Lymphoblastoid cell lines – a suitable tool in diagnostics of primary immunodeficiencies

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Abstract

Primary immunodeficiencies (PIDs) are congenital disorders caused by defects in different elements of the immune system. In the last 60 years, advances in molecular genetics and immunology have resulted in the identification of a growing number of genes causing PIDs in human subjects and a better understanding of the pathophysiology of these disorders. Early diagnosis is essential for referral to specialized care centers and the prompt initiation of appropriate therapy. As availability of primary cells can be limited, lymphoblastoid cell lines (LCLs) are common sources of proteins and genomic DNA used for PID diagnosis. In this article the clinical, laboratory, and molecular elements of the most common antibody and cellular PIDs are described, and LCLs are presented as a suitable tool in a diagnostic approach.

Key words: lymphoblastoid cell lines, antibody immunodeficiency, cellular immunodeficiency

Introduction

The true incidence and prevalence of immunodeficiencies are unknown, as no large prospective studies have been performed. The incidences and occurrence of all forms of primary immunodeficiency (PID) estimated from reports of surveys or registries in over 40 countries range from 1:10,000 to 1:2,000 individuals [35]. However, this is suspected to be an underestimate since it is likely that many cases of immunodeficiency are undiagnosed [7]. The prevalence of PIDs in Poland is not known. Since 1980 there have been 1100 cases of PIDs in the registry of Department of Immunology The Children’s Memorial Health Institute. Primary antibody deficiencies (PAD) account for 50 to 60% of all immunodeficiencies [35]. Recurrent infection is the predominant presenting complaint for PAD disorders. The majority of patients with PAD have syndromes defined by clinical and laboratory criteria and whose molecular basis remains unknown. Among cellular deficiencies severe combined immunodeficiencies (SCIDs) are the biggest group of the most severe forms of congenital immune defects. Hypogammaglobulinemia and impaired antibody responses are the hallmark of PAD dis-
orders yet can also be due to combined immunodeficiency and can be part of the presentation of a number of specific immunodeficiencies that affect primarily the cellular immune compartment. Patients with significant hypogammaglobulinemia should have their lymphocyte subset numbers and distributions determined [5]. Flow cytometric analysis is now widely available to enumerate T-cell, B-cell, and NK (natural killer) cell subsets and will identify patients with agammaglobulinemia. Patients with common variable immunodeficiency (CVID) might benefit from a further characterization of their specific phenotype. Several classification schemes for CVID have been proposed on the basis of B-cell and T-cell immunophenotyping [39]. Clinical or laboratory features that are suggestive of defects of cellular immunity or of a combined defect, including T-cell lymphopenia, opportunistic infections, or failure of young children to thrive, might warrant an assessment of T-cell function by testing of lymphocyte proliferative responses to recall antigens and mitogens.

In this review, we begin with a discussion of antibody and cellular immunodeficiencies with reference to specific diseases. We will discuss in some detail the clinical, laboratory, and molecular elements defining each diagnosis. Finally, lymphoblastoid cell lines (LCLs), as a supportive tool in PID diagnosis is presented.

Primary antibody deficiencies

Agammaglobulinemias are rare antibody deficiencies caused by defects in early B-cell development and characterized by low numbers of or absent B cells, marked hypogammaglobulinemia, and increased susceptibility to infections.

