

# Cytokine mRNA in Gaucher Disease

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**ABSTRACT:** Gaucher disease, the most common glycolipid storage disease, is caused by glucocerebrosidase deficiency, resulting in accumulation of glucocerebrosides within the macrophages of the reticuloendothelial system. The disease is characterized by great phenotypic heterogeneity, which can be explained only in part by the various mutations in the glucocerebrosidase gene, and by the amount of storage material in affected organs and tissues. Therefore, it has been postulated that some of the biochemical and clinical features may be related to the fact that "Gaucher" cells, as activated macrophages, express and release cytokines such as IL-1 $\beta$ , IL-8, IL-6 and TNF- $\alpha$  which play a role in different physiological processes. In the present study, cytokine mRNA expression was measured in monocytes isolated from Gaucher patients and from healthy controls, using RT-PCR methodology with semiquantitative analysis. We found significantly increased expression of IL-1 $\beta$  mRNA, as well as a trend to elevated TNF- $\alpha$  mRNA in Gaucher patients relative to healthy individuals. There were no statistically significant differences between Gaucher disease patients and controls with respect to two other tested cytokines (IL-6 and IL-8).

**Keywords:** Gaucher disease, glucocerebroside, glucocerebrosidase, cytokine, TNF $\alpha$ , IL-1 $\beta$ , monocytes/macrophages, polymerase chain reaction, mRNA expression

## INTRODUCTION

Gaucher disease, inherited as an autosomal recessive trait, is the most prevalent sphingolipid storage disorder, with a particularly high incidence among Ashkenazi Jews (1). The reduced glucocerebrosidase activity is due to different mutations within the gene encoding the lysosomal enzyme glucocerebrosidase, and results in glucocerebroside accumulation within the lysosomes of the phagocytic cells of the reticuloendothelial system. The storage process produces a multi-system disease that clinically manifests as progressive hepatosplenomegaly, anemia, thrombocytopenia, skeletal deterioration,

and less commonly, in neurological abnormalities. However, the molecular and the biochemical mechanisms underlying these defects in Gaucher disease remain to be elucidated.

We investigated the hypothesis that in Gaucher disease the accumulation of glucocerebroside maintains the macrophages or their cellular precursors, the monocytes, in a unique state of activation. Activated macrophages express and release various characteristic cytokines such as IL-1 $\beta$ , IL-8, IL-6 and TNF- $\alpha$  (2). These cytokines may influence tissues and cells not directly associated with accumulation of glucocerebroside, thus explaining various processes such as abnormal neutrophil

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chemotaxis (3) or increased incidence of hypergammaglobulinemia (4,5) and multiple myeloma among Gaucher patients (6). To study cytokine expression, the conjunctural cell sources should be measured. Since macrophages are inaccessible, their precursor monocytes, which are present in the blood circulation, were used for these studies. Reverse transcriptase - polymerase chain reaction (RT-PCR) technique was utilized to compare cytokine mRNA expression in Gaucher patients to that of healthy volunteers.

## MATERIALS AND METHODS

### *Patients*

Eighteen adult Ashkenazi Jewish patients with type I Gaucher disease were selected for this study and 19 healthy Ashkenazi Jews served as controls. The diagnosis of Gaucher disease was confirmed in all patients by low activity of leukocyte  $\beta$ -glucosidase, as well as by mutation analysis. Severity of the disease was assessed by a severity score index (SSI), which includes age at presentation, degree of organomegaly, hematological parameters, liver function tests and severity of bone involvement (7). The latter component was separately used as a "bone score" for comparing the results of cytokine expression with skeletal involvement. The range for the bone score was 0 (no clinical and radiological evidence of bone involvement) to 9 (severe skeletal involvement, including avascular necrosis of large joints). The study was approved by the Shaare-Zedek Helsinki Committee, and all subjects signed informed consent forms.

