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Diagnosis of chronic granulomatous disease and of its mode of inheritance by dihydrorhodamine 123 and flow microcytofluorometry

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Abstract. Dihydrorhodamine 123 (DHR) attached to membranes of granulocytes (PMN) and monocytes is caused to fluoresce by reactive oxygen intermediates (ROI) indicating the ability of phagocytes to produce these microbicide metabolites in a flow microcytofluorimeter. Whole blood samples from five boys with known chronic granulomatous disease (CGD) and from their mothers (and from one father and one grandmother), were examined following erythrocyte lysis in order to test this new method. An incubation period of 10 min with phorbol-myristate-acetate, followed by another 15 min incubation period with DHR before flow microcytofluorimetric analysis of 5 or 10×10^3 phagocytes, was sufficient to obtain the following results. PMN and monocytes from four patients with CGD could clearly not produce any ROI whereas cells from one patient displayed decreased activity in ROI production as compared to cells from a healthy donor. The X-linked mode of inheritance was detected in six carriers by the presence of two different cell populations (one normal ROI-producing and one negative or less active population). All the phagocytes from one mother produced ROI in normal amounts suggesting an autosomal mode of inheritance. All in all, the method presented provides a fast and most simple tool to diagnose CGD, to determine a decrease or total lack of ROI production and to establish the mode of inheritance of the disease.

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Abbreviations: CGD = chronic granulomatous disease; DCF = dichlorofluorescin; DHR = dihydrorhodamine 123; FSC = forward scatter; NBT-test = nitroblue tetrazolium reduction assay; PBS = phosphate buffered saline; PMA = phorbol-myristate-acetate; PMN = polymorphonuclear leucocytes; ROI = reactive oxygen intermediates

Key words: Chronic granulomatous disease – Dihydrorhodamine 123 – Diagnosis – Inheritance – Flow microcytofluorimetry

Introduction

Different laboratory methods such as chemiluminescence or cytochrome c reduction assay have been used to detect chronic granulomatous disease (CGD). However, if the cytochrome b activity is diminished but not absent, especially when only the nitroblue-tetrazolium reduction assay (NBT-test) is used, patients can be missed. On the other hand there is some danger of a false-positive diagnosis of CGD if cells are deficient in myeloperoxidase alone and for example a luminol-dependent chemiluminescent assay is used. Up to now the NBT test has been used most frequently to detect carriers, although alternative methods have recently been developed: cytochrome b is expressed on the cell surface of phagocytes and can be detected by monoclonal antibodies. Two cell populations (one cytochrome b^+ and one cytochrome b^-) could be determined in carriers by flow cytometry [4]. Another indicator of reactive oxygen intermediates (ROI) production, dichlorofluorescin, (DCF) [1,8] has also been used to detect these two carrier cell populations by flow cytometry [3], but this dye was less sensitive than dihydrorhodamine 123 (DHR) [6]. A rapid and simple method is described which provides basic diagnostic information concerning CGD.

Patients

All CGD patients had been diagnosed previously using standard assays: lucigenin- and luminol-dependent chemiluminescence [2],

cytochrome c reduction, NBT testing [5] (200 polymorphonuclear leucocytes (PMN) were counted) and sodium dithionite induced differential spectra [7].

All patients had characteristic symptoms of CGD e.g. all developed hepatic abscesses. Patients I and II presented as young infants whereas patient III was healthy until the age of 8 years. Disease onset was mild but then he also suffered severely.

Materials and methods

Chemicals and buffers

Dihydrorhodamine 123 was purchased from Molecular Probes Inc. (Eugene, Oregon, USA) and dissolved in N,N-dimethyl formamide (Sigma Chemicals, Munich, FRG) at a concentration of 3 µg/ml. This concentration was achieved by modification of the method of Rothe et al. [6] and was necessary due to the more sensitive optical system of our scanner.

Phorbol-myristate-acetate (PMA) (Sigma Chemicals, Munich, FRG) was diluted in dimethylsulphoxide to obtain a stock solution of 2 mg/ml, which was further diluted 1:100 with phosphate-buffered saline (PBS).

Zymosan (Sigma Chemicals, Munich, FRG) was suspended in PBS at a concentration of 12.5 mg/ml.

Enrichment of peripheral blood leucocytes

Peripheral blood was heparinized (10 IU/ml) and mixed with hydroxyethyl starch (Plasmasteril, Fresenius, Bad Homburg, FRG), (2:1) to promote erythrocyte sedimentation. After 30 min at room temperature the leucocyte containing supernatant was carefully harvested and washed twice with cold PBS supplemented with antibiotics (100,000 IU penicillin; 100 mg streptomycin/l). Remaining erythrocytes were lysed using doubly distilled water and 1.8% (w/v) sodium chloride solution.

