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Early Transmembrane Potential Changes of Lymphocytes in Mixed Lymphocyte Cultures as Detected by Flow-Cytometry

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Human peripheral blood lymphocytes (PBL) respond to allogeneic cells in the mixed lymphocyte culture (MLC) with proliferative as well as nonproliferative reactions. These responses are related to the degree of disparity between the human lymphocyte antigens (HLA) of allogeneic cells and determined by genes located within the human major histocompatibility complex (MHC) [1, 2]. Proliferation in the MLC can be quantitated by the amount of radioactive thymidine incorporated in the DNA of dividing cells. The incorporation is usually determined after a culture period of 96 to 120 h which can be shortened under special circumstances to 28 to 36 h [3]. Proliferative reactions as blast transformation and cell division are late steps in a chain of physical and metabolic reactions which follow the initial antigen recognition. The membrane permeability for various ions such as H^+, K^+, Na^+, Ca^{2+} and Mg^{2+}, sugars, nucleosides and amino acids are changed seconds to minutes after binding of the antigenic molecules to surface receptors of lymphocytes [4, 5]. It is conceivable that the transmembrane potential (TMP) is altered as a consequence of the altered permeabilities. The transmembrane potential of cells can be determined by implanted microelectrodes, a technique which is slow and technically not possible for lymphocytes. Indirect methods use the distribution of radiolabeled weak acids or bases or fluorescent cationic dyes [6] between the interior and exterior of the cell. The indirect methods provide only estimates of the average potential of all cells in suspension and are not sensitive if only a small fraction of the cells depolarizes.
Flow-cytometry in combination with the

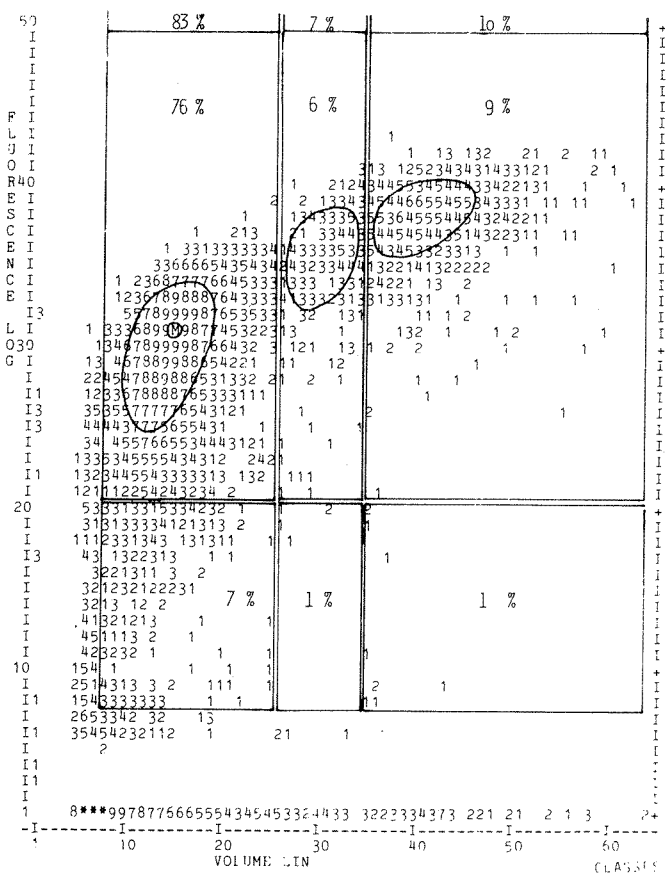

Fig. 1. Isoamplitude print of cell volume versus fluorescence of human PBL of blood donor A stained with DiOC6(3) for 5 min at 22 °C. The cell volume scale is linear and one cell volume class corresponds to 13.14 μm^3. The fluorescence scale comprises 2.5 logarithmic decades. The logarithmic cell frequency in each histogram class was calculated for the amplitude display. The print out was standardized to the maximum logarithmic frequency indicated by "M". The three-decade range of logarithmic cell frequencies was equally divided into 10 steps (numbered *1* to *9*, zeros are suppressed). The total cell number in the histogram was 66.561 cells, "M" corresponds to 303 cells. The encircled areas were drawn for the better visualization of peaks of distinct cell subpopulations. 7% of the total cells were depolarized in the compartment of small cells. A similar number of cells was observed in the autologous control of blood donor B (not shown)

