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Article

Impaired glucose tolerance is associated with increased serum concentrations of interleukin 6 and co-regulated acute-phase proteins but not TNF-α or its receptors

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Abstract

Aims/hypothesis. A population-based sample was studied to define immune abnormalities in individuals at risk of Type II (non-insulin-dependent) diabetes mellitus because of impaired glucose tolerance.
Methods. A total of 1653 individuals aged 55 to 74 years participated in a population based survey in Southern Germany (KORA Survey 2000). Those without a history of diabetes were subjected to an OGTT. Randomly selected subjects with IGT (n=80) were compared with non-diabetic control subjects (n=77) and patients with Type II diabetes (n=152) of the same population-based sample after matching for age and sex. Immune parameters were analysed in serum with rigidly evaluated ELISA.
Results. Serum pro-inflammatory cytokine interleukin 6 (IL-6) concentrations were higher in subjects with IGT and Type II diabetes than in the control subjects (median 1.8 and 2.5 vs 0.8 pg/ml, p<0.0001). Soluble IL-6 receptors potentiate IL-6 bioactivity and their concentrations were mildly increased in Type II diabetes (p<0.05). These immune changes seem relevant because IL-6 dependent acute-phase proteins C-reactive protein, serum amyloid A protein and fibrinogen were also increased in IGT and Type II diabetes. Circulating concentrations of TNF-α and its two receptors sTNF-R60 and sTNF-R80 were not increased in IGT subjects compared with the control subjects.
Conclusion/interpretation. Our study shows systemic up-regulation of selected inflammatory mediators in patients with Type II diabetes and IGT. The pattern observed is non-random and fits with an IL-6 associated rather than TNF-α associated response. [Diabetologia (2002) 45:805–812]

Keywords Type II diabetes, impaired glucose tolerance, systemic inflammation, interleukin 6, tumour necrosis factor α, C-reactive protein, serum amyloid A protein, innate immunity, cytokine, acute-phase protein.

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Abbreviations: CVD, Coronary vascular disease; CRP, C-reactive protein; WHO-MONICA, Monitoring of Trends and Determinants in Cardiovascular Disease project; KORA, Kooperative Gesundheitsforschung im Raum Augsburg; OD, optical density R^2 regression coefficient; SAA, serum amyloid A protein; sIL-6R, soluble IL-6 receptor; sTNF-R, soluble TNF receptor

Several studies have reported signs of chronic activation of the innate immune system in patients with Type II (non-insulin-dependent) diabetes mellitus. Abnormalities included small but definite increases of serum or plasma concentrations of several acute-phase proteins, including C-reactive protein (CRP), serum amyloid A (SAA), fibrinogen, α1-acid glycoprotein and plasminogen activator inhibitor-I [1, 2, 3, 4, 5]. Acute-phase reactants are primarily produced by hepatocytes and their chief inductor is the pro-inflammatory cytokine interleukin 6 (IL-6). There is also a contribution by other inflammation-associated cytokines such as TNF-α or IL-1 [6]. Indeed, increased circulat-

ing concentrations of IL-6 were found in Type II diabetes [7, 8] as well as serum TNF-α [9, 10, 11].

Thus, it has been suggested that chronic activation of innate immunity might be the cause of Type II diabetes [12]. This is further supported by observations that changes of serum lipid patterns typical of Type II diabetes are also seen in conditions of pro-inflammatory immunity such as infections or malignancies [13, 14, 15]. Experimental induction of an acute-phase response elicits similar lipoprotein abnormalities [16].

A major argument against this pathogenic concept of Type II diabetes is that immune abnormalities or chronic slow infections with concomitant immune activation could occur as a result of metabolic disturbances in Type II diabetes rather than representing its cause. Therefore, it seems important to determine if signs of activated innate immunity are present before the onset of overt Type II diabetes. One such approach is to analyse individuals at risk of Type II diabetes because of IGT [17, 18, 19]. Recently, a first study of adults with IGT did not show abnormal plasma concentrations of TNF-α [20]. Also, circulating concentrations of IGF-1 were normal, though IGF-1 concentrations often decrease during an acute-phase response [21, 22].

