset of clinical symptoms lowered the increased blood glucose levels, reversed the established inflammatory infiltrate of pancreatic tissue, and profoundly inhibited the development of diabetes for prolonged time. c-hCG also induced profound inhibition of the functional activity (i.e. production of IFN- γ) of Th1 cells. Transfer of spleen cells from c-hCG-treated NOD

mice into immunocompromised NOD.SCID mice inhibited the development of diabetes in these otherwise non-treated mice. This shows that the treatment of the donor NOD mice induced persistent changes in the immune system. The antidiabetic activity of c-hCG was not caused by heterodimeric hCG or its subunits. Instead, this antidiabetic activity resided in a fraction of c-hCG preparation that contains a 400–2000 Dalton natural (immuno)modulatory pregnancy factor (NMPF). *Human Immunology* 62, 1315–1323 (2001). © American Society for Histocompatibility and Immunogenetics, 2001. Published by Elsevier Science Inc.

KEYWORDS: pregnancy/urine; diabetes; immunomodulation; gonadotrophins chorionic; Th1/Th2 cells

ABBREVIATIONS

NMPF natural (immuno)modulatory pregnancy factor
hCG human chorionic gonadotrophin
IFN- γ interferon- γ

IL-4 interleukin-4
TGF- β tumor growth factor- β
HPLC high performance liquid chromatography

INTRODUCTION

T-cell-mediated autoimmune diseases belong to the commonest chronic diseases in industrialized countries and increase worldwide. It imposes an enormous burden on Western economies. These diseases, including type I diabetes, Graves' disease, rheumatoid arthritis (RA), and multiple sclerosis (MS), generally have a dominant Th1 immune response in common [1, 2]. An adjustment of the innate and adaptive immune system during pregnancy, characterized amongst others by a Th2 cytokine profile, could maintain normal immune competence against microorganisms and also account for the clinical

improvement of Th1 autoimmune disease [3–6]. A better understanding of the immune modulation during pregnancy, and the identification of the natural factor(s) responsible for this, could help to develop new strategies for the prevention and treatment of Th1 autoimmune diseases with less detrimental effect to the treated individual than currently used broad range immunosuppressive drugs. Since hormonal changes in pregnant women precede and accompany immune changes, we hypothesized that human chorionic gonadotrophin (hCG), an early pregnancy hormone, might account for the regression of Th1 mediated autoimmune diseases during pregnancy. Therefore, we tested a commercial clinical grade hCG (c-hCG) preparation derived from first trimester pregnancy urine on the development of diabetes in the NOD mouse model [7]. Female NOD mice spontaneously develop type I diabetes with remarkable similarity to human type I diabetes (insulin-dependent diabetes

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mellitus [IDDM]) [8, 9]. The main clinical feature of this model is the elevated blood glucose level, which is caused by Th1 cell-mediated autoimmune destruction of insulin-producing β cells in the islets of Langerhans of the pancreas [10]. The disease severity in NOD mice is correlated with high levels of interferon-gamma (IFN- γ), a signature cytokine for Th1 cells.

MATERIAL AND METHODS

NOD Mice and BALB/c Mice

All mice used in these studies were maintained in a pathogen-free facility at the Department of Immunology, Erasmus University Rotterdam, The Netherlands. NOD and NOD.SCID mice were bred in our facility. Female BALB/c mice were purchased from Harlan (Horst, The Netherlands) and were also maintained in a pathogen-free facility at the Department of Immunology. All mice were given free access to food and water. The experiments were approved by the Animal Experiments Committee of the Erasmus University Rotterdam.

Diabetes

Diabetes was assessed by measurement of the venous blood glucose level using an Abbott Medisense Precision glucometer. Mice were considered diabetic after two consecutive glucose measurements ≥ 11 mmol/l (200 mg/dl). Onset of diabetes was dated from the first consecutive reading.

In vivo Treatment

Fourteen-week-old female NOD mice ($n=12$) were treated with c-hCG (Pregnyl Organon, Oss, The Netherlands). Treatment was done by injecting 300 IU c-hCG diluted in PBS three times per week intraperitoneally (i.p.). Control mice were treated with PBS only. The treatment was discontinued when all PBS treated NOD mice in the experiment, all at 19 weeks of age, had developed diabetes. This implies that the c-hCG and PBS treatments were done for five weeks. In instances of sustained hyperglycaemia of 20 mmol/l, the mice were killed to avoid prolonged discomfort. The remainder mice were kept alive up to the age of 35 weeks without any further treatment. For Th polarization assessment, mice ($n=6$) were treated i.p. with 300 IU c-hCG, 10 μ g lyophilized material obtained from first trimester pregnancy urine (u-hCG) or their fractions for 4 days. The u-hCG was prepared according to the procedures described below. Control mice were treated with PBS only. Spleen cells were pooled from each group and were used for CD4⁺ T-cells preparation (see below) or were stimulated with anti-CD3 and IL-2. RPMI 1640 medium was used supplemented with 25 mM HEPES, 100 IU/ml penicillin, 50 μ g/ml streptomycin, 1 mM pyruvate, 50

μ M 2-ME, and 10% heat inactivated FCS. All cultures were performed in triplicate or quadruplicate.

Glucose Tolerance Test

Glucose tolerance test was performed at 17 (during treatment, $n=6$) and 35 weeks of age (after treatment, $n=6$) by injecting 0.5 g/kg D-glucose intravenously (i.v.). At 3, 5, 10, and 15 min, blood samples were collected from the tail and tested for glucose content.

