THE ANALYSIS OF VIRUS-CAUSED ALTERATIONS OF CELLULAR AND HUMORAL IMMUNORESPONSIVENESS DURING THE FIRST DAYS OF MORBILLI RUSH IN THE YOUNG ADULT POPULATION

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Summary. – The parameters of cellular and humoral immune responsiveness were studied during the first days of morbilli rush in the group of 34 young adult patients. The analysis of cell-mediated immune response included T cell relative and absolute numbers, mitogen-induced T cell proliferative response, as well as the relative and absolute numbers of mononuclear phagocytes. The tested parameters of humoral immune response included B cell relative and absolute numbers, serum concentrations of immunoglobulins IgG, IgA and IgM, and serum level of immune complexes (IC). The reduction of total leukocyte number, T cell and B cell relative and absolute numbers, as well as diminished mitogen-induced T cell proliferative response, associated with the elevation of IgM and IC serum levels were found in the majority of analyzed patients. Moreover, the subsequent analysis revealed positive correlations between T cell percentage and T cell proliferative response, as well as between serum concentrations of IgM and IC. These data confirmed the presence of acute morbilli infection-caused disturbances of patients' immunocompetence.

Key words: cell-mediated immunity; humoral immunity; acute morbilli infection; young adults

Introduction

The predominant role of either humoral or cellular immune response (IR) in the elimination of primary viral infection, and further protection against subsequent infection with the same virus, is not fully understood. Generally, after initial phase of an antiviral IR which includes recognition of viral pathogens as foreign entities in the context of major histocompatibility complex (MHC) antigens on the surface of antigen-presenting cells, there is a subsequent cascade of cellular and humoral IR which complement each other (Zurbriggen and Fujinami, 1990). Accordingly, the extensive antigen stimulation present in disseminated phase of acute measles infection provides pronounced activation of immunity of different qualities (Norrby, 1985). Concerning both humoral and cellular IR, one of the major antiviral processes is neutralization by antibodies. The presence of antibodies capable to neutralize free circulating viruses or destroy virus-infected cells, even correlates with the host protection against measles reinfection (Graziano *et al.*, 1975). However, in order to clean an infection, functioning cell-bound immunity is requested, especially since measles virus spread must take place by direct cell-to-cell transmission (Norrby, 1985). According to that, in contrast to agammaglobulineamia children who are able to ward off measles in a normal fashion (Graziano *et al.*, 1975), children with inherited or aquired defective cell-bound immunity (various forms of thymic deficiency, leukemic children treated with cytotoxic drugs...) did not survive measles or developed a progressive forms of disease involving organs like lung, kidney or brain (Aicardi *et al.*, 1977). The pathogenic events occuring during the incubation period are yet speculative, but may include local virus replication, as well as viremic spread of virus. The latter may be accentuated by viral replication in selected reticuloendothelial cells, and by the use of lymphocytes as transport vehicles for viruses (Norrby, 1985), very likely damaging the immunocompetent cells.

Considering all mentioned data, in order to analyze the immunocompetence of patients in the first days of morbilli rush, this study was performed in young adult population during an outbreak of morbilli infection.

Materials and Methods

Patients. We included a total of 34 patients aged from 15 to 31 years (mean age 23.2 years) with general features of the acute symptomatic morbilli infection, confirmed by serologic diagnostic procedures. The clinical symptoms included lesions Koplik's spots (red ulcers with bluish white center in the bucal mucosa), and subsequently developed morbilli macular or maculopapular rush. The patients were admitted to the Institute for Infectious and Tropical Diseases, „Dr. Kosta Todorović", Clinical Center of Serbia, where clinical diagnosis (by Dr. E. Gvozdenović and her associates), and serologic diagnosis (by Dr. Žerjav and her associates) were done. The immunological profile of each patient was analyzed in the Laboratory of Immunology.

