Parvovirus B19: recent insights and implications for pathogenesis, diagnosis and therapy

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Summary

Parvovirus B19 is a human pathogenic virus, a ssDNA member of the family Parvoviridae, characterized by a selective tropism for erythroid progenitor cells (EPCs) in the bone marrow and an ample pathogenetic potential. The selective tropism for EPCs can be explained both in terms of receptor-mediated tropism and of an intracellular permissive environment conditioned by the cell differentiation and proliferation stage. Infection of EPCs is productive, induces apoptosis and leads to a temporary arrest of erythropoiesis, which can usually be manifest in cases of underlying erythropoietic disorders or immune system deficiencies. Endothelial cells constitute an additional diffuse target, whose infection is mediated by ADE phenomenon, but is normally non-productive and mainly leading to inflammatory processes. The relevance of parvovirus as a cardiotropic virus is recently emerging, while its capability of intrauterine transmission and consequences on the fetus is known and should not be overlooked. To the purpose of diagnosis, a combination of molecular and immunological methods offers the best discrimination of active infectious processes, and an application of these methods especially in cases of atypical presentations should be encouraged. Ongoing research is directed towards the development of a vaccine and the discovery of antiviral drugs that may be useful in the prevention and treatment of parvovirus B19 infections.

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Introduction

Parvovirus B19 (B19V), a ssDNA virus in the family Parvoviridae, is characterized by several properties that convey interest (36,81). It is a virus with a small and compact genome organization, with a relatively limited molecular repertoire (Figure 1). A pathogenic human virus, it is characterized by a selective but not exclusive tropism for erythroid progenitor cells (EPCs) in the bone marrow, and by a strict dependence on the cellular environment for its replication, so that both the target cell types and their differentiation stages are critical to the diverse outcomes of infection. In the population, infection is widespread and can be associated with an ample range of pathologies and clinical manifestations, whose characteristics and outcomes depend on the interplay between viral properties and the physiological and immune status of the infected individuals. As a consequence, B19V can be responsible for different clinical manifestations, from the asymptomatic or mild to the severe and in some cases life-threatening, affecting the hemopoietic system as a primary target tissue, but also an ample spectrum of secondary target tissues and organs. The virus can establish a complex relationship with the immune system, whose capacity of controlling infection can be critical to the course of infection and the development of pathological processes. As a general scheme, it is now accepted that following a primary infection the virus can establish persistence in many tissues, probably lifelong, and questions then arise about the characteristics of this long-term relationship, whether the virus maintains its full biological potential, and what consequences if any this relationship can have on the host. At the state of our research, still more questions are open than knowledge established.

An expanding spectrum of target cells

The strict tropism of B19V for cells of the erythroid lineage can explain most of its pathogenetic characteristics, and a first debated issue is whether this tropism and the ability to complete a productive replicative cycle is mainly dictated by receptor-mediated cell-specificity, or by restrictive intracellular events. Probably, B19V has evolved towards a striking specialization for erythroid lineage cells by exploiting both mechanisms, but their relative relevance still needs to be defined.

The membrane glycolipid globoside has early been recognized as the main, docking receptor, for B19V. Sufficient evidence accumulated so far indicate its necessary function in a first, reversible binding of virus to cells. Such binding has been shown to induce a series of structural modifications in the capsid shell leading to

extrusion of the VP1u, minor capsid protein unique region, then allowing irreversible binding to a specific penetration coreceptor and subsequent intracellular translocation of the nucleocapsid (54). Recent work suggest that this coreceptor is highly restricted to cells of the erythroid lineage and plays a key role in determining viral internalization within a specialized cell type, characterized by a potentially permissive cellular environment apt to support viral replication (53,55).