Agammaglobulinemia X linked (XLA). XLA accounts for 85% of known cases of agammaglobulinemia and is caused by a deficiency of the tyrosine kinase BTK [12]. BTK mutations have been identified throughout the five functional domains of the gene, which is located in the middle portion of the X chromosome at Xq22. Some mutations that permit the production of a small amount of functioning BTK protein lead to milder disease, but a completely consistent genotype-phenotype correlation has not been found [12]. The incidence of XLA in the United States has been estimated to be at least 1:190,000 male births. The age of onset of symptoms for most patients is between 3 months and 3 years, with over 50% of patients becoming symptomatic by 1 year of age and more than 90% of patients becoming symptomatic by 5 years of age [41]. T-cell numbers and function are normal for patients with XLA. Neutropenia, often severe, is found in 15 to 25% of patients at the time of diagnosis [15]. Characteristic physical examination findings of XLA are paucity or absence of lymphatic tissue such as tonsils or palpable lymph nodes. Autoimmune diseases such as Crohn’s disease and type 1 diabetes mellitus have been described for a few patients with XLA, albeit much less frequently than for CVID [4]. At the time of diagnosis, most, but not all, patients have low serum immunoglobulin levels, with IgG levels of 200 mg/dl, IgA levels of 15 mg/dl, and IgM levels of 40 mg/dl [41]. Almost all patients with classic XLA have markedly decreased B-cell numbers, with 2% of CD19- or CD20-positive lymphocytes in the blood. B-cell lymphopenia with normal T-cell numbers can be considered diagnostic in the context of a positive family history or if the mother is an identified carrier for the disease. Maternal carrier status can be established by an evaluation of maternal B cells for a pattern of nonrandom X-chromosome inactivation. If there is no family history of XLA, it can be helpful to measure BTK protein expression by Western blotting or by flow cytometric analysis of the patient’s monocytes or platelets [18]. Flow cytometric analysis for cytoplasmic BTK expression can also detect carrier females, revealing BTK-positive and BTK-negative populations [19]. In cases of atypical XLA, in which a poorly functional BTK protein is expressed, genetic analysis of the BTK gene might be necessary to distinguish it from other forms of severe antibody deficiency such as CVID [23]. In an estimated 10% of cases, agammaglobulinemia is not due to mutations in BTK; rather, it is inherited as an autosomal trait and is therefore classified as autosomal-recessive agammaglobulinemia. Underlying molecular defects are heterogeneous, with several key components of early B-cell development. Clinical and laboratory features of autosomal-recessive agammaglobulinemia are essentially identical to those of XLA [5, 12].

Immunodeficiency with low IgG and normal or high IgM levels (Class Switch Recombination Defects – CSRD). The term hyper-IgM has been used to describe a group of disorders of CSRD with characterized elevated IgM levels and low levels of switched isotypes (IgG, IgA, and IgE).

X-linked hyper-IgM is caused by mutations of the gene encoding ligand for CD40 molecule (CD40L). A rare autosomal-recessive forms with an identical phenotype are due to mutations in the gene encoding CD40 (TNFRSF5), AID or UNG. Patients usually present in infancy severe and/or recurrent bacterial respiratory and gastrointestinal infections as well as opportunistic infections such as Pneumocystis jirovecii pneumonia, disseminated fungal infections, disseminated cytomegalovirus and herpes simplex virus infections, and cholangitis due to Cryptosporidium parvum. Other clinical features are neutropenia, chronic anemia associated with erythrovirus (parvovirus B19) infections, and an increased incidence of gastrointestinal tumors [28, 40]. Patients have an increased incidence of autoimmune disease such as autoimmune hemolytic anemia and autoimmune thrombocytopenia [33].