### *Monocyte Isolation*

Peripheral blood mononuclear cells (PBM) were separated from heparinized blood by standard Ficoll-Paque density gradient centrifugation (Pharmacia). The mononuclear cells were suspended in RPMI - 1640 media (Biological Industries, Beit Haemek, Israel) supplemented with 2% heat inactivated human

AB serum (Sigma) at a concentration of  $4 \times 10^6$  cells per ml, cultured in petri dishes and incubated for 90 minutes at 37 C in the presence of 5% CO<sub>2</sub>. Non-adherent cells (90% of the original mononuclear cells) were removed by aspiration and the plate was washed 3 times with PBS. The adherent cells (monocytes) were cultured for 4 hours in the same media in the presence of 2 mg/ml of lipopolysaccharide (LPS). The adherent cell population contained > 85% monocytes, as verified by morphological determination (Wright's stain) and by cytometry (FACSscan; Becton and Dickinson).

### *Isolation of Cellular RNA*

Total cellular RNA was extracted from the adherent monocytes with TRI-REAGENT (Molecular Research Center, Inc) according to the manufacturer's instructions, and stored at -20 C. The integrity of all RNA samples was determined by electrophoresis through 1.2% agarose gel.

### *Reverse Transcription*

The obtained RNA was reverse transcribed with 100 U of Moloney murine leukemia virus reverse transcriptase (GibcoBRL) in a total volume of 30  $\mu$ l containing 1  $\mu$ g of oligo (dT)<sub>15</sub> primer (Promega), 40 U of RNasin (Promega), 0.25 mM of each dNTP (Boehringer), 25 mM Tris-HCL, pH 8.3, 37.5 mM KCL, 1.5 mM MgCl<sub>2</sub> and 5 mM dithiothreitol (DTT) at 42 C for 1 hour.

### *Primer Selection*

Oligonucleotide primer pairs for each gene amplification were designed based on published cDNA sequences. The primer sequences were chosen from two exons of the studied genes in order to differentiate cDNA amplification products from any contaminating genomic DNA products. The following are the sequences of the oligonucleotides used:

**Human IL1- $\beta$  (249 bp product):**

Sense primer:

5'- GGCAGACTCAAATTCAGCT-3',

antisense primer:

5'-GGACAGGATATGGAGCAAC A-3',

**Human IL-8 (289 bp product):**

sense primer:

5'- ATGACTTCCAAGCTGGCCGTG-3',

antisense primer:

5'-TCTCAGCCCTCTTCAAAAACTTCTC-3',

**Human TNF- $\alpha$  (355 bp product):**

sense primer:

5'- CGGGACGTGGAGCTGGCCGAGGAG-3',

antisense primer:

5'-CACCAGCTGGTTATCTCTCAGCTC-3',

**Human IL-6 (628 bp product):**

sense primer:

5'- ATGAACTCTTCTCCACAAGCGC-3',

antisense primer:

5'-GAAGAGCCCTCAGGCTGGACTG-3'.

**The primer sequences for the human (constitutively expressed) ribosomal L19 gene (194 bp product) were:**

sense primer:

5'-CTGAAGGTGAAGGGGAATGTG-3',

antisense primer:

5'-GGATAAAGTCTTGATGATCTC-3'.

**Polymerase Chain Reaction (PCR)**

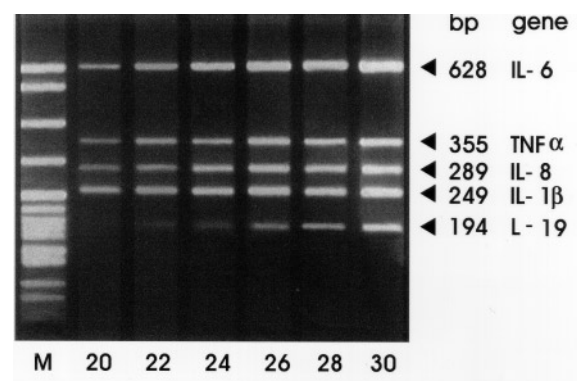
To determine the four cytokine mRNA levels simultaneously, a specific amount of each cDNA sample (as a template) was separately amplified with each pair of primers: 10  $\mu$ l for IL-6, 10  $\mu$ l for TNF- $\alpha$ , 5  $\mu$ l for L19, 2  $\mu$ l for IL-1 $\beta$  and 2  $\mu$ l for IL-8, according to their individual gene expression frequencies, as was determined by preliminary studies conducted in our experimental system. Each PCR was performed in a 50  $\mu$ l (final volume) using standard PCR technique. The amplification cycle (1 min at 95 C, 1 min at 60 C, and 2 min at 72 C) was carried out in a DNA Thermal Cycler (Perkin Elmer Cetus). Amplified PCR products were sampled (5  $\mu$ l of each PCR mixture) after 20, 22, 24, 26, 28 and 30 cycles.

