

Increased neutrophil adhesive capability in Cohen syndrome, an autosomal recessive disorder associated with granulocytopenia

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Abstract

Background and Objective. Cohen syndrome is a multiple congenital anomalies-mental retardation syndrome associated with granulocytopenia. To date, the mechanisms involved in causing the neutropenia are unknown. In order to get insight into the mechanisms of neutropenia, we studied both the bone marrow and the functional properties of neutrophils obtained from peripheral blood (PB) or skin window (SW) exudate of a patient affected by Cohen syndrome.

Design and Methods. Assays of superoxide anion release (as reduction of cytochrome C) and cell adhesion (quantified by measuring membrane acid phosphatase) were carried out according to a microplate method whereby both parameters can be evaluated (Bellavite *et al.*, 1992). Neutrophil surface integrins and CD62L (selectin) were evaluated by flow cytometry.

Results. Bone marrow did not show relevant morphological abnormalities in either erythroid or myeloid precursors. Cohen neutrophils exhibited a greater adhesive capability than control leukocytes in all the conditions studied (PB or SW, unstimulated or agonist-stimulated leukocytes). Cytofluorometric evaluation of neutrophil $\beta 2$ integrin (CD11b) and selectin (CD62L) showed a lower mean fluorescence intensity and a lower percentage of fluorescence conjugate monoclonal Ab-positive cells in the patient than in control subjects. Moreover, a double population of neutrophils, with different affinities to the specific monoclonal antibody anti-CD11b, was observed in the patient. Superoxide anion release, expression and distribution of fluorescence conjugate MoAb anti-human CD11a were normal.

Interpretation and Conclusions. Neutrophil adhesive capability was greatly increased in a case of Cohen syndrome. Cytofluorimetric expression of CD11b and CD62L molecules was consistent with a generalized neutrophil activation *in vivo*.

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Key words: Cohen syndrome, granulocytopenia, neutrophils, integrins, adhesion

Cohen syndrome was originally described as a multiple congenital anomalies-mental retardation (MCA/MR) syndrome with autosomal recessive inheritance.¹ Further delineation of the syndrome has included chorioretinal dystrophy and neutropenia as additional distinctive markers.²

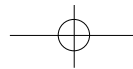
As the other numerous signs and symptoms are nonspecific and found in several MCA/MR syndromes, neutropenia is often an important finding in the diagnosis of Cohen syndrome, especially in infancy and adolescence.³ However, the mechanism(s) involved in causing the neutropenia is (are) unknown.

In a very recent report,⁴ aimed at elucidating this aspect, bone marrow cellularity, growth of cultured CFU-GM progenitors, granulocyte response to adrenaline and hydrocortisone, and sensitivity to granulocyte colony stimulating factor (rhG-CSF) were studied in 26 Cohen patients. Although "the basic mechanism remains unsolved", the authors suspect that "granulocytopenia in Cohen syndrome is of bone marrow origin", mainly on the basis of the finding that a left-shifted granulopoiesis was noted in some patients.

In the present report we describe a leukopenic patient with multiple congenital anomalies and mental retardation who fulfils the criteria for the diagnosis of Cohen syndrome. In order to get insight into the mechanisms of neutropenia, we studied bone marrow and functional properties of neutrophils obtained from peripheral blood (PB) or skin window (SW) exudate from the patient. Neutrophil surface $\beta 2$ integrins and CD62L were also evaluated by flow cytometry.

Materials and Methods

The patient, a 22-year-old white girl, was referred to our out-patient clinic in May 1996 for investigation of neutropenia and recurrent gingivitis. In infancy, a tentative diagnosis of Prader-Willi syndrome (PWS) was proposed by the pediatricians in order to account for the complex pattern of multiple congenital anomalies (obesity, hypotonia, microcephaly, chorioretinal dystrophy, high nasal bridge, narrow hands and feet, narrow and high-arched palate, prominent central incisors) and mental retardation presented by the patient. Till our examination the diagnosis of PWS was never disputed; moreover, despite recurrent laboratory evidence, neutropenia was never considered as an additional feature of the



syndrome complex presented by the patient. Apart from several episodes of gingivitis, the remaining clinical history was uneventful.