Immunohistochemistry

At the age of 21 weeks, 6 NOD females from the two experimental groups (PBS vs c-hCG) were killed. For detailed immunohistochemistry, pancreases were removed, embedded in Tissue-tek, and frozen in liquid nitrogen. Tissues were stored at -80°C until immunohistochemistry was performed. Before sectioning, microscopic slides were coated with 0.1% gelatin/0.01% chromium-alum. Thereafter, at least ten 5- μ m cryosections at four noncontiguous levels (around 50 μ m apart) of the stored tissue specimens were cut, dried in air overnight, and fixed with the appropriate fixatives depending on the monoclonal or polyclonal antibody used [11].

Immunohistochemistry was performed essentially as described previously [11]. Pancreas cryostat sections were prepared, coded and fixed for 2 min in 2% pararosaniline. After a wash with phosphate buffered saline with 0.1% Tween-20 (Merck-Schuchardt, Hohenbrunn bei Munchen, Germany) (PBS/Tween), slides were incubated with first step antibodies for 30 min at room temperature. The antibodies used were RA3.6B2 for B cells, KT3 for T cells, MOMA-1 and BM8⁺ for macrophages, and ER-MP23⁺ for dendritic-like cells. Anti-insulin (DAKO, Glostrup, Denmark) was used diluted 1:100 in PBS/Tween. All other monoclonal antibodies used in this study were supernatants from hybridoma cultured at our department and used undiluted. Subsequently, slides were washed with PBS/Tween and incubated with peroxidase-conjugated rabbit- α -guinea pig-Ig (to detect insulin) or rabbit- α -rat-Ig (to detect all others) second-step antibodies in the presence of 2% normal mouse serum for 30 min at room temperature. After an additional wash with PBS/Tween, slides were incubated with 0.05% (w/v) Ni-di-amino-benzidine (Ni-DAB) with 0.02% H₂O₂ and washed in water after 3 min. Finally, slides were counterstained for 3 min in nuclear fast red, dehydrated in a graded ethanol series, and mounted. For each staining run, one slide was stained with a second antibody only as a control for endogenous peroxidase activity and nonspecific binding of the second step, and a section of spleen was included as positive control.

Spleen Cell Transfer

Spleens of PBS and c-hCG treated mice were removed under aseptic conditions and single-cell suspensions were made. Erythrocytes were removed by incubating with Gey's medium for 5 min on melting ice. Isolated pooled spleen cells (20×10^6 in 0.3 ml PBS) from c-hCG treated mice ($n=6$) and control mice ($n=6$) were injected i.v. into the tail vein of 8-week-old female NOD.SCID mice ($n=6$). In other experiments, spleen cells were recovered from diabetic female NOD mice and stimulated *in vitro* with IL-2 (50 U/ml) along with 300 IU/ml c-hCG (Pregnyl, Organon; Profasi, Serono; APL, Wyeth Ayerst) or 10 $\mu\text{g/ml}$ from HPLC purified fractions of c-hCG or hCG directly purified from pregnancy urine (u-hCG, see below), recombinant hCG (10 $\mu\text{g/ml}$) (r-hCG, Sigma, St. Louis, MO, USA), its subunits (r- α -hCG or r- β -hCG), or purified hCG (from pregnancy urine; 10 $\mu\text{g/ml}$). After 48 h of culture, cells were collected and evaluated for their viability by trypan blue staining. Cells were washed twice with PBS and 20×10^6 cells were i.v. transferred into 8-week-old female NOD.SCID mice.

In Vitro Stimulation of Splenocytes

Splenic cell suspensions (2×10^5 cells/well) were cultured in 96-well flat-bottom plates (0.2 ml) and stimulated with plate bound anti-CD3 (145-2C11, 25 $\mu\text{g/ml}$) and IL-2 (50 U/ml) along with c-hCG or u-hCG or fractions derived from it. After incubation for 48 hrs, supernatants were collected for cytokine analyses. RPMI 1640 medium was used supplemented with 25 mM HEPES, 100 IU/ml penicillin, 50 $\mu\text{g/ml}$ streptomycin, 1 mM pyruvate, 50 μM 2-ME, and 10% heat inactivated FCS. All cultures were performed in triplicate or quadruplicate.

Preparation and Stimulation of Purified CD4⁺ T Cells

Purified CD4⁺ T cells from the spleen were obtained by complement depletion with antibodies to heat stable antigen (HSA), CD16/32, MHC class II (BALB/c, M5/114; NOD, M10/216) and GR-1. Cells were further purified using magnetic activated cell sorting with a cocktail of biotinylated mAbs against CD11b (M1/70), B220 (RA3 6B2), CD8 (YTS-169), and CD40 (FGK-45.5), followed by incubation with streptavidin-conjugated microbeads (Milteny Biotech, Bergisch Gladbach, Germany). CD4⁺ T-cells used for experiments were always 90–95% purified as determined by flow cytometry.