Mutations in the genes encoding the I-κB kinase γ chain (also called NF-κB essential modulator-NEMO) or IκB kinase γ chain result in a distinct phenotype characterized by variable manifestations of ectodermal dysplasia (conical or absent teeth, sparse hair, frontal bossing, and decreased eccrine sweat glands) and susceptibility to mycobacterial infections in addition to antibody deficiency with often high serum IgM or IgA levels [30]. Patients with a CD40L or CD40 deficiency have markedly reduced IgG levels (IgG
common variable immunodeficiency (CVID). CVID is a heterogeneous disorder of B-cell differentiation and maturation with dysfunctional antibody production. Patients have markedly reduced serum concentrations of IgG, impaired specific antibody responses, and recurrent infections. With an estimated prevalence of 1 in 25,000 to 1 in 75,000 individuals, it is the most common of the more severe primary immunodeficiencies that come to medical attention. A familial pattern of inheritance has been demonstrated for approximately 10 to 20% of patients with CVID [20]. The variability of its phenotype and the incomplete penetrance of some of its associated molecular defects suggest that the etiology of CVID is multifactorial, with a combination of defects of B-cell differentiation resulting in antibody deficiency. While the etiology remains unknown for the majority of patients, gene defects have been revealed for approximately 10 to 15% of CVID patients in recent years. Thus far, mutations or polymorphisms in four genes, TACI, Msh5, and two of which appear to affect only very few patients (ICOS and gene encoding CD19) have been described [38]. However, mutations in TACI and Msh5 are also present in some healthy individuals; hence, it has been proposed that additional genetic and/or environmental factors are needed to develop disease [37]. The age of presentation of CVID varies widely. According to a recent large European registry study of 334 patients, the most common age at the onset of symptoms was in the third decade, with a mean of 26.3 years and a median of 24 years. Patients often come to medical attention due to acute or chronic bacterial and, less frequently, viral infections. Noninfectious complications are also frequent, encompassing autoimmune diseases, lymphoproliferative disease, granulomatous disease, a spectrum of gastrointestinal disorders, as well as malignancies. Significant morbidity and mortality arise from chronic lung disease as a consequence of recurrent and chronic infections as well as granulomatous and lymphoid interstitial pneumonitis [13]. Granulomatous disease is present in 5 to 10% of patients with CVID. Granulomas are of the noncaseating type and resemble those found in sarcoidosis. Although granulomas most commonly affect the lungs, they are also found in almost any other organ including skin, liver, spleen, bone marrow, and the gastrointestinal tract of patients [26]. Hypertrophy of lymphoid tissue is common in CVID. Splenomegaly has been reported for 26% of patients according to one study [34]. The overall prevalence of autoimmune disease is estimated to be 20 to 30% and includes a wide spectrum of disorders: idiopathic thrombocytopenic purpura, autoimmune hemolytic anemia, rheumatoid arthritis, and pernicious anemia [13, 34]. Both hematologic malignancies and solid tumors have an increased prevalence in patients with CVID [5, 27]. For patients with CVID, IgG levels are reduced by greater than 2 standard deviations (SD) below the mean for age. An associated reduction in either the IgA or IgM level has been included as part of the diagnostic criteria by some authors. Also essential for establishing the diagnosis is evidence of impaired specific antibody responses to infection or vaccine challenge. Peripheral B-cell numbers can be either normal or reduced. T cell numbers and function are also reduced in some patients. CVID remains predominantly a diagnosis of exclusion. Other specific immunodeficiency diagnoses have to be ruled out by careful evaluation of clinical features and laboratory phenotypes. In some cases, molecular testing may be required to distinguish other diseases with similar phenotypes, including CSR, agammaglobulinemia, and X-linked lymphoproliferative syndrome (caused by mutations in SH2D1A) [14]. ICOS expression on T cells can be assessed by flow cytometric analysis; however, it is not routinely done due to the extraordinarily rare occurrence of this mutation. The expression of CD19 is routinely assessed as part of the lymphocyte subset evaluation. In a rare individual with other clinical and laboratory features of CVID in whom no B cells are detected after staining for CD19, the presence of measurable numbers of B cells with analysis of another marker, such as CD20, may suggest the defect. TACI gene sequence analysis is commercially available. The number of isotype-switched memory B cells in the peripheral blood has been found to be one of several useful parameters to distinguish distinct phenotypes of CVID [39].

IgA deficiency. Selective IgA deficiency is a common immunological variant with a prevalence of 1:400 to 1:600 in the healthy US population. Affected individuals are asymptomatic in up to 90% of cases [10]. There is familial clustering of IgA deficiency with CVID, with 20 to 25% of individuals having a positive family history of either IgA deficiency or CVID. Some IgA deficient patients progress over time to CVID. The pattern of inheritance of selective IgA deficiency is unclear [20]. The underlying defect has also not been determined for the majority of patients. Despite its association with a benign clinical course in most patients, a subgroup of IgA-deficient patients develops recurrent sinopulmonary and gastrointestinal infections, but invasive infections such as meningitis or sepsis generally do not occur. IgA-deficient individuals are at an increased risk of developing autoimmune disease, particularly systemic lupus erythematosus and rheumatoid arthritis, and gastrointestinal disease such as inflammatory bowel disease and celiac disease [1]. A higher prevalence of asthma and allergies has also been reported. Selective IgA deficiency is defined as a serum IgA level below 7 mg/dl in the presence of normal serum IgG and IgM.
levels for a patient older than 4 years of age after other causes for hypogammaglobulinemia have been excluded. Young children can have a physiological delay in IgA production that they will outgrow and therefore are not considered deficient. The clinical presentation together with an evaluation of specific antibody responses should determine further management [5].