One shortcoming of these earlier studies on immune parameters in Type II diabetes is that subjects analysed were not population-based and thus more severe cases could have been recruited by diabetes centers and healthy control subjects might not have been representative. We therefore revisited the issue by analysing a random sample of the adult general population between 55 to 74 years of age in the region of Augsburg in Southern Germany. Both, IL-6 and TNF-α as well as circulation cytokine receptors were measured. In addition, the major acute-phase reactants CRP, SAA and fibrinogen were analysed.

Subjects and methods

Subjects. In the KORA Survey (Kooperative Gesundheitsforschung im Raum Augsburg 2000) a representative sample of the adult general population of German nationality in the region of Augsburg was recruited from October 1999 to April 2001. The sampling design followed the guidelines of three previous surveys conducted in this region within the frame of the multinational Monitoring of Trends and Determinants in Cardiovascular Disease (WHO-MONICA) Augsburg project [23]. The investigations were carried out in accordance with the Declaration of Helsinki as revised in 1996, including written informed consent of all participants. The survey comprised 4261 men and women between 25 and 74 years of age with a response of 67%. Our report is based on subjects aged 55 to 74 years. A total of 1653 participants were submitted to a standardized questionnaire carried out by trained interviewers. Coronary heart disease (CHD) was assessed by the Rose questionnaire for angina pectoris and by self report of past myocardial infarction. Cardiovascular disease (CVD) was assessed by asking for a previous stroke or for symptoms or diagnosis of claudicatio intermittens. Blood samples were obtained, which were fasting in non-diabetic subjects and in newly detected diabetic patients, and non-fasting in subjects with a history of diabetes.

Oral glucose tolerance test (OGTT). All individuals without a known history of diabetes were subjected to a 75 g OGTT. Because of the variability of OGTT [24, 25] a standardized protocol was followed which required fasting from 22.00 hours in the evening before the visit until 8.00 to 11.00 the next day. Individuals with signs of infections were not eligible. Subjects with IGT and newly diagnosed with diabetes were identified following current World Health Organisation (WHO) criteria [26].

Serum analyses were done in 80 subjects with IGT. For comparison, 152 patients with Type II diabetes and 77 non-diabetic control subjects, matched for age and sex, were selected from the total random study sample. Diabetic patients comprised 71 individuals diagnosed with Type II diabetes by the treating physician (mean duration from diagnosis 10.7± 8.1 years) and 81 subjects with diagnosis of non-insulin-dependent diabetes during the survey. The latter two groups are reported together because no significant differences in median concentrations of immune mediators were observed.

Blood biochemistry. Blood samples were drawn and prepared according to the recommendations of the International Committee for Standardization in Haematology [27]. The following parameters were measured on fresh samples. Serum glucose was measured by Gluco-quant (hexokinase-method, Roche Diagnostics, Mannheim, Germany). The percentage of HbA_{1c} was analysed by Tina-quant (turbidimetric immunologic method, Roche Diagnostics). Total cholesterol was measured by enzymatic methods (CHOD-PAP, Roche Diagnostics). HDL cholesterol was measured after precipitation with phosphotungstic acid/Mg^{2+} (Roche Diagnostics), LDL cholesterol after precipitation with dextran sulfate (Quantolip LDL, Immuno AG, Vienna, Austria). Serum triglyceride concentrations were analysed by the GPO-PAP method (Roche Diagnostics). In addition, serum was stored at –80°C for further analyses.

