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PHENOTYPIC ANALYSIS OF DIFFERENTIATED HL-60 CELLS FOR USE IN STREPTOCOCCUS PNEUMONIAE OPSONOPHAGOCYTIC-KILLING ASSAY

Immunology

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ABSTRACT

Aim & Background Use of differentiated Human leukemia cells HL-60, as a source of phagocytes in opsonophagocytic-killing assay to test protective and functional efficacy of pneumococcal vaccines is well established. 5 to 6 days chemically differentiated granulocytic HL60 cells with the expression of CD35 ≥ 55%, CD71 ≤ 20% of markers and ≤ 25% apoptotic cells are used as effector cells. The study analyses the phenotypic characters of granulocytic HL60 cells to identify the optimal days for their use in OPA assay. **Method** HL60 cells were differentiated with 0.8 % N, N-dimethylformamide (DMF) for 10 days. Phenotypic change examined by flow cytometry using a FACS Calibur (Becton-Dickinson, San Jose, CA, USA) with specific antibodies to CD11b, CD35, CD71, IgG1, and IgG2a. Apoptosis and necrosis determined using Propidium Iodide and FITC respectively. The assay was repeated for 10 times to observe inter assay variability. **Results** The expression of CD11b, CD35, IgG1 and IgG2a gradually increased significantly from day 1 to day 7 reaching the optimal concentration by day 4 ($P \leq 0.05$). CD71 decreased from day 1 to 4 and had optimal levels on day 4 to 7. Apoptotic and necrotic markers were within the optimal range during 4 to 7 days. The study shows expression of HL60 cells phagocytic, proliferation and apoptotic markers from day 4 to 7 can be used as effector cells in OPA assay. **Conclusion** The advantage of using the differentiated effector cells for 4 days in place of 2 days is demonstrated. Further research is needed to understand the functional efficiency of the differentiated cells.

KEYWORDS

Opsonophagocytic assay (OPA), Human leukemia cells (HL-60 cells), N,- N-dimethylformamide (DMF), Streptococcus pneumonia(S. pneumonia).

INTRODUCTION

HL-60 cell characteristics to use in in-vivo phagocytosis

Hl60 cells have the ability to grow in suspension culture. The doubling time vary from 20h to 45h, depending on the subline. All sublines display stages of a myeloblastic/promyelocytic morphology: large, blast-like cells with characteristic large, rounded nuclei containing 2-4 distinct nucleoli, and a basophilic cytoplasm with azurophilic granules [,]. Cultures of HL60 cells comprise 90-95% of cells with this morphology; the remaining cells display morphologies resembling those of more mature myeloid cells (mainly myelocytes, with some neutrophils and monocytes).

HL60 cells have the property to differentiate in vitro either to granulocyte-like or to monocyte/macrophage-like cells depending on the nature of the inducing agent []. Polar-planar compounds such as dimethyl sulphoxide (DMSO), and other compounds such as all -trans retinoic acid (ATRA), butyrate, hypoxanthine, dimethyl sulfoxide (DMSO) or dimethylformamide (DMF)[], all induce differentiation to granulocytes, while 1, 25-dihydroxyvitamin D3, phorbol esters and sodium butyrate induce monocyte/macrophage differentiation[,].

The property of HL60 cells that has probably attracted most interest is the ability of a variety of agents to increase the proportion of cells differentiating in vitro to as great as 90%, and the capacity of cloned cell populations to differentiate either to granulocyte-like or to monocyte/macrophage-like cells depending on the nature of the inducing agent[]. Polar-planar compounds such as dimethyl sulphoxide (DMSO), and other compounds as diverse as retinoic acid and actinomycin D, all induce differentiation to granulocytes, while 1,25-dihydroxyvitamin D3, phorbol esters and sodium butyrate induce monocyte/macrophage differentiation[,].

