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T lymphocytes and NK cells in X - linked agammaglobulinemia

Seven boys with diagnosis of X-linked agammaglobulinemia on regular substitution of immunoglobulins were included into study. The patients showed episodes of infections but the clinical course was mild with good response to antibiotics. All patients developed, with time, the chronic sinusitis with proliferation of mucous membrane, two patients showed bronchiectases. The number of T lymphocytes, ratio of CD4:CD8 subpopulations, response to stimulation and NK number were assayed with flow cytometry and cell culture. Results showed CD4:CD8 ratio within normal value in majority of patients, reverse ratio in 2 boys, increased number of activated T cells (CD3/HLA-DR) in one of them. The number of NK cells was different from lack of these cells to high number. Response of T cells to stimulation (mitogens and CD3) were normal in majority of assays. There were no associations between clinical course and observed changes in T or NK cell populations. Further studies on number and function of NK cells are needed.

Introduction

The chromosome X-linked agammaglobulinemia (XLA, Bruton's disease) belongs to primary deficiencies in humoral immunity [5]. The disease is triggered by a gene point mutation located on long arm of X chromosome (Xq21.3-q22) encoding tyrosine kinase enzyme (Btk – Burton's tyrosine kinase). The lack of this enzyme is associated with deregulation of lymphocyte B maturation and its inhibition at pre-B stage of B lymphocyte ontogeny.

X-linked agammaglobulinemia is diagnosed in boys based on clinical symptoms, laboratory data (lack of B lymphocytes and trace of IgG, lack of IgA and IgM immunoglobulins in serum) confirm with mutation within Btk gene, lack of Btk mRNA (Northern blot), lack of Btk in monocytes and platelets [3,5]. Typical clinical symptoms included recurrent purulent otitis, pneumonias and

Do badań włączono 7 chłopców z rozpoznaniem agammaglobulinemii sprzężonej z chromosomem X pozostających na regularnej substytucji immunoglobulin. Pacjenci przechodzili epizody infekcji ale o łagodnym klinicznym przebiegu i dobrej odpowiedzi na stosowane antybiotyki. W miarę upływu czasu u wszystkich chłopców stwierdzono przewlekłe przerostowe zapalenie zatok zamykające ich światło a ponadto u 2 pacjentów rozwinęły się rozstrzenie oskrzelowe. Liczebność limfocytów T, proporcje subpopulacji CD4:CD8, odpowiedź na stymulację i liczba komórek NK były badane za pomocą cytometrii przepływowej i hodowli komórkowej. W wynikach obserwowano zachowane proporcje subpopulacji limfocytów T u 5 pacjentów, odwrócone tylko u 2 pacjentów, podwyższony odsetek aktywowanych limfocytów T (CD3/HLA-DR+) u jednego z nich. Liczba komórek NK była zmienna od braku tych komórek do podwyższonej ilości. Odpowiedź limfocytów na stymulację była porównywalna do wartości prawidłowych. Nie stwierdzono zależności pomiędzy przebiegiem klinicznym, a obserwowanymi zmianami w populacjach limfocytów T i komórek NK. Ocena zmian ilości funkcji komórek NK wymaga dalszych badań.

skin abscesses. Pathogens like *Staphylococci*, *Streptococci*, *Neisseria meningitidis* and *Haemophilus influenzae* are the dominant types similar to others deficiencies of humoral immunity [3]. In majority of bacterial infection the response to antibiotics is good. These infections are started after decrease of mother's IgG protection (4-6 months of life) [6,9].

The basic therapeutic procedures is life-long substitution of immunoglobulins with 5.0 g/l IgG as safety level [1,6,9]. Subcutaneous form of substitution is preferred for these patients as procedure resulting in higher level of IgG, sparing veins and supporting good comfort of life as home therapy. Infections (mainly bacterial) are treated with antibiotics usually in prolonged course. However, the longitudinal observations of XLA patients showed occurrence of bronchiectases, chronic sinusitis with tendency

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Table I
Patients' characteristics.
 Charakterystyka grupy pacjentów.