Analyses of acute-phase proteins and cytokines. Plasma CRP concentrations were assessed by a high sensitivity latex enhanced nephelometric assay on a BN II analyser (Dade Behring, Marburg, Germany) [28]. SAA and fibrinogen concentrations were analysed by immunonephelometry [29]. Serum concentrations of cytokines and circulating receptors were measured by sandwich ELISA [TNF-α, BD Pharmingen, Heidelberg, Germany; sTNF-R60 (CD120a), sTNF-R80 (CD120b), Bender MedSystems, Vienna, Austria; IL-6, CLB, Amsterdam, Netherlands; IL-6R, Bender MedSystems, Vienna, Austria]. All sandwich ELISAs were established to meet the following criteria: linearity of signal for the standard curve between optical density (OD) 0.05 and 2.0, difference between expected and measured signal in spiking experiments less than 15%, mean intra-assay variation below 10%, mean interassay variation below 10%, loss of signal after freezing and thawing of sera three times less than 20%. An interference of heterophile antibodies was not observed. If sera gave signals above OD 2.0, measurements were repeated with higher diluted samples. All sera were analysed in duplicate. Measurements were repeated if there was more than 20% difference between the two parallel measurements. Detection limits of cytokine ELISAs were 0.24 pg/ml for IL-6, 0.16 pg/ml for TNF-α, 1.54 ng/ml for sIL-6R, 0.02 ng/ml for sTNF-R60 and 0.2 ng/ml for sTNF-R80.

GAD antibody measurement. Autoantibodies to GAD were measured using radiolabelled human recombinant antigen in a 96-well assay format as described [30]. The cutoff for GAD antibody positivity was set at the 99th percentile in healthy control subjects (6.5 GAD antibody units). The assay was evaluated in the IDS Combined Autoantibody Workshop and yielded a disease sensitivity (Type I (insulin-dependent) diabetes mellitus) of 81% and a specificity of 99%.

Statistical analysis. Basic variables and serum parameters were described by mean ± SD (age, total cholesterol, LDL and HDL cholesterol) or median and range (all other variables). Differences between groups concerning total cholesterol, LDL or HDL cholesterol were analysed by analysis of variance including Fisher's exact test. For other variables comparisons between groups were done by Kruskal-Wallis tests (three groups) and, if significant, followed by the Wilcoxon test for multiple testing. Spearman rank correlation coefficients were used to analyse associations between continuous variables, in particular between the various inflammatory and immune markers.

Between group comparisons of inflammatory markers were carried out firstly by non-parametric analyses of variance using Kruskal-Wallis followed by Wilcoxon test as described above. The problem of multiple testing was also addressed by carrying out 21 pair wise Wilcoxon tests followed by Bonferroni-Holm procedure at the multiple level of 5%. Furthermore, normal values were estimated as 95 percentiles in the control group, and the percentage of non-normal values in the IGT or diabetic group were compared with the control group using Fisher's exact test (left-sided). Linear regression models were fitted to adjust group differences for BMI.

The level of significance was 5%. Calculations were carried out using the SAS statistical package version 6.12 TS020.

Results

Basic characteristics of groups studied. A comparison of basic characteristics of the cohorts studied is given in Table 1. As age and sex were matching variables, no difference was seen, whereas mean or median values of metabolic parameters differed between Type II diabetic patients and non-diabetic control subjects. Subjects with IGT showed statistically significant differences in BMI, fasting plasma glucose, triglyceride concentrations and blood pressure, but not for HbA_{1c}, total and lipoprotein associated cholesterol compared with non-diabetic control subjects (Table 1). The prevalence of reported CHD did not differ between the three groups (means 11.8–19.2%). The same was found for the prevalence of reported CVD (means 17.5–25.8%).

Acute phase protein concentrations. Median plasma concentrations of CRP were higher in subjects with IGT or with Type II diabetes than in the control group (Fig. 1A). There was no significant difference between IGT and Type II diabetes. When excluding individuals with a possibly activated and transient acute phase response (CRP >10 mg/l, n=21), differences between groups remained statistically significant. A similar outcome was noted for SAA and fibrinogen concentrations, albeit less pronounced (Fig. 1B, C). When the control group was used to define a normal range, 19/80 subjects with IGT (23/152 patients with Type II diabetes) had plasma CRP concentrations above 6.76 mg/l (95th percentile of normal) compared with only 3/77 non-diabetic subjects (p<0.001 for IGT, p<0.01 for Type II diabetes group).