**mRNA Expression Estimation**

The PCR products were electrophoresed through 2.5% agarose gel. Following ethidium bromide staining, the intensity of the DNA fragments was densitometrically scanned by the Fugi-PCBAS Biological Analysis Software. Reaction cycle-intensity curves of each tested cytokine as well as for the ribosomal L19 gene (used as an internal control) were plotted. Only the reaction cycles, which did not plateau, were used in the analysis to ensure quantitative evaluation of the PCR. To determine the relative abundance of the multiple cytokine mRNAs among the individual samples, the ratio of each cytokine to L19 was averaged over a range of cycles in the logarithmic phase of amplification. The majority of the samples were analyzed at least twice.

**Statistical Methods**

Analysis of variance statistical software was used to assess significance of the observed differences between the two examined groups (Gaucher patients and healthy controls). The differences were considered significant when the probability (*p*) value was < 0.05.



**Figure 1.** Cytokine gene mRNA expression measured by RT-PCR analysis. cDNA synthesized from total RNA isolated from Gaucher monocytes incubated for 4 hours with 2 mg/ml LPS, was analyzed by PCR, in the presence of oligonucleotide primers for IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  or L19 as control. After 20, 22, 24, 26, 28, 30 cycles, amplified PCR products were run through 2.5% agarose gel, and visualized under UV light after ethidium bromide staining. bp = base pairs, M = DNA marker (PBR-MspI).

## RESULTS

The results of the cytokine mRNA expression obtained in both the Gaucher patients and the controls are presented in tables 1-3. A representative gel is shown in figure 1. The PCR analysis demonstrated that the transcription level of IL1- $\beta$  gene is increased in the monocytes of the Gaucher patient group. The difference in IL-1 $\beta$  mRNA abundance in Gaucher patients was statistically significant ( $p=0.03$ ). The mean value for Gaucher patients was 1.4 fold higher than that obtained for non-Gaucher patients. In the case of TNF- $\alpha$ , a slight trend toward increased mRNA in

the Gaucher patient group was detected: the mean value of the Gaucher patient group was 1.7 fold higher than in the controls ( $p=0.07$ ). IL-6 and IL-8 mRNA levels were not statistically different between Gaucher patients and controls; however, the mean values of the patients were higher.

We did not find a correlation between any of the cytokine levels and the status of spleen (intact or splenectomized) (Table 4) or the SSI (data not shown), yet relative to the small sample number, there was a trend to correlation between the bone score and TNF $\alpha$  ( $r=0.38$ ;  $p=0.1$ ) and IL1 $\beta$  ( $r=0.41$ ;  $p=0.087$ ).

**Table 1.** Individual results of LPS inducible effects on monocyte cytokines mRNA expression of 18 Gaucher patients and 19 healthy individuals (controls).