Parameter/patient	K.A.	D.A.	S.D.	S.K.	C.D.	W.B.	Sz.D.
Age of diagnosis	9/12	1/365	9/12	1/12	36/12	4y 4/12	16/12
Indications for diagnosis		family history	family history	S.D. brother			
Actual age (years)	11	13	13	10	14	14	13
Initial IgG level (g/l)	0.12	3.86 (mother)	0.1	3.1 (mother)	0.32	<0.7	0.21
Clinical symptoms	pneumonia pharyngitis	bronchitis	respiratory infections	otitis purulental	purulental otitis rec.	pneumonia	pneumonia Pneumocystis
Substitution (IVIG) age	11/12	1/12	9/12	3/12	3 y 8/12	4 y 5/12	16/12
Substitution (SCIG) age	5 y	7 y	9	5 y 6/12	8 y	12 y	8 y
Other symptoms		asthma			ASA		ASD II asthma
Chronic/obstructive sinusitis	++/++	+/+	+	+	+/+	+/+	++
Respiratory infections	++	++	++	++	+	++	++
Bronchiectases			++	++			
Weight and growth	normal	normal	normal	normal	normal	underweight	normal

to proliferation of mucous membrane closing the sinuses in majority of patients. These complications are independent of frequency of acute infections or exacerbation episodes (e.g. pneumonias, bronchitis). Moreover, the regular substitution of immunoglobulins is without influence on development of these symptoms.

The cellular immunity seemed to be unchanged in X-linked agammaglobulinemia so the studies showing T lymphocytes counts, function and NK cells in these patients are rare [2,7,8]. The purpose of this study was longitudinal observation and analysis of T lymphocytes and NK cells in XLA patients on regular immunoglobulins substitution.

Patients

The study included 7 boys diagnosed as XLA (btk mutation) observed no less than 10 years. Two of them (S.D. and S.K.) are brothers. In one family (D.A.) the previous death of boy within first year of life suggested primary immunodeficiency in next boy in this family. Following this, patients S.K., and D.A. were diagnosed as newborn (D.A.) and infant (S.K.). All of them presented infections (pneumonias, otitis, bronchitis) before substitution. The clinical status, claimed symptoms, history of infections and therapy effects in the period between hospitalizations were carefully examined during every planned hospital visit. Additionally, 2 patients demonstrated allergy – D.A. bronchial asthma, C.D. – atopic skin allergy. After the period of IVIG all boys are treated with SCIG form of immunoglobulins substitution. IgG level was monitored every month (IVIG), now is controlled every 2-3 months (SCIG) during hospital visit.

The parameters of cellular immunity were tested 1-2 times per year. X-ray or CT of sinuses and lungs were performed according clinical indications or once per year as routine. Despite of substitution of immunoglobulins the respiratory infections are present but the course was rather mild

and response to antibiotics were good. All patients developed chronic sinusitis with proliferating and overgrowing mucous membrane closing the sinuses. Two brothers (S.D. and S.K.) showed bronchiectases in HRCT of lungs. These complications are developed as process independent to regular substitution of immunoglobulins. Generally, the physical and mental development is normal in all studied patients (with one exception – underweight W.B. patient) (Table I).

Methods

Complete blood counts were measured according standard procedure (Coulter). The level of C-reactive protein (CRP) was assayed with nephelometry (Nephelometer II DADE Behring, Germany). Biochemistry (liver enzymes, total proteins level, kidney parameters and others) were tested according standard procedure every 3-4 months.

Immunological tests

IgG level was assayed with nephelometry (Nephelometr II, DADE Behring, Germany) and compared to age-adjusted normal value in children.

The presence and number of T lymphocytes, subpopulations, B lymphocytes and NK cells in peripheral blood was assayed with multicolour flow cytometry after staining with standard monoclonal antibodies kit (Becton-Dickinson) against CD45/CD3/CD19, CD45/CD3/CD4/CD8, CD45/CD3/CD16+CD56, CD3/HLA-DR (activated T lymphocytes) conjugated with FITC, PE, APC, PerCp, followed with erythrocytes lysis. For analysis no less than 10 000 events was acquired to cytometer (FACSCalibur, Becton-Dickinson, San Jose, Ca, USA). Analysis of presence of subpopulations, percentage and number of cells within given subpopulations were analyzed based on CD45 expression and FSC parameter.