This specific internalization process on the other hand is unlikely involved in the infection of other cell types, that might result non-permissive to viral replication. A wide distribution of globoside has been advocated in support of the capacity of virus to infect many diverse cell types, for example endothelial or connective tissue cells, but the demonstrated absence of the specific coreceptor implicates that either a different coreceptor is involved, or that mechanisms different than receptor-mediated endocytosis are involved. In this respect, a relevant phenomenon also observed for B19V is the Antibody Dependent Enhancement of infectivity (ADE), that has been shown relevant for the infection of endothelial cells (through C1q receptor) (89), and recently for B lymphocytes from tonsil tissue (80). Endothelial cells constitute a diffuse target, and their involvement together with C1q may account for the involvement of endothelia in the secondary phase of infection, when in the presence of an immune response, as well for diffuse persistence of virus in the organism. Notably, the ADE pathway usually leads to internalization of virus in a non-permissive cellular environment, and possible effects on cells can be more a consequence of innate immune recognition than due to expression or replication of the viral genome.

Different cells, diverse outcomes of infection

Following attachment and internalization, the pathway to an intracellular compartment supporting viral macromolecular synthesis is long and the efficiency of the process appears very low (82). Moreover, pathways differ depending whether uptake it is receptor-mediated or not, such as in ADE, and outcomes will depend on the cell type, differentiation stage and cell cycle phase.

In a productive cycle, as observed in EPCs, the viral genome is translocated to the nucleus and first converted to a double stranded form by cellular DNA repair enzymes, then replicates via a rolling hairpin mechanism, effected by cooperation of the viral NS protein and cellular machinery, this event provoking a shift in the expression profile leading to prevalent expression of viral capsid proteins (23). Thus, early expression of viral NS is critical, and in turn this requires a transcriptional active template, that is a double stranded DNA with an active promoter. Initial conversion of the incoming ssDNA into dsDNA requires cellular proteins involved in DNA repair, and it might be a critical bottleneck for the virus (60). The B19V promoter, although most efficient in erythroid cells, is relatively generic and conversely might promote expression of the viral genome even in otherwise non-permissive environment.

Within a heterogeneous population of expanding (proliferating and differentiating) erythroid progenitor cells, several levels of intracellular restriction steps can apply to regulate the viral lifecycle (Figure 2). These levels relate to the capacity of virus to make a second strand of the incoming genome and thus start macromolecular synthesis, to the capacity of virus to replicate its genome and switch its expression profile from an early (prevalent expression of NS protein) to late (prevalent expression of VP proteins) pattern, and finally to the capacity of completing assembly and release of infectious virus. In all of these steps, not only cell-type specificity, but mainly the differentiation stage and physiological state of cells are critical to complete a productive cycle, whose effect is to induce apoptosis of infected cells. EPCs are fully permissive within a relatively narrow window in the differentiation pathway, corresponding to the proerythroblast stage, so that both cells with staminal characteristics or terminally differentiating cells are either unsusceptible or not able to sustain viral replication. It is known that Epo pathway activation and a hypoxic environment, such as present in the bone marrow, are requisites for a permissive environment (60). Thorough identification of the mechanism required to sustain a viral replication in proerythroblasts opposed to other erythroid differentiation stages is still to be obtained, and alternative possibilities to be investigated are that permissiveness to viral replication might be related either to the expression of specific intracellular factors, or to a relative inefficiency of cellular restriction factors.

Lifelong persistence and pathogenetic implications

As a DNA virus, ssDNA within virions but dsDNA when replicative intermediates are formed in infected cells, B19V is a member of right of the expanding paradigm of the human virome (51). Presence of virus in the peripheral blood, in particular detection of viral DNA in the plasma/serum fraction (69), has always been considered as the evidence of a productive infection localized in the erythroid compartment in the bone marrow. Following acute