Th Polarization Assay

For Th polarization assay, primary stimulation of purified CD4⁺ cells was done by culturing 1×10^5 cells/well in 96-well flat-bottom plates (Nalge Nunc Int.,

Naperville, IL., USA). The cells were stimulated with plate-bound anti-CD3 mAb (145-2C11; 25 $\mu\text{g/ml}$) in the presence of soluble anti-CD28 mAb (37-51; 10 $\mu\text{g/ml}$) and IL-2 (50 U/ml). For differentiation of Th1 cells (Th1 polarizing condition), anti-IL-4 mAb (11B11; 10 $\mu\text{g/ml}$) and IL-12 (10 ng/ml) were added to the cultures. For differentiation of Th2 cells (Th2 polarizing condition), IL-4 (35 ng/ml) and anti-IFN- γ mAb (XMG 1.2, 5 $\mu\text{g/ml}$) were employed. Neutral (nonpolarized) condition cultures contained only anti-CD3, anti-CD28, and IL-2. All doses were optimized in preliminary experiments. After 4 days of culture, the cells were washed three times and transferred to new anti-CD3-coated 96-well plates and restimulated in the presence of IL-2 (50 U/ml) and anti-CD28 (10 $\mu\text{g/ml}$). All cultures were performed in triplicate or quadruplicate. Forty-eight hours later, supernatants were collected and assayed for IL-4, IFN- γ , IL-10, and (total) TGF- β production by ELISA as a read-out for Th1 versus Th2 polarization.

Cytokine ELISA

Flat bottom microplates (96-wells, Falcon 3912, Microtest II Flexible Assay Plate, Becton Dickinson, Oxnard, CA, USA) were coated with capture antibody diluted in PBS (SXC-1; 1 $\mu\text{g/ml}$, 11B11 and XMG 1.2; 5 $\mu\text{g/ml}$) at 4°C for 18 h. After coating, plates were washed (PBS, 0.1% BSA, 0.05% Tween-20) and blocked with PBS supplemented with 1% BSA at room temperature for 1 h. After washing, samples and standards were added and incubation was continued for at least 4 h at room temperature. Thereafter, plates were washed and biotinylated detection antibodies were added (2A5.1 for IL-10 and BVD624G for IL-4, 0.1 $\mu\text{g/ml}$; R46A2 for IFN- γ , 1 $\mu\text{g/ml}$) and incubated overnight at 4°C. After washing, streptavidin-peroxidase (1/1500 diluted, Jackson ImmunoResearch, West Grove, PA, USA) was added. After 1 h, plates were washed and the reaction was visualized using 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS, 1 mg/ml, Sigma, St. Louis, MO, USA). Optical density was measured at 414 nm, using a Titertek Multiscan (Flow Labs, Redwood City, CA, USA). The amount of TGF- β was measured with commercially available ELISA kit (Genzyme Corp, Cambridge, MA, USA) according to the protocol provided by the manufacturer. The detection limits of the various ELISA were: IL-4: 80 pg/ml, IFN- γ : 800 pg/ml, IL-10: 80 pg/ml and TGF- β : 32 pg/ml.

Purification of u-hCG From First Trimester Pregnancy Urine

First trimester pregnancy urine (2 l) was collected in a bottle from a healthy volunteer and was refrigerated until delivered at the laboratory within 2 days. Upon delivery, the pH was adjusted to 7.2–7.4 with sodium hydroxide

and allowed to sediment for 1 h at room temperature. Approximately 75% of the supernatant was decanted and the remainder close to the precipitate was centrifuged (10 min at 25000 rpm at 4°C) to remove sediment and added to the rest of the supernatant. The supernatant was filtered through 0.45 µm in a Minitan (Millipore) transversal filtration setup. Subsequently, the filtrate (2 l) was concentrated in an Amicon ultrafiltration setup equipped with a YM Diapore membrane with a 10 kilo Dalton (kDa) cut-off. The final volume (250 ml) was dialyzed against 2 changes of 10 litres of Milli Q water. Next, the sample was further concentrated by 10 kDa cut-off in an Amicon ultrafiltration to a final volume of 3 ml. The material was further lyophilized for purification and for other experiments.

Gel Permeation

c-hCG or u-hCG was fractionated by gel filtration using a fast-protein liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden) equipped with Superdex 75 (10mm ID × 300 mm L) gel permeation column. Superdex columns have superb resolution and reproducibility for separating monomeric and dimeric forms of proteins and peptides. The separation range of this column was 70,000–3000 Dalton. 50 mM of ammonium bicarbonate in combination with 4% methanol was used as a running buffer and the selectivity profile of the column was determined. FPLC fractions of c/u-hCG in 50 mM of ammonium bicarbonate in combination with 4% methanol were further analyzed by gel filtration using a high performance liquid chromatography (HPLC) system (Shimadzu Co., Japan) equipped with a macrosphere size exclusion (GPC) 60Å (7.5mm ID × 300mm L) column (Alltech, Illinois, USA). This is an appropriate column for separation of not only proteins, but also peptides. The separation range for this column was 28,000–250 Dalton and the flow rate was 0.5 ml/min. External molecular weight standards were employed to calibrate the column elution positions. The eluted fractions from FPLC and HPLC columns were lyophilized for the use in experiments.

Statistics

Data was analyzed by Student's T test and differences were considered significant at $p < 0.05$.

RESULTS

In order to determine whether hCG treatment affects the diabetes development in NOD mice, we tested c-hCG on prediabetic female NOD mice. In our female NOD colony, the majority of β cells (>80%) are histologically and functionally destroyed between 15 and 21 weeks of age and clinical symptoms appear between 15–21 weeks

of age. c-hCG treatment of 14-week-old female NOD mice was done with a titrated dose of 300 IU i.p. three times per week. In the experiments reported here, all PBS treated NOD control mice had developed diabetes and showed blood glucose levels over 20 mmol/l at 20 weeks of age. In contrast, c-hCG treated mice remained nondiabetic as evidenced by blood glucose levels not higher than 7 mmol/l (Figure 1A). This remained so up to the termination of the experiment at the age of 35 weeks.