The leucocytes were counted and adjusted to a final concentration of 2.5×10^6/ml.

Induction of ROI production

Human peripheral blood leucocytes (5×10^5/200 µl) were seeded into polypropylene tubes (Falcon 2063, Becton Dickinson, Heidelberg, FRG), stimulated by adding 20 µl of a PMA solution (20 µg/ml) and incubated for 10 min at 37°C.

Zymosan was used to assess the phagocytosis triggered ROI production. For this purpose 10 µl of the stock solution (12.5 mg/ml) was added to the samples.

At the end of the incubation period 10 µl of a DHR solution (15 µg/ml) was added and the cells incubated for 15 min at 37°C. In parallel, DHR was added to a sample not treated with PMA. This sample served as negative control for the DHR staining and was also incubated at 37°C for 15 min.

At the end of this second incubation 500 µl of PBS were added to each sample and the ROI production measured in the flow cytometer.

Measurement of ROI production using the FACScan

By using the FACScan (Becton Dickinson, Heidelberg, FRG) for analysis of ROI production, monocytes and granulocytes could be clearly defined and separated by setting a gate on each population. By this means both phagocyte populations could be analysed without need for further purification.

To set the gates, the forward scatter (FSC, size) and side scatter (granularity) of the cells were determined and recorded. The phagocyte populations of the whole blood sample were then identified and gated as shown in Fig. 1. Granulocytes are characterized as medium-sized cells with high granularity (gate 1). Monocytes are the largest cells in the blood sample with lower granularity (gate 2). Lymphocytes (3) and cell fragments, thrombocytes and erythrocyte fragments (4) can also be identified.

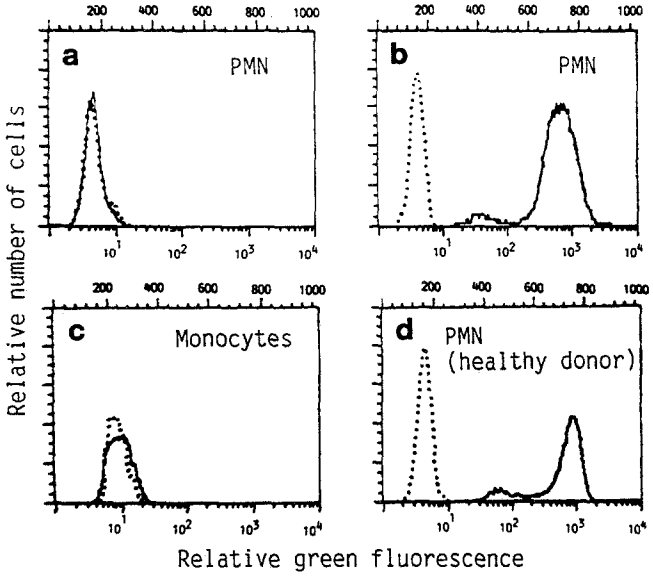

Fig. 2. Failure of phagocytes from patient I to produce ROI (**a, c**). Normal ROI producing populations of PMN from his mother (**b**). ·····, staining with DHR alone (negative control); ——, additional activation of cells with PMA

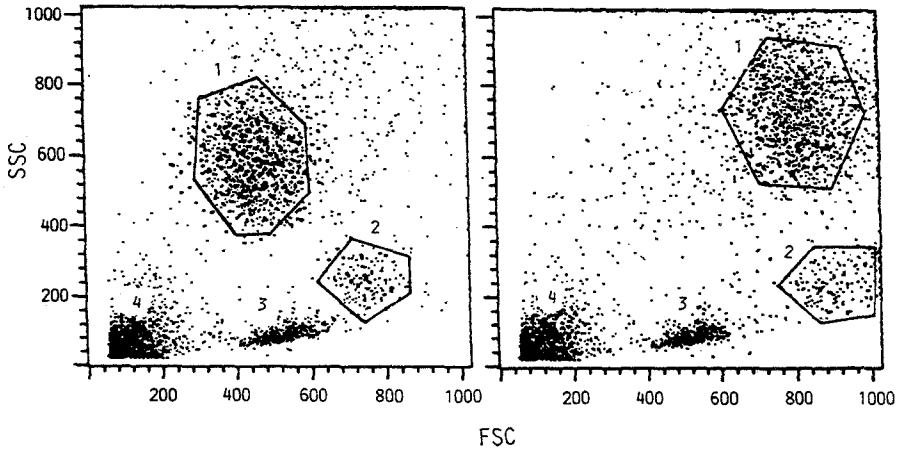

Fig. 1. Gating for further analysis by flow cytometry: *1*, PMN; *2*, monocytes; *3*, lymphocytes; *4*, erythrocytes, cell debris, thrombocytes. Shift after activation with PMA regarding FSC has to be considered for gating *(right side)*. SSC, side scatter

