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Research Article

Serum Sphingomyelin Levels in Patients with HIV Infection

Fred Chasalow[1,2]*

[1]*Chief Scientist, IOMA LLC, Belmont, CA, USA*

[2]*Visiting Professor, Department of Laboratory Medicine, VA Medical Center, San Francisco, CA, USA*

***Corresponding Author:** Fred Chasalow, Chief Scientist, IOMA LLC, Belmont and Visiting Professor, Department of Laboratory Medicine, VA Medical Center, San Francisco, CA, USA.

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Abstract

We report a simple LC-MS method to measure serum sphingomyelin levels. In brief, we observed that sphingomyelin levels in serum are less variable than are levels of thyroxine. There was no evidence for circhoral variation and levels were not altered after a meal of sphingomyelin-rich foods, including eggs, cheese or pizza. In adults, within 2 days of HIV infection, serum levels of sphingomyelin were not within the normal range. When (if) patients developed HIV-specific antibodies, sphingomyelin levels were restored. Ultimately, when the virus 'escaped' from the antibody, sphingomyelin dropped again. In infants at birth, low levels of sphingomyelin predicted which infants of HIV- infected mothers would later develop infection. Sphingomyelin levels were normal in those children with HIV- infection who benefited from AZT therapy but levels did not return to normal in children that did not benefit. In summary, changes in sphingomyelin levels might be a useful marker to evaluate HIV disease status in children. We propose that one of the first biochemical events after HIV infection is stimulation of sphingomyelinase and release of ceramides. One of the ceramides might be an apoptotic factor for CD4 cells.

Keywords: *Sphingomyelin; Sphingosine-Phosphocholine; Ceramide; HIV; HTLV; Covid-19*

Abbreviations

AUC: Area Under the Curve; AZT: Zidovudine; C-8.2: Compounds eluting at 8.2 minutes by HPLC; Da: Dalton - Unit of Mass; EIA: Enzyme Linked Immuno Assay; FAB-MS: Fast Atom Bombardment Mass Spectroscopy; FTIR: Fourier Transform Infra Red Spectrometry; HPLC: High Performance (Pressure) Liquid Chromatography; HIV: Human Immunodeficiency Virus; HTLV: Human T-lymphotropic Virus; LC-MS: Liquid Chromatography - Mass Spectrometry; s. d.: Standard Deviation; UV: Ultraviolet Spectrometry

Introduction

My laboratory developed new HPLC and LC-MS methods specifically to identify novel, water-soluble, compounds [1]. Initially, the novel compounds were detected by RIAs for either steroid sulfates or for digoxin [2,3]. As part of our efforts to identify a rich source for the endogenous Digoxin-Like Material (DLM), we obtained serum from patients with many diseases. Typically, these were residua from samples collected in the normal course of patient care. In addition to the peaks of interest, there was an HPLC peak which eluted at 8.2

minutes. Until we identified the compound, we designated its name by its retention time - 'C-8.2', intended as an abbreviation for "the compound(s) eluting at 8.2 minutes". C-8.2 could not be detected with the RIA methods that detected either DLM or androgen sulfates but could be detected by its UV absorbance.

This paper reports the isolation of C-8.2 and establishes its normal serum concentration range. The standard deviation (s.d.) was less than 10% of the mean. One component of C-8.2 has a mass spectrum, FTIR spectrum and HPLC retention time equivalent to that observed with authentic sphingomyelin. Sphingomyelins were known to be in serum, but it was not known that the total levels were not very variable.

In our search for rich sources of the endogenous DLM, we also obtained a few samples from patients with HIV infection. C-8.2 levels were extremely low in those samples. This paper describes our method of measuring C-8.2 and our initial observations of C-8.2 in patients with HIV infection. The data formed the basis of a patent [4].

Materials and Methods

Sample preparation

Serum samples were mixed with 4 volumes of acetonitrile (HPLC grade acetonitrile; Fisher, Springfield, NJ). The extract was centrifuged at 750xg and filtered (LC13 Acrodisk obtained from Gelman Laboratories, Ann Arbor, MI); the organic layer was used for analysis. This method denatures any virus present in the original sample. Extracts were kept at 2 - 4°C until analyzed.