lipophilic cationic dye 3,3′-dihexyl-oxacarbocyanine iodide [DiOC6(3)], in contrast, permits the fast measurement of changes of the TMP of individual cells. DiOC6(3) has been used for the estimation of the TMP of individual lymphocytes following mitogen stimulation [7]. The aim of this study was to determine whether changes of the TMP occurred in the allogeneic MLC. Fresh PBL were isolated from heparinized (50 U/ml) human blood by a Ficoll step gradient as described previously [8], washed twice, and resuspended at a final concentration of 1.5×10^6 cells/ml in RPMI 1640 medium (Grand Island Biological Co, Grand Island, N.Y.) with 10 m*M* HEPES (hydroxyethyl-piperazine-ethanesulfonic acid), penicillin (100 U/ml), streptomycin (100 µg/ml) and L-glutamine (2 m*M*). The cells were kept without protein addition at room temperature (22 °C) until use. Cells from unrelated individuals and from families were selected from a local panel which was characterized in the VIII. International Histocompatibility Workshop. The indicator dye DiOC6(3) (Eastman Kodak Co., N.Y.) [9] was dissolved in absolute ethanol at a concentration of 15 µg/ml. 5 µl of this solution were added to 250 µl of cell suspension (1.5×10^6 cells/ml) in RPMI 1640 medium without protein. The assay was incubated for 5 min with the stain at room temperature before the flow cytometric measurement. The fluorescence and the cell volume of the stained cells were measured simultaneously in a Fluvo-Metricell flow cytometer which was developed in our laboratory [10]. The cell volume was measured electrically, and the fluorescence was excited with a HBO 100 high-pressure mercury lamp using a 450 nm high pass and a 500 nm low pass filter (Schott, Mainz, FRG). The emitted fluorescence was collected between 500 and 580 nm. An orifice of 85 µm diameter and 100 µm length with a current of 0.40 mA was used for the electrical cell volume determination. The electrical conductivity of the RPMI medium was 15 mS (22 °C). Between 500 and 1000 cells/s were measured. Two-parameter histograms of cell volume versus fluorescence were collected in a 64×64 array of a multichannel analyzer and stored on magnetic tape for further evaluation. Display and quantitative evaluation of the stored histograms was performed with FORTRAN IV computer programs [11].

Figure 1 shows a representative histogram of cell volume versus intracellular DiOC6(3) fluorescence of PBL from a single individual after 5 min staining. Two cell types with high and low stain were consistently observed. Cells with higher fluorescence than fluorescence channel 20 represented $90.2 \pm 3.3\%$ of all cells as determined in 15 independent experiments. The remaining cells contained less stain. Three subpopulations of cells could be distinguished by cell size. $85.8 \pm 2.5\%$ of the cells were small with a mean size of $195.8 \pm 6.4\ \mu m^3$, $7.1 \pm 1.7\%$ had an intermediate volume of $387.6 \pm 2.6\ \mu m^3$, and $8.0 \pm 1.6\%$ had a large cell volume of $547.9 \pm 11.8\ \mu m^3$. Each of the small, intermediate and large cells occurred with high (greater than channel 20) or low (smaller than channel 20) fluorescence which means that six subgroups of cells with different cell volume and fluorescence could be determined in each two-parameter histogram (Fig. 1, 2).

In the two-way MLC, 3×10^5 cells of each partner (A, B) were incubated at a concentration of 1.5×10^6 cells/ml for 5 min at 22 °C, and then stained with DiOC6(3) for 5 min (Fig. 2). A significant increase in the number of depolarized cells in the fluorescence channels below 20 was observed in all three cell volume subpopulations. The most obvious depolarization following the incubation of allogeneic cells occurred in the fraction of small cells. 7% of all cells showed a low stain in the compartment of small cells if cells of individual A (Fig. 1) and individual B were incubated separately, while 19% of cells were found in this compartment after 10 min allogeneic co-incubation of cells from individual A and B (Fig. 2).