Histological examinations of the pancreatic tissue of the c-hCG treated mice at 21 weeks of age showed large insulin-producing islets and virtually no insulinitis (Figure 2A–D, upper panel). Control-treated mice showed no insulin producing cells, except for one PBS treated mouse where only few weakly positive insulin producing cells and a massive infiltrate were found containing large numbers of T and B lymphocytes, macrophages (MOMA-1⁺ and BM8⁺ cells), and dendritic-like (ER-MP23⁺) cells (partly shown in Figure 2A–D, lower panel) in the islets.

Impairment of the glucose tolerance test (GTT) is positively correlated to insulinitis, but negatively correlated to the number of functional β cells [12, 13]. This test showed that NOD mice during the 5 weeks of c-hCG treatment and during the follow-up period till 35 weeks of age were tolerant for glucose (Figure 1B).

Splenocytes of diabetic NOD mice contain CD4⁺ and CD8⁺ diabetogenic T cells, which can effectively transfer the disease into immunocompromised NOD.SCID mice [14, 15]. We transferred splenocytes from 21-week-old PBS and c-hCG treated NOD mice into NOD.SCID mice. Four weeks after transfer, all NOD.SCID mice that had received splenocytes from control (PBS) NOD mice were diabetic (Figure 1C), but mice that had received splenocytes from c-hCG treated NOD mice remained nondiabetic.

The effect of 4 days of *in vivo* c-hCG treatment on the Th1 and Th2 cell populations of NOD mice was studied in an *in vitro* Th polarization assay. For comparison BALB/c mice were similarly tested. Under Th1 polarizing conditions (thus in the presence of IL-12 and anti-IL-4), purified CD4⁺ T-cells from c-hCG treated NOD mice showed a lower capacity to produce IFN-γ producing cells than PBS treated mice (19±2 vs 28±3 ng IFN-γ/ml). Under neutral conditions purified CD4⁺ T-cells from c-hCG treated NOD mice also showed a significantly lower IFN-γ production as compared to PBS treated mice (Figure 3A), while no significant differences were observed between the groups in IL-4 production under neutral conditions (Figure 3B). The same was true for the effect of c-hCG treatment of NOD mice on the *in vitro* IL-4 production by purified CD4⁺ T-cells under Th2 polarizing conditions (2 ng IL-4/ml). The