HL-60 cells when treated with DMF, expression of genes for differentiation occur and halt proliferation process. Changes in genes during differentiation includes complement receptor 1 (CR1; C3b receptor), indicators of successful differentiation [] . The expression percentage of receptors can be monitored by flow cytomteric technique. Morphological alterations during differentiation include shrinkage in cell size, decreased nuclear-cytoplasmic ratio, increased nuclear pyknosis and segmentation, decreased cytoplasmic basophilia, replacement of the coarse azurophilic granules with smaller specific granules[], and alterations in gene and protein expression along macrophage [,] and granulocytic [] lineages. The differentiated cells such as granulocytes have the property of phagocytosis which can be used as source of phagocytes in in-vitro opsonophagocytic assay (OPA). The ability of granulocytes to opsonize pneumococci is the key measure of vaccine-induced immunoprotection, diverse in vitro methods of measuring opsonic capacities of antibodies have been devised; these assays are termed opsonophagocytic assays (OPAs).

The successful differentiation of HL60 for an OPA may be measured by the acquisition of attributes of a phagocyte such as receptor sites, which recognize opsonins. Phagocytic cells, primarily granulocytes, recognize the Fc portions of the bound antibody and the C3b and iC3b complement deposited onto the bacterial surface via specific cell receptors.

For the determination of vaccine-induced opsonophagocytic capacity, the primary receptors of interest are the Fcγ types I, II, and III. FcγRI (CD64) is the high affinity receptor for immunoglobulin G1 (IgG1), IgG3, and IgG4; FcγRII (CD32) is the low-affinity receptor for aggregated IgG1, IgG2, and IgG3; and FcγRIII (CD16) is the low-affinity receptor for IgG1 and IgG3. Although Fc receptors are important in opsonization, the complement-dependent opsonophagocytic pathway remains central to the clearance of Streptococcus pneumoniae in vivo []; complement receptors for C3b (CR1 and CD35) and iC3b (CR3 and CD11b) are the primary receptors mediating opsonophagocytosis of pneumococci and other encapsulated bacteria[]. The presence of complement receptors in HL-60 cells and HL60 granulocytes has been previously documented []. Expression of CD11b, which binds noncovalently to CD18 to form the Mac-1 integrin[] , is a useful marker of granulocytic differentiation and

of suitable cell lines for OPA [,,]. Because of its simplicity and reproducibility, flow cytometric analysis is the practical way of monitoring the differentiation of HL60 cells to obtain granulocytes with phagocytic function. Differentiation of HL-60 can be readily identified by monitoring the expression of cell surface markers and may therefore be used to quality control the effector cells.

MATERIALS AND METHODS

Cell culture

Human promyelocytic leukemia cell line HL60 (obtained from ATTC) was cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal clone I serum (HyClone, Logan, UT, USA), 100X (50 mg⁄ml) penicillin/streptomycin (Gibco) and GlutaMax-1 (Gibco).

Differentiation of HL60 Cells

80 ml of cells removed from CM1 flask and transferred to 50 ml centrifuge tubes. Centrifuged at ~350g for 5 minutes at RT and supernatant removed carefully without disturbing the pellet. Pellet suspended in 1ml CM2 (containing 0.8% DMF) media[], taken for cell count. The concentration of cells will be adjusted to ~4 x 10^5 cells/ml after the cell count. To the above cells 100 ml of CM2 (containing 0.8% DMF) media added and mixed well and transferred carefully to tissue culture flask without touching the neck of the flask. Then flask is carefully placed in lying flat position in a tissue culture incubator (37ºC, 5% CO2. Differentiated cells will be used on day 5 and 6 days. Cells not used within 6 days will be discarded[].

Flow cytometry assay for percentage expression of HL60 cell phagocytic marker.

Phenotyping of HL60 cells of undifferentiated and differentiated. Tubes labelled A, B, C, or D and 22 FACS tubes labelled 1 to 22. Approximately 4 ml of undifferentiated (A & B) cells (2 X 10^6) and 3 ml of differentiated cells (C & D) (2 X 10^6) were taken in tubes labelled A, B, C and D and were centrifuged at 350 g for 5 minutes at 4 °C. Cell pellets were suspended in 1 X PBS and 50 microlitres of these cells were aliquoted to Facs tubes labelled with respective antibodies. 10 microliters of PE labelled antibodies CD11b, CD35, CD71, IgG 1/5, IgG 1/80, IgG 2a pipetted to respectively labelled tubes. Tube without antibodies considered unstained. All the tubes incubated at 4 °C for 30 mins. 150 microliters of Facs/buffer added and centrifuged at 350 g for 5 minutes at 4 °C. Cell pellets were suspended in 250 microliters of Facs/buffer and analysed in flow cytometry instrument (Beckman Coulter Dx Flex B5-RO-V Flow Cytometer one laser 488 nm). Viability of cells determined by annexin V FITC and PI labelled apoptosis and necrosis.