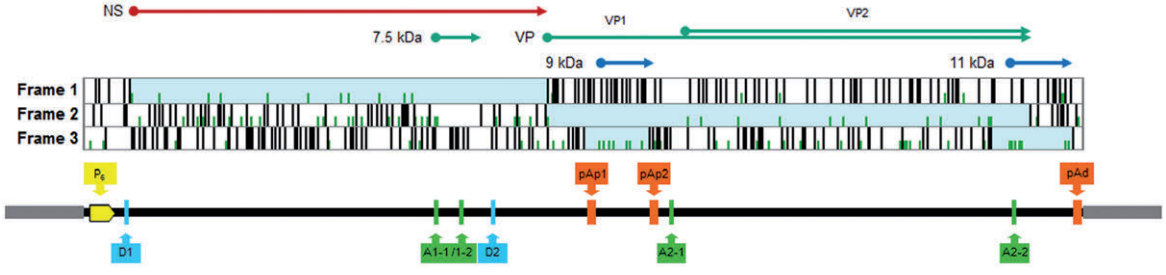

Figure 1. Outline of parvovirus B19 genome structure and organization. Above, ORF distribution within B19V internal coding region and related coding sequences. Below, functional map of B19 virus genome and distribution of regulatory signals: grey boxes, inverted terminal regions; P6, promoter region; pAp1, pAp2, pAd: proximal and distal cleavage-polyadenylation sites; D1, A1.1/2, D2, A2.1/2, donor and alternative acceptor sites for introns 1 and 2, respectively. Modified from Gallinella, 2013 (34).

infection, virus is cleared from blood with a relatively slow kinetics, and prolonged viremia is indicative of a persistent infection not adequately controlled by the immune system. However, it has recently been shown that the detection of viral DNA in peripheral blood may not be always ascribable to persistent infections, since DNA may not be protected from nuclease digestion and so presumably not incapsidated in mature virions (67). Thus, what is found in peripheral blood may be just DNA released from sites of persistence in the tissues, in a concept similar to that of liquid biopsy.

In the quest for sites of viral persistence, viral DNA was first sought and found in bone marrow, the primary target of infection, but strikingly it was also progressively detected in almost all solid tissues and organs (1,64). While the aim was to link viral presence to tissue- and organ-specific pathologies, the results indicated that the presence of viral DNA as resident in the diverse tissues is a very likely common outcome of the natural course of infection, whose characteristics need to be defined in order to propose any plausible hypothesis on any pathological role. To this purpose, detection of viral DNA must be linked to specific molecular and cellular features, with respect either to viral lifecycle or to pathological consequences on the host, to assert any viral pathogenetic role.

An issue correlated to the establishment of persistence and molecular detection of virus/viral DNA in tissues, is that we can gain insights in the molecular evolution of B19V. Three genotypes are known for B19V, whose distinction was proposed based on isolates detected in peripheral blood, but it was then realized that genotype 2 was an ancient virus, mainly resident in tissues and possibly ancestral to genotype 1 (74). Its prevalent detection in skin of elder individuals has been paralleled by its almost exclusive detection in bone tissues from WWII casualties (86), and recently also by its detection in tonsil tissues from elder people (80). In this latter case, the virus has been detected in B lymphocytes, another unexpected finding that poses additional questions on the range of B19V target cells and the regulation of viral lifecycle in dependence of the specific intracellular environment.

Concerning the form of viral DNA persistent in cells, two possibilities are that the incoming viral DNA is inaccessible to DNA repair enzymes and maintained as silent ssDNA, or that it can be converted to a dsDNA form, with a potential for expression or replication. Quantitative determination of the amount of viral DNA in cells or tissues can be obtained, but only relative comparisons can be made as an indirect indication of viral replication. Quantitative detection of viral mRNAs could yield a better clue to investigate the activity of virus within infected cells. Considering the viral expression profile in erythroid progenitor cells as a common paradigm, then expression of a resident viral genome could be confirmed by detection of spliced mRNAs in higher abundance with respect to unspliced mRNAs, while the determination of the relative abundance of early (left half of the genome, including NS) to late (right half of the genome, including VPs) messengers could yield information relative to the abortive or productive characteristic of the viral lifecycle. In this respect, in vitro experimental systems suggest that limited expression may occur even on a non-replicating template (23,94). In most instances, reports of *ex-vivo* detection of viral mRNAs are limited by technical difficulties, are only qualitative or incomplete in their quantitative determination of mRNAs abundance, but there are instances suggesting low-level expression even in non-permissive environments. Presence of viral mRNAs does not implicate production of viral proteins, that would possibly be effectors of pathological processes in persistently infected cells, so ideally a reliable characterization of the viral proteome would also be necessary.