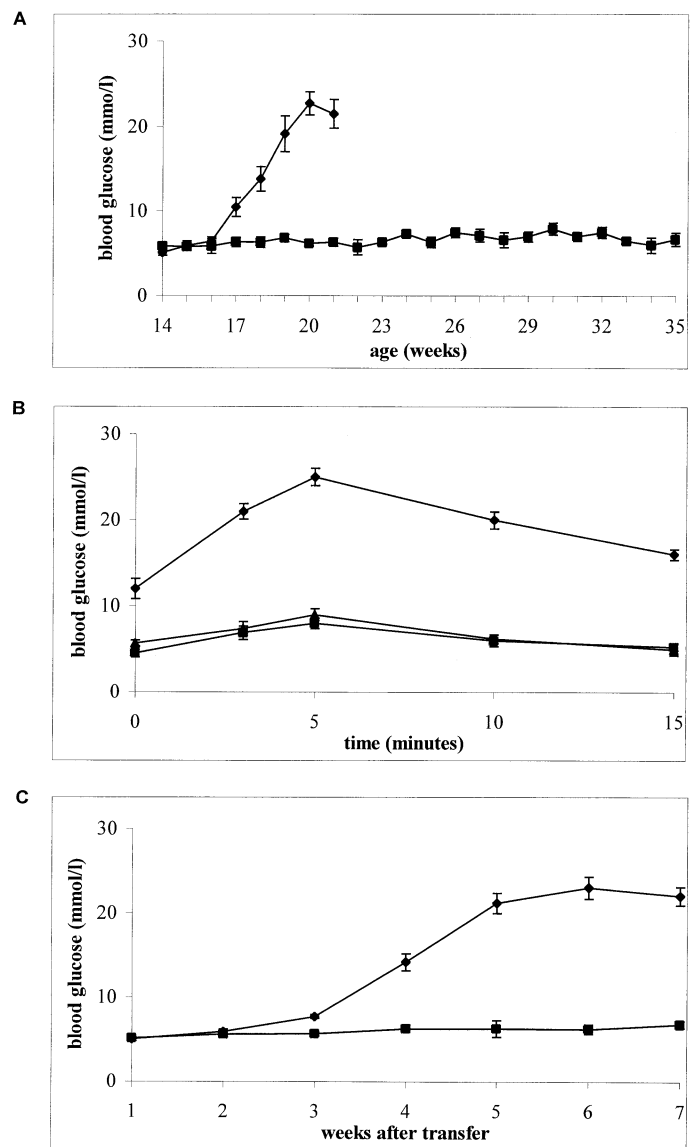


FIGURE 1 Effect of 5 weeks of c-hCG treatment (three times a week, 300 IU c-hCG starting at the age of 14 weeks) in female NOD mice on blood glucose levels (n=12). (A) Blood glucose levels of c-hCG (■) and PBS (◆) treated NOD mice. From 17 weeks and onwards the values between the groups were significantly different. After treatment the c-hCG treated mice (n=6) were kept alive for an additional 16 weeks without any further treatment. (B) GTT in c-hCG and PBS treated NOD mice (n=6) at 17 (during treatment) and 35 weeks of age (after treatment). Blood glucose levels of PBS (◆) and c-hCG (■) treated NOD mice after glucose challenge at 17 weeks of age and c-hCG (▲) treated NOD mice at 35 weeks of age are shown. The values of PBS and c-hCG groups are significantly different for all data points. (C) Transfer of diabetes resistance from c-hCG treated NOD mice to NOD.SCID mice. Spleen cells were isolated two weeks after termination of the 5-week c-hCG or PBS treatment (n=6). Viable pooled cells were infused i.v. into 8-week-old NOD.SCID mice (n=6) in a dose of 20×10^6 cells/mouse. Transfer of splenocytes from PBS-treated NOD mice to 8 weeks-old female NOD.SCID mice consistently induced diabetes after 22 days. Blood glucose levels of NOD.SCID mice receiving splenocytes from PBS-treated NOD mice (◆) and c-hCG treated NOD mice (■) are shown here. Data points from 4 weeks and onwards are significant by different. The results shown here are from a single experiment and representative of three independent sets of experiments.

IL-10 (Figure 3C) and TGF- β (Figure 3D) production by purified CD4⁺ T-cells from c-hCG treated NOD mice *in vitro* under neutral conditions was significantly increased as compared to PBS treated NOD control mice.

In contrast to NOD mice, PBS and c-hCG treated BALB/c mice did not show differences in IFN- γ production under neutral conditions (Figure 3A) as well as Th1 polarizing (18 ± 3 vs 17 ± 3 ng IFN- γ /ml) conditions, while a significant upregulation of IL-4 production was observed under neutral conditions (Figure 3B). No significant differences in IL-4 production were observed under Th2 polarizing conditions between PBS and c-hCG treated BALB/c mice (21 ± 4 vs 26 ± 2 ng IL-4/ml).

hCG is a heterodimeric glycoprotein hormone composed of two noncovalently bound glycosylated α and β

subunits. During pregnancy it is produced by the placenta and occurs in high concentration in circulation and urine in many different molecular forms such as heterodimer, free subunits, proteolytically cleaved forms, and fragments [16, 17]. Commercial hCG preparations are derived from first trimester pregnancy urine and vary in their content of hCG and related molecules. To define better the active moiety in first trimester pregnancy urine (u-hCG) and in the heterodimeric hCG itself, we fractionated c-hCG and u-hCG by gel filtration using an FPLC system. Neither c/u-hCG fractions greater than 25,000 Dalton nor r-hCG, highly purified urinary hCG, r- α -hCG, and r- β -hCG inhibited the anti-CD3 stimulated IFN- γ production *in vitro* by spleen cells from diabetic NOD mice. Also, the transfer of diabetes to