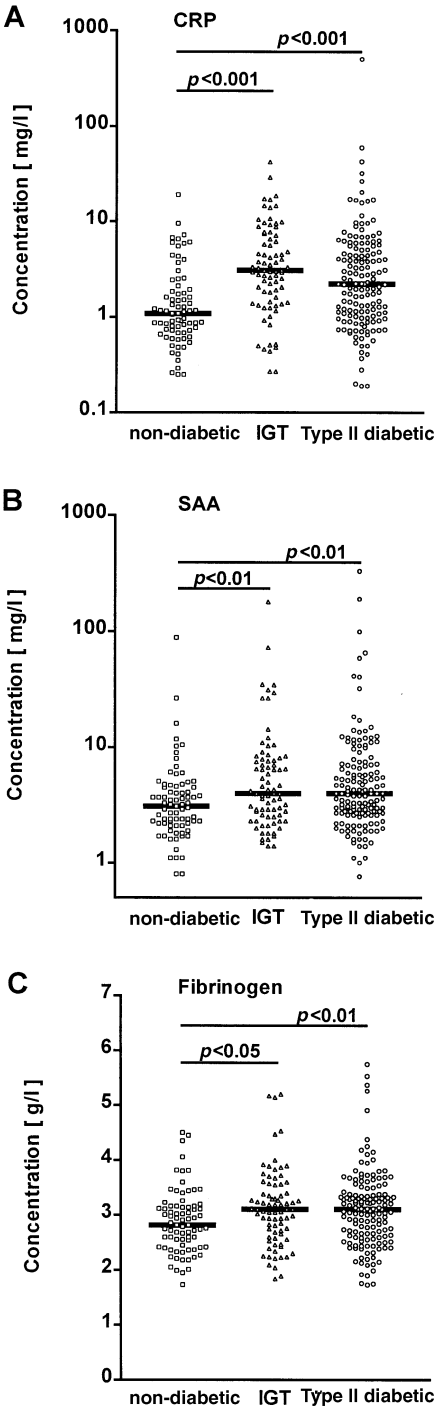

Fig. 1A–C. Plasma concentrations of acute phase proteins. **A** Plasma concentrations of CRP, **B** Plasma concentrations of SAA, **C** Plasma concentrations of fibrinogen. Each symbol represents one individual, the *bar* shows the median per group

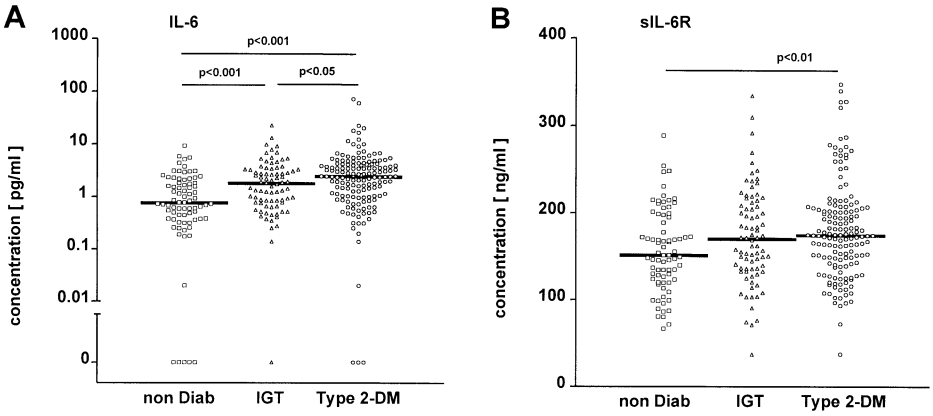

Fig. 2A, B. Serum concentrations of IL-6 and sIL-6R. **A** Serum concentrations of IL-6, **B** Serum concentrations of sIL-6R. Each symbol presents one individual, the *bar* indicates the median per group