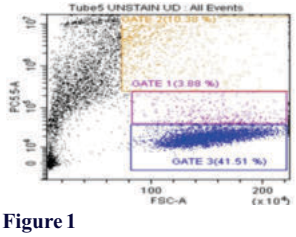
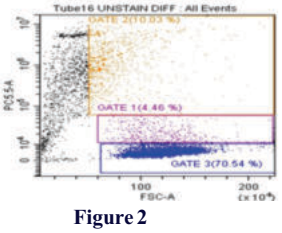


Figure 1



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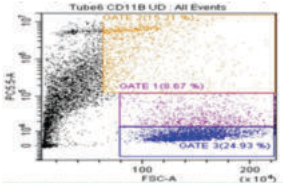

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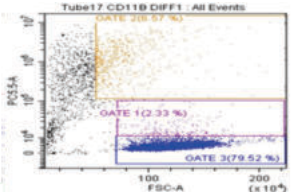

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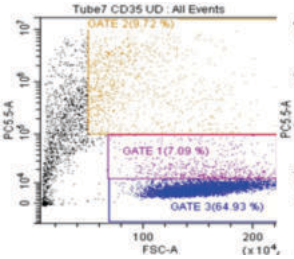

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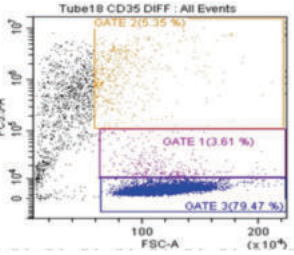

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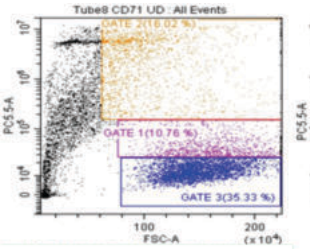

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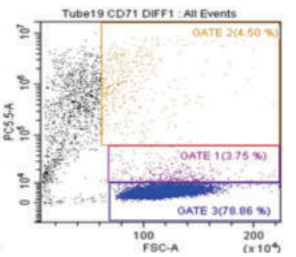

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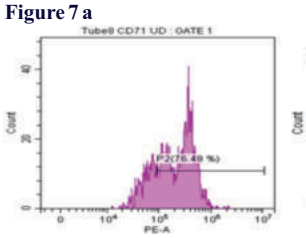

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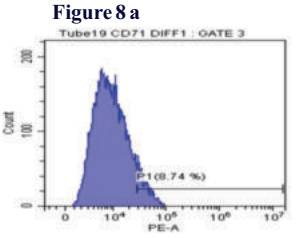

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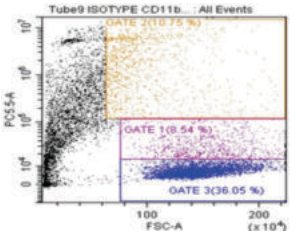

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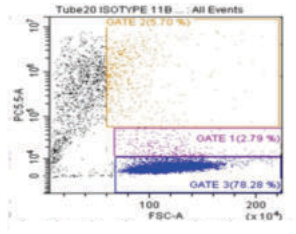

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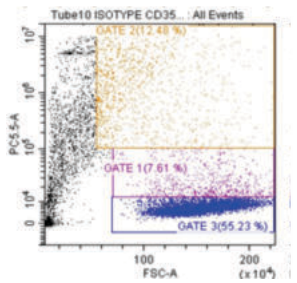

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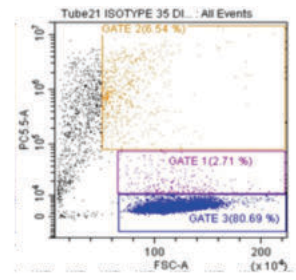

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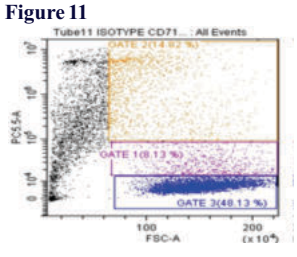