HPLC method

The HPLC was Model 401T from BioRad, Richmond, CA. Amino-Carbohydrate columns (4.6x250 mm) were obtained from Alltech, Chicago, IL. Samples were eluted with a gradient comprised of acetonitrile and water as defined by these points: [i] t = 0 minutes - 95% acetonitrile; [ii] t = 2 minutes - 75% acetonitrile; [iii] t = 10 minutes - 55% acetonitrile; [iv] t = 12 minutes - 40% acetonitrile; [v] t = 14 minutes - 40% acetonitrile; [vi] t = 15 minutes 95% acetonitrile [3]. After completion of the gradient and prior to the next sample, the column was washed for 10 minutes with 95% acetonitrile. Column eluates were monitored at 210 nm. Note that, in contrast to most HPLC solvent gradients, this gradient increases water content. Typical HPLC results are shown in figure 1.

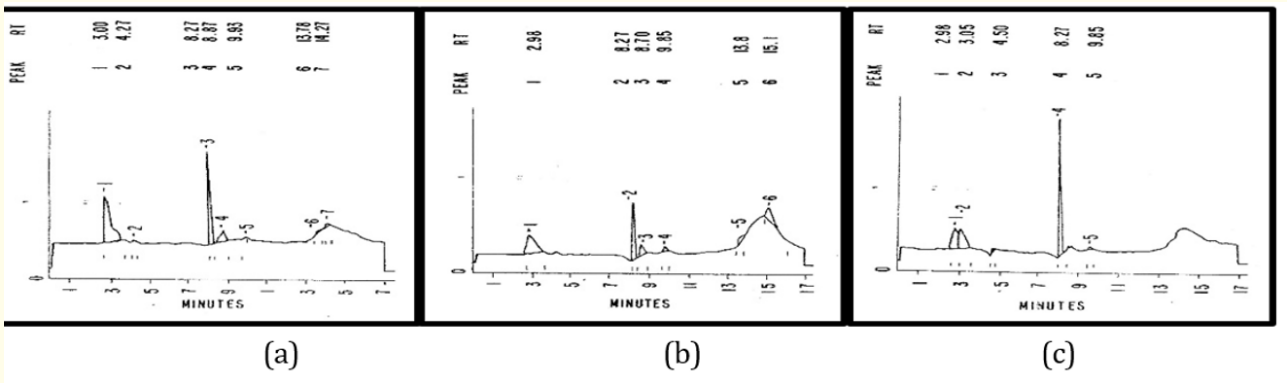

Figure 1: *HPLC analysis of serum extracts: (a) from a HIV sera-negative adult; (b) from an HIV zero- positive adult; (c) from a HIV-sera-positive adult.*

Isolation of C-8.2

HIV-negative, outdated plasma (1.5L) was obtained from the local blood bank. It was mixed with 6L of acetonitrile and filtered to remove denatured, insoluble materials. The extract was treated with 3.75L of benzene (HPLC grade, Fisher, Springfield, NJ). The lower aqueous phase was discarded. The organic phase was evaporated to dryness, in batches, on a rotary evaporator (Bucher, Fisher, Springfield, NJ). Each batch was dissolved in a minimum amount of methanol and pooled. Finally, the volume was reduced to 10 ml of methanol.

Column purification of C-8.2

The second step was chromatography in 5 ml batches on Sephadex™ LH-20 (Pharmacia, Piscataway, NJ.) with methanol as the solvent. The column fractions that contained C-8.2 were combined. The final step in purification was preparative HPLC with an NH_2-Lichosorb column (10 x 250 mm, Alltech). The sample was applied in 95% acetonitrile- 5% distilled water. The column was eluted with a 10 minutes linear gradient to 40% acetonitrile and then with that mixture until the C-8.2 eluted. Peak fractions were combined. The absence of major, unexplained mass ions on Fast Atom Bombardment Mass Spectroscopy (FAB-MS) suggested there were no major contaminants.

Fourier transform infrared spectrometry (FTIR)

FTIR analysis was performed by Hauser Chemicals (Golden, CO). The spectrum of the compound that eluted at 8.2 min is shown in figure 2.