Hematologic investigation showed a normal number of red cells and platelets, but an absolute neutrophil count of $0.26\text{--}0.60 \times 10^9/\text{L}$. All the other routine parameters were normal, as was a serologic search for anti-PMN antibodies. Bone marrow biopsy showed a normal overall cellularity without relevant morphologic abnormalities in either erythroid or myeloid precursors. At the prometaphase chromosome study, the patient resulted to be euploid (46,XX); a fluorescence *in situ* hybridization (FISH) study with specific microsatellites for chromosome 15 failed to confirm the diagnosis of PWS.

A computerized search for multiple congenital anomalies-mental retardation and neutropenia led us to suspect the diagnosis of Cohen syndrome, the literature's description of which fully agrees with all the clinical features presented by our patient.³ In order to evaluate the capacity of her defensive response against infective and exogenous agents, we decided to study the functions of neutrophils obtained from peripheral blood and skin window exudate.

Neutrophil functions

Informed consent was obtained from parents of the patient; 20 subjects, recruited between the medical and laboratory staff members, were also studied as controls. Neutrophils were obtained from peripheral blood (PB) and skin window (SW) exudate on the same day. Blood cells were prepared from 20 mL of ethylene diamine tetra-acetate-anticoagulated blood by centrifugation over discontinuous 63%/72% Percoll gradients. After hypotonic lysis of contaminating erythrocytes and two washings with phosphate buffered saline (PBS, Gibco Ltd., Paisley, Scotland), cells (>95% neutrophils, >99% viable as judged by the trypan blue exclusion test) were finally suspended in Hank's balanced salt solution (HBSS, Gibco Ltd., Paisley, Scotland), containing 0.2% human serum albumin (H-A), and kept at room temperature until used.

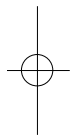
Exudate neutrophils were isolated according to the method described by Senn and Jungi⁶ with modifications.⁷ Briefly, an abrasion of 1 cm² was obtained with a rotating sterile abrasive cylinder on the volar surface of the non-dominant forearm: abrasion did not cause bleeding as only epidermis was removed. A bell-shaped, sterile, disposable plastic skin chamber (FAR Italia, Verona, Italy) was placed over the skin abrasion and fixed with fenestrated sticking plaster. One milliliter of autologous serum was then injected into the chamber through a hole, and a similar volume of exudate was removed by aspiration 24 hours later. Cells from exudate (>95% neutrophils, >99% viable) were centrifuged at 1200 rpm, washed twice in PBS, and finally suspended in HBSS + 0.2% H-A and kept at room temperature until used.

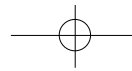
Assays of superoxide anion release and adhesion were carried out according to a microplate method whereby both parameters can be evaluated.⁸ Briefly, sterile 96-well microtitre plates with flat-bottomed wells were pre-coated with fetal bovine serum, in order to avoid non-specific adhesion of neutrophils to plastic. After washing out the coating medium, the microplate wells were supplemented with: i) 25 mL of HBSS containing 5 mM glucose, 0.2% H-A, 0.5 mM CaCl₂ and 1 mM MgSO₄ (H-GACM), for assays on resting cells, or 25 mL of the agonist dissolved in H-GACM; ii) 25 mL of 0.45 mM cytochrome C (Boehringer, Mannheim, Germany) dissolved in H-GACM; iii) 50 mL of neutrophils, suspended in H-GACM at the concentration of $3 \times 10^6/\text{mL}$. The concentrations of agonists used were: fMLP 5×10^{-7} M, STZ 0.1 mg/mL, PMA 10 ng/mL. Assays were done in triplicate for each experimental condition.