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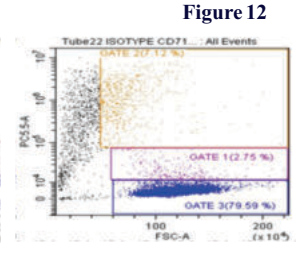
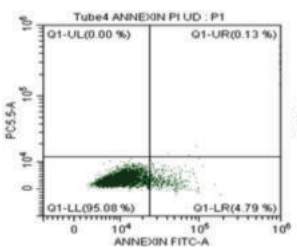


Figure 14



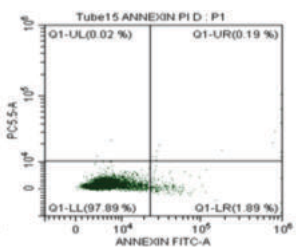


Figure 1: Representative images showing gating for CD11b, CD35, CD71, IgG1, IgG2a for undifferentiated and differentiated HL60 cells.

Statistical analysis

The phenotypic expressions of differentiated HL-60 cells were expressed as means±SD and were statistically analyzed by two-tailed non parametric man Whitney U-test.

RESULTS

To investigate the differentiation efficiency of HL-60 cells, CD11b, CD35, CD71, IgG1, IgG2a were used as the phagocytic and complement surface markers and Apoptosis Detection kit used the evaluation of cell viability. Earlier studies suggest differentiation need five days to produce efficient expression of CD11b, CD35, CD71, IgG1, IgG2a. Our study showed that with early day 4 and later day 7 showed significant expression of surface markers and increasing differentiation time i.e from day 8, the expression decreased drastically. The association between the differentiation time and the survival rate of cells was determined and the results indicated that the cell viability was similar from the fourth to the seventh day; however, cell death increased significantly from the eight day.

Table 1. Effects of Dimethyl formide induced differentiation on HL-60 cell towards granulocytes

	n=10	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
1	CD11b	32.08 ±6.76	39.59* ±5.57	51.49* ±5.69	75.42* ±4.19	72.83* ±7.66	77.78* ±4.64	79.1* ±4.01	71.785* ±5.95	34.03 ±12.41	29.05 ±8.08	30.35 ±6.94
2	CD35	32.08 ±6.83	39.59* ±5.77	47.10* ±6.29	71.85* ±4.69	76.39* ±4.64	78.63* ±3.67	75.51* ±6.79	76.43* ±4.80	27.74 ±5.47	30.64 ±8.95	25.40 ±5.74
3	CD71	35.11 ±5.67	41.74* ±6.08	57.65* ±14.09	67.68* ±14.12	15.75* ±6.42	8.52* ±6.13	11.80* ±5.69	12.49* ±4.81	24.77* ±5.49	25.36* ±5.67	25.36* ±4.27
4	IgG1/5	31.54 ±6.83	39.78* ±6.45	46.67* ±6.80	73.18* ±4.77	76.39 ±4.64	78.63* ±3.67	75.55* ±7.22	7.16* ±5.49	27.74 ±5.47	29.75 ±8.10	25.31 ±6.04
5	IgG1/80	29.14 ±5.54	47.50* ±6.46	53.10* ±8.45	70.21* ±6.89	76.88 ±8.61	79.27* ±3.53	76.04* ±5.06	60.29* ±7.36	26.96 ±5.93	25.56 ±4.40	26.39 ±5.95
6	IgG2a	26.52 ±6.95	43.73* ±8.98	53.67* ±7.85	70.21* ±6.89	76.68 ±8.42	80.03* ±4.67	77.0* ±4.00	61.67* ±7.36	27.01 ±10.16	29.36 ±5.42	28.39 ±4.84
7	Annexin V+/PI-	4.17 ±4.79	2.72 ±1.31	2.70 ±1.24	6.50 ±1.23	7.37 ±0.97	1.81 ±0.631	1.87 ±0.76	2.23 ±0.99	20.97* ±6.40	28.21* ±5.57	25.9* ±5.23

HL-60 cells cultured with DMF over 10 days were assessed for the above.

*p<0.05 vs day 0.