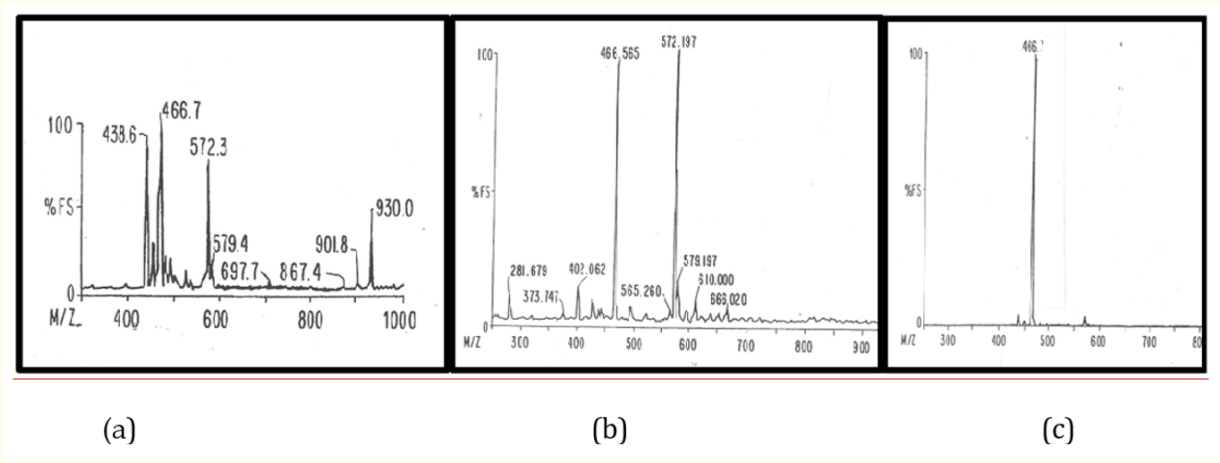

Figure 2: *Fast Atom Bombardment of the compounds included in the C-8.2 peak: (a) HPLC from fraction #24 obtained from normal serum; (b) HPLC fraction 24 from C-8.2 purified from normal serum; (c) sphingosine phosphocholine diester. Gramicidin was added as an internal control and generates the ion at m/z = 572 Da.*

Time and diet studies

Sequential samples showed no evidence of circhoral or other episodic changes in serum levels of C-8.2. We also confirmed that serum levels did not change even after a meal rich in sphingomyelin, such as eggs or cheese.

Statistical analysis

The results were expressed as mean + standard deviation (s.d.). Differences were evaluated by Student's T test. Samples were considered positive if they were not within 3 s.d. of the mean for the age-matched control group. X^2 test was used to test if the samples

could be from the same data set. Samples were considered concordant if [a] HIV-EIA negative samples had values of C-8.2 within 3 s.d. of mean for the age-matched control group or if [b] HIV-EIA positive samples had values of C-8.2 that were not within 3 s.d. for the age-matched control group.

Results

While investigating endogenous DLM in human breast cyst fluids, we used the same methodology to determine if there were similar materials in serum [3]. Figure 1 shows the HPLC results from one of these studies. In brief, The DLM of original interest eluted at 8.77 minutes [4] but there was another peak that eluted at 8.2 minutes, C-8.2. The C-8.2 peak was not a DLM. But what was it? The extraction method eliminated proteins, aromatic amino acids, and most neutral lipids from consideration. It did not eliminate lipid phosphoesters or phosphodiesters. We addressed the problem in two ways: [i] isolation and identification and [ii] quantitation in many serum samples, including HIV sero-positive patients. For quantitation, we used area under curve (AUC). The AUC from the HIV sero-positive samples were not within 3 s.d. of the mean AUC of the control group.

Isolation and characterization of C-8.2

There are several things of note in figure 2. First, the FAB-MS that we isolated from serum extracts had only one major ion - m/z = 466 Da. Second most of the ions in the crude extract had a retention characteristic of C-8.2 and had m/z = 466 Da, If the m/z = 466 Da peak was the phosphocholine ester, then the ester component would have a molecular mass of 299 Da [4]. The m/z of sphingosine is 299 Da. Note that the mass is an odd number, confirming the presence of an odd number of nitrogen and phosphorus atoms. Sphingosine phosphocholine ester has one phosphorus atom and two nitrogen atoms (See figure 3, panel a).

