Elevated Levels of M-CSF, sCD14 and IL8 in Type 1 Gaucher Disease

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ABSTRACT: In type 1 Gaucher disease, decreased activity of glucocerebrosidase results in accumulation of glucosylceramide in macrophages. Infiltration of liver, spleen and bone marrow by lipid-laden macrophages leads to hepatosplenomegaly, bone lesions and cytopenia. These abnormal macrophages may produce and release macrophage derived factors and cytokines, which could contribute to the pathophysiology of the disease. Whether these cytokines and factors are elevated in Gaucher disease is currently unknown. In 29 type 1 Gaucher disease patients we measured serum levels of the macrophage derived cytokines IL8, IL6, TNF α , M-CSF and the monocyte/macrophage activation marker sCD14. These factors were studied in relation to disease severity and during treatment with enzyme supplementation therapy. Most patients showed remarkably elevated levels of M-CSF (2-8 fold) and sCD14 (2-5 fold) as compared to normal controls. Levels of IL8 were elevated in all patients (2-20 fold), whereas levels of IL6 and TNF α were normal. There was a significant correlation between severity of the disease as determined by the severity score index (SSI), and M-CSF, sCD14 and IL8 levels. M-CSF and sCD14 levels also correlated with the excess liver and spleen volumes. During treatment with alglucerase, levels of M-CSF and sCD14 declined, but IL8 remained unchanged. The relative reduction in excess liver and spleen volume did not correlate with the relative reduction in M-CSF or sCD14 levels. We conclude that serum levels of M-CSF, sCD14 and IL8 are increased in type 1 Gaucher disease. The biological activities of M-CSF and IL8 may add to the pathophysiology of the disease.

Keywords: Gaucher Disease, cytokines, macrophages, alglucerase, enzyme supplementation therapy

INTRODUCTION

Gaucher disease, the most common lysosomal storage disorder, is caused by a deficiency of the lysosomal enzyme glucocerebrosidase (1). Accumulation of glucocerebroside (glucosyl-ceramide), the substrate for this enzyme, occurs preferentially in macrophages. Massive infiltration of the bone marrow, spleen and liver with large numbers of characteristic lipid laden macrophages (Gaucher cells) can be found by histopathological examination (1). In type 1 Gaucher disease hepatospleno-

megaly, pancytopenia and bone involvement are the most common manifestations. The variability of the clinical spectrum of the disease is intriguing and not explained. Since the clinical symptoms of the disease are not strictly correlated to either properties of mutant glucocerebrosidase or genotype (1,2), epigenetic factors probably influence the disease manifestations. In addition, several clinical phenomena in Gaucher disease are poorly understood, such as hypermetabolism and wasting (3), and an increased incidence of monoclonal gammopathies, multiple myeloma and

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autantibodies (4-6). Many of these phenomena might be ascribed to activities of macrophage derived cytokines. A role for cytokines in the pathophysiology of the disease has been suggested previously (1,3,7). Indeed, *in vitro* stimulation of murine macrophages with glucosylceramide has revealed enhanced production of IL1 (8). Recently, elevated plasma levels of TNF α were reported in some patients with Gaucher disease (9). In another study, increased levels of IL6 and IL10, but not of TNF α and IL1, were established (10).

In the present study we investigated whether serum levels of macrophage derived cytokines (M-CSF, IL6, IL8, TNF α) and a macrophage marker protein, sCD14, were elevated in type 1 Gaucher disease patients. We studied whether these levels correlated with disease severity in Gaucher disease as determined by the Severity Score Index (SSI) (11) or spleen and liver volumes. Enzyme supplementation therapy with alglucerase (CeredaseTM, Genzyme, Boston MA) for type 1 Gaucher disease has shown to improve the common clinical manifestations of type 1 Gaucher disease, such as cytopenia and organomegaly. Levels of M-CSF, sCD14 and IL8 were also determined during treatment with enzyme supplementation therapy and studied in relation to clinical response.