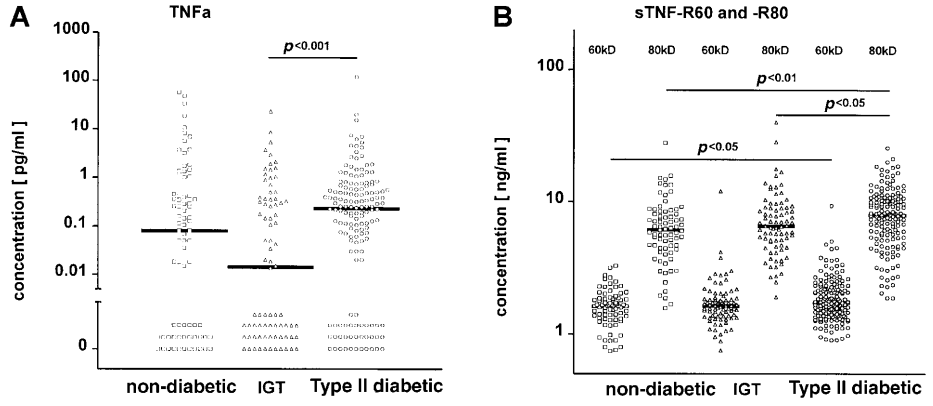

Fig. 3. **A** Serum concentrations of TNF-α, **B** Serum concentrations of sTNF-R60 and sTNF-R80. Each symbol represents one individual, the *bar* indicates the median per group

Table 1. Basic characteristics of the study population

	Non-diabetic subjects	IGT subjects	Type II diabetic subjects
Age (years)[a]	65.4±5.8	65.5±5.4	65.4±5.1
Sex (men/female)	47/30	50/30	95/57
BMI (kg/m^2)[b]	27.1 (20.4–37.0)	29.4 (19.9–40.3)[e]	29.8 (21.8–51.2)[e]
Fasting PG (mmol/l)[b]	5.43 (4.37–6.10)	5.88 (4.93–7.00)[e]	not done
HbA_{1c} (%)[b]	5.7 (5.1–6.5)	5.8 (5.1–7.2)	6.4 (4.9–13.2)[e]
Total cholesterol (mmol/l)[a]	6.28±1.10	6.27±1.05	6.14±1.14
LDL-cholesterol (mmol/l)[a]	4.08±1.06	3.92±0.93	3.84±1.03
HDL-cholesterol (mmol/l)[a]	1.45±0.39	1.45±0.46	1.29±0.37[e]
Triglycerides (mmol/l)[b]	1.12 (0.5–5.5)	1.38 (0.6–7.9)[e]	1.79 (0.3–11.5)[c]
Systolic blood pressure (mmHg)[b]	131 (99.5–180)	145 (94–205)[e]	143 (78–217)[e]
Diastolic blood pressure (mmHg)[b]	80 (61.5–112.5)	84.5 (59.5–110)	82 (51–120)
Increased blood pressure (%)[d]	42.1	63.75[e]	56.6[e]

PG, Plasma glucose
Data are [a]means ± SD or [b]median (range)
[c] 71 patients with known history of Type II diabetes provided non fasting blood samples are not included
[d] Percentage of patients with increased blood pressure (≥140/90 mmHg) at the date of the visit
[e] Significant difference to the non diabetic group

Cytokines and cytokine receptor concentrations. Median circulating IL-6 concentrations were increased in the IGT and Type II diabetes group, with higher concentrations in the latter (Fig. 2A). Since IL-6 function is supported by soluble IL-6 receptors we analysed whether decreased sIL-6R concentrations might counteract the increase of serum IL-6. This was not the case. Rather, there was an increased concentration of sIL-6R in subjects with Type II diabetes and a trend towards increased concentrations in the IGT group

(Fig. 2B). There was an overall correlation between IL-6 and sIL-6R concentrations (r=0.15, p<0.01). For serum IL-6 concentrations, 11/80 subjects with IGT (24/152 patients with Type II diabetes) were above 5.13 pg/ml (95th% percentile of normal) compared with 3/77 non-diabetic subjects (p<0.05 and p<0.01, respectively). Control experiments with mixtures of defined quantities of IL-6 and sIL-6R as found in sera, verified that the presence of IL-6 did not affect the measurement of sIL-6R and vice versa.