**IgG subclass deficiency.** IgG subclass deficiency is defined as an abnormally low level of one or more IgG subclasses (IgG1, IgG2, IgG3, or IgG4) with normal levels of total IgG and normal levels of the other immunoglobulin isotypes. It is sometimes associated with IgA deficiency [5]. The diagnosis of IgG subclass deficiency is controversial since low levels of one or more IgG subclasses can be found in 2 to 20% of healthy individuals. Diagnosis is further complicated by the variation of IgG subclass levels with age and by different methods used in individual laboratories for determining the serum levels [6]. Since IgG2 is the isotype that is primarily responsible for responses against polysaccharides, it was postulated that a deficiency of this subclass in children would predispose patients to infections with encapsulated bacteria. The presence of symptoms seems to correlate rather with an impairment of specific antibody responses to vaccines or to natural exposure to pathogens [8]. If patients are symptomatic, they present with recurrent sinopulmonary bacterial infections. Association with atopy and autoimmune disease has been reported, similarly to IgA deficiencies [5]. IgG subclass deficiency can be established if one or more IgG subclasses are 2 SD below the age-adjusted norm, with normal total serum IgG levels. Only if associated infections are present is further immunological workup warranted, including evaluation of specific antigen responses to protein and polysaccharide antigens [8].

**Specific antibody deficiency (SAD).** SAD is characterized by impaired IgG responses to polysaccharides in the presence of normal serum concentrations of IgG, IgM, and IgA. It is therefore also called selective antibody deficiency with normal immunoglobulins. Patients have poor responses to polysaccharide antigens such as pneumococcal polysaccharide and *Haemophilus influenzae* type b capsular polysaccharide. The underlying molecular defect is not known. The prevalence of SAD is also unknown but is estimated to be high, with a few studies reporting 5 to 10% of children who are evaluated for recurrent infections being affected [21]. Patients present with infections with a predominance of recurrent upper and lower respiratory tract bacterial infections. A diagnosis of SAD requires a demonstration of poor responses to polysaccharide vaccines in the context of normal serum immunoglobulin concentrations. It may be difficult to establish the diagnosis with confidence for young children below the age of 2 years, as they have less consistent responses to polysaccharide vaccine challenge. Abnormal responses to protein vaccines or evidence of further abnormal laboratory findings can be indicative of a more extensive defect and should prompt evaluation for other immunodeficiencies.

**Transient hypogammaglobulinemia of infancy (THI).** Transplacentally acquired maternal antibodies protect the infant against pathogens until his or her own antibody production has reached sufficient levels. The hiatus between the loss of maternal antibodies and the onset of a robust antibody synthesis represents a physiological period of hypogammaglobulinemia, usually lasting from 3 months to about 6 months of age. Prolongation and accentuation of this phase with decreased levels of IgG and, in some cases, also IgA and IgM production until early childhood are considered to account for cases THI. The delay in antibody production is sometimes associated with recurrent infections. Infectious manifestations include mostly upper respiratory tract infections and, less commonly, pneumonia. Rarely, invasive infections such as sepsis or meningitis have been reported [5]. Laboratory evaluation reveals serum IgG levels 2 SD below the mean for age-matched controls. Serum levels of IgA and, less frequently, IgM can also be decreased. Evaluation should include specific antibody responses to vaccines and also flow cytometric quantitation of lymphocyte subsets to rule out more substantial defects. Specific antibody responses are most often normal in patients with transient hypogammaglobulinemia. Patients should be monitored over time until levels have normalized. The disease is self-limited by definition. Initial laboratory testing includes quantitative serum immunoglobulin levels (IgM, IgG, and IgA) and evaluation of specific antibody responses to both protein and polysaccharide antigens [4]. It is important to interpret serum immunoglobulin levels according to age of pediatric patients.

**Cellular deficiencies**

It is possible to identify children with a cellular immune defect with a few relatively simple tests and procedures. Many cases of T cell immunodeficiency can be predicted on the basis of obtaining an absolute lymphocyte count either at birth or in the first 3 months of life as T cells normally comprise 70% of circulating lymphocytes.