PATIENTS AND METHODS

Patients

Serum and plasma samples of 29 adult type-1 Gaucher disease patients (15 males and 14 females; age 16-66 yrs) were obtained. All patients studied were referred to the Academic Medical Center for treatment with enzyme supplementation therapy. A diagnosis of Gaucher disease was confirmed on the basis of deficient glucocerebrosidase activity in leucocytes and/or urine samples (12,13). Table 1 summarizes the pre-treatment characteristics. In patients 1-4, no liver and spleen volumes were obtained. Thirteen patients had

undergone splenectomy. The assessment of the severity of the disease is performed by using the Severity Score Index (SSI), which is based upon the extent of cytopenia, organomegaly and bone infiltration (11). No patients had acute manifestations of the disease. Plasma samples were collected before the initiation of enzyme supplementation therapy and after 6 and 12 months of treatment.

Enzyme supplementation therapy with alglucerase was initiated in patients 1-4 using a dose of 4 U/kg three times a week (50 U/kg/month; n=4) and in patients 5-28 using a dose of 1.15 U/kg, three times a week (15 U/kg per month, n=24) according to the earlier described study protocol (14). One patient, a child, received an initial dose of 30 U/kg every two weeks (patient 29).

Measurement of Liver and Spleen Volumes

Liver and spleen volumes were measured by spiral computerized tomography before treatment and after 6 and 12 months of treatment. Conventional CT has an accuracy for liver and spleen volume measurements of 3-5% (15,16). The use of spiral CT allows measurement of 26 cm of the length of an organ within a single breath-hold, eliminating misregistration of adjacent scan slices (17). The excess liver and spleen volumes were calculated by the subtraction of the estimated normal volume (0.2% and 2.5% of bodyweight for spleen and liver, respectively) from the measured volumes. Reductions in excess volumes were calculated as percent reduction from the initial excess volume and corrected for a change in body weight (18).

Cytokine Assays

M-CSF. Gaucher patient serum samples were tested by radio-immuno assay, kindly provided by A. Creasy (Chiron Corporation, CA, USA). To avoid interassay variability, all longitudinally obtained patient samples, standards and controls

Table 1. Characteristics of the patients

Patient	Sex nr	Age yrs	SSI	Liver volume	Spleen volume	Liver & Spleen Excessvolume	M-CSF ng/ml	sCD14 U/ml	IL8 pg/ml
1	M	43	19	.	.	.	21,6	361	.
2	M	42	14	.	.	.	19,7	461	20
3	F	41	7	.	.	.	15,9	292	27
4	M	29	10	.	.	.	14,8	560	44
5	F	36	19	6220	.	5020	23,5	737	203
6	M	35	14	2257	.	532	10,6	396	59
7	F	53	3	1528	470	336	8,6	219	29
8	M	28	16	6542		4817	17,6	495	100
9	M	48	14	3195	.	1020	11,7	279	93
10	F	39	5	1873	.	323	10,5	141	26
11	M	43	15	3828	.	2178	19,1	214	105
12	F	31	15	4451	4821	7787	20,9	537	38
13	F	51	7	4201	4526	6027	18,3	262	63
14	F	66	12	1504	.	0	17,8	360	70
15	F	36	11	3451	.	2076	15,8	534	244
16	F	43	12	3036	732	1878	18,8	258	111
17	M	52	15	3424	.	1774	14,6	537	.
18	M	41	9	3351	2092	3364	13,8	459	26
19	F	47	8	3139	1042	2156	14,9	222	36
20	M	23	6	2911	3354	3835	18,6	214	49
21	M	29	7	3695	1400	3151	16,1	236	42
22	F	45	8	2387	1905	2456	10,3	353	22
23	M	51	17	2393	.	693	18,8	380	70
24	M	30	17	3691	.	2516	16,3	414	114
25	F	63	8	2241	2267	2861	.	.	38
26	F	33	11	4914	.	3514	12,6	479	83
27	M	47	7	3334	3118	4427	14,4	.	31
28	M	37	5	1830	574	404	11,6	96	47
29	F	15	7	1597	864	1111	12,5	189	29

were assayed simultaneously. Patient samples were diluted 1:40 in PBS, 0.02% NaN_3 . ^{125}I E.coli CSF-150 was diluted to approximately 200,000 cpm/ml in assay diluent (PBS, containing 0.5% BSA, 0.02 % sodium azide, pH 7.4) and 100 μl was added to 100 μl of sample. After the addition of 100 μl 1:20,000 diluted antiserum (rabbit anti CVCSF), samples were incubated for 18-24 hrs at 4 °C. Precipitation was performed by adding 500 μl of goat-anti-rabbit IgG in 7.38% PEG to each sample. After incubation for 1 hr at room temperature, samples were centrifuged for 20 min at 3000 g at 2-8 °C. Supernatant was removed immediately and pellets were counted in a Gamma counter for 1 min. Sample concentrations were computed from a point-to-point analysis of the standards and values were expressed in ng CSF per ml.