Median serum TNF-α concentrations were lower in the IGT group than in Type II diabetic patients but both groups did not differ from the control subjects (Fig. 3A). The same outcome was found by a Student's t test but there was a trend towards increased TNF-α concentrations in the Type II diabetes versus the control group (p=0.12). Since circulating TNF-α receptors can antagonise TNF-α activity we also analysed serum concentrations of both soluble TNF receptor types. Both sTNF-R60 and sTNF-R80 differed in their median concentrations between the control group and the Type II diabetic group (Fig. 3B). sTNF-R80 concentrations in Type II diabetic subjects were slightly increased compared with the IGT group. Individual sTNF-R60 concentrations did not correlate with those of TNF-α, and sTNF-R80. Control experiments were done with mixtures of soluble receptors and TNF-α as found in serum. The presence of receptors did not interfere with TNF-α measurements and vice versa.

When adjusting for multiple testing the differences for IL-6 and CRP were still significant between healthy control and diabetic subjects or with IGT subjects. Furthermore, TNF-α differs between the IGT and the diabetic group, and sTNF-R80 and SAA between the control and the diabetic group at the multiple 5% level. This did not apply to comparisons of other groups.

We analysed for islet autoimmunity in subjects with IGT because one might expect abnormal immune parameters. Two of the 80 subjects with IGT were positive for GAD autoantibodies. They did not show extreme values for any of the endpoints and their omission did not affect the outcome.

Correlation between immune markers. Both, IL-6 and TNF-α are known to induce the production of acute-phase proteins from hepatocytes. Since only IL-6 concentrations were increased in both IGT and Type II diabetes it was expected that only IL-6 concentrations would correlate with concentrations of acute-phase proteins. This was the case as shown for TNF-α versus CRP (r=0.09, p=0.11, Fig. 4A) and IL-6 versus CRP (r=0.49, p<0.0001, Fig. 4B). IL-6 concentrations also correlated with SAA concentrations (r=0.35, p<0.0001) and fibrinogen concentrations (r=0.27, p<0.0001). There was also correlation of individual serum concentrations between each of the three acute-phase proteins analysed, CRP versus SAA (r=0.60, p<0.0001,

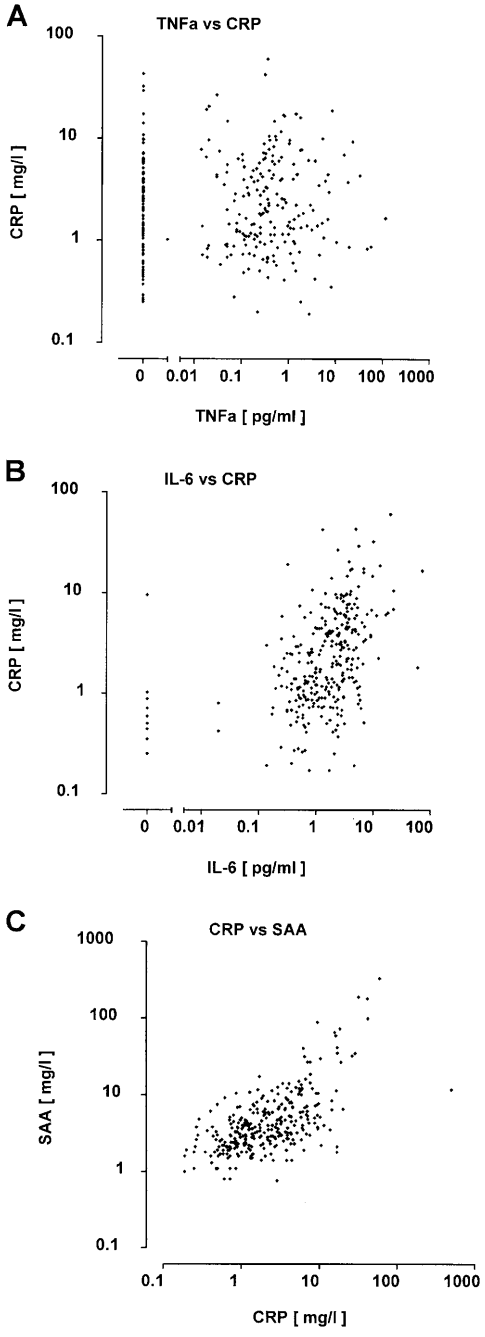

Fig. 4A–C. Correlation of marker in individuals. **A** TNF-α vs CRP concentrations (r=0.09, p>0.05), **B** IL-6 vs CRP concentrations (r=0.49, p<0.0001), **C** CRP vs SAA concentrations (r=0.59, p<0.0001). Each symbol represents one individual