The physical state and epigenetic modification of the viral genome resident in cells may add levels of regulation. By now, it is assumed that B19V DNA can establish persistence in episomal from, while the possibility that the viral genome might integrate its genome in the host genome is raised in analogy to Adeno-Associated Viruses (AAVs). This occurrence has recently received its first experimental support in EPCs, and it should be considered that an integration/rescue mechanism would yield opportunity for the virus to establish a true latency and reactivations (40). Additionally, the terminal regions have the characteristics of CpG islands and are a possible target of cytosine methylation (15), an epigenetic modification able to regulate expression of the viral genome. This modification has been shown to occur in vitro in non-permissive cell systems, and has also been detected to different extents in bioptic samples. Modification of the methylation status within terminal regions might be a means of modulating and silencing viral expression by the host tissues, and might effectively contribute to establishment of lifelong persistence of viral DNA in tissues. More investigation is however needed to confirm the actual relevance of epigenetic modifications and regulation of the viral genome.

All this considered, the actual capacity of B19V to establish true latency and reactivation is not established. In vitro cellular models suggest as more plausible the persistence of viral genomes with some expression potential, rather than establishment of a latency

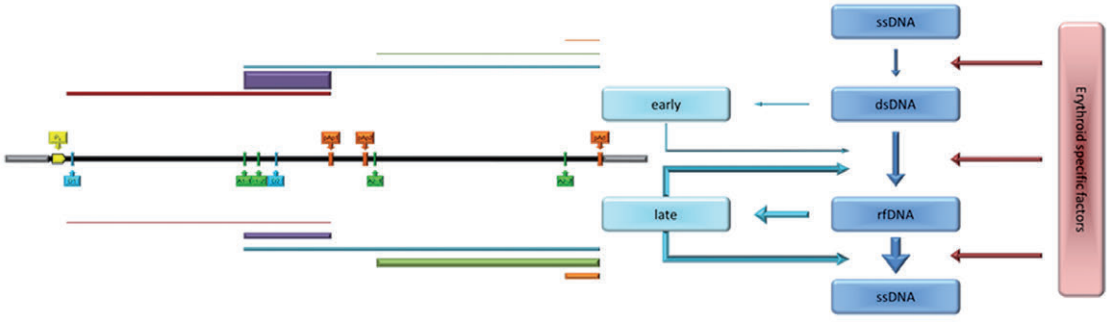

Figure 2. A model for B19V replication and expression. Viral genome can be present in four consecutive states, connected by three state transitions: input ssDNA, parental dsDNA, replicating rfDNA, and product ssDNA. Two functional profiles are identified as *early* (from dsDNA) and *late* (from rfDNA), characterised by a differential abundance and relative composition of transcriptome (compare map in Figure 1). Erythroid specific factors are critical in regulating state transitions and are dependent on the differentiation and physiological state of the cell. Modified from Bua *et al.*, 2016 (23).

with possible reactivation, but the situation might be more complex *in vivo*. The eventuality of reactivation of a latent infection has been sparsely suggested based o case reports, but in many instances in terms that would not discriminate reactivation from viremic blips in the course of persistent infections, or from exogenous reinfections. Critical attention is needed when introducing such concepts with the unique support of limited laboratory and clinical data.

How many pathologies?

Following contact via the respiratory route, the virus gain access to the bloodstream, and then to the primary target organ, the bone marrow, where it can infect erythroid progenitor cells achieving a productive infection and exerting cytotoxic effects. In this phase, the bone marrow can show erythroid aplasia and the presence of characteristic giant erythroblasts, that are considered patognomonic of B19V infection. The effects on bone marrow are derived from the ability of virus to induce cell-cycle arrest, block of erythroid differentiation and eventually apoptosis of susceptible and infected cells on one side, and from the dimension and turnover rate of the erythroid compartment on the other. The fact that the more undifferentiated precursors are resistant to infection ensures that the block in erythropoiesis is temporary, and can be relieved by the development of a neutralizing immune response.