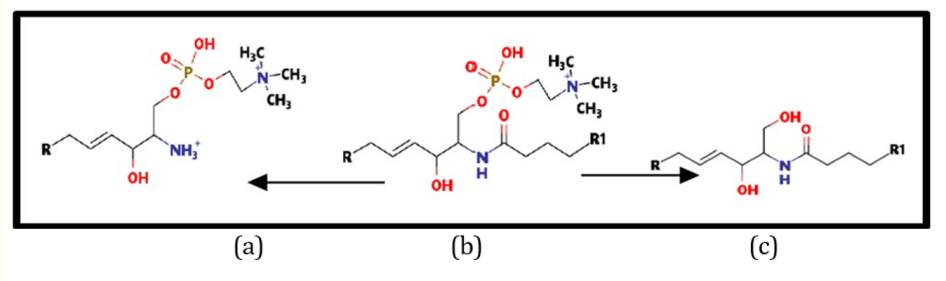

Figure 3: *Structures. (a) Sphingosine phosphocholine diester; (b) Sphingomyelin; (c) Ceramide. R=$(CH_2\text{-}CH_2)_6$-H -- sphingosine; R1=$(CH_2\text{-}CH_2)_n$-H -- long chain fatty acid.*

Figure 4 shows spectra of the purified C-8.2. The UV spectrum indicated the absence of conjugated double bonds. The FTIR spectrum provided several clues about the structure: [a] there is a carbonyl, [b] the absorbance at 3278 cm- suggests a hydroxyl group and [c] the 1249 cm- indicates the presence of a phosphate group. As shown in figure 3, enzymatic hydrolysis of sphingomyelin (shown in panel b) by sphingomyelinase cleaves off the phosphocholine component, releasing ceramide (shown in panel c). In contrast, FAB-MS breaks the amide linkage and releases the ion with m/z = 466 Da (shown in panel a) [4]. Note that there are multiple ceramides, each derived from a sphingomyelin with a different long chain fatty acid (designated as R1) but all generate the characteristic sphingosine fragment at m/z = 466 Da.

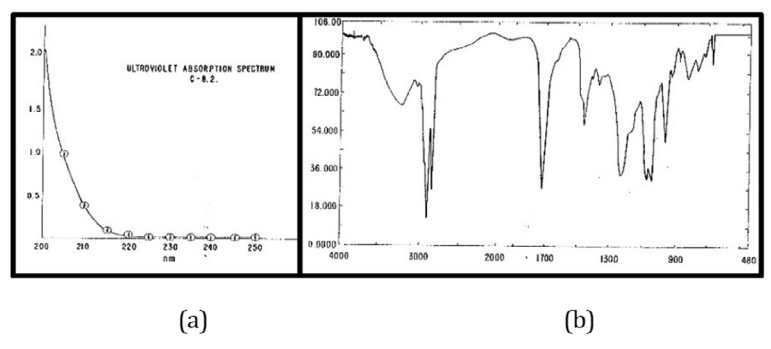

Figure 4: *Spectra of purified C-8.2 isolated by HPLC. Panel a - UV spectrum; Panel b FTIR spectrum.*

Adult serum levels of C-8.2 (sphingomyelin)

When we first observed the variation in AUC for C-8.2 in serum from HIV infected patients, we investigated the level of C-8.2 in normal patients (n-14). Without knowing its identity, we found AUC for C-8.2 (n = 14) was 38 ± 3.5 AUC (area units; mean ± s.d.). We used this data to establish a normal range (mean ± 3 s. d.) of 27-48 AUC. Samples with AUC values not within this range were defined as positive responses. For comparison, normal levels of thyroxine are 5 - 11 µg/dl. Thus, in contrast to glucose and most steroids, serum level of C-8.2 are as tightly regulated as thyroxine.

Table 1 shows results with clinical samples obtained from two commercial laboratories. In group 1, 30 of 33 samples were concordant. In group 2, 26 of 37 test results were concordant. Neither clinical laboratory further identified the health status of their patients or if they were being treated with AZT, which was the only HIV specific drug that was available.

Group 1	EIA Test		Group 2	EIA Test	
	Neg	Pos		Neg	Pos
HPLC Neg	14	3	HPLC Neg	13	4
HPLC Pos	0	16	HPLC Pos	7	13
$X^2 = 22.9$; $p < 0.0001$			$X^2 = 6.36$; $p < 0.01$		

Table 1: *Correlation between HIV antibody test and HPLC Test.*

HIV EIA tests were done by the commercial blood bank. The criterium for a negative HPLC result was based on AUC between 27 and 48. Positive tests were AUC less than 27 or over 48.