Enzyme-linked immunosorbent assay (ELISA). The specific serologic diagnosis of morbilli infection was done by ELISA (Lievens and Brunell, 1986) determining the specific IgM and IgG antibodies. The commercial microtiter plates (Behring) were coated with morbilli virus antigen (Test-plate Antigen coated for Enzygnost Measles, Behring). For detection of IgM antibodies rabbit anti-human IgM conjugated with alkaline phosphatase (Anti-Human IgM/AP Conjugate, Behring) was used while for detection of IgG antibodies rabbit anti-human IgG conjugated with alkaline phosphatase (Anti-Human IgG/AP Conjugate, Behring) was employed. Chromogen TMB (Chromogen TMB + Buffer/Substrate TMB, Behring) was used as appropriate substrate. A_{405} (A_{492}) for both IgM and IgG antibodies were read using Behring ELISA Processor with cut-off value of 0.200.

Dilution of sera to detect specific IgM antibodies was 1/40 (with previous adsorption of IgG antibodies with RF adsorbens). If serum sample at dilution 1/40 gave absorbance value equal or higher than the cut off value, it was considered that specific IgM were detected. The results were expressed in qualitative manner (positive or negative). The IgG antibodies were detected semiquantitatively. The starting dilution of sera was 1/40, final dilution 1/640, while dilution of 1/80 was considered as a measure of IgG positivity. The maximal dilution which gave absorbance value equal or higher than the cut-off value (characteristic for antigen-antibody complex formation), was used as a semiquantitative measure of specific IgG antibodies.

Isolation of peripheral blood mononuclear cells (PBMC). PBMC were separated from heparinized whole blood by Lymphoprep (Nycomed AS Diagnostica, Oslo, Norway) gradient centrifugation. Interface cells were washed 3 times in Haemaccel (Jugoremedija, Zrenjanin, Yugoslavia), and resuspended at concentration of 4×10^6 cells/ml.

Total T cells. The total T cell number in peripheral blood was assessed by their capacity to form rosettes with sheep red blood cells (SRBC), as described before (Wybran and Fudenberg, 1973). Briefly, PBMC suspension mixed with SRBC suspended in Haemaccel, was incubated in water bath at 37 °C for 15 mins. After centrifugation the incubation was continued overnight at 4 °C. The cell pellet was placed into a haemocytometer and 2×100 cells were counted. Any cell binding three or more SRBC was considered positive.

Lymphoproliferative response to phytohaemagglutinin (PHA). *In vitro* lymphocyte reactivity was estimated by their proliferative response to mitogen PHA using the whole blood method as it was described before (Spužić *et al.*, 1971). Briefly, to sterile bottles containing RPMI medium supplemented with 20% heat-inactivated foetal bovine serum, penicillin and streptomycin, 10 drops of heparinized blood, as well as PHA (reagent grade - PHA, Wellcome, England) to optimal concentration of 80 μg/ml were added. After 4 days of incubation at 37 °C, colchicine (20 μg/ml, Sigma Chemicals, St. Louis, USA) was added to arrest mitoses, and incubation was continued for further 4 hrs. After centrifugation, addition of 0.76% sodium citrate and another centrifugation, the cell pellet was resuspended in methanol-glacial acetic acid (1:3)fixative, put on glass slides and stained with Giemsa solution. The number of mitoses was counted per 2×1000 cells, and the results were expressed as percentage of normal response (of healthy persons).

B-cells. The number of B cells was determined by rosetting with yeast particles coated with complement as it was previously described (Rivero *et al.*, 1979). Briefly, PBMC suspension mixed with yeast particles suspended in Haemaccel, was incubated in water bath at 37 °C for 15 mins. After centrifugation the incubation was continued at room temperature for 1 hr, and overnight at 4 °C. The cell pellet was stained with 1% fuchsin and transfered into a haemocytometer. 2×100 cells were counted and any cell binding three or more yeast particles was considered positive.

Serum immunoglobulins. Concentrations of serum immunoglobulins IgG, IgA and IgM were determined by radial immunodiffusion (RID) method (Mancini *et al.*, 1965) using Behring NOR Partigen plates.

Circulating immune complexes (cIC) were determined by spectrophotometric method (Riha *et al.*, 1979). As measure of cIC presence the A_{450} of patient serum in the presence of 3.75% polyethylene glycol (PEG, M_r 6 000) solution was used.