In fact, in the bone marrow, a balance is reached between cell population dynamics and viral replication, considering also possible stresses on the erythropoietic compartments and the immune response (22). In a normal individual, with physiological erythopoiesis and normal immune response, infection is limited in extent and temporal frame, and is controlled by the development of neutralizing antibodies. Levels of haemoglobin only marginally decrease and infection is usually asymptomatic from the haematological point of view. Clearance of infection, determined by detection of virus in the blood, takes usually 3-4 months with constantly decreasing levels, even if very low levels of virus can be detected for years following primary infection (57,72). Persistence of viral DNA in peripheral blood implicates active chronic infection, but recent caveats as reported above should be taken into consideration (52,67).

Infection becomes manifest as pure red-cell aplasia (PRCA) and anaemia when pre-existing alterations in the erythropoiesis process, or defects in the immune response, alter the balance between viral replication and cellular turnover (22,92). In case of stressed and expanded erythropoietic compartment, because of a reduced lifespan of erythrocytes or increased need, infection by B19V can lead to an acute episode of profound anaemia, that presents as the classical aplastic crisis in patients with underlying haematological disorders. On the opposite, when the immune system has not the capability to control, neutralize and clear viral infection, infection can become persistent, with active viral replication and the involvement to different degrees of erythroid compartment. These last situations can be typical of congenital or acquired immunodeficiency, such as HIV-infection (46), in cases of malignancies (20), in the course of chemotherapy (56), or in the course of immunosuppressive treatments, such as in bone marrow (75) or solid organ transplant recipients (31). Depression of erythropoiesis can be manifest, with anaemia of different grade, but also compensated and unapparent. A general correlation may be present between viraemic levels and anaemia, but a clinical threshold has not been univocally defined, as very high-level viraemia may be compensated by active erythropoiesis, while many normal and asymptomatic individuals can support prolonged low-level persistent replication of the virus. The distinctions between a normal course and active chronic infections are smooth, and we should take in mind that our picture of B19V as a virus capable of acute, self-limiting infections has been replaced with a more complex picture of a virus capable of establishing long-term relationship with the host.

Bone marrow supports a productive infection and leads to a secondary viraemia that is the mark of active infection and that in the initial phase, before the development of an effective immune response, can reach exceedingly high viraemic levels (10^{12} virus/mL). This secondary viraemic phase leads to the systemic distribution of the virus and preludes to possible late clinical manifestations of infection. In this phase, the two classical manifestations of B19V infection are erythema infectiosum, typical of children (2,25), and arthropathies, typical of adults (83,91). Then, since the initial reports, the range clinical manifestations associated to B19V infection has been constantly increasing, to involve almost all organs and tissues, and descriptions of clinical presentations have progressively stressed atypical aspects. Strict criteria and sound methodologies should be always adopted to link B19V infection and definite atypical pathological processes, by demonstrating the presence and expression of virus in pathological tissues, or by rigorous association of laboratory, clinical and epidemiological parameters. Unfortunately, large part of the scientific literature on B19V is not sound enough in this respect.

When investigating the pathogenetic processes possibly linked to the systemic phase of B19 infection, problems arise regarding the identification of secondary target cells, the definition of the viral expression profile and virus-induced alterations within these cells, and the characterization of the degree and role of the immune response. In some instances, the pathogenic process may depend upon direct cytotoxic or proapoptotic effects of viral proteins, and in some it may depend upon stimulation of an inflammatory response by viral proteins, such as the NS protein or the VP1u-associated phospholipase PLA(2). The interplay with the immune system, by its innate and adaptive recognition mechanisms, may lead to the development of immunopathological mechanisms, or autoimmune processes that also have been described (43,59).

In the systemic phase of infection, endothelial cells may play a central role. Endothelia constitute a diffuse tissue that can account for the wide distribution of virus and the detection of its genome in disparate organs. Endothelial cells can be infected by B19V (89) and, normally non-permissive, can be a site of persistence of the viral genome. In some cases, however, markers of viral activity have been precisely localized to endothelial cells within diverse tissues and organs, and causally linked to pathological processes, so the question is in what situations endothelial cells can become permissive to the virus. Another general assumption is that some typical manifestations of infection, such as the erythema, are due to immune complex formation and deposition, with development of inflammatory responses. In fact, immune recognition mechanisms leading to pathological processes have been described in several instances and will contribute to the general clinical picture of B19V infection.