Table 2 shows analysis of sequential samples obtained from two patients. Each had experienced a high-risk event within the previous 2 days. The details of the event were not disclosed to us (privacy considerations). At the time they came to medical attention, both patients showed a positive assay result for C-8.2 but neither developed a positive EIA result during the first week after the event. During the second week after exposure, Patient #1 developed HIV specific antibodies, presumably IgM, C-8.2 levels increased. For Patient #2, there was no

immediate development of IgM antibodies and the serum levels of C-8.2 remained depressed. During the first week, both patients would have been scored as false negatives by the EIA test. The conclusion was that C-8.2 serum levels change during HIV infection and that the changes begin prior to the development of HIV specific antibodies.

Sample #		HPLC Assay		HIV specific Antibody	
	Day #	Patient #1	Patient #2	Patient #1	Patient #2
1	1	25.5	25.1	0	0
2	4	19.8	15.0	0	0
3	7	23.5	23.4	+	0
4	10	24.5	----	++	0
5	14	26.2	16.4	+++	0
6	17	30.0	15.0	+++	0
7	21	24.3	4.5	+++	0
8	24	25.2	----	+++	0

Table 2

Table 3 shows the results of a study of patients (n = 10) known to be positive for either HTLV-1 or HTLV-2. Eight of the patients were negative for C-8.2. Patient 9 was retested and shown to be positive for HIV infection. Patient 10 was lost to follow-up could be retested for HIV infection.

HTLV	Pos 10	Neg 0	
HIV	Pos 1	Neg 8	Unknown 1
HPLC Assay results	Pos 2	Neg 8	

Table 3: *Serum samples obtained from patients with documented HTLV 1 or HTLV 2 infection.*

Infant serum levels of C-8.2 (sphingomyelin) [5]

Infants (n = 11) less than 18 months of age had slightly lower levels of C-8.2 than did adults. The mean was 25.5 AUC with a s.d. of 2.5 AUC. The 3 s.d. range was 17.5 - 33 AUC. Children (n = 11) in the age range 2 - 11 years of age had a normal range for C-8.2 of 20-33 AUC.

Serum sphingomyelin levels in infants of HIV infected mothers

Table 4 shows the results for high-risk infants (n = 27) less than 18 months of age. All of these infants had HIV-specific antibodies at birth. All of the infants with normal C-8.2 (n = 15) had not developed symptoms of HIV infection by 18 months of age. Nine of the 12 infants with positive responses developed symptoms. The remaining 3 patients with positive C-8.2 levels were asymptomatic but continued to have HIV specific antibodies in their serum. C-8.2 measurement might be a useful screening test for these infants. In summary, measurement of C-8.2 discriminated infants at risk for developing symptoms of HIV infection from the infants with maternal antibodies.

In this age group, the HIV-infected children (n = 4), who had not been treated with AZT, had elevated levels of C-8.2. In AZT-treated children (n = 16), 5 had normal levels and 11 had elevated C-8.2 levels. The latter group continued to have neurological symptoms typical of HIV infection.

C-8.2 level	No symptoms	Symptoms	AUC
Normal	15	0	> 18 and < 33
Abnormal	3*	9	< 18 or > 33
$X^2 = 16.9$; $p < 0.001$			

Table 4: *Infants less than 18 months age who were at high risk for HIV infection (n = 27).*
**Continuing presence of HIV specific antibodies; ultimate diagnosis unknown.*

Children (n = 12) in the age range of 4 - 11 years of age with neurological symptoms of HIV infection and abnormal levels of C-8.2 were treated with AZT. Four of the children did not benefit from the AZT therapy and C-8.2 remained abnormal. Eight of the children benefited from the therapy and their C-8.2 levels returned to the normal range. In summary, the studies of AZT identified the children who benefited from the therapy. We don't know if newer therapies could also be monitored with this method.

Virtually all infants of HIV-infected women have HIV specific antibodies in their serum [5]. Thus, measuring antibodies is not a useful marker in newborn infants or young children. Earlier, we showed that elevated cord hCG levels correlate with HIV infection [5]. However, this generates an ethical dilemma: false negatives lead to not treating an infant in need of therapy and false positives lead to treating newborn infants unnecessarily. Measuring sphingomyelin (C-8.2) might be a useful biomarker to help identify both false patterns.