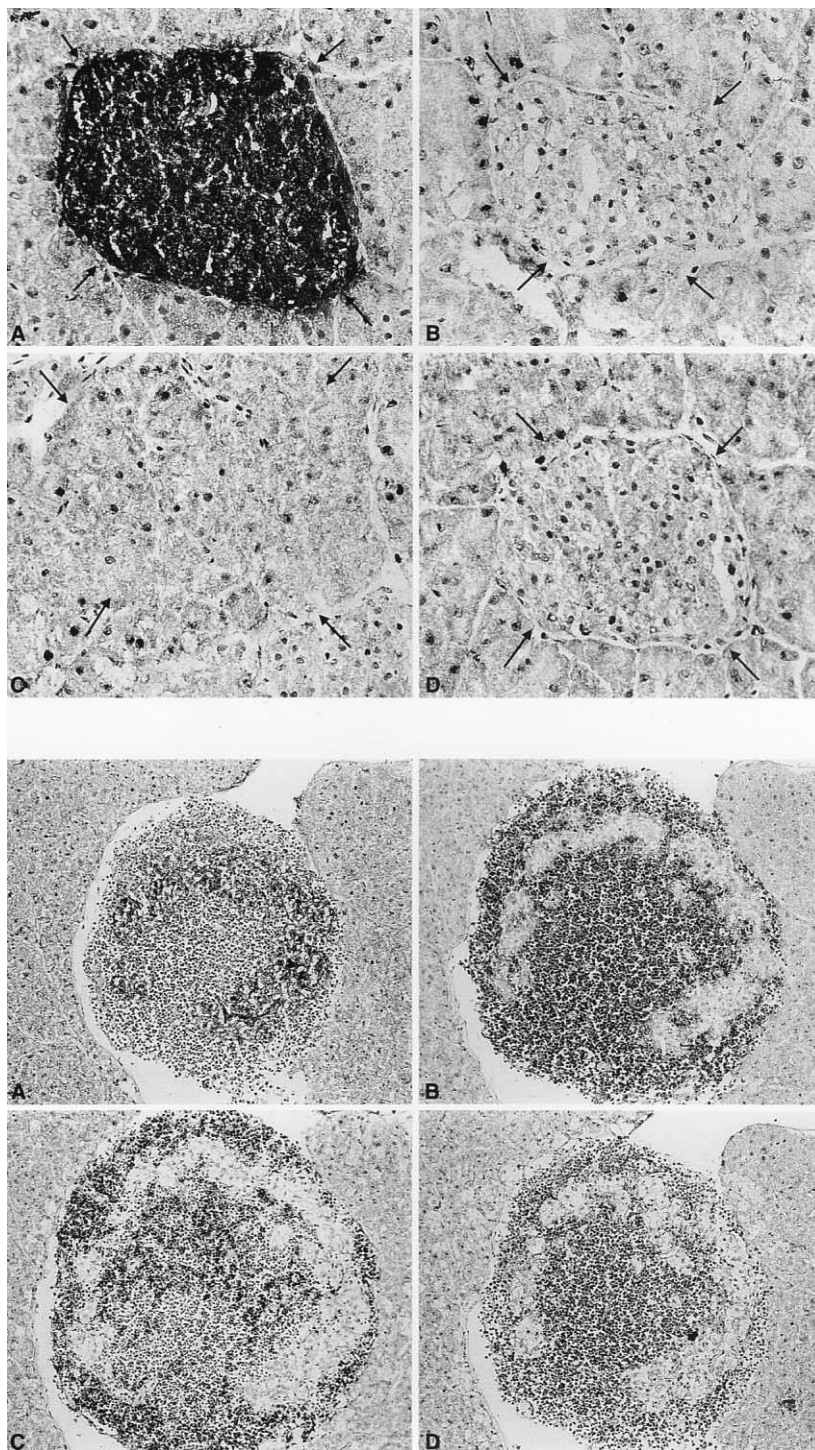


FIGURE 2 Immunohistochemistry of the pancreas of NOD mice after five weeks of c-hCG treatment. Representative islets of PBS and c-hCG treated mice are shown. Staining of pancreatic tissue for insulin (A), B cells (B), T cells (C) and macrophages (D) of c-hCG (magn. 320x, upper panel) and PBS (magn. 128x, lower panel) treated NOD mice is shown. At least ten independent microscopic fields were examined from at least three animals per group. At least 50 islet sections were examined per mouse.

NOD.SCID mice by such treated spleen cells from diabetic NOD mice was not inhibited (data not shown). We further fractionated *c/u*-hCG on a HPLC system and these fractions were also tested for their antidiabetic effect. These experiments showed that the active moiety eluted at molecular range 400–2000 Dalton when assayed for IFN- γ inhibiting capacity and transfer of dia-

betes to NOD.SCID mice, employing spleen cells from diabetic NOD mice (Figure 4). This 400–2000 Dalton fraction from *c/u*-hCG also inhibited the development of diabetes in NOD mice, while no such inhibitory effect was observed with 400–2000 Dalton fraction from the urine of nonpregnant women (data not shown). Similar to c-hCG treatment *in vivo*, under Th1 polarizing con-

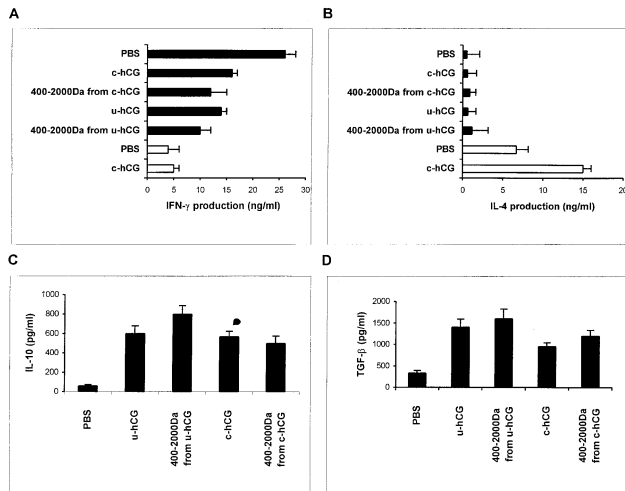


FIGURE 3 Effect of c/u-hCG treatment on Th polarization in NOD and BALB/c mice under neutral (nonpolarizing conditions). CD4⁺ T-cells were purified from spleens of mice (n=6) treated with c/u-hCG or its 400–2000 Dalton fraction. Cells were stimulated with anti-CD3/anti-CD28 and cultured for 4 days with IL-2. Subsequently, the cells were washed and restimulated for 2 days with anti-CD3, anti-CD28, and IL-2 only. Supernatant levels of IFN- γ (A) and IL-4 (B) were measured by ELISA. Black bars correspond to CD4⁺ T-cell cultures of NOD mice and white bars of BALB/c mice. The supernatant levels of IL-10 (C) and TGF- β (D) in these cultures are also shown. These results are from one experiment and are representative of at least three independent sets of experiments (* p < 0.05).

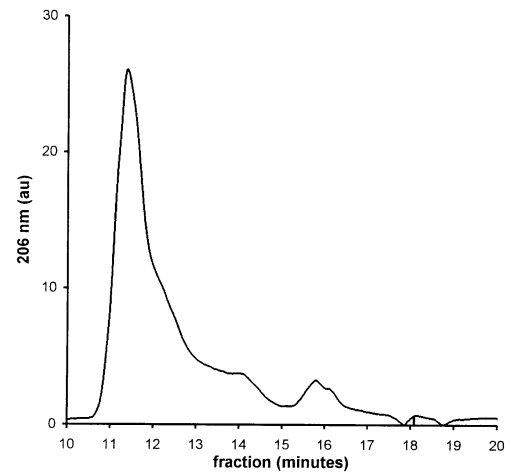
ditions the c-hCG and u-hCG 400–2000 Dalton fractions inhibited the IFN- γ production (16 \pm 3 vs 28 \pm 3 ng IFN- γ /ml and 17 \pm 3 vs 28 \pm 3 ng IFN- γ /ml, respectively) as well as under neutral conditions by NOD CD4⁺ T cells (Figure 3A). The IL-4 production, however, was not affected under neutral (Figure 3B) as well as Th2 polarizing conditions (data not shown).