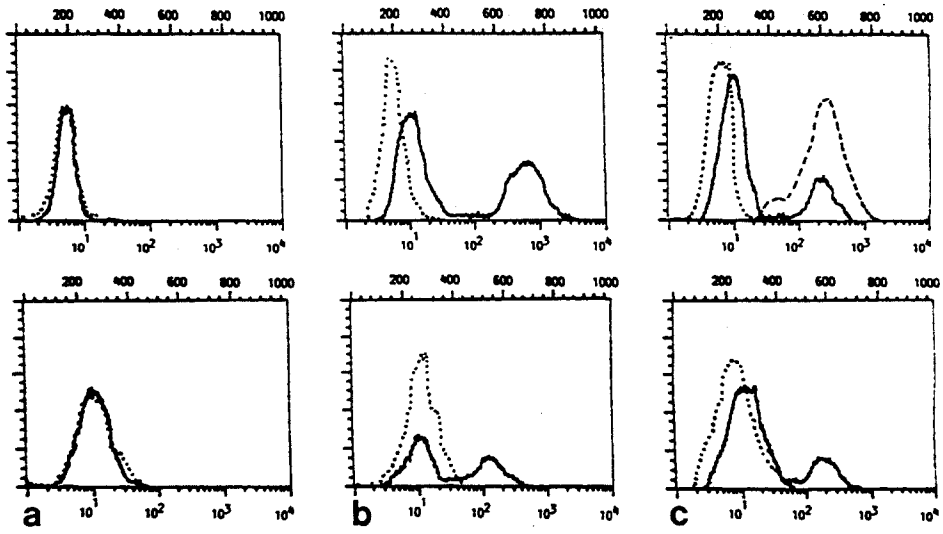

Fig. 3. X-linked CGD of patient II with failure of phagocytes to produce ROI (**a**). Mother (**b**) and grandmother (**c**) are carriers as revealed by two different cell populations. ·····, ——, abscissa, ordinate, as in Fig. 2. – – –, PMA-activated PMN of the father (for comparison). *Above:* PMN; *below:* monocytes

Having set the gate on phagocytes 5000–10,000, events were recorded whereby cells incubated with DHR only were measured first.

To analyse cells treated with PMA, the correct gate setting had to be confirmed and newly adjusted. This was necessary since PMA led to a change in both FSC and SSC (Fig. 1, right side), whereby neither autofluorescence nor binding or conversion of DHR was changed. This is clearly demonstrated by Figs. 2a and 3a.

Inducing ROI production by zymosan, no change in the FSC of granulocytes/monocytes was observed. Therefore the gate of the negative control could also be used for the measurement. Care was taken to avoid measurement of autofluorescent zymosan particles instead of PMN. Therefore, small particles and events occurring on the diagonal line in the dot plot "green fluorescence vs red fluorescence" were excluded by double gating.

Analysing the ROI production of the phagocytes, the shift in fluorescence 1 (green) was determined. This could be done by using multiple histogram analysis or calculation of the percentage of positive cells using the statistical option of the FACScan software.

Results

The method was initially tested with samples from healthy volunteers. As negative controls, the phagocytes were stained with DHR alone, but not activated by PMA.

Flow microcytofluorimetry always revealed a single "narrow" population of cells with very weak fluorescence (e.g. Fig. 2d). In contrast, populations of PMN from healthy donors, which were activated by PMA, shifted to the right, because they became strongly fluorescent due to ROI production. Very often, two cell populations were detected: a major one with a high degree of fluorescence and a minor one with a lower degree of staining. If the blood samples were handled properly, there was no overlap with the negative control.

Neither PMN nor monocytes from patient I became stained after activation by PMA (Fig. 2). The absence of any shift with regard to green fluorescence intensity after activation demonstrated the inability of the patient's cells to produce ROI in detectable amounts. In contrast, all PMN from his mother produced normal ROI and no negative cell population was present (Fig. 2b). Therefore an X-linked mode of inheritance is very unlikely in the case of patient I.