Interleukin 6, Interleukin 8 and Tumor Necrosis Factor α . Levels of Interleukin 6, Interleukin 8 and Tumor Necrosis Factor α were measured by ELISA, using commercially available kits (Central Laboratory of the Netherlands Red Cross Blood Transfusion Services; The Netherlands).

Soluble CD14. s-CD14 levels were determined by enzyme linked immunosorbent assay (sandwich-ELISA) using monoclonal antibodies directed against non-overlapping epitopes on CD14. MoAb 8G3 (IgG2a) and KL77 (IgG2a) were purified from ascitic fluid by protein A affinity chromatography (protein A sepharose CL-4B, Sigma). MoAb KL77 was biotin labelled using standard procedures. The Elisa was performed in 96 wells plates (Maxisorb, Nunc, Denmark) precoated with 100 μl MoAb 8G3 (5 $\mu\text{g}/\text{ml}$) in PBS pH 7.4. Non specific binding sites were blocked by incubation for 1 hr at room temperature with PTG (PBS/0.02% Tween/0.4% gelatin). The wells were washed with PBS/0.05% Tween-20 and incubated 1 hr at 4 °C with 100 μl sample of human serum diluted in Tris-buffer (pH 8.0). After washing, the wells were incubated with 100 μl of

biotinylated MoAb KL77 (5-10 $\mu\text{g}/\text{ml}$) for 1 hr at 4 °C and subsequently, a 1:1000 dilution of streptavidine- peroxidase (100 ng/ml) (Sigma) was added and incubated for 1 hr at 4 °C. The final washing step was followed by addition of 100 μl of substrate containing 0.4 mg/ml O-phenylenediaminedihydrochloride (Sigma) in 0.05 M phosphate-citrate buffer (pH 5.0), containing 7 μl 30 % H_2O_2 . After 20 min., the peroxidase reaction was stopped by adding 4N H_2SO_4 (50 $\mu\text{l}/\text{well}$). The absorbance was measured at 492 nm by a Titertek Multiskan ELISA reader (Flow laboratories).

Controls

Levels of M-CSF, IL6, IL8, $\text{TNF}\alpha$ and sCD14 were determined in healthy controls, usually hospital staff members. These samples had served earlier as controls for the different assays.

Statistics

The results are presented as median and range. Differences between data from patients and controls were tested by the Mann-Whitney test. Differences between data obtained before and after alglucerase treatment were tested by the Wilcoxon signed rank test. Correlations were calculated by using the Spearman rank correlation coefficient. A p-value of < 0.05 was considered to represent a significant difference.

RESULTS

SSI, (Excess) Liver and Spleen Volumes and Levels of M-CSF, sCD14, IL8, IL6 and $\text{TNF}\alpha$ Before Treatment

Table 1 shows the SSI, liver and spleen volumes, excess liver and spleen volumes and levels of M-CSF, sCD14 and IL8 before treatment.

Elevated serum levels of M-CSF were found in 24 of 28 Gaucher disease patients (median 15.8

ng/ml, range 8.6–23.5 ng/ml) as compared to normal controls ($n=17$, normal range ($\text{mean} \pm 2\text{SD}$): 1.25–11.25 ng/ml; $p < 0.001$). In 25 of 27 Gaucher disease patients, increased levels of sCD14 were established (median 360 U/ml, range 96–737 U/ml), as compared to the normal controls ($n=52$, normal range ($\text{mean} \pm 2\text{SD}$): 45–153 U/ml; $p < 0.0005$). Although patients with the highest or the lowest levels of M-CSF generally also had the highest or the lowest levels of sCD14, no correlation between these factors was established ($\rho=0.30$; $p=0.1$).