	GAUCHER PATIENTS				CONTROLS				
	IL-1 $\beta$	IL-8	TNF- $\alpha$	IL-6	IL-1 $\beta$	IL-8	TNF- $\alpha$	IL-6	
1	8.37	0.3	2.81	4.38	1	5.85	0	1	1.21
2	3.6	0	1.74	2.64	2	6.0	1.2	5.36	4.2
3	4.5	1.33	2.4	3.43	3	7.0	0	0.47	0
4	8.2	3	2.85	4.27	4	4.14	0.62	1.59	2.75
5	4.18	0.67	1.48	2.3	5	4.9	2	1.4	3.36
6	6.39	0.66	1.11	3.3	6	7.1	0.55	1.04	2.25
7	6.75	1.23	1.72	5.8	7	6.53	0.81	1.7	5.1
8	10.2	0.37	5.8	9.13	8	2.23	0	5.57	4.6
9	3.16	1.12	1.6	3.47	9	4.7	1.69	3.66	4.5
10	11.4	1.39	3.98	10.5	10	2.25	1.32	1.31	1.61
11	6.3	0.84	3.4	4.43	11	3.17	1.49	0.15	6.75
12	2.58	0.04	0.73	0.41	12	2.75	1.1	1.46	2.25
13	12.3	3.7	13.7	4.7	13	3.45	0.15	0.94	1.3
14	4.8	0.63	2.8	2.63	14	5.8	2.6	1.0	3.6
15	5.8	0	2.53	2.83	15	3.87	0.82	1.77	6.8
16	5.52	3.49	4.49	6.87	16	2.43	2.91	1.6	5.9
17	3.9	1.45	4.72	7.39	17	3.26	0.17	1.5	6.39
18	3.05	0.25	3.8	8.0	18	6.52	0	2.8	2.51
					19	2.5	0	3.65	4.7

The IL1- $\beta$ , IL-8, TNF- $\alpha$  and IL-6 gene expression of 37 individuals (Gaucher patients and the control group) are expressed as a ratio of the cytokine to L19 mRNA levels, as a result of four hours incubation of the monocytes with LPS. Values represent the average of at least two separate experiments carried out on each of the individuals.

**Table 2.** Expression of cytokine mRNA in LPS treated monocytes of Gaucher patients and healthy volunteers.

	IL-1 $\beta$ /L19		TNF $\alpha$ /L19		IL-6/L19		IL-8/L19	
	G	C	G	C	G	C	G	C
Range	2.58-12.3	2.23-7.1	0.73-13.7	0.15-5.57	0.41-10.5	0-6.8	0-3.7	0-2.915
Mean	6.174	4.445	3.426	2	4.805	3.695	1.138	0.918
Std. Dev	2.914	1.726	2.899	1.5	2.642	1.998	1.149	0.907
n	18	19	18	19	18	19	18	19
p	0.0337		0.0684		0.1572		0.522	

A summary and comparison of the cytokine expression genes results between the two tested groups

G = Gaucher patient group, C = Healthy-control group, Std. Dev = standard deviation, n = patient number, p = probability

**Table 3.** The relative expression of cytokine mRNA in monocytes of the Gaucher patients group compared to the healthy control group.

IL-1 $\beta$ /L19	TNF $\alpha$ /L19	IL-6/L19	IL-8/L19
1.39	1.7	1.3	1.23

Values represent the ratio of Gaucher patient group mean values to the healthy control group mean values for each of the studied cytokine.

**Table 4.** Correlation between cytokine mRNA levels and spleen status among the Gaucher patients.

	IL-1 $\beta$ /L19		TNF $\alpha$ /L19		IL-6/L19		IL-8/L19	
	SPX	IS	SPX	IS	SPX	IS	SPX	IS
	n = 7	n = 11	n = 7	n = 11	n = 7	n = 11	n = 7	n = 11
Mean	6.731	5.807	3.723	3.091	3.775	5.144	1.6029	0.841
Std. Dev	2.843	3.017	4.449	1.752	1.227	3.695	1.243	1.034
Std. Err	1.075	0.910	1.682	0.528	2.642	3.498	0.470	0.312
p	0.527		0.675		0.256		0.177	

SPX = status post splenectomy

IS = intact spleen

Std. Dev = standard deviation

Std. Err = standard error of mean

n = number of patients

p = probability

## DISCUSSION

Since many of the common features of Gaucher disease cannot be explained solely by the undegraded glucocerebroside mass content in the macrophages of the involved tissues, efforts were made to study secondary biochemical abnormalities which may be contributory. These studies revealed elevated plasma levels of certain enzymes including acid phosphatase (8), lysosomal hydrolases (9) and chitotriosidase (10). Other observations such as abnormal neutrophil

chemotaxis (3), hyper gammaglobulinemia (4,5) and monoclonal gammopathies (11,12) were reported. It was also suggested that Gaucher patients have an increased risk for hematological malignancies including chronic lymphocytic leukemia, multiple myeloma and others (13). However, the relationship between these abnormalities and the pathophysiology of the disease remains unclear. It has been suggested that the excess glucocerebroside accumulation may cause a chronic stimulation of the immune system through macrophage activation (14).