The effect of the 400–2000 Dalton c/u-hCG fractions on the *in vitro* IL-10 (Figure 3C) and TGF- β production (Figure 3D) was similarly enhanced when compared to PBS, as found after culture with c-hCG and u-hCG.

We also tested two other c-hCG preparations (Profasi, APL) for their antidiabetic activity: profasi and APL showed differential and only partial dose-dependent inhibitory effects (data not shown).

DISCUSSION

The study presented here demonstrates that short-term (5 weeks) treatment of female NOD mice starting prior to the onset of the symptoms, with hCG preparations derived from first trimester pregnancy urine, inhibits the development of diabetes. This was supported by GTT testing, showing that the treated NOD mice were tol-



Anti-diabetic activity	-	-	-	-	-	+	+	-	-	-
IFN- γ inhibition	-	-	-	-	-	+	+	-	-	-

FIGURE 4 Analysis of c-hCG by gel filtration using an HPLC system. GPC 60 \AA column was run at a flow rate of 0.5 ml/min and 1 ml fractions were collected. Each fraction was lyophilized and assayed for IFN- γ inhibiting capacity and transfer of diabetes to NOD.SCID mice, employing spleen cells from diabetic NOD mice. This figure shows that the IFN- γ inhibitory and anti-diabetic activity elutes in fractions that correspond to a molecular weight between 400 to 2000 Dalton.

erant for glucose during 5 weeks of c-hCG treatment and during the follow-up period till 35 weeks of age. This and histochemical analysis indicated the presence of sufficient numbers of insulin-producing β cells. The treatment even reversed established inflammatory infiltrates of the pancreas, as evidenced by histology. Previous studies of our department have shown [11] that in our NOD colony at the age of 14 weeks, the mice have abundant infiltrates and severe insulinitis (stage 4/5) in their pancreas correlating with the start of clinical symptoms of diabetes. These infiltrates consist of T and B lymphocytes, macrophages, and dendritic-like cells. Others have demonstrated that short-term low-dose anti-CD3 treatment can also induce a complete remission of the diabetes when applied to adult NOD females within 7 days of full-blown diabetes [18]. However, in the latter, the remission was not associated with the disappearance of the insulinitis.

Splenocytes of diabetic NOD mice contain diabetogenic T cells, which can transfer the disease into immunocompromised NOD.SCID mice [14, 15]. Splenocytes from NOD mice treated with c-hCG for five weeks and from c-hCG-treated NOD mice 16 weeks after termination of the c-hCG treatment were not able to induce diabetes in NOD.SCID mice. This shows the fast and prolonged inhibitory effect of c-hCG-treatment on dia-

betogenic NOD cells. *In vivo* and *in vitro* c-hCG treated diabetogenic NOD splenocytes produced low levels of IFN- γ , which correlated with the inability of these cells to transfer the disease to NOD.SCID mice.

Further investigation also revealed the direct or indirect effect of c/u-hCG treatment on CD4⁺ T-cells of NOD mice. Such *in vivo* treated purified CD4⁺ T cells showed lower IFN- γ production levels under Th1 polarizing and neutral conditions. This not just implicates intrinsic changes in T cells to develop into Th1 cells, but also suggests a smaller pool of IFN- γ producing Th1 cells *in vivo* due to the c/u-hCG treatment. No significant differences were observed between the groups in IL-4 production under Th2 polarizing and neutral conditions. Instead, upregulation of IL-10 and TGF- β production was observed under neutral conditions, showing that *in vivo* c/u-hCG treatment inhibited the IFN- γ production and the preferential outgrowth of Th1 cells by inducing increased levels of anti-inflammatory cytokines.

Splenocytes from c/u-hCG treated BALB/c mice did not show differences in IFN- γ production in the Th polarization assay as compared to control mice, but did show a significant upregulation of IL-4 production under neutral conditions. This excludes the possibility of a general inhibitory effect of c/u-hCG treatment on cytokine production and substantiates the immunomodulatory effect of the treatment.

Several groups have reported anti-Kaposi's Sarcoma (KS) and anti-HIV activity of some hCG preparations [19]. This was not due to the native hCG heterodimer. In these studies, the active moiety was thought to be β core or an as yet unidentified hCG associated factor. The mechanism of action was proposed to be the selective induction of apoptosis or direct cytotoxic effects on the tumor cells [20]. No infiltration of the tumor with mononuclear cells was observed, leading to the conclusion that the anti-KS activity could not be due to an immune-mediated response [21].

As evident by HPLC, our 400–2000 Dalton bioactivity from pregnancy urine and thereof derived hCG preparations eluted at a position which is far removed from the elution positions of hCG (38 kDa), β -hCG free subunit (>15 kDa), and β -core (>10 kDa). In addition, neither c/u-hCG fractions greater than 2000 Dalton nor r-hCG, highly purified urinary hCG, r- α -hCG, and r- β -hCG showed inhibition of IFN- γ production and anti-diabetic activity. This argues against the possibility that functional heterodimeric hCG, its subunits, or β -core could be responsible for the immunomodulatory and anti-diabetic effects. We named this 400–2000 Dalton bioactivity natural (immuno)modulatory pregnancy factor(s) (NMPF). Heat (30 min at 56°C) and protease enzyme (elastase, pronase) treatment abrogated the immunomodulatory effect of NMPF suggesting a peptide

nature of the factor(s). In an upcoming paper on the effect of NMPF on septic shock in mice, we show that the synthetic peptide VLPALP has the same therapeutic effect on the outcome of septic shock as the 400–2000 Dalton NMPF fraction. This peptide sequence is part of the primary structure of the hCG β chain. Studies in NOD mice employing this peptide are in progress. Our data excludes that the mechanism, through which NMPF with a proven molecular weight between 400 and 2000 Dalton exerts its antidiabetic effect, and the c-hCG associated component, which exerts the anti-KS/anti-HIV effect (20), are the same.

