The usefulness of microculture assay system in differential diagnosis and therapeutic management in children. Part 1: the evaluation of reference ranges

DARIA AUGUSTYNIAK[1], ADAM JANKOWSKI[1,2]

[1]Immunology Unit, Department of Microbiology, Institute of Genetics and Microbiology, University of Wrocław, Poland; [2]Clinic of Children's Immunology and Rheumatology, Medical University of Wrocław, Poland

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Abstract

Polyclonal activators are widely used as surrogate antigens in analysis of human proliferative response in vitro. *The great advantage of microculture system is that it belongs to one of the most powerful diagnostic tool that make possible to characterize some markers of cellular immune response. The relevant diagnostic markers are useful indicators of many different disorders. Part 1 of this study was designed to evaluate the reference ranges of four immunological parameters involved in microculture assay system to estimate the functional activity of cellular PBMC* in vitro *response in healthy immunocompetent children. The studied parameters were: suppressive activity of T cells (SAT index), the degree of proliferative response against phytohaemagglutinin (PHA) and concanavalin A (ConA) mitogens (PC index), the immunogenic activity of monocytes (LM index) and accessibility of T cell growth factor (IL-2 index). Authors have presented the reference ranges for all parameters included into this system for healthy immunocompetent children that are necessary to carry out the correct analysis of peripheral blood mononuclear cells* in vitro *in clinical samples. In part 2 we will estimate and discuss the clinical importance, diagnostic accuracy and application each of those parameters in pediatric practice.*

Key words: *microculture assay, proliferation, mitogens, PHA, ConA, PBMC, healthy children.*

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Introduction

Cellular immunity is important and integral part of human immune response. The process of T cell activation can be initiated by diverse agents such as antigens, mitogens, cytokines and monoclonal antibodies. These stimuli acting through T cell receptor cause complex series of ordered interactions and events, that lead to T cell proliferation, differentiation, cytokine production, and induction of T-cell tolerance [1-5]. The mononuclear cell population (PBMC) isolated from peripheral blood contains the number of lymphocytes and monocytes representative for the respective blood values of the donor. Under appropriate experimental conditions, the results of tests performed on the *in vitro* cultures offer a great possibility to obtain insight into the actual functional state and sometimes even in actual immune status of the individual patient. Thus, basic immunoregulatory activities which can be observed and precisely quantified in microcultures of immune cells, represent an unique and objective model that enables us to estimate proper homeostatic function of our organism [6].

Correspondence: Daria Augustyniak, PhD, Institute of Genetics and Microbiology, University of Wrocław, Przybyszewskiego 63/77, 51-148 Wrocław, Poland. Phone number: +48 71 375 62 96, fax number: +48 71 325 21 51, Email: august@microb.uni.wroc.pl

In general it is well established that impairment or augmentation of immune function plays a contributing role for many different disorders during childhood. Hence, the evaluation of cellular immunity, apart from humoral ones, has very important diagnostic and prognostic implications. There are many functional assays performed to evaluate both innate and adaptive immune responses such as phagocytosis, natural killer cell activity or mitogen-induced lymphocyte transformation assay. Regarding to the latter at present the broad panel of stimulators including both polyclonally acting mitogens (PHA, ConA, PWM) and specific antigens (Candida antigens, Tetanus antigens, PPD) are used. These methods are one of the most powerful tool for the characterization of cellular immune response *in vitro* that in turn, enables the clinician to make the proper diagnosis and to administrate the peculiar therapeutic management, as well.

The described by Dąbrowski et al. [7, 8] microculture *in vitro* system is based on the measurement of four cellular immune response parameters including SAT index, PC index, LM index and IL-2 index and, as the authors emphasized, was designed and carried out using PBMC isolated from adults and among adulthood population was extensively studied. For several years mentioned above microculture system has been successfully used in our laboratory focused on children's diagnosis. Because every diagnostic procedure requires the reference to proper normal values that are calculated for population with similar demographic characteristics, first of all, we were obliged to evaluate the ranges of normative values of measured parameters among that particular population.

The purpose of the present study was to evaluate the reference ranges of immunological parameters assessing the functional activity of cellular response in healthy immunocompetent children. In the part 2 of this article we will estimate and discuss the clinical importance, diagnostic accuracy and application each of those parameters in pediatric practice.

Material and Methods

Blood donors

Blood samples were obtained from 26 healthy immunocompetent children aged 5 to 16 years (mean ± SD, 10.8±3.17; median 11.5). Males and females were represented in numbers of 9M:17F. Children were enrolled for the study with institutional ethics committee approval and parental agreement. Children were all in good health, had physical and laboratory finding within normal range and have not been receiving any antibiotics during the preceding 4 weeks. None of the participant's parents reported medical history of asthma, other allergic, parasitic or immunologic diseases nor any chronic medical problems in anamnesis. Informed consent was obtained from all children's parents. All children were volunteers. Heparinized blood was obtained at volume 5 ml, and PBMC were isolated according to the techniques described below.

