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Cytofluorometric assessment of phagosomal oxidation and the mode of inheritance in patients suffering from chronic granulomatous disease

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Introduction

Patients with chronic granulomatous disease (CGD) are mostly suffering from severe infections with catalase positive bacteria (e.g. Staphylococcus aureus, Escherichia coli, Pseudomonas). The main symptoms, beside mycotic infections with candida or aspergillus, are affections like pyoderma, recurrent pneumonia or abscess formation in lung, liver, spleen or bone marrow. This clinical picture is caused by a defective ability of the patient's cells to produce reactive oxygen intermediates (ROI), resulting in the incompetence of neutrophils and macrophages to kill phagocytised bacteria or fungi [1].

In the past, different laboratory methods have been used to detect CGD including chemiluminescence, nitroblue tetrazolium reduction and others. But most of these assays failed in cases of residual activity of cytochrome b or with reference to the diagnosis of X-linked heterozygous women.

In 1988 Rothe and colleagues described a simple method for the measurement of the ROI producing capacity by flow cytometry. Dihydrorhodamine 123 (DHR), a nonfluorescent dye, incubated with peripheral mononuclear cells (MNC) passes the cell membrane and becomes fluorescent through degradation by free reactive oxygen intermediates. Thus the resulting rhodamine, a well known fluorescent dye, can indicate the ability of neutrophils and monocytes to produce microbicidal metabolites [2]. The fluorescence intensity is proportional to the amount of produced ROI.

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Methods

In our investigations we obtained leucocyte suspensions by sedimentation of heparinized whole blood samples using dextran. The buffy coat was washed three times in cold PBS and adjusted to approximately 10^6 MNC/ml. Afterwards samples of 200 µl were incubated at first for 15 min with 10^{-8} Mol phorbol-myristate-acetate, followed by another 15 min incubation with DHR ((add 5 µl of DHR 123 dissolved at a concentration of 15 µg/ml in N,N-dimethyl-formamide) 37 °C, humidity 95%, 5% CO_2). Thereafter the intracellular production of ROI was assessed by flow cytometry using an EPICS C (Coulter Corp.), expressed as shift of green fluorescence intensity of each cell after stimulation. Neutrophils and monocytes were measured separately by setting a gate on each population depending on their different light scatter signals.

Results

The Figure (part A) shows the green fluorescence histograms of the healthy wife of a CGD patient. The left panel shows the unstimulated autofluorescence. In the right panel the mean peak of fluorescence intensity of PMA stimulated cells has moved