In summary, our data shows that a low molecular weight fraction from first trimester human pregnancy urine and some commercial hCG preparations can prevent diabetes development in NOD mice and possibly other Th1 mediated autoimmune diseases. Further identification of the presumed peptide(s) accounting for the antidiabetic effect and elucidation of its precise mechanism(s) of action are in progress.

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REFERENCES

1. Beeson PB: Age and sex associations of 40 autoimmune diseases. *Am J Med* 96:457, 1994.
2. Whitacre CC, Reingold SC, O'Looney PA: A gender gap in autoimmunity. *Science* 283:1277, 1999.
3. Wilder RL: Hormones, pregnancy, and autoimmune diseases. *Ann N Y Acad Sci* 840:45, 1998.
4. Wegmann TG, Lin H, Guilbert L, Mosmann TR: Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? *Immunol Today* 14:353, 1993.
5. Piccinni MP, Romagnani S: Regulation of fetal allograft survival by a hormone-controlled Th1- and Th2-type cytokines. *Immunol Res* 15:141, 1996.
6. Nelson JL, Hughes KA, Smith AG, Nisperos BB, Branchaud AM, Hansen JA: Maternal-fetal disparity in HLA class II alloantigens and the pregnancy-induced amelioration of rheumatoid arthritis. *N Engl J Med* 329:466, 1993.
7. Wilde L: *Chorionic Gonadotrophin*, S.J. Siegal, editor. Plenum, New York, 1980.
8. Andre I, Gonzalez A, Wang B, Katz J, Benoist C, Mathis D: Checkpoints in the progression of autoimmune disease:

- lessons from diabetes models. *Proc Natl Acad Sci U S A* 93:2260, 1996.
9. Lampeter EF, Signore A, Gale EA, Pozzilli P: Lessons from the NOD mouse for the pathogenesis and immunotherapy of human type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* 32:703, 1989.
 10. Katz JD, Benoist C, Mathis D: T helper cell subsets in insulin-dependent diabetes. *Science* 268:1185, 1995.
 11. Jansen A, Homo-Delarche F, Hooijkaas H, Leenen PJ, Dardenne M, Drexhage HA: Immunohistochemical characterization of monocytes-macrophages and dendritic cells involved in the initiation of the insulinitis and beta-cell destruction in NOD mice. *Diabetes* 43:667, 1994.
 12. Alberti KG: Impaired glucose tolerance. *Lancet* 2:211, 1980.
 13. Reddy S, Liu W, Thompson JM., Bibby NJ, Elliott RB: First phase insulin release in the non-obese diabetic mouse: correlation with insulinitis, beta cell number and autoantibodies. *Diabetes Res Clin Pract* 17:17, 1992.
 14. Delovitch TL, Singh B: The nonobese diabetic mouse as a model of autoimmune diabetes: immune dysregulation gets the NOD. *Immunity* 7:727, 1997.
 15. Yagi H, Matsumoto M, Kunimoto K, Kawaguchi J, Makino S, Harada M: Analysis of the roles of CD4⁺ and CD8⁺ T cells in autoimmune diabetes of NOD mice using transfer to NOD athymic nude mice. *Eur J Immunol* 22:2387, 1992.
 16. Kardana A, Elliott MM, Gawinowicz MA, Birken S, Cole LA: The heterogeneity of human chorionic gonadotropin (hCG). I. Characterization of peptide heterogeneity in 13 individual preparations of hCG. *Endocrinology* 129:1541, 1991.
 17. Lunardi-Iskandar Y, Bryant JL, Blattner WA, Hung, CL, Flamand L, Gill P, Hermans P, Birken S, Gallo RC: Effects of a urinary factor from women in early pregnancy on HIV-1, SIV and associated disease. *Nat Med* 4:428, 1998.
 18. Chatenoud L, Primo J, Bach JF: CD3 antibody-induced dominant self tolerance in overtly diabetic NOD mice. *J Immunol* 158:2947, 1997.
 19. Albin A, Paglieri I, Orengo G, Carlone S, Aluigi MG, DeMarchi R, Matteucci C, Mantovani A, Carozzi F, Donini S, Benelli R: The beta-core fragment of human chorionic gonadotrophin inhibits growth of Kaposi's sarcoma-derived cells and a new immortalized Kaposi's sarcoma cell line. *AIDS* 11:713, 1997.
 20. Samaniego F, Bryant JL, Liu N, Karp JE, Sabichi AL, Thierry A, Lunardi-Iskandar Y, Gallo RC: Induction of programmed cell death in Kaposi's sarcoma cells by preparations of human chorionic gonadotropin. *J Natl Cancer Inst* 91:135, 1999.
 21. Gill PS, Lunardi-Iskandar Y, Louie S, Tulpule A, Zheng T, Espina BM, Besnier JM, Hermans P, Levine AM, Bryant JL, Gallo RC: The effects of preparations of human chorionic gonadotropin on AIDS-related Kaposi's sarcoma. *N Engl J Med* 335:1261, 1996.