Plates and cells were prewarmed to 37°C and incubations were carried out for 40 min. in a humidified thermostat at 37°C. The reduction of cytochrome c was measured with a microplate reader at 550 nm as a reference wavelength. Immediately after reading cytochrome c reduction, the plates were transferred to an automatic washer and subjected to two washing cycles with PBS. Adherent cells were quantified by measuring the membrane acid phosphatase and the percentage adhesion was calculated on the basis of a standard curve obtained with known numbers of neutrophils from the same subject.⁸

Cell surface integrins and CD62L (selectin) were detected by direct immunofluorescence evaluated by flow cytometry (FACS). Freshly isolated PB neutrophils (10^7 cells/mL) were stimulated with fMLP (5×10^{-7} M final concentration), STZ (0.1 mg/mL) and PMA (10 ng/mL) for 30 min. at 37°C in a shaking water bath. Agonist-activated and resting (unstimulated) cells were tested for the following monoclonal antibodies (MoAbs): BEAR1 (FITC anti-CD11b), 25.3.1 (Phycoerythrin anti-CD11a) and anti-mouse control 679.1Mc7 (FITC/Phycoerythrin IgG1/IgG1) (Immunotech Coulter Co., Marseille, France). Neutrophils (1×10^5) were resuspended in 20 mL of MoAbs diluted in PBS containing 2% BSA and 0.1% sodium azide and incubated for 30 min. at 4°C, washed in HBSS and immediately analyzed.

Additional experiments with BEAR1 (FITC anti-CD11b) and MEL-14 (FITC anti-CD62L) monoclonal Ab were performed on leukocytes from whole blood, to avoid any *in vitro* manipulation. These monoclonal Abs were incubated at the final concentration of 10 mL per 100 mL of whole blood for 30 min. on ice, washed twice before and after lysis of erythrocytes by adding 2 mL of ice-cold erythrocyte-lysing solution (NH₄Cl 2.08 g; Na₂EDTA 0.0108 g; NaHCO₃ 0.21 g in 250 mL H₂O), and analyzed. Non-specific immunofluorescence was determined using the control MoAb, and the cell purity for granulocytes was evaluated on the bitmap gating of the flow



**Table 1. Adhesive capability of peripheral blood (PB) and skin window (SW) neutrophils from controls (n=20) and the Cohen patient.**

	Unstimulated		Adhesion (% adherent cells)					
	PB	SW	fMLP		STZ		PMA	
	PB	SW	PB	SW	PB	SW	PB	SW
controls	7.28±4.4	13.44±7	22.5±7.1	28.61±8	23.94±10	27.31±8	49.5±12	48.29±9
patient	15.1	69.84	49.07	84.35	51.25	75.73	47.04	79.84

cytometer. Analysis was performed by flow cytometry using a fluorescence-activated cell sorter (Epics Elite, Coulter Electronics Inc., Hialeah, FL, USA) equipped with a 488 nm air-cooled argon ion laser; gating for granulocytes was determined by dot blot generated by forward-angle versus right-angle scatter. Fluorescence intensity of each cell was recorded as a mean channel number over the logarithmic range of 1 to 1024.

Results

As reported in Table 1, Cohen neutrophils exhibited a spontaneous (unstimulated) adhesive capability which was twofold higher than that of the control leukocytes; this difference was also more striking for SW leukocytes (Table 1). Likewise, adhesive response elicited by the stimulation with several agonists was strongly increased in the PB and SW neutrophils of

the patient as compared with the cells of the controls (Table 1). In order to avoid erroneous conclusions due to unrecognized inflammatory conditions, experiments using PB cells were repeated three times over a six months period, when the patient had different white cell counts (within the range reported above); results did not, however, change substantially.

Superoxide anion release from the patient's leukocytes was similar to that from the control cells, both of PB and SW origin, with or without agonist-stimulation (data not shown).

Cytofluorometric evaluation of CD11b and CD62L expression is reported in Table 2. Neutrophils from the Cohen patient showed a lower percentage of fluorescence conjugate monoclonal CD11b Ab-positive cells and a lower mean fluorescence intensity than neutrophils from control subjects, both for resting and for agonist-stimulated (fMLP, STZ, PMA) cells. Two experiments on whole blood (without any cell manipulation) from the patient produced similar results. Moreover, the patient showed an abnormal bimodal distribution of fluorescence conjugate MoAb anti-human CD11b-positive resting cells (Figure 1); a similar pattern was also observed on agonist-stimulated (fMLP, STZ, PMA) leukocytes (Figure 1).