Accordingly, no ROI-production by phagocytes from this patient could be detected by chemiluminescence, cytochrome c reduction or NBT testing. In addition, PMN membranes were negative for cytochrome b_{558}. All of these assays resulted in normal values for his maternal phagocytes.

After activation with PMA and addition of DHR, phagocytes of patient II remained unstained as those from patient I (Fig. 3a). But in contrast to the mother of patient I, phagocytes from the mother of patient II clearly included two different cell populations: 45% of the PMN remained unstained whereas 55% became normally stained (Fig. 3b). Furthermore, PMN from the grandmother also demonstrated the two populations: 68% unstained vs 32% normally stained cells (Fig. 3c). The monocytes from the mother (Fig. 3b) and from the grandmother (Fig. 3c) revealed a very similar pattern as compared to the respective PMN. (The somewhat higher percentage of negative cells as compared to PMN is probably due to lymphocytes straying into the gate for monocytes.) The very weak shift of the unstained cell populations with regard to the negative control could be due to some "leakage" of fluorescent dye from stained cells to the negative populations (Fig. 3b, c), but this shift does not interfere at all with the recognition of the carriers of X-linked CGD. Thus, the mother and the grandmother of patient II are such carriers and further related women at risk were recommended for testing.

Again, the results from DHR-staining were compared to those from the four standard assays (see above): no ROI-production by phagocytes from patient II could be revealed and the cell membranes from PMN were cytochrome b_{558} negative. The results from NBT testing of PMN from the mother and from the grandmother were in good agreement with those from DHR-staining: in the mother 49% of the PMN remained unstained whereas 51% became normally stained. In the grandmother the NBT test revealed 65% unstained vs 35% normally stained cells.

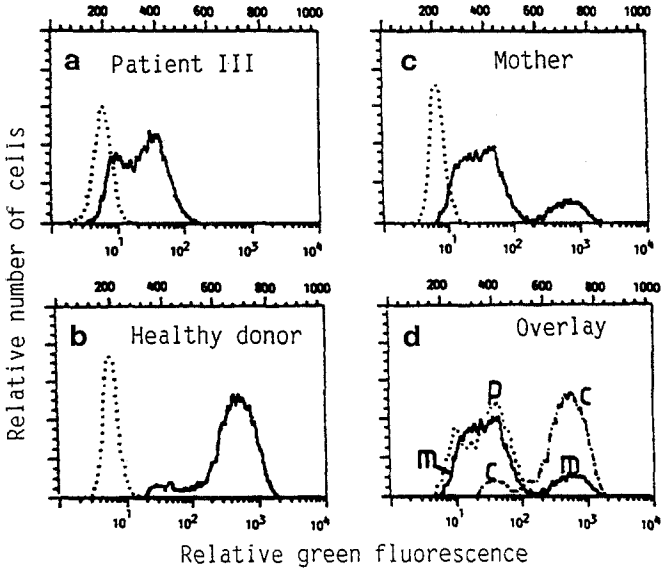

Fig. 4. X-linked CGD of patient III. His PMN retained significant ability to produce ROI. X-linkage is revealed again by two different cell populations from the mother. ·····, ——, as in Fig. 2, except "Overlay" (**d**) which reveals the position of the mother's cell populations (**c**) with regard to the son's (**a**) PMN and control's (**b**) PMN positions

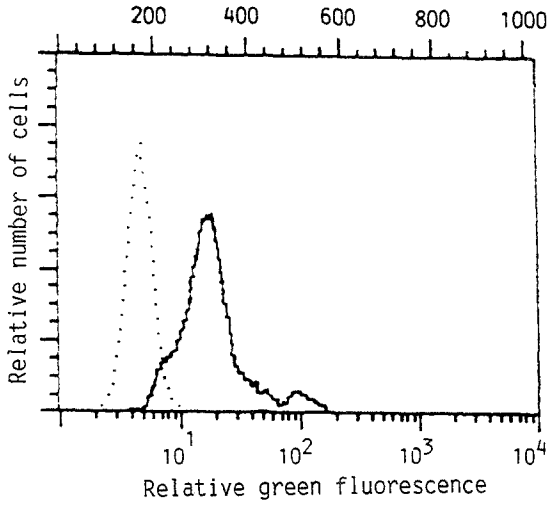

Fig. 5. Activation of ROI production in PMN of a healthy donor by phagocytosis of zymosan particles