B19V as a cardiotropic virus

In recent years, B19V has gained interest as a cardiotropic virus, being detected at ever increasing frequencies in endomyocardial tissues, and replacing other cardiotropic viruses as the most prevalent virus detected in the heart (3,45,76,88). B19V has been directly involved as an etiologic agent in acute myocarditis both in paediatric (19) and adult populations (61). The course of disease may be severe and not readily diagnosed. In the course of myocarditis, active B19V

infection has been shown in myocardial endothelial cells, then B19V probably acts by inducing endothelial dysfunction which in turn triggers inflammatory responses in the cardiac tissue (10). The rare occurrence of clinically relevant myocarditis compared to the widespread diffusion of B19V infections underscores the relevance of coincident factors, that are presently ignored.

The frequency of B19V DNA detected in cardiac bioptic samples is constantly high, and a marked cardiotropism should be mentioned as one of the main characteristics of B19V. The definition of a possible role of B19V in the development of chronic cardiomyopathies and cardiac dysfunction is a matter of on-going debate. Correlation between B19V and cardiomyopathies, in particular dilated cardiomyopathy and ventricular dysfunction, has been proposed by several studies comparing selected groups of patients versus controls (30,77,87), but on the opposite, a lack of significant clinical association has been supported by other studies (47,48,85). A positive association and a role of B19V in the development of chronic cardiomyopathies has been proposed because of significant higher frequencies of detection in patient versus control groups, or of the presence of higher mean viral loads suggesting active viral replication. The degree of immune response, either activation of innate immune system as hinted by cytokine levels, or specific anti-B19V immunity, can also be considered as a clue to a role for B19V in the development of cardiomyopathies.

B19V and intrauterine infections

A relevant property of B19V is its ability to cross the placental barrier and infect the foetus. The viral receptor, globoside, is present on the villous trophoblast layer of human placenta (41). While trophoblasts are not permissive to the virus they may bind viral capsid via the globoside receptor, hence facilitating transcytosis of virus to the foetal circulation (90). Endothelial placental cells can be productively infected, facilitating the establishment of fetal infection and contributing to placental damage (78). When in the foetal circulation, the virus can infect erythroid progenitor cells, in liver and/or bone marrow depending on the gestational age, and can be detected in cells circulating in the vessels of several tissues as well as in the amniotic fluid (17). The block in foetal erythropoiesis can be severe, because of the physiologically expanded erythropoietic compartment combined to an immature immune response, and lead to profound foetal anaemia.

The natural course of foetal infection is affected by several factors. The immune status of the mother plays a prominent role, as the presence of specific IgG in maternal serum is assumed to be protective towards infection of the foetus. Therefore, intrauterine infections should be confined to the case of primary infections in non-immune pregnant women. In this situation, the effective rate of transmission will depend on the gestational age, being higher in the first two trimesters possibly because of different expression of globoside on the placenta. The effect on the foetus will also depend on its developmental stage, depending on the rate of expansion of its erythroid compartment and maturity of foetal immune response. As a consequence, infections occurring at earlier stages carry a higher risk of leading to foetal deaths, while the development of hydrops is more frequent in the central part of pregnancy. Hydrops may lead to foetal death, or the foetus may more frequently recover without persistent damage. In the third trimester, transmission is more difficult and foetuses are relatively resistant, so the overall risk of foetal damage decreases to background values (33).

Some case series also reported a high frequency of detection of B19 DNA in late intrauterine deaths (73), a finding not confirmed in other case series, that did not show such correlations and indicate that late intrauterine foetal death is a rare event [56]. Finally, the infected foetus may show presence of virus at birth (79). Congenital infections have been sporadically associated to neonatal anaemia or anomalies, while possible consequences on the neurological development are currently investigated as a possible event (28,58).