Recently, there have been a few reports regarding elevated serum levels of cytokines in Gaucher patients. Allen et al. found elevated concentrations of IL-6 and IL-10, without significant increases in TNF $\alpha$  or IL-1 $\beta$  (15). However, Hollak et al. reported elevated levels of the monocyte/macrophage activation marker sCD14, IL-8 and M-CSF in Gaucher patients, whereas levels of IL-6 and TNF- $\alpha$  were in normal range (16). These results, although contradictory, support the hypothesis of activation state of Gaucher macrophages.

Unlike cytokine measurements in the serum, our study was focused on detection of cytokine gene expression by specific cells (monocytes). Cytokine genes are generally expressed at low levels and their mRNA has a short half-life (17,18). Therefore, we treated the monocytes with LPS - a potent activator of both monocytes and macrophages - in order to elevate the low expression level of the tested cytokines. In addition, we used a quantitative RT-PCR methodology (19,20).

Of interest is our finding of a significant increase of IL-1 $\beta$  mRNA in Gaucher patient monocytes. These results are supported by the previous observation concerning LAF (Lymphocyte Activating Factor, today designated IL-1) secretion from normal circulating monocytes treated by glucocerebroside (21). While we did not find a correlation between IL-1 $\beta$  levels and severity of clinical manifestations as assessed by SSI, there were indications of relation between the degree of bone involvement and the mRNA levels of TNF- $\alpha$  and IL-1 $\beta$ .

IL-1 was the first cytokine identified for its powerful stimulatory effect of osteoclastic bone resorption (22), and hence was implicated in the pathophysiology of osteoporosis, multiple myeloma and other diseases (23,24). It is possible therefore that the elevated IL-1 $\beta$  mRNA levels in Gaucher patients may be relevant to the skeletal abnormalities associated with the disease.

Since IL-1 $\beta$  also plays an important role in

the stimulation of the immune response and is involved in normal cellular processes such as proliferation and differentiation (25), imbalance in production and activation of IL-1 $\beta$  precipitation may contribute to the development of hematological abnormalities often seen in Gaucher disease.

We observed a trend toward increased TNF- $\alpha$  mRNA in the Gaucher patients group relative to controls. TNF- $\alpha$  shares several biological properties with IL-1 $\beta$  and also acts synergistically with the latter cytokine (25-27).

Our data suggest that in Gaucher monocytes there are higher levels of IL-1 $\beta$  and TNF- $\alpha$  mRNA transcripts. However, transcription levels do not necessarily correspond with higher levels of protein (28). The relationship between mRNA expression and the biologically active cytokine secreted by cells is dependent on other factors, including mRNA stability, maturation, transport, and rate of release from cells.

Increasing the sample size of the studied groups and combining the mRNA testing with the serum cytokine levels, should allow better definition of the relations between cytokine mRNA levels and their translation and secretion in Gaucher disease. Similarly, studying cytokine mRNA and protein levels of untreated Gaucher patients and patients treated with enzyme replacement may help explain the relevance of our findings to the improvement in some of the clinical manifestations with treatment.