Levels of IL8 were elevated in 25 of 27 patients (median 47 pg/ml, range 20–244 pg/ml) as compared to 38 controls (normal range ($\text{mean} \pm 2\text{SD}$): <25 pg/ml; $p < 0.0005$). There was a weak correlation with both M-CSF and sCD14 levels ($\rho=0.40$; $p=0.05$ and $\rho=0.49$; $p=0.01$ resp.)

Levels of IL6 were all within the normal range (median 0.75 pg/ml, range 0–12.6 pg/ml; normal levels <20 pg/ml). The same was found for levels of TNF α (median 0 pg/ml, range 0–5 pg/ml; normal levels <10 pg/ml). No correlation between the levels of IL6 and TNF was found and no correlation with the other factors existed.

Relation Between SSI, Excess Liver and Spleen Volumes and M-CSF, sCD14 and IL8

The levels of M-CSF, sCD14 and IL8 correlated with disease severity as assessed by the SSI ($\rho=0.59$, $p=0.002$; $\rho=0.65$, $p=0.001$ and $\rho=0.58$, $p=0.003$ resp.; figure 1). Both levels of M-CSF and sCD14 correlated with the excess liver and spleen volumes ($\rho=0.60$, $p=0.005$ and $\rho=0.49$, $p=0.02$; figure 2), but levels of IL8 did not correlate with excess liver and spleen volume ($\rho=0.16$, $p=0.5$). IL8 levels were highly variable. Two patients with extreme elevations were noted (patients 5 and 15, table 1). Patient 5 is a very seriously affected female with massive hepatomegaly and severe bone disease

with extensive marrow infiltration leading to transfusion dependent anemia. This patient had no bone crisis or infection at the time the samples were taken. Levels of M-CSF and sCD14 were also extremely high in this patient. Patient 15 is a female, who just recovered from a severe bone crisis. There seems to be a slight discrepancy between the extremely high IL8 level and the M-CSF and sCD14 levels. However, during follow-up, the patient recovered fully, but IL8 levels remained elevated (figure 3). Interestingly, IL8 levels were clearly much higher in splenectomized patients than in non-splenectomized patients (mean 106.1 ± 63.6 pg/ml, $n=11$ and 40.8 ± 21.8 pg/ml, $n=16$, respectively; $p < 0.0001$). Although there was a trend indicating that levels of sCD14 were higher in splenectomized patients as compared with non-splenectomized patients (mean 410 U/ml, $n=13$ and 311 U/ml, $n=14$, respectively), this difference was not significant ($p=0.09$). Levels of M-CSF were almost equal in splenectomized and non-splenectomized patients (mean 16.19 ± 4.1 ng/ml, $n=13$ and 15.28 ± 3.58 ng/ml, $n=15$, respectively).

Effect of Enzyme Supplementation Therapy on the Levels of M-CSF, sCD14 and IL8

Both M-CSF and sCD14 levels and excess liver and spleen volumes declined significantly during enzyme supplementation therapy (0–6 and 0–12 months: $p < 0.0005$ for M-CSF, sCD14 and excess liver and spleen volumes; figure 3). IL8 levels fluctuated slightly during treatment with alglucerase, but did not decrease significantly (figure 4). The absolute and relative (percent) reductions of excess liver and spleen volumes and the reductions in M-CSF and sCD14 levels were calculated between 0 and 6 months and between 0 and 12 months of treatment. Only a weak correlation was found between the percentage reduction in liver and spleen volume between 0 and 6 months and the percentage decrease in sCD14 level ($\rho=0.43$;

p=0.05; figure 5). This correlation was not found for M-CSF ($\rho=0.29$, $p=0.2$). Between 0 and 12 months, no correlation of the absolute and percent reductions in both M-CSF and sCD14 levels with the reduction in excess liver

and spleen volumes was apparent. The relative decreases in M-CSF and sCD14 showed no significant correlation ($\rho=0.32$, $p=0.1$ between 0 and 6 months; $\rho=0.34$, $p=0.1$ between 0 and 12 months).