Neutrophil CD62L expression, evaluated on whole blood and in resting condition, was lower in the Cohen patient than in control subjects (Table 2).

Analysis for CD11a fluorescence stained cells did not show differences between the Cohen patient and control subjects (data not shown).

Discussion

We describe a case of Cohen syndrome with the typical multiple congenital anomalies, mental retardation and neutropenia. This last is often an important distinctive marker for the differential diagnosis between Cohen and other MCA/MR syndromes, as for example Prader Willi syndrome⁹ which was an early diagnosis in our patient. As demonstrated by Kivittie-Kallio *et al.*,⁴ granulocytopenia is an essential feature of the Cohen syndrome, but it may be intermittent and remains unnoticed or undervalued in some of patients (as in our case).

Despite its diagnostic relevance and frequency in these patients, granulocytopenia in Cohen syndrome has scarcely been studied, the pathogenetic mechanisms remaining so far largely unknown. With the

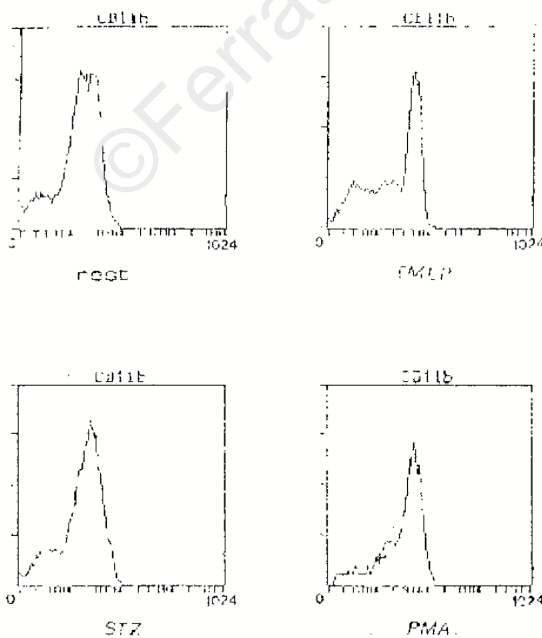


Figure 1. FACS evaluation of neutrophil integrin CD11b in resting and agonist-stimulated cells of Cohen patient. Concentrations of the agonists are reported in the text; mean fluorescence intensity and percentage of CD11b-positive cells are shown in Table 2.

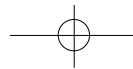


Table 2. Percentage positivity of neutrophils and mean fluorescence intensity for the anti-CD 11b and anti-CD62L MoAbs.

		Cohen	Controls	
CD11b	% positive-cells	68.2	97.2±2.9	resting
		78.4	96.7±2.1	fMLP
		73.9	95.5±0.6	STZ
		84.5	90.2±3.5	PMA
	MFI	2.19	6.11±2.7	resting
		3.63	9.24±3.1	fMLP
		2.56	6.76±3.6	STZ
	4	5.46±2.7	PMA	
CD11b	% positive-cells	72.1	97±2.8	resting (WB)
	MFI	6.1	8.8±0.9	resting (WB)
CD62L	% positive-cells	56.5	96.2±2	resting (WB)
	MFI	3.7	12±1.8	resting (WB)

MFI: mean fluorescence intensity; WB: whole blood.

sole exception of the recent paper from Kivitié-Kallio *et al.*,⁴ there is no report on this topic in the literature published during the last twenty years. Our study is therefore the first demonstrating abnormal leukocyte function in the course of Cohen syndrome.

Neutrophils have various functional states, which may remarkably influence the parameters that we considered. Consequently, their activation state was evaluated by reproducing the different conditions occurring *in vivo* during the life of the cell, i.e.: in *resting* (as occurs in peripheral blood) and *primed* condition (as occurs after transmigration through the endothelium) and under stimulation by specific chemoattractants.