Mononuclear phagocytes. The number of mononuclear phagocytes was estimated by the yeast particles phagocytosis method (Vujanović *et al.*, 1982). Briefly, PBMC suspension, mixed with yeast particles suspension in Haemaccel containing 0.0125% neutral red, was centrifuged and incubated at 37 °C for 1 hr. The cells were washed twice in cold EDTA 0.02%, PBS and Haemaccel, stained with trypan blue and transfered into haemocytometer. 2×100 cells per sample were counted, and any cell phagocyting yeast particles was considered positive.

Statistical analysis. Data for tested parameters were expressed as median values, and also as percentages of patients within the whole group tested, in whom observed values were above or below the normal range previously estimated for each parameter. The correlation analysis between the pairs of analyzed immunological variables was examined by the Spearman correlation test (non-parametric). Any p value below 0.05 was considered significant.

Results

Serologic testing

As already metioned results of specific IgM antibodies were expressed in qualitative manner (positive or negative), while results of specific IgG antibodies were expressed semiquantitatively in titers. While specific IgM antibodies were determined in all included patients, specific IgG antibodies, as additional analysis, were tested in majority of included patients (n=26).

In all tested patients the specific IgM antibodies were found to be positive. The IgG antibodies were negative in sera of 5 patients, and positive (at dilutions ranging from 1/80 to 1/320) in sera of the rest of analyzed patients (n=21).

Immunological testing

For 34 analyzed patients who developed morbilli infection, the immunological findings are presented in Table 1. In the analysis of Ig and cIC serum levels results of 31 patients were available. In more than half of patients (in 53%)leukopenia was found. In the majority of analyzed subjects reduced relative and absolute number of T cells (in 85% relative, and in 62% absolute number), as well as of B-cells (in 53% relative, and in 68% absolute number), deeply reduced PHA-induced T cell proliferative response (in 85%), but increased both IgM (in 58%) and cIC (in 61%) serum levels were observed.

In order to reveal any relationship between tested parameters the correlation analysis was done. The positive correlation with correlation coefficient $\rho=0.570$ and highly significant slope $p<0.005$ was found between T cell relative number and mitogen-induced T cell proliferative response (Fig. 1). Concerning the variations of analyzed parameters, the whole group of patients was further subdivided to pa-

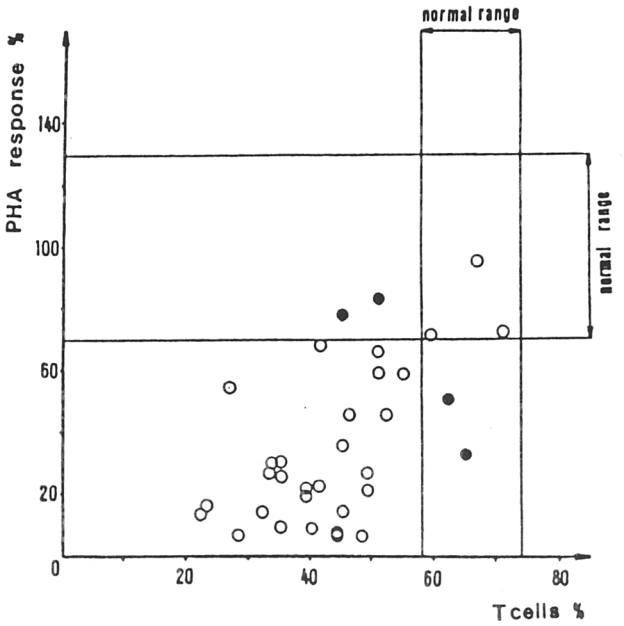

Fig. 1
The correlation analysis of lymphoproliferative response to T cell relative number (n=34, ρ=0.570, p<0.005)
Each symbol represents an individual patient
○ The patients with both normal or both decreased variables.
● The patients with one normal variable and decreased other one.