Fig. 4C), CRP versus fibrinogen (r=0.47, p<0.0001) and SAA versus fibrinogen (r=0.29, p<0.0001).

Correlation of immune markers to other biochemical or clinical markers. There was no influence of sex on the concentrations of immune parameters, except for serum TNF-α (median for men of all groups 0.25, women 0.02 pg/ml, p<0.01). IL-6 and sTNF-R60 concentrations in serum increased slightly with age

(r=0.12, p<0.05 and r=0.20, p<0.001, respectively), whereas this was not seen for concentrations of sIL-6R, TNF-α or sTNF-R80. Despite the association of IL-6 with age, no correlation with age was found for any of the acute-phase protein analysed.

In a further analysis the diabetes group was subdivided in 20 patients showing three or more features of the insulin resistance syndrome (HDL cholesterol <1.1 mmol/l; blood pressure ≥140/90 mmHg; CHD; BMI ≥30 kg/m^2) and 78 patients showing one or none of these symptoms. Though there was a trend for most immune parameters, only median IL-6 concentrations were increased in the insulin resistance syndrome group (median 2.41 vs 2.08 pg/ml, p<0.001).

Of the markers associated with IGT all were positively correlated with BMI (IL-6, r=0.26, p<0.0001; CRP: r=0.24, p<0.0001; SAA: r=0.16, p<0.01, fibrinogen: r=0.18, p<0.01). To search for immunological associations independent of BMI, linear regression analyses were carried out. Dependent variables were log values of IL-6, CRP, SAA and fibrinogen, independent variables were BMI (continuous) and IGT or diabetes (control group as baseline). In case of IL-6, CRP and SAA, the association with IGT and diabetes were still significant (IL-6 and CRP p<0.01, SAA p<0.05), and BMI itself was also associated with the acute-phase response (p<0.01 and p<0.05, respectively). Both multiple linear regression models (with the dependent variables IL-6, CRP or SAA) did not fit well. The model merely explains R^2=10%, R^2=13% and R^2=0.05% of the total sum of squares of the observed IL-6, CRP and SAA values, respectively. In case of fibrinogen, the association with IGT and diabetes was lost after adjusting for BMI (p=0.14 and p=0.26, respectively), however, the R^2 value of 5% was poor. Overall, the association between increased concentrations of IL-6 and CRP with IGT or Type II diabetes cannot be completely explained by BMI.

Discussion

These data provide clinically important information on systemic immune abnormalities in Type II diabetes.

A key finding is that systemic IL-6 concentrations are increased in both IGT and Type II diabetes. Individual serum concentrations of IL-6 varied considerably, with the normal range covering a 40-fold difference. Exactly the same distribution was seen in IGT and Type II diabetes, though at a higher level. An increment was seen for low as well as for high IL-6 producers in comparison with healthy control subjects suggesting an increased inflammation state throughout the two groups. Since not all individuals with IGT progress to Type II diabetes [17, 18, 19] an increase in systemic IL-6 concentrations seems to be associated with IGT rather than with incident Type II diabetes.

The concentrations of serum IL-6 were only slightly higher in Type II diabetes than in IGT, suggesting that there is little further increase of cytokine production towards the clinical manifestation of Type II diabetes. With the development of diabetic complications, however, a substantial rise of systemic IL-6 is seen [31, 32]. Hence, analyses of the association between cytokine concentrations and Type II diabetes have to cope with diabetic complications as a possible confounder. We did not analyse the possibly modulating effect of drug therapy on cytokine concentrations. Many of the treatments of features of the metabolic syndrome or of Type II diabetes bear some anti-inflammatory potential. Hence, an adjustment for drug therapy could augment the difference observed between control subjects and subjects with IGT or Type II diabetes.