The function of lymphocytes was estimated as stimulation index after 3 days culture of isolated mononuclear cells from

peripheral blood in presence of medium (unstimulated control), or following stimulation - phytohaemagglutinin (PHA, Wellcome, Dartford, UK), CD3 monoclonal antibody (Becton-Dickinson, Biosciences, San Jose, CA) and pokeweed mitogen (PWM, Gibco, Paisley, UK). Culture was finished with H^3 thymidine uptake (6 hrs) assayed in beta counter (Beckmann). Stimulation index was calculated as ratio of cpm stimulated cells to unstimulated cells (S.I.) Stimulation index higher than 20 was considered as normal response to stimulation.

Results

The average level of IgG immunoglobulin before the substitution was 0.56g/l (ranging from 0.15 g/l to 0.67g/l) with exception of maternal IgG in D.A. and S.K. patients. The regular IVIG in dose 0.4 – 0.5 g/kg body weight resulted in obtaining the safety level (IgG above 5.0 g/l) within first 6 months of substitution. The SCIG substitution led to more stable level of IgG with mean value – 8.4 g/l.

The analysis of cellular immunity showed high percentage and number within normal value for age of CD3+ T lymphocytes in all boys. Ratio of CD4:CD8 was between 1.0 – 3.0 in 3 patients during all time of observation (K.A., D.A., S.D.), partially in one patient (C.D.). The reverse ratio was noted as stable characteristic in 2 boys (W.B., S.K.). Patient K.A. demonstrated high level of CD3+/CD4-/CD8- TCR γ/δ cells. The number of CD3/HLA-DR+ cells (activated T lymphocytes) was increased in 2 patients (C.D.), normal in 1 patient (Sz.D.).

NK cells showed wide range of percentage and number from lack of this cells (patient W.B.) to high number above upper limit of normal value (brothers S.K. and S.D.). The clinical significance of these phenomenon is not clear, moreover, boys with low number of NK did not demonstrated higher frequency of viral infections. Boy with lack of NK cells (W.B.) showed high number of activated T lymphocytes (CD3/HLA-DR) what may

suggest the role of activated T lymphocytes in antiviral protection.

The response of T lymphocytes to mitogens (PHA, PWM) and CD3 stimulation was different in individual patients and varied from high value of stimulation index (e.g. 103) to low value (e.g. 2). These low values were noted in single tests in individual patients (Table II).

Discussion

Analysis of T lymphocytes and NK cells in 7 boys with XLA during years showed normal proportions between subpopulations in 5 patients, reversed ratio in 2 patients during all time of observation. One of these patients demonstrated lack of NK cells. The number of NK cells was increased or normal in remaining patients. The response to stimulation was preserved and indexes were lower in one patient or lower in singular assays in others patients. There were no associations between clinical symptoms, frequency of infections, development of complications (chronic sinusitis) and disorders in T lymphocytes and NK cells population. The results showed the different pattern of immune system disorders in XLA patients, however, the influence of these disorders on clinical course of XLA is no obvious and need further observations. The study of T lymphocytes in 16 XLA patients was performed to solve the question of influence of congenital lack of B cells on development of T lymphocytes subpopulations (Martini). Despite of individual variability of total T cell

counts the mean value was comparable to control. Analysis of T cells subsets showed naïve CD4 T cells and CD4 recent thymic emigrant number was unchanged. The only difference between XLA patients and healthy control was reduced in XLA patients CD4 T cells memory subset. This results suggested that not the btk mutation but the lack of B cells is associated with impaired CD4 T cell maturation [4]. These results are based on amount of given subsets of T lymphocytes without functional study of CD4 T cells. Other data showed T lymphocytes functions as specific memory cells to hepatitis B envelope antigen in XLA patients similar to control group of healthy people. It was important to show that B lymphocytes are not obligatory required for the generation of effective T memory cells as was previously suggested [8]. Moreover, T lymphocytes from XLA patients stimulated with tetanus toxoid showed predominant Th1 type of response and higher number of activated T cells than control [2]. These results and our observations suggest preserved function of T cells compared to healthy people.

The role of NK cells in response to viral infection is well known. Our patients showing higher number of NK cells did not suffer from frequent or persistent viral infection. Moreover, patients with lack of NK cells are not more prone to this type of infections and frequency of viral episodes is low. These observations are initial and further studies of NK subpopulations and function are needed.

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