Isolation and propagation of human PBMC

PBMC were separated from heparinized human blood by ficoll density gradient centrifugation (Lymphoflot, Biotest) and washed three times in Hanks medium (BIOMED--Lublin) with 18 U/ml of neomycin. PBMC were cultured in RPMI-1640 (Sigma) supplemented with 15% inactivated autologous human plasma and 2 mM L-glutamine at a final concentration 10^6 cells/ml (10^5 cells per well/200 µl) in the presence of different mitogens. The mitogens used as stimuli for PBMC were PHA at final concentration 2 µg/ml (Murex Diagnostics Ltd) and ConA (Sigma) at final concentration 40 µg/ml.

Proliferation assay

Freshly isolated PBMCs were cultured in 96 round--bottomed microplates (Falcon) at 37°C in a humidified atmosphere containing 5% CO_2. Non stimulated as well as mitogen-stimulated cells were cultured in triplicates and each data point represented the mean of triplicate cultures. In all experiments PBMC were stimulated using the optimal concentrations of used mitogens which were previously defined in dose-response experiments.

Cell proliferations were determined after 72 hours of culture. Sixteen hours before the end of the culture, cells were pulsed with 0.4 µCi/well of [^{3}H]thymidine (Lacomed, Czech Republik) that was used as the parameter for cellular activation. After cell harvesting the incorporated radioactivity was measured by a liquid scintillation counter (Beckman LS 100C). Viability was ensured by trypan-blue staining.

Some data were expressed as the stimulation index (SI): the ratio of the mean dpm value (disintegrations per minute) of experimental cultures in the presence of mitogen to mean dpm of unstimulated control cultures.

Parameters of cellular immunity

The microculture system designed to evaluate features of T cells and monocytes that are important for the regulation of the immune response was previously described [6-8]. Briefly, the elements involved into this system were:

A. Evaluation of ConA induced suppressive activity of T lymphocytes (SAT) calculated according to formula: SAT index = 1- [cPHA+cConA]/[cPHA+sConA] + ½ [cConA+sPHA] ×100 where cPHA+cConA – proliferative response of PBMC stimulated with PHA to T lymphocytes activated previously by ConA; cPHA+sConA – proliferative response of PBML stimulated with PHA to cell-free supernatant (CFS) from cultures activated by ConA; cConA+sPHA – proliferative response of ConA stimulated PBMC in the presence of CFS from PHA activated culture. The possible mutual influences of "mixed" microculture supernatants on proliferation titer were include in the formula as described elsewhere [7].
B. Evaluation of PBMC response to PHA and ConA (PC index): the mean proliferative response of cultures stimulated for 72 hours with PHA versus the mean proliferative response of cultures stimulated for 72 hours with

Con A were calculated. The relative predominance of the response to PHA or to ConA in comparison with values determined for healthy children should be interpreted as the presence of a more or less advanced stage of maturity of T lymphocytes present in PBMC population [8].

C. Monokine influence on T cell proliferative response (LM index): Two PBMC triplicates were incubated with medium alone and third with PHA for 24 hours. Then the 100 µl of CFS of each well of second triplicate was discarded and replaced with an equal volume of CFS from PHA stimulated PBMC. The cultures were next seeded additional 48 hours before harvesting. The ratio of proliferative response between the second and the first triplicate was calculated to express the LM index that reflects the immunogenic activity of monocytes.

D. Evaluation the efficiency of Con-A stimulated PBMC in assessment of IL-2 index: Two PBMC triplicates were stimulated with ConA and third triplicate with PHA for 24 hours. Then the 100 µl of CFS of each well of second triplicate was discarded and replaced with an equal volume of CFS from PHA stimulated PBMC. The cultures were next seeded additional 48 hours before harvesting. The ratio of proliferative response between the second and the first triplicate was calculated as the IL-2 index. The results of addition of PHA-CFS or purified IL-2 were the same as described elsewhere [7]. The IL-2 index above or below reference range should be assumed as indicative of decreased or increased ability of lymphocytes to produce endogenous IL-2 and their dependence on delivery of the exogenous IL-2, respectively.