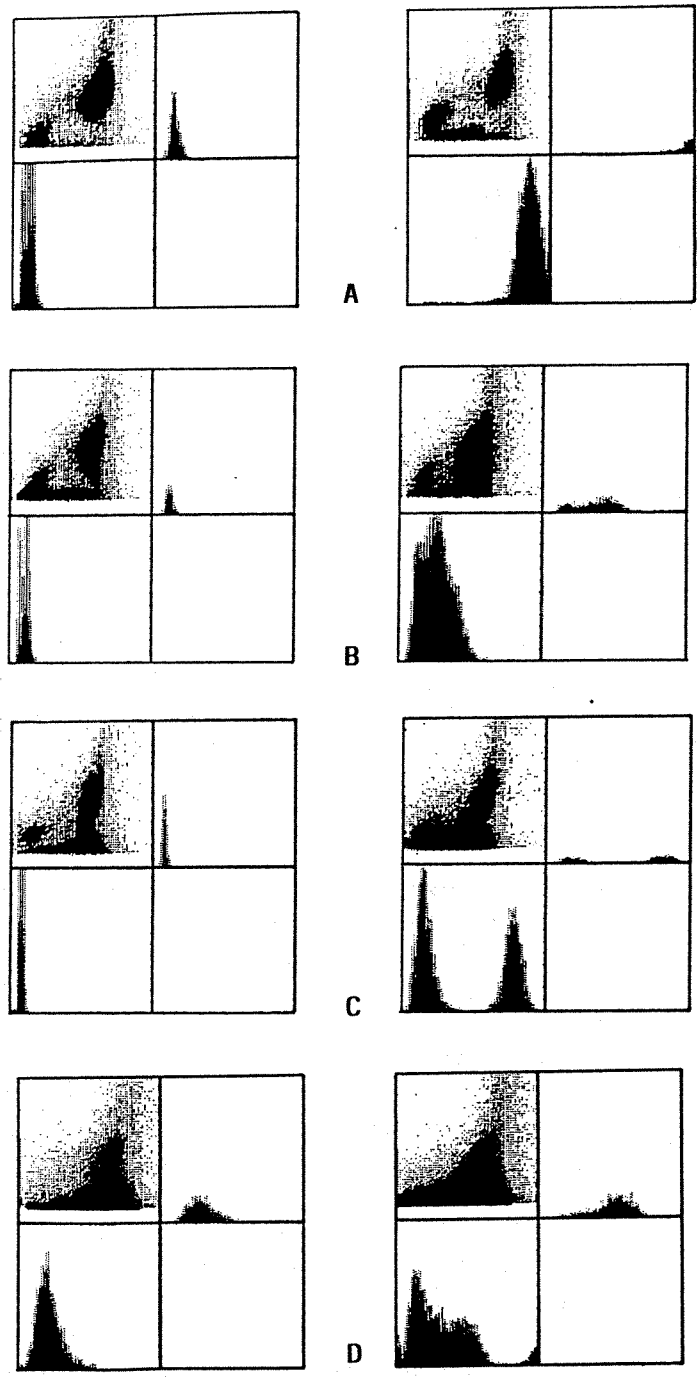

Figure 1
EPICS-C Cytoflowmeter histograms of DHR stained monocytes and granulocytes before (left panels of the figure) and after (right panels) stimulation with 10^{-8} PMA. The fluorescence intensity indicates the ability of the cells to produce reactive oxygen intermediates, "A" indicates the cells of the healthy donor, "B" indicates the CGD patient, "C" indicates the X-linked heterozygous daughter of the patient, "D" indicates the patient's cells after incubation with 200 U/ml IFN gamma.

to the upper fluorescence intensity canals (log scale).

In a 20-year old patient with CGD (part B) there is no shift in green fluorescence intensity of the neutrophils after stimulation. This indicates that these cells are not able to produce ROI. Interestingly, his monocytes show some but only little ROI production, visible as a very broad mean peak of fluorescence intensity.

His mother's as well as his daughter's cells show two different cell populations with differing capabilities to produce ROI as an expression of an X-linked mode of inheritance (part C, histograms of the daughter's cells).

The healthy two sisters and one brother of the patient have normal ROI production by all cells (data not shown).

In order to find out whether IFN gamma might be useful for treating this patient, we measured ROI production after *in vitro* incubation of the patient's MNC with IFN gamma (200 U/ml RPMI, 1 h, 37 °C). Afterwards 2.5% of the neutrophils showed normal ROI production and about 35% a slightly enhanced one, whereas the monocytes, after this short-term incubation period, changed their capability to produce ROI only a little (part D).

Discussion

In many cases it is easy to diagnose CGD with one of the conventional methods. Even though the diagnosis can be false negative if ROI production is diminished, but still sufficient amounts of ROI are present. Additionally, it is often difficult to find heterozygous carriers (women) as seen in the patient's daughter. In this case, we got border line pathological results in multiple investigations by chemiluminiscence because, in this technique, the reactivity of both populations is summarized. In addition, it is possible to determine the absolute count of "healthy neutrophils". 45% of the patient's daughter's neutrophils became normally stained after stimulation with PMA. From her blood count we knew that she had 8.7 Gpt/l neutrophils, i.e. she had an absolute count of "healthy neutrophils" of 3.9 Gpt/l in her circulation. So the method appears to be easier and more reliable for diagnosing X-lined carriers than other tests.

Furthermore, the induction or enhancement of the ability to produce intracellular ROI by IFN

gamma, which is now used in clinical protocols of CGD, could be tested *in vitro* and *ex vivo* [3, 4]. The results obtained in our patient suggest that he could be an IFN-gamma responder.

Summarizing, our results demonstrate that (i) the method should be useful in screening neutrophil function in CGD families as well as in cases of other granulocyte dysfunctions e.g. in septic disease, in immunosuppressed patients of after bone marrow transplantation and (ii) the ability or various substances (e.g. recombinant cytokines) to induce or enhance ROI production *in vitro* or *in vivo* could be tested.

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