Figure 1. Levels of M-CSF, sCD14 and IL8 in relation to SSI

Figure 2. Correlation between levels of M-CSF, sCD14 and excess liver and spleen volumes in type 1 Gaucher disease patients.

Figure 3. IL-8 levels during alglucerase treatment in patient 15.

Figure 4. Reduction in levels (percentiles) of M-CSF, sCD14 and excess liver and spleen volumes in type 1 Gaucher disease patients upon treatment with alglucerase.

Figure 5. Correlation between percentage reduction in sCD14 and excess liver and spleen volume between 0 and 6 months of alglucerase treatment.

DISCUSSION

Among clinical and fundamental researchers of Gaucher disease, it is generally believed that cytokines secreted by the lipid-laden macrophages may play a role in the pathophysiology of the disease (1). Our investigation demonstrates the occurrence of increased plasma levels of two cytokines, M-CSF and IL8 as well as of a monocyte/macrophage activation marker, sCD14. On the contrary, levels of IL6 and TNF α are normal. The levels of M-CSF, sCD14 and IL8 correlate with the severity of the disease as estimated by the SSI. In addition, levels of M-CSF and sCD14 correlate with excess liver and spleen volumes and decline during enzyme supplementation therapy, whereas IL8 remains elevated.

Macrophages have a significant role in the production of cytokines involved in immunological responses and inflammation, such as IL1, IL6, IL8, TNF α , GM-CSF and M-CSF (19). sCD14 is derived from activated monocytes and macrophages (20,21). It is tempting to speculate that the lipid-laden Gaucher macrophages are activated to directly produce and release these factors. The correlation of M-CSF, sCD14 and IL8 levels with disease manifestations suggests this. Further sup-

port is found in the fact that *in vitro* activation with LPS of murine macrophages, pre-incubated with glucosylceramide, are capable of enhanced IL1 production (8). However, it should be stressed that sCD14 can also be released by monocytes and IL8 and M-CSF can be produced by many other cell-types. If the Gaucher macrophages are indeed the source of these factors, their levels may both reflect the enormous mass of macrophages and increased activation of macrophages. In support of this “activation hypothesis”, elevated levels of sCD14 and IL8 are found in association with diseases in which monocyte/macrophage activation is present. For example, *in vivo* studies have shown that elevated levels of sCD14 are present in sepsis, malignancies and sarcoidosis (22–24). CD14 is a by PI-linkage membrane-bound myeloid differentiation antigen, present on monocytes and macrophages (25). Both the membrane bound CD14 molecule and sCD14 are capable of binding LPS and are involved in phagocytic activity (26). Production of sCD14, or the expression of CD14 on the cell surface and subsequent release from the cell membrane, occurs also upon induction of phagocytosis. Elevated levels of interleukin 8 are also found in systemic inflammatory

diseases such as sepsis (27). Interleukin 8 is a cytokine, produced by monocytes, macrophages, endothelial cells, hepatocytes and other cell-types which has specific chemotactic and activating effects on neutrophils (28). Mononuclear phagocytes express IL8 in response to stimulation with TNF α or IL1 (29) and after phagocytosis (30). It is therefore also possible that the extreme phagocytosis of glycolipids in Gaucher disease triggers the release of IL8 and sCD14.

M-CSF, a growth factor that stimulates the proliferation and stimulation of cells of the monocyte-macrophage lineage (31), is secreted by monocytes and macrophages in response to activating agents such as TNF α , IFN γ , GM-CSF and other factors [for review see (32)]. Elevated levels have been reported in immune thrombocytopenic purpura (33).

The effects of treatment with alglucerase on the levels of M-CSF, sCD14 and IL8 are of interest. Levels of M-CSF and sCD14 decrease significantly upon treatment with alglucerase. The decrease in M-CSF and sCD14 could reflect a reduction in Gaucher cell mass. However, no correlation is established between reduction in M-CSF and sCD14 levels and reduction in liver and spleen size. Several factors can contribute to this observation: first, the supplementation of Gaucher cells with glucocerebrosidase might primarily affect their state of activation and capacity to release M-CSF and sCD14; second, reduction in organomegaly may not reflect the decrease in Gaucher cell mass, since an important part of the volume is taken up by fibrosis and altered vascularization, and because changes in other storage sites (e.g. the bone marrow) are not considered; third, M-CSF is cleared principally by binding to its receptor on macrophages (32) and a decrease in macrophage mass after treatment could reduce its clearance, resulting in less decline in plasma levels. Because a clear relation with reduction in organomegaly can not be established, caution has to be taken to judge an improvement in Gaucher disease manifestations on the basis of a decline in these factors.