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## REFERENCES

1. Beutler E, Grabowski GA. Glucosylceramide lipidoses. In: Scriver CR, Beaudet AL, Sly WS et al. (eds). *The Metabolic Basis of Inherited Diseases*. 7th ed. McGraw-Hill. New York, 1995.
2. Janeway CAJR, Travers P. In: *Immunobiology: The Immune System in Health and Disease*. Second ed. Current Biology Ltd. Garland Publishing Inc, 1996.
3. Aker M, Zimran A, Abrahamov A, Horowitz M, Matzner Y. Abnormal neutrophil chemotaxis in Gaucher disease. *Br J Hematol* 83:187-191, 1993.
4. Goldfarb RA, Atlas DH, Goberman P. Electrophoretic studies in Gaucher's disease. *Am J Clin Pathol* 20:963-965, 1950.
5. Pratt PW, Estren S, Kochwa S. Immunoglobulin abnormalities in Gaucher's disease. Report of 16 cases. *Blood* 31:633-640, 1968.
6. Garfinkel D, Sidi Y, Ben-Bassat M, et al. Coexistence of Gaucher's disease and multiple myeloma. *Arch Intern Med* 142:2229-2230, 1982.
7. Zimran A, Sorge J, Gross E et al. Prediction of severity of Gaucher's disease by identification of mutations at DNA level. *Lancet* 2:349-353, 1989.
8. Robinson DB, Glew RH. Acid phosphatase in Gaucher Disease. *Clin Chem* 26: 371-382, 1980.
9. Mottiff KD, Chambers JP, Diven WF, et al. Characterization of lysosomal hydrolases that are elevated in Gaucher Disease. *Arch Biochem Bioph* 190:247-260, 1978.
10. Hollak CEM, Van Weely S, Van Oers MHJ, et al. Marked elevation of plasma chitotriosidase activity. *J Clin Invest* 93:1288-1292, 1994.
11. Pinkhas J, Djaldetti M, Yaron M. Coincidence of multiple myeloma with Gaucher's disease. *Isr J Med Sci* 1:537-540, 1965.
12. Benjamin D, Joshua H, Djaldetti M, et al. Non-secretory IgD kappa multiple myeloma in a patient with Gaucher's disease. *Scand J Hematol* 22:179-184, 1979.
13. Shiran A, Brenner B, Laor A, et al. Increased risk of cancer in patients with Gaucher disease. *Cancer* 72:219-224, 1993.
14. Shoenfeld Y, Gallant LA, Shaklai M et al. Gaucher's disease. a disease with chronic stimulation of the immune system. *Arch Pathol Lab Med* 106:388-391, 1982.
15. Allen MJ, Myer BJ, Khokher AM, et al. Pro-inflammatory cytokines and the pathogenesis of Gaucher disease: increased release of interleukin-6 and interleukin-10. *Q J Med* 90:19-25, 1997.
16. Hollak CEM, Aerts JMFG, van Weely S, et al. Increased energy expenditure and elevated levels of macrophage derived factors in type 1 Gaucher Disease. *Second EWGGD Workshop* Maastricht, Holland, p. 54. May 1-3, 1997.
17. O'Garra A, Vieira P. Polymerase chain reaction for detection of cytokine gene expression. *Curr Opin Immunol* 4:211-215, 1992.
18. Shaw G, Kamen R. Conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* 46:659-667, 1986.
19. Byrne BC, Li JJ, Sninsky J, Poiesz BJ. Detection of HIV-1 RNA sequences by *in vitro* DNA amplification. *Nucleic Acid Res* 16:4165-4170, 1988.
20. Wang AM, Doyle MV, Mark DF. Quantitation of mRNA by the polymerase chain reaction. *Proc Natl Acad Sci USA* 86:9717-9721, 1989.
21. Gery I, Zigler JS, Brady RO. Selective effects of glucocerebroside (Gaucher's storage material) on macrophage culture. *J Clin Invest* 68:1182-1189, 1981.
22. Boyce BF, Aufdemorte TB, Garrett IR, et al. Effects of interleukin 1 on bone turnover in normal mice. *Endocrinology* 123:1142-1150, 1989.
23. Mundy GR. Cytokines and growth factors in the regulation of bone remodeling. *J Bone Min Res* 8 (Suppl 2): S505-S510, 1993.
24. Whicher JT, Evans SW. Cytokines in disease. *Clin Chem* 36(7):1269-1281, 1990.
25. Dinarello CA. Biology of interleukin 1. *FASEB J* 2:108-115, 1988.
26. Beutler B, Cerami A. Cachectin and tumor necrosis factor as two sides of the same biological coin. *Nature* 320:584-588, 1986.
27. Dinarello CA. Interleukin-1: amino acid sequences, multiple biological activities and comparison with tumor necrosis factor (cachectin). *Year immunol* 2:68-69, 1986.
28. Assoian RK, Fleurdelys BR, Stevenson HC et al. Expression and secretion of type B transforming growth factor by activated human macrophages. *Proc Natl Acad Sci USA* 84:6020-6024, 1987.