The number of depolarized cells in the co-incubated A+B assays of unrelated donors increased in the subpopulation of small cells by $10.6 \pm 2.2\%$, by $0.88 \pm 0.3\%$ for the cells of intermediate size, and by $0.95 \pm 0.3\%$ for the large cells as compared to the autologous controls. The means were calculated from a total of 15 separate experiments. We also examined TMP

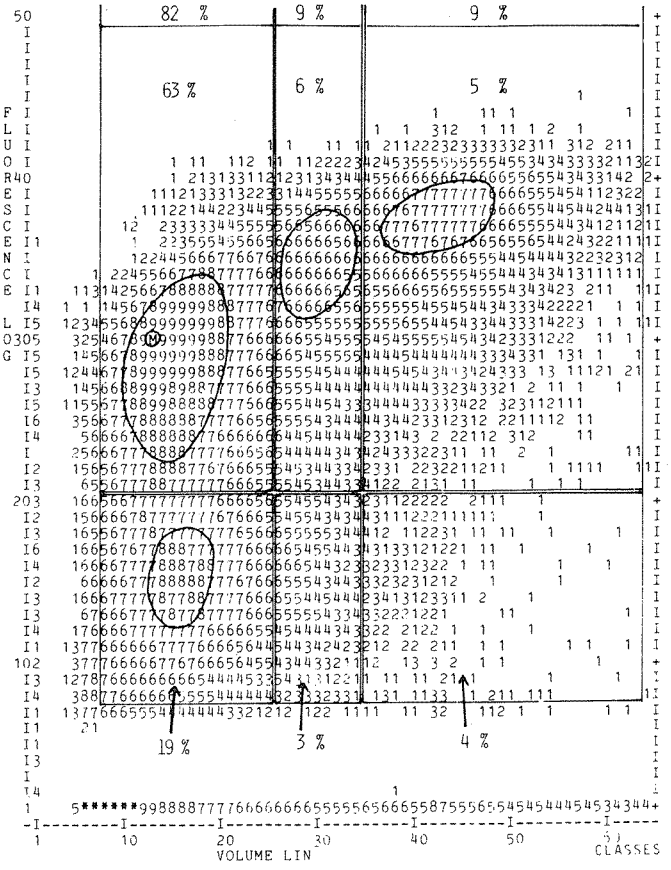

Fig. 2. Isoamplitude print out of cell volume versus DiOC6(3) fluorescence of an allogeneic two-way MLC with equal numbers of cells of blood donors A+B, co-incubated for 5 min and stained for additional 5 min at 22 °C. A subpopulation of small cells (19%) was depolarized after this time. Their number corresponds to an increase of 12% depolarized cells over the autologous control shown in Fig. 1

changes in two-way MLC of HLA-identical and -different siblings in a family. The parents in this selected family carried four different HLA-D specificities as determined by typing with HLA-D homozygous typing cells. Two identical siblings showed almost no increase in depolarized cells ($+0.3 \pm 0.1\%$) for the small cells, one haplotype-different sibling showed $+3.0 \pm 0.2\%$, and two haplotype-different siblings $+8.1 \pm 0.3\%$ additional depolarized small cells after 10 min co-incubation. These results suggest a close relationship between TMP changes and cellular recognition of HLA antigen disparity.

DiOC6(3) has been well characterized as a specific dye for indicating the TMP of individual living cells [6, 7, 9, 12]. DiOC6(3) detects the changes of the TMP following mitogen stimulation [7], and our results suggest that DiOC6(3) can also be used for the detection and rapid quantitation of lymphocyte activation by allogeneic cells in the MLC. A constant fraction of small lymphocytes, representing about 10% of all cells in a two-way MLC, depolarizes within 10 min co-incubation at room temperature. Furthermore, the numbers of cells undergoing depolarization increase in correlation with HLA haplotype disparity. In contrast to other methods, the flow cytometric measurements provide information not only about the average degree of stimulation but also about the exact number of responding cells and their degree of depolarization. An advantage of the flow-cytometric assay is that no radioactivity is required. An even more important advantage seems to be that MLC reaction can be performed much faster than by any other method. This is of particular interest in cases where quick decisions concerning the histocompatibility of donor and recipient tissues in transplantation are necessary. Independently from the clinical requirements, this method provides a new possibility to study the initiation of the immune response because the early reacting cells are funtionally identified by the TMP changes and should be sortable by a cell sorter. This would allow the identification of the lymphocyte subsets which are involved in the early immune reaction events.

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