Statistical analysis

The reference (normative) ranges for variables: SAT index, PC index, LM index, IL-2 index were calculated as mean extended or diminished by 2SD value. The normal distribution and variance analysis was performed by Shapiro-Wilk and Levene's test, respectively. The statistical analysis between males and females was determined using parametric t-test for independent variables. *P* values of <0.05 was selected to indicate significance. Inter-assay variation was determined by assessing identical triplicate samples from 3 adult's donor in three assays measured in different days. Intra-assay variation was calculated by the software using series of triplicate samples. Analysis was performed with StatView software.

Results

The childhood population enrolled for the study involved 9 males and 17 females at the age 5-16 years old. Comparing the sexual status of study population we did not found (except of one) any significant differences for tested parameters between males and females indicating that studied group was homogeneous population. Detailed statistics of demographic and cellular characteristics are presented in table 1.

Table 1. Statistics of demographic and cellular characteristics

		Totals	**Males**	**Females**	**p#**
№		26	9	17	M:F
age (years)	mean ± SD/median	10.8±3.17/11.5	10.11±3.68/10	11.12±2.93/12	NS
PHA*	mean ± SD	27.95±11.25	35.53±9.9	23.95±9.97	p<0.01
	(95% CI)+	(23.4-32.5)	(27.92-43.14)	(18.82-29.07)	
	median (min-max)	30.38 (4.09-49.25)	36.93 (17.5-49.25)	20.12 (4.09-40.91)	
ConA*	mean ± SD	20.77±10.21	24.63±10.42	18.72±9.78	NS
	(95% CI)	(16.64-24.89)	(16.62-32.64)	(13.68-23.75)	
	median (min-max)	17.95 (8.25-40.91)	21.53 (12.15-40.91)	16.8 (8.25-38.27)	
SAT index	mean ± SD	27.34±6.19	28.37±8.55	26.79±4.74	NS
	(95% CI)	(24.84-29.84)	(21.79-34.94)	(24.36-29.23)	
	median (min-max)	26.47 (17.58-40.81)	26.54 (17.58-40.81)	26.41 (19.84-36.17)	
PC index	mean ± SD	1.55±0.35	1.55±0.44	1.55±0.31	NS
	(95% CI)	(1.41-1.69)	(1.12-1.89)	(1.39-1.71)	
	median (min-max)	1.46 (0.98-2.31)	1.45 (0.98-2.31)	1.47 (1.07-2.13)	
LM index	mean ± SD	2.14±0.96	2.29±0.85	2.05±1.08	NS
	(95% CI)	(1.74-2.54)	(1.64-2.95)	(1.5-2.16)	
	median (min-max)	1.64 (0.97-4.52)	2.08 (1.24-3.69)	1.59 (0.97-4.52)	
IL-2 index	mean ± SD	1.08±0.14	1.08±0.14	1.08±0.14	NS
	(95% CI)	(1.03-1.14)	(0.97-1.19)	(1.0-1.15)	
	median (min-max)	1.06 (0.86-1.35)	1.03 (0.87-1.35)	1.08 (0.86-1.29)	

** – data are represented as stimulation index (SI) described in* Material and Methods*; # – statistics were performed with t- student test for independent variables; NS – not significant; + – 95% confidence interval.*

The magnitude of proliferative response of PBMC following the stimulation with PHA or ConA showed differences between individuals reflecting an actual functional state and proliferative potency of particular PBMCs. In general PHA induced stronger proliferative response than ConA and interestingly we observed significantly higher responsiveness of male in comparison to female PBMCs. The main principle in determination of SAT parameter was *in vitro* preactivation of PBMC with ConA in order to generate the population of T cells with suppressive activity. The PBMC response to stimulation with PHA at the presence of Con A-pretreated autologous lymphocytes was suppressed in all cases. The mean level of suppression was 27.34±6.19 (median 26.47, min. 17.58 – max. 40.81). The normal range of SAT index in healthy children that lie between end points of mean ± 2 SD was from 14.96 to 39.72. The SAT index below or above this range should be considered as the decrease or increase in suppressive activity of T cells, respectively.

The reference ranges that we have similarly calculated for the remaining parameters of the microculture assay system were as follows: for PC index – from 0.85 to 2.25, for LM index – from 0.22 to 4.06, for IL-2 index – from 0.8 to 1.36. The values being out of these ranges for well-defined parameter should be considered as abnormal (explain in *Material and Methods*). The calculated intervals that we have chosen as the most relevant in estimation of normal values are conventionally accepted in medical studies indicating that more than 95% of tested normal cases were included within the particular range.

To confirm assay reliability intra- and inter-assay coefficients of variations were determined. Intra-assay coefficients of variation for proliferation assay were typically between 5 and 10%, whereas inter-assay variation fell below 20%.