Phagocytes from patient III retained significant ability to produce ROI, as demonstrated by a clear staining (Fig. 4a), but the shift of the cell population after activation with PMA with regard to green fluorescence was not as intense as that of healthy donor cells (Fig. 4b). Accordingly, very small peaks could be detected from PMN membranes by a dithionite-induced differential spectrum at 428 nm and 558 nm and the amount of ROI production by the whole cells was about 5%–10% of PMN from a healthy donor as revealed by lucigenin-dependent chemiluminescence and cytochrome c reduction. However, the correct diagnosis in this patient was missed on NBT testing because his PMN could be stained clearly. Therefore the outcome of a first severe episode of his disease was nearly fatal. Abscesses in the liver were not treated appropriately and became more and more severe (including many complications) until the correct diagnosis was revealed by cytochrome c reduction and appropriate antibiotics were introduced.

This patient also had an X-linked CGD since maternal phagocytes contained the two different populations: weak staining of 79% and normal staining of 21% of the PMN (Fig. 4c). An overlay reveals that the weak-staining population has the same position regarding green fluorescence as the son's PMN, whereas the normal stained population has the same position as the main peak of the control PMN. This pattern of two populations could only be revealed clearly by this method, whereas the NBT test was unsuitable: all maternal PMN were stained in a continuum of intensity and different populations could not be discerned. However, the mother has another son by a different father with the same variant form of CGD. Therefore, she is undoubtedly heterozygous.

Two other boys with classical X-linked CGD could be easily detected by DHR staining and flow cytometry. In addition, three related female carriers could be revealed by this method. The results were very similar to those obtained from patient II and from his mother, respectively. Again, these results were in complete accordance with those obtained by the conventional tests. Especially in the carriers, the ratios of the numbers of stained to the numbers of unstained cells were nearly identical to those resulting from NBT testing (max. deviation 5%). Moreover, an additional 26 healthy volunteers and patients with suspicious symptoms were tested: DHR staining and cytochrome c reduction were always in complete accordance and CGD could be excluded. No ambiguous result was obtained despite at least 30 measurements being performed in all CGD-patients.

Measurement of ROI production by DHR is not limited to activation of phagocytes by PMA. Addition of zymosan to the cell suspension also resulted in conversion of DHR into the fluorescent form (Fig. 5). Thus, the method is suitable for determining whether activation mechanisms of ROI production by phagocytosis are intact.

Discussion

The results obtained by staining of phagocytes with DHR and subsequent flow microcytofluorimetry were in complete accordance with those from conventional tests (cytochrome c reduction and chemiluminescence) in five CGD patients (altogether at least 30 tests) and in more than 26 healthy donors. Moreover, the method described was in complete accordance with NBT testing in four patients and in five female carriers (X-linked form).

However, using the NBT test alone, the diagnosis of variant CGD was missed in one patient with nearly fatal consequences. This diagnosis could then be revealed and confirmed by cytochrome c reduction, chemiluminescence, spectroscopic measurement of cytochrome b_{558} and by the method described here (Fig. 4). Since false-negative results using the NBT test are not unique (per-

sonal communication) this test should not be used for screening for CGD.

The mother of this patient (III) is undoubtedly heterozygous because another son from her and from another father suffers from the same variant form of CGD. This was revealed neither by NBT testing nor by any other conventional test. However, the two different PMN populations could easily be detected by DHR staining and flow cytometry. Therefore, this method appears to be more reliable in detecting variant CGD and X-linked carriers of this form than the NBT test.

Some similar information concerning CGD could be obtained by staining phagocytes with monoclonal antibody against cytochrome b also using flow cytometry [4]. However, this method is not suitable for screening, because cytochrome b positive CGD would be missed. The two methods compete in some aspects (e.g. determination of carriers) and staining with DHR is done with less expense, but they are supplementary in other aspects e.g. indication of the biochemical defect.

One example for determination of heterozygous PMN by another indicator of ROI, DCF, also using flow cytometry, has already been described [3]. But the two different cell populations could only be discerned beyond doubt after addition of catalase. Presumably, sensitivity of DCF alone is not sufficient for a test used routinely to detect X-linked carriers. DHR was described to be about three times more sensitive than DCF [6].

Our results determine the possible future place of DHR staining as a diagnostic tool amongst the conventional methods. It could be used: 1. As a screening test. 2. For confirmation of an otherwise prediagnosed CGD. 3. For determination of retained ability to produce ROI with high sensitivity. 4. For determination of a defective activation mechanism for ROI production by phagocytosis. 5. For detection of X-linked carriers (perhaps of carriers of other forms of CGD?). 6. For prenatal diagnosis (blood samples of 100–200 µl or even less are sufficient).

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