Discussion

This investigation began in 1991 when Chasalow became Director of a clinical laboratory at the Maimonides Medical Center. He had approval of the Institutional Review Board to use samples collected in the normal course of clinical service to investigate new methods, with appropriate privacy considerations. On several occasions, nurses or physicians reported there had been a needle 'stick' and they were concerned about HIV infection. The initial strategy is best described by, "If the only tool you have is a hammer, everything looks like a nail". Figure 1 shows the initial results. To maintain privacy, each person at risk collected serum from several colleagues. The samples were coded and the code was broken only by the person at risk. Chasalow never met the people at risk. As shown in table 2, serum levels of C-8.2 dropped within the first few days after exposure, long before antibodies or symptoms developed. Fearon describes current methods of diagnosing HIV infection based on detection of HIV specific antibodies [6]. But the antibodies do not appear for several weeks after initial exposure. Thus, therapeutic intervention is delayed. Routine application of a sphingomyelin assay might be a timely approach. The sequence of biochemical changes that lead to decreases in serum sphingomyelin levels begins with TNFα. TNFα synthesis is an early event in HIV infection and increases the activity of sphingomyelinase [7]. Increased ceramides leads to apoptosis [8] and HIV dementia [9]. Kumar is developing novel TNF-α inhibitors for therapy [10]. Monitoring sphingomyelin levels might also be a useful marker to evaluate effectiveness of that therapy. There are three common diseases associated with low sphingomyelin levels - HIV infection, Alzheimer's dementia [11,12], and Covid-19 infection [13]. All three have neurological disorders commonly described as 'brain fog'. We propose that serum sphingomyelin levels might be a useful biomarker in these forms of dementia.

Conclusion

Correlation of serum sphingomyelin levels needs to be divided into two parts: 1) adult onset and 2) infants born with HIV infected mothers.

In adults, serum sphingomyelin levels are changed early in the HIV-dependent infection process. Literally, within hours of exposure, sphingomyelin levels decrease. Our method did not confirm corresponding increases in ceramide levels. A test group of samples from patients with HTLV 1 or HTLV 2 did not show decreases in sphingomyelin levels, confirming the specificity for HIV. Based on about a

hundred serum assays, there were three phases in the changes: [i] a decrease in the immediate period after exposure, [ii] a rebound when HIV specific antibodies were initially detectable and becomes supra-physiological while the HIV is suppressed, and [iii] decreases when AIDS develops. The rebound seems to coincide with IgM production while the supra-physiological is associated with IgG production. Finally, in some patients on AZT therapy (the only drug that was available at the time), sphingomyelin levels indicate therapeutic utility. In the long term, this may a useful method of individualizing specific therapeutic protocols.

Whether or not infants of HIV-infected mothers have maternal HIV-specific antibodies, does not predict which infants are at risk for developing HIV infection or AIDS. Table 4 summarizes the sphingomyelin levels in 27 infants. 15 of the 27 infants had sphingomyelin with in the normal range and none of these infants had developed symptoms by 18 months of age. Of the 12 infants with abnormal sphingomyelin levels, 9 had developed symptoms by 18 months age. The ultimate 'fate' of the three infants with abnormal sphingomyelin levels was unknown when the data was tabulated. Finally, several groups of symptomatic infants were treated with AZT and sphingomyelin levels were monitored. Sphingomyelin levels failed to return to normal in the infants that did not respond to AZT therapy. Altogether sphingomyelin assay may be a useful criterion to identify infants with antibodies who do not need to be treated. This may help solve the ethical problem of unnecessary therapy with powerful drugs while providing a basis for therapy for patients at risk.

Author Contributions

Conceptualization, F.C.; H.L.B.; and S.N.; methodology, F.C.; investigation, F.C. and S.N.; writing-original draft preparation, F.C.; writing-review and editing, F.C. and S.N.; funding acquisition, F.C. and S.N. All authors have read and agreed to the published version of the manuscript.

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This research received no specific external funding. Dr. Joseph Rubinfeld, CEO of SuperGen, prearranged for the commercial samples. SuperGen had no role in the design of the study; analyses, interpretation of data; in the writing of the manuscript, or the decision to publish the results.

Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki. In 1991, The Institutional Review Board of Maimonides Medical Center, Brooklyn, NY approved the use of residua from serum samples obtained in the normal course of medical practice. There was a specific requirement that samples must not be identifiable by name. Each collaborator (Drs. Nachman, Bradlow and Rubinfeld) satisfied the Institutional Review Board of their respective institutions.

Informed Consent Statement

Each collaborator obtained informed consent from all of their patients in the study. For the children, Dr. Nachman obtained informed consent from the responsible parent. No participating patients can be identified by the data presented in this paper.

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Conflicts of Interest

The author declares no current conflict of interest.

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