Interestingly, levels of IL 8 do not change in response to therapy. Since IL8 binds to the Duffy antigen present on erythrocytes, it is possible that IL8 is excessively cleared by an enlarged spleen (34). This is in line with the observation that IL8 was significantly higher in splenectomized patients as compared with non-splenectomized patients. A compensatory reduction in clearance by a smaller spleen could explain the absence of a reduction upon treatment. However, this is not supported by the finding that IL8 levels did not decrease in splenectomized patients either.

Although the presence of elevated levels of M-CSF, sCD14 and IL8 suggests the presence of macrophage activation, other cytokines that are released during an inflammatory response, such as IL6 and TNF are not found to be elevated in this study. The fact that the plasma levels of IL6 and TNF α are normal does not exclude the possibility that concentrations of these cytokines in the vicinity of the Gaucher macrophages are abnormally high. In this respect, it is of interest to mention that elevated plasma levels of TNF α have been found in the more severe type 2 and 3 forms of Gaucher disease and to a lesser extent in some type 1 Gaucher disease patients (9). In another recent study no elevations in TNF α concentrations in type 1 disease could be established (10). These researchers also measured levels of IL1 β , IL6 and IL10 and found that the latter two were slightly elevated in most patients. Although inter-assay variabilities may have played a role in the different results from these studies, it is also possible that the cytokine levels are related to the severity of the disease, which may have been different in the various study populations. The clinical relevance of the finding that macrophage derived factors are increased in type 1 Gaucher disease is an important issue to be addressed. The abnormally elevated levels of IL 6 that were found in the study by Allen and co-workers were studied in relation to the prevalence of monoclonal or biclonal gammopathies (10). It was found that the higher IL 6

concentrations indeed occurred in the patients showing a clonal expansion of B-cells. Since IL6 is a well known potent stimulator of B-cells [see (35)], it is possible that the increased incidence of autoantibodies, monoclonal gammopathies and multiple myeloma in Gaucher disease (4-6) might result from stimulation of B-cells by IL6. M-CSF may enhance the apparent proliferation of macrophages in Gaucher disease (31). The presence of osteopenia (7) and increased bone resorption (36) in Gaucher disease could also be influenced by M-CSF, since it has been observed that rhMCSF promotes bone resorption (37). In our study, M-CSF levels were indeed particularly high in patients with extensive bone disease (data not shown), but since these patients are usually the most severely affected patients, the elevated levels could also be a reflection of more severe disease. IL8 may be associated with activation of coagulation: *in vitro* coagulation activation in a whole blood culture system induced significant IL8 production in mononuclear cells (38). We recently established that low grade activation of coagulation appears to be present in Gaucher disease (39). The observation that neutrophil chemotaxis is impaired in Gaucher patients contrasts with the expected biological effects of IL8 (40). Another feature of Gaucher disease is hypermetabolism and increased glucose production (3). The hypermetabolic state of the patients, resulting in wasting and growth retardation, may be explained by the involvement of cytokines (41,42). We have recently observed that enzyme supplementation therapy partly restores the hypermetabolism (43).

Further research will be needed to establish the precise cellular source of M-CSF, sCD14 and IL8 and to investigate the presence of increased production of other cytokines such as IL6 and TNF α . In principle the factors could originate from the mature storage cells, but they might also be produced by activated monocytes in various stages of their maturation into Gaucher macro-

phages or even by other cell types. In situ hybridization techniques could be used to address these questions.

In conclusion, abnormally elevated plasma levels of macrophage derived factors are demonstrable in type 1 Gaucher disease, which might contribute to several aspects of the disease. However, the mechanisms involved in the production and release of these factors are as yet unknown. More detailed studies on the role of cytokines and cellular activation processes in the pathophysiology of Gaucher disease seems warranted for a better understanding of the complex clinical manifestations of the disorder.

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