We focussed on the parallel analysis of immune mediators which are known to constitute an immunoregulatory network in inflammation. IL-6 actions are strongly modulated by soluble IL-6 receptors (soluble form of IL-6Rα) which potentiate IL-6 bioactivity by mediating binding to cells which express the signal transducing β chain gp130 [33]. It is therefore important to assess both IL-6 and sIL-6 receptor concentrations. We find here that sIL-6 receptor concentrations are also increased in Type II diabetes and a similar trend is seen in IGT.

Major components of the IL-6 associated immune network are acute-phase proteins for which the chief inducer of production in hepatocytes is IL-6 [6]. We therefore analysed systemic concentrations of three major acute-phase proteins and found all of them increased in IGT and Type II diabetes. Individual concentrations of acute-phase proteins closely correlated with those of IL-6 and each other. This observation shows the biological relevance of mildly increased concentrations of IL-6 in IGT and Type II diabetes as these changes are accompanied by considerable increases of acute-phase protein production.

Another component of the inflammatory cytokine network is TNF-α whose activity in turn is regulated by the two soluble TNF receptor types both of which antagonise TNF-α bioactivity [6, 34]. We did not observe any increase of systemic TNF-α nor of sTNF-R concentrations in IGT, suggesting that TNF-α is not, or is less affected in these subjects. Hence, TNF-α does not seem to contribute to the rise of circulating IL-6 and acute-phase proteins in IGT. However, in overt Type II diabetes, there was an increase in median serum TNF-α concentration when compared with the IGT group. This increase seems counterbalanced by some increase of sTNF-R80 concentrations. The data do not confirm reports on increased systemic TNF-α concentrations in Type II diabetes compared with healthy control subjects [9, 10, 11, 36]. Factors accounting for this difference might be the smaller sample size of these studies, possible selection bias, or

the inclusion of patients with diabetic complications, because a rise in systemic TNF-α concentrations in the latter case is known [11, 37, 38].

The observation that IL-6 regulated immune mediators are increased not only in Type II diabetes but also in IGT clearly dissociates immune changes from overt hyperglycaemia and points to a contribution of IL-6 and acute-phase proteins to pathogenic events prior to the onset of Type II diabetes. These findings concur with recent reports of increased CRP concentrations in individuals with insulin resistance syndrome [39, 40] and of increased CRP and IL-6 in incident Type II diabetes [41].

The parallel analysis of many interconnected inflammatory mediators in our study indicates that there is a co-ordinated and enhanced production of some immune mediators in both Type II diabetes and IGT; i.e., neither did we find general up-regulation of all inflammatory mediators analysed, nor a random up-regulation of some mediators. Rather, there is co-ordinated overexpression of IL-6-dependent acute-phase proteins, whereas TNF-α and its two soluble receptor species are neither consistently up-regulated nor associated with IL-6 or CRP concentrations. Hence, there is a bias towards IL-6-dependent inflammatory-type changes. This concurs with reports showing that the cross talk between IL-6 and TNF-α is largely unidirectional in that TNF-α is a potent inducer of IL-6 but not vice versa [42].

Data from previous studies suggest a complex interaction between IL-6 and features of the metabolic syndrome. IL-6 could affect functions of lipid and muscle cells [43, 44, 45]. In contrast lipid tissue is a major source of IL-6 [46, 47] and possibly also of sIL-6R [48]. IL-6 is also produced by pancreatic beta cells [49]. Thus, it will be difficult to resolve this complex network of immune and metabolic mediators and to distinguish between primary and responding mediators, which can also vary depending on the genetic background. However, the fact that positive associations of systemic IL-6 and CRP concentrations persist after adjustment for BMI render a contribution of an IL-6 dependent innate immune response to the pathogenesis of Type II diabetes plausible and hence suggests a new target for approaches for disease prevention.

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