Table 1. Immunological parameters tested in patients with acute morbilli infection

Tested parameters and their normal range	Median values	Percentage of values below the normal range	Percentage of values above the normal range
Leukocytes × 10^9/l (6–10)	5.6	53	18
T cells % (58–74)	44.5	85	0
T cells × 10^9/l (1.18–2.24)	1.01	62	15
PHA response % (70–130)	28	85	0
B cells % (5–13)	4	53	6
B cells × 10^9/l (0.12–0.36)	0.10	68	9
IgG g/l (10.9–17.0)	14.3	16	23
IgA g/l (1.55–3.35)	2.9	0	23
IgM g/l (1.23–2.87)	3.1	0	58
cIC A_{450} (<0.22)	0.29	–	61
Phagocytes % (8–18)	15	3	24
Phagocytes × 10^9/l (0.16–0.50)	0.41	18	32

tients with reduced (n=27) or normal (n=3) values of both correlated parameters, and also to patients with normal T cell percentage but suppressed PHA-induced T cell response (n=2), as well as to patients with the quite opposite results (n=2). The positive correlation with correlation coefficient $\rho=0.417$ and highly significant slope $p<0.01$ was also found between increased serum levels of IgM and cIC (Fig. 2). The variations of these two parameters allowed further subdivision of the whole patients group to the patients with elevated (n=13) or normal (n=9) values of both variables, as well as to patients with normal serum level of cIC but elevated IgM (n=3), or the patients with the opposite results (n=6).

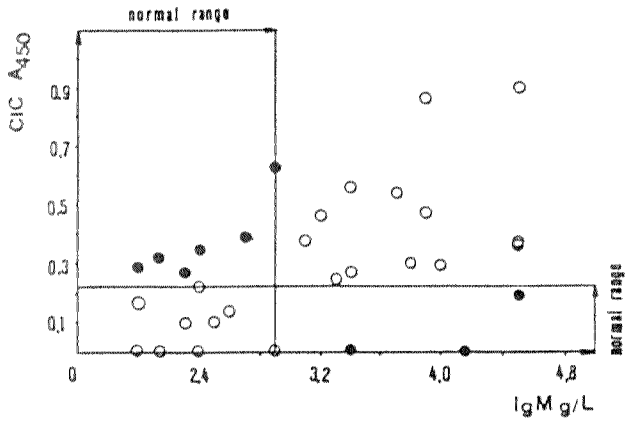

Fig. 2
The correlation analysis of cIC A_{450} level to IgM serum concentration (n=31, $\rho=0.417$, $p<0.01$)
○ The patients with both normal or both elevated variables.
● The patients with one normal variable and elevated other one.

Discussion

The viremic spread of measles, as it was mentioned above, may include the viral replication in reticuloendothelial cells and lymphocytes. The replication of virus leads to disruption of cellular cytoskeleton, and can cause rapid destruction of cells (Norrby, 1985). Measles virus RNA was detected in PBMC isolated from measles patients (Zurbriggen and Fujinami, 1990), although in papers dealing with restriction of viral replication to certain immunocytes, some disagreements exist. It was reported that *in vitro* up to 80% of cells expressing viral antigens were CD14+ monocytes, and less than 10% CD3+ T cells (mostly CD4+ T helper/inducer cells) (Salonen *et al.*, 1988), but also that *in vitro* infection included monocytes, T and B-lymphocytes (Chesney *et al.*, 1986). Really, the involvement of lymphatic system in disseminated stage of measles infection, as well as the subsequent damage of the same system were evidenced by leukopenia (Norrby, 1985), confirmed also by our own results.

In order to elucidate the complexicity of viral-induced disturbances of lymphocyte activity, it was essential to add more than one antigen and/or mitogen to lymphocyte culture. While active synthesis of viral RNA and proteins with undetectable release of infectious virus were found in *in vitro* infected, but unstimulated human PBMC, the productive infectious cycle comparable to lytic infection was detected after the stimulation with PHA (Hyypia *et al.*, 1985 Salonen *et al.*, 1988). Thus, although our finding of deeply depressed lymphocyte proliferative response obtained in the first days of morbilli rush in the most of analyzed patients is in concordance with previous data (Dagan *et al.*, 1987 Graziano *et al.*, 1975), one might speculate that such result may strictly represent an *in vitro* phenomenon.