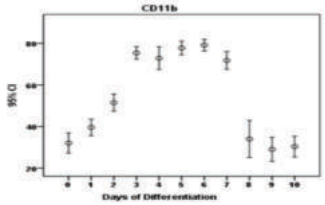


Fig1: CD11b expression from day 0 to day 0 to day 10 differentiated HL-60 cells

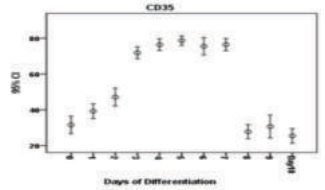


Fig2: CD11b expression from day 0 to day 10 differentiated HL-60 cells

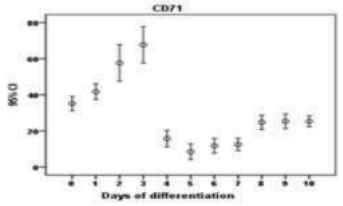


Fig3: CD71 expression from day 0 to day 10 of differentiated HL-60 cells

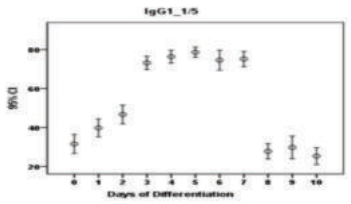


Fig4: IgG1_1//5 expression from day 0 to day 10 of differentiated HL-60 cells

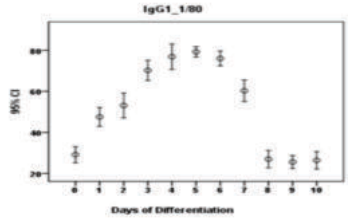


Fig5: IgG1_1/80 expression from day 0 to day 10 of differentiated HL-60 cells

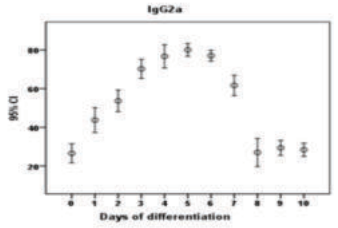


Fig6: IgG2a expression from day 0 day 10 of differentiated HL-60 cells

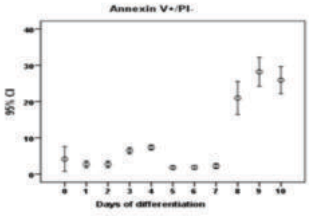


Fig7: Annexin V+/PI- expression from day 0 to day 10 of differentiated HL-60 cells

DISCUSSION

Approximately 5 to 10% of HL-6 cells appear to be mature myelocytes; many resemble mononuclear phagocytes, metamyelocytes, banded cells, or fully segmented neutrophils[]. Similarly, about 5 to 10 % of cultured HL-60 cells have properties of differentiated granulocytes, such as phagocytic ability and the ability to respond to chemotactic peptides (e.g.,N-formylmethionyl-leucine) [,]. The course of the differentiation induced by chemical agents is accompanied by a large number of changes in the cells, and is easily monitored by morphological, histochemical and immunological criteria[]. Thus, incubation with DMSO or retinoic acid leads, over a period of 5 days, to a progressive decrease in the size of HL60 cells and condensation of nuclear material with the appearance of kidney-shaped nuclei characteristic of the myelocyte and, later, lobed nuclei characteristic of banded and segmented neutrophils[]. The nuclear/cytoplasmic ratio decreases, and the cytoplasm becomes more diffuse. In parallel with these changes there occur marked changes in histochemistry, including decreased myeloperoxidase activity and the appearance of cells capable of reducing nitro blue tetrazolium (a granulocyte marker)[29].

Our study suggests the expression of phagocytic surface markers CD11b, CD35, CD71, IgG1, IgG2a has been seen optimal levels on day 4 to 7 and depletion of expression with increase in days upto 10 []. Arthur Millius et al. suggest the cells achieve a density of 1–2 million cells/mL at 7 days of post-differentiation []. However, cells are most active at 5–6 days post-differentiation, but can still respond even at day 8. These differentiated cells can be used as granulocytes for opsonophagocytic assay (OPA). Evidences suggest cells at day 5 and 6

used for opsonophagocytic assay [, ,]. However, current study inkling the use of DMF treated HL-60 cells at day 4 and 7 as well. Although finding suggest the use of cells at 4th and 7th days, factors such as viability and density need to be considered critically. The limitation of present study is a comparison of use of these cells at different days and impact on OPA is not seen.

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