Superoxide anion release from Cohen leukocytes was adequate in all the experimental conditions we tested (data not shown), thus suggesting these patients having a normal defensive capability against microbial agents. Accordingly, there is no mention of severe infections complicating the clinical history of these patients.³ Our patient had never had infections with the only exception of recurrent gingivitis, a frequently reported complication of Cohen syndrome.^{4,10} The reasons for such periodontal-specific susceptibility remain obscure.

Blood and skin window neutrophils from our patient showed a several-fold higher spontaneous adhesion than control cells; agonist-induced adhesion was also more elevated in Cohen neutrophils than in control cells (Table 1). Cell response elicited by the different agonists is mediated by independent mechanisms, in particular fMLP- and STZ-stimulations act by mobilization of free calcium ions, whereas PMA involves the activation of protein kinase C.¹¹ Despite these specific receptorial and post-receptorial pathways elicited by fMLP, STZ and PMA, agonist-stimulated adhesion of Cohen leukocytes was simi-

larly increased in comparison with control cells. This suggests that the event responsible for the abnormal adhesion observed in Cohen syndrome could involve a final step common to the different pathways activated by the agonists.

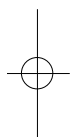
Firm adherence of circulating neutrophils to the vascular endothelium or an other biological substrate is an essential component of a multistep adhesion cascade that results in the accumulation and eventual migration of neutrophils through the vessel wall.¹² Circulating leukocytes are able to regulate adhesive interactions by controlling the expression of specific receptor and adhesive molecules.⁵ A first class of molecules involved in these interactions is represented by the selectins expressed by activated endothelium and by the leukocytes themselves. The binding of these molecules decelerates the flow of the circulating leukocytes by rolling on endothelial cells and allowing a second β_2 integrin-mediated step of adhesive interaction.^{12,13} Activated neutrophils shed L-selectin (CD62L) and modify affinity of CD11b/CD18 integrins.¹⁴

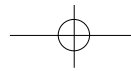
Our adhesion assay investigates leukocyte capacity to adhere to biological substrates, such as bovine fetal serum, by means of β_2 integrin-mediated interactions.⁸ To determine whether the increased adhesion observed in Cohen leukocytes was dependent on an altered expression of β_2 integrins or L-selectin, we also evaluated cell surface CD11a, CD11b and CD62L by direct immunofluorescence and flow cytometry.

Experiments were performed on both separated and whole blood leukocytes. Actually, granulocyte separation on a gradient induces a higher expression of CD11b, as demonstrated by Kuijpers *et al.*¹⁵ As we found a reduced mean fluorescence intensity and percentage of CD11b-positive cells in Cohen patient, the procedure used for purification of neutrophils could have underestimated the entity of the observed change. Moreover, experiments on whole blood (without any cell manipulation) from the patient furnished qualitatively similar results (Table 2). Finally, as shown in Figure 1, a double cell population was stained by fluorescence conjugated Ab anti-CD11b BEAR1. All of these data are consistent with the view that, on Cohen leukocytes, CD11b molecules have an abnormal binding affinity for the specific monoclonal BEAR1 Ab.

Expression of L-selectin on neutrophils is rapidly down-regulated and residual molecules are partly shed after activation.¹⁴ Cytofluorimetric expression of CD62L on Cohen leukocytes from whole blood was remarkably reduced in comparison with the expression on corresponding cells from control subjects (Table 2), once more suggesting that an activated state is a prominent feature of these neutrophils.

If the complex biochemical mechanism responsible for cell adhesion is to some extent permanently activated in Cohen syndrome, as our findings seem to suggest, granulocytopenia could be the final conse-





quence of such a process.¹⁶ As our findings do not prove a direct relationship between leukocyte count and the demonstrated functional abnormalities, future investigations will be necessary to confirm and extend these preliminary observations.

Contributions and Acknowledgments

OO and RC formulated the design of the study; OO wrote the paper and was the principal clinician involved; SL and CR performed the neutrophil function assays and cytofluorimetric analysis, respectively.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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