Discussion

This study determined the normal ranges for four parameters that characterize an actual functional state of peripheral blood mononuclear cells in microculture system, in healthy immunocompetent children. Since different stimuli act on different cell types and via different receptors, two accessory cell dependent polyclonal activators were studied in detail: phytohemagglutinin (PHA) that acts on both the CD2 molecule and T cell receptor complex (TCR, CD3), and Con A exerts its action in T cell induction through TCR [9, 10]. Using an *in vitro* restimulation system, we have found that a primary activation with ConA effectively suppressed the subsequent response to PHA in all tested samples with manifestation of SAT index in the range from 14.96 to 39.72. The given range of normal SAT value typical for healthy children is markedly below the normal range of 30-50 described in literature for healthy adults [7]. This observation indicates that adult's range of SAT can not be used as the reference range for pediatric patients. The reason for the lower suppressive activity in children's population in comparison to adulthood one is not known, as it was not the purpose of the present study.

The ratio of PHA-induced proliferative response versus ConA-induced proliferative response of PBMCs in culture was expressed as PC index. The values of that index ranged from 0.85 to 2.25 with mean of 1.55±0.35 indicate that in studied population the more effective proliferation was seen in response to PHA than to ConA action. Predominance of proliferative response after PHA action seems to be a general phenomenon, however in terms of analogous determination of PC index for adults with range from 2 to 4 [7], pediatric values are near two-fold lower. It is well established that spectrum of target T cells for each mitogen is known to be somewhat similar but with different specificity against surface oligosaccharides. Cona A is reported to stimulate primarily suppressor inducer T cells or "virgin" (naïve) T cells [11, 12] and acts through its specific recognition of mannose structures, primarily trimannosides [13], whereas PHA is specific for complex oligosaccharides including mannose, N-acetylglucosamine, and sialic acid [14]. It was also documented that kinetics and profiles of surface expression molecules such as CD69, CD25, and CD71 – essential for T cell proliferation, following PHA- or ConA-mediated stimulation of human PBMCs, showed major differences neither between $CD4^+$ and $CD8^+$ subsets nor between PHA and ConA activation [1]. Hence, for now we can only speculate that possible predominance of functionally different subsets together with different mitogen affinity could be responsible for observed phenomenon. It is noteworthy that CD4/CD8 ratio of studied by us lymphocyte populations was within normal range (data not shown).

Proliferative response of ConA-induced PBMCs in the presence of supernatant from 24h-PHA-induced PBMC versus proliferation stimulated via ConA for 72 h served for calculation of IL-2 index, which the reciprocal may be calculated as percentage of IL-2 receptor saturation [15]. It is well established that IL-2 and IL-4 are main growth factors responsible for T cell activation and proliferation triggered by their interaction with specific receptors [16, 17]. It is also well documented that the mitogen stimulation of PBMCs result in high levels of many cytokine production over a relatively short time period and that peak levels are recorded where the balance between production, consumption, degradation and homeostatic suppression is achieved. In addition, the earliest production is restricted primarily to IL-2, and in terms of PHA stimulation, maximal amount is observed after 24 hours of culture and is followed by a transient decline [18]. Moreover, the maximum levels of IL-2 mRNA correlated significantly with levels of cell proliferation during response of normal human lymphocytes, as reported elsewhere [19].

In addition to IL-2 index also LM index of studied microculture system takes into account the influence of cy-

tokine milieu. As recently described, LM index expressed from PBMC cultures may serve as a suitable parameter for evaluation of the monocyte immunogenic activity indicating that the delayed stimulation with PHA is inversely proportional to IL-1ra/IL1β supernatant concentration ratio [6]. In contrary to IL-2 index for which the reference range was rather narrow, the reference range of LM index was considerably wider. It was the result of the slight and greatest dispersion of tested values within the whole studied population, respectively.

Two main questions should be addressed when considering the above data. These are: 1. What is the significance of the particular parameter in terms of functional properties reflecting the immune competence of PBMCs? 2. What applications are there for clinician from measurements of microculture system *in vitro*?

The magnitude of the proliferative response that appears following mitogen stimulation almost certainly indicates the predominant functional state of the responding T cell population. The strongest responses are recorded where the balance between proliferation and homeostatic suppression is achieved. We hope that detailed analysis we have just been performing, enable us to estimate the diagnostic accuracy of the whole microculture system in pediatric practice. Moreover, this model may have been usefully employed in examining the proliferative response changes during therapy for a number of diseases where the dysregulation of T cell subset function is known or suspected. It refers for example to allergy [20], autoimmune diseases [21] or immunodeficiences [22]. We had been investigated the diagnostic accuracy of presented here microculture system as well as the effects of immunomodulatory drugs on normalization of presented here parameters of cellular immunity in pediatric patients with different disorders. These results will be discussed in the part 2 of the article.

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