However, in majority of patients besides T-cell proliferative response both T and B cells relative and absolute numbers were reduced too. Such decreases were certainly included in reduction of overall leukocyte number as a consequence of viremic spread and cellular destruction, but we have to discuss the involvement of other factors too. Firstly, marked reduction of T-cell number may also be caused by their mobilization reflecting as a measles rush. This rush develops after interaction of T cells with virus-infected cells in small blood vessels, and, in the absence of normal cellular IR it does not appear (Graziano *et al.*, 1975). Secondly, decrease of both T and B cell numbers might develop as a consequence of insufficient T cell-T cell, or T cell-B cell cooperation, especially considering previously emphasized fact that virus-induced T cell reduction is mainly due to decrease of T helper/inducer cells (Dagan *et al.*, 1987; Salonen *et al.*, 1988). Really, acute measles infection or vaccination with live attenuated measles vaccine can impair or even eliminate delayed hypersensitivity reaction to tubercullin even for weeks after recovery from infection (Graziano *et al.*, 1975; Norrby, 1985), proving damage of Th1 clones which mediate delayed type of the hypersensitive inflammatory response to activation *in vivo*. Also, one consequence of *in vitro* measles infection is the failure of T and B lymphocyte mixture to cooperate in secreting Ig in PWM-driven system, suggesting a suppression of B cell development at the activation of proliferative stages (Chesney *et al.*, 1986), and indicating a damage of Th2 clones which efficiently stimulate B cell growth and differentiation (Gazzinelli *et al.*, 1992). According to these data diminished T cell proliferative response found in majority of our patients may represent an outcome of real lymphocyte damage. The positive correlation found between T cell relative number and T cell proliferative response further suggests that in the most of the patients in the first days of morbilli rush we actually might expect mutual

variations of these parameters, reduction of both variables and vice versa. Nevertheless, preserved value of T cell proliferative response despite reduced T cell relative number observed in 2 patients may be more promising in regard to the significance of cell mediated antiviral IR. On the contrary, quite opposite results in other 2 patients do not fit this interpretation.

Concerning humoral antiviral IR, high titer of IgM is used nowadays in measles diagnostic procedure as an indicator of the primary IR in active infection (Faulk and Greenwood, 1977; Lievens and Brunell, 1986), as it was found also in our study. The positive correlation between serum levels of IgM and cIC indicates that increase of one variable implicates the increase of other one, suggesting predominant participation of IgM antibodies in the formation of circulating antibody-antigen complexes. It is known that under circumstances of slow antibody response or viral replication, the duration and amount of cIC may vary, not always to the advantage of the host. Accordingly, the measles are assigned as possible cause of IC-cutaneous vasculitis (Blandford, 1979), whereas in children who had received killed measles vaccine, but sustained later natural infection, sometimes a more generalized and more dangerous disease developed (Faulk and Greenwood, 1977). The subdivision of our whole group revealed 3 patients with normal cIC level despite elevated IgM, in contrast to other 6 patients with the opposite results. The results found in the latter group of 6 patient suggest the participation of other Ig classes in the formation of antibody-antigen complexes as well as a worse prognosis of disease (related to the probability of cIC-caused complications to occur), compared to the first group of 3 patients.

In the majority of analyzed patients no disturbance of monocyte phagocytic capability was found, albeit capability of monocytes to secrete cytokines required for successful antiviral protection (Kohl and Loo, 1988) should be assessed too. Considering PHA as monocyte-dependent T cell mitogen (Nouri-Aria *et al.*, 1988), the decrease of T cell proliferative response might be additionally aggravated because of depressed monocyte activity caused either by viral replication (Salonen *et al.*, 1988), or by increased level of cIC (Rao *et al.*, 1982).

In conclusion, we actually established changes of both cellular and humoral immunocompetence in the first days of morbilli rush. However, although these results mostly refer to the whole tested group of patients, one should not intend to identify them to every single patient. It means that the significance of such findings, as revealed by tested parameters, incorporates observations of various subsets of patients. Further clinical interpretations may be offered after correlation analysis of clinical outcome of measles infection with previously estimated patients' immunocompetence.

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