The heterogeneous genetic background of SCIDs result in different clinical and immunological phenotype are presented [29]. In all patients both cellular and humoral immunity is severely affected. In most patients the onset of symptoms occurs between 3 and 6 months of life. Early diagnosis makes possible early definitive therapy and avoids the complications of pretreatment infections that damage the lungs, liver, kidneys, and other vital organs [2, 3, 31]. With the X-linked form representing nearly 45% of cases of SCID, occurrence in maternal male relatives is particularly important [32]. There is high incidence of almost all infections: bacterial, viral, fungal and others. Opportunistic infections, the most often chronic or recurrent oral thrushes, infections caused by cytomegalovirus, *Pneumocystis jiroveci*, *Aspergillus* spp., raise suspicions toward immunodeficiency. Particular and almost exclusive complication in SCIDs is serious adverse event following vaccination against tuberculosis, with the most severe form of disseminated BCG – it
is with poor prognosis. A chest radiograph is indicated to look for a thymic shadow, which is often absent in patients with SCID. Some children can present phenotype of Omenn syndrome with severe generalised erythrodema, lymphadenopathy and hepatosplenomegaly from first weeks of life. On the other hand some patients have mild phenotype of disease, called “leaky SCID” and often the current diagnosis in such cases is delayed. These children can suffer from recurrent, but not severe infections and other complications like autoimmune diseases for example; autoimmune haemolytic anaemia. Because most infants with SCID lack T cells, they are often (but not always) lymphopenic. The diagnosis of SCIDs also consist of full assessment of humoral and cellular immunity. IgG is not useful in young infants because it is largely maternal. With referral to the immune deficiency specialist, the patient will have a more extensive evaluation of immunoglobulin production and lymphocytes assessment by means of flow cytometry using antibodies to CD3, CD4, CD8, CD19, and CD16/CD56 to determine whether the infant has normal percentages of T cells and subsets, B cells, and NK cells and to determine whether there are naïve, i.e. CD45RA positive, T cells present [17]. The function of T cells will be determined by means of lymphoproliferation in vitro in response to stimulation with mitogens, such as phytohaemagglutinin, concanavalin A, and pokeweed mitogen, and to antigens, such as Candida species and tetanus (the latter only if the infant has been immunized).

Depending on number of lymphocyte subpopulation SCIDs are divided in two major immunophenotypes: SCID T-B- and SCID T-B+. In some situations the results of immunological investigations are not so clear. Increased number of T lymphocytes and/or dysgammaglobulinaemia, elevated IgE can be observed in patients with Omenn syndrome or in SCID with maternal T-cell engraftment. Increased radiosensitivity is a feature of some SCID patients including: RAG1/ RAG2, Artemis, Cernunnos deficiencies. Very important, particularly before decision on stem cell transplantation, is final confirmation of disease causing mutations. Universal newborn screening for SCID is not yet available, although pilot trials are in progress.

**Lymphoblastoid cell lines in PID diagnosis**

Early diagnosis of PIDs is important for genetic counseling, appropriate medical care, and avoidance of complication after infection. PID diagnostics often requires analysis of genes in order to find mutation underlying the disease or study of production and activity of proteins involved in immune cell functions. Whereas amounts of DNA enough to sequence the gene of interest may be isolated from peripheral blood mononuclear cells, it is not feasible to extract sufficient amounts of proteins for further analysis. LCLs that can be easily established from patients’ blood may be used for flow cytometry analysis as well as highly efficient source of DNA, RNA or proteins. Thanks to the preserved genomic stability, one may also use LCLs to study the karyotype. The method of establishment of LCLs is well studied and can be easily implemented in most of the laboratories. LCLs are established from peripheral blood B lymphocytes immortalized with human herpes virus 4 more commonly known as EBV. EBV infects 90% of human population causing infectious mononucleosis but can also be associated with Burkitt lymphoma, nasopharyngeal carcinoma, Hodgkin’s disease, hairy leukoplasia and lymphoproliferative disorder in immunocompromised patients. EBV shows tropism towards circulating B lymphocytes as its envelope protein gp350/220 is recognized by C3d complement receptor type 2. i.e. CD21 [16] and HLA class II molecules on the surface of B-cells [24]. Latently infected B cells proliferate continuously what results in their immortalization that can be obtained in vitro as well. Virtually no virus particles are produced by LCLs. On the other side it was shown that LCLs regress after approximately 160 population doublings and only few of them are stably immortalized with enhanced telomerase activity [25]. Upon the B-cell infection resulting in the latent cycle, linear EBV DNA forms circular multicopy episome and 10 out of the approximately 100 viral genes are expressed [9, 11]. Viral proteins EBNA-1,2 and 3, LMP-1, BCRF1 and BARF1 promote maintenance of viral DNA, control the replication and prevent anti-EBV immune response [22, 31, 36].

Since 2007 the method of establishing LCLs has been successfully implemented in the Department of Pathology the Children’s Memorial Health Institute. Thanks to it some Polish patients with PIDs were genetically diagnosed.

**Conclusions**

Analysis of specific protein expression by Western blotting or flow cytometry is required for the diagnosis of some PIDs. Gene sequencing is commercially available for many of the known gene defects and, depending on the presenting clinical and laboratory features, may be indicated to confirm or rule out specific diagnoses. Whenever possible, molecular diagnosis is desirable for accurate genetic counseling. A potent of LCLs is widely appreciated in advanced PID diagnosis.

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References