Clinical manifestations in B19V infected pregnant women do not differ from those commonly observed in healthy individuals and there is no correlation among presence of maternal symptoms, occurrence of vertical transmission and severity of foetal disease. During pregnancy, B19V infection can be entirely asymptomatic or associated with either polyarthralgia and/or rash. When symptoms are present, an acute biphasic illness is reported with fever, headache, and myalgia, followed within 1-3 weeks by the onset of joint inflammation, most commonly of hands, knees, wrists and ankles, or the erythematous eruption (18). The time between maternal infection and observation of clinical findings suggesting B19V congenital infection varies between 1 and 20 weeks. Typical foetal effects include foetal anaemia and hydrops, cardiomegaly and pericardial effusion, hydropic or nonhydropic intrauterine foetal death (IUFD) (29). Hydrops, defined as fluid accumulation in foetal compartments, (subcutaneous, pericardial, pleural, and abdominal) has been mostly reported when maternal infection occurs in the central part of the pregnancy, and only occasionally at the beginning and at the end. Commonly hydropic fetuses present ascites, skin oedema, pericardial effusions, hydrothorax as well as placental oedema. Hydrops may lead to poor outcomes, death in utero or at birth, or the foetus may recover without congenital abnormalities. Hydrops is rarely reported in early pregnancy loss while foetal death accompanied by hydrops fetalis is commonly observed in case of maternal infection during 13 to 20 gestational weeks.

Besides foetal anaemia, the main hematologic feature due to the viral apoptotic effect on foetal erythroid precursor cells, thrombocytopenia has been reported in many case reports, however only few studies included a larger number of cases. When retrospective studies are designed on a significant cohort, the rate of severe foetal thrombocytopenia has been estimated between 38% and 64%, with the highest incidence in the selected group of hydropic foetuses. Foetal thrombocytopenia occurs mainly during the second trimester but, in some cases, persists into the third trimester, and may be associated with an increased risk procedure-related foetal loss after foetal blood sampling and/or intrauterine transfusion of red blood cells (IUT), that constitutes a therapeutic option in case of symptomatic B19V foetal infection (27,65).

The virus is not regarded as a teratogen (50). Sporadic B19V-related foetal complications are described in documented intrauterine infections; they include chronic anaemia, and other defects affecting gastrointestinal, ocular, cardiac and central nervous systems, but in most cases these can be considered coincidental. Presently, there is insufficient data assessing a role for B19V in inducing neurological effects in congenitally infected children. The limited existing studies report an increased incidence of delayed neurodevelopment in long-term survivors after IUT for B19V-induced severe foetal anaemia compared to those treated for maternal red cell alloimmunisation, thus possibly indicated that intrauterine infection might be a contributor to neurodevelopmental delay.

Diagnostic awareness

Definition of an analytical profile

A clinical diagnosis of B19V infection can only be suspected. Infections can be asymptomatic, or present with unspecific symp-

toms, while the typical symptoms attributed to B19 infection can be the results of other infective processes. Then, a laboratory diagnosis is required in the presence of a specific or generic request.

The diagnostic approach can now rely on the standardized detection of specific viral components, mainly viral genome, that is a valuable complement to the more traditional approach of detection of a specific immune response, mainly antibodies of IgM and IgG classes. Serum or plasma specimens are the material of choice for the detection, obtained from the same sample, of specific antibodies and viral DNA in the viraemic phase. Viral DNA, or proteins, can be detected also from bone marrow aspirates, or bioptic samples. In case of suspected foetal infections, amniotic fluid is suitable for the detection of viral DNA, while in case of foetal death analysis of foetal or placental tissues may allow to indicate viral aetiology (93).

As discussed, B19V is a virus capable of infections presenting with different courses, so that the acute phase infection can be followed by a delayed clearance, active chronic infections or silent persistence in tissues, depending on the interplay with host factors and the efficacy of the immune system response. Therefore, an accurate laboratory diagnosis of B19V infection will necessary rely on a multiparametric approach, combining as much as possible both molecular detection of viral components and immunological detection of virus-specific antibodies (Figure 3) (35).

Molecular diagnosis

The detection of the viral genome in peripheral blood, bone marrow or tissues can be considered the more direct and appropriate approach to the diagnosis of infection. Since the beginning, the technical advancements in the development of molecular analytic techniques have always found a complete paradigm in the development of applications to the detection of B19V.

In the progress towards a rapid and accurate molecular diagnosis, a wide array of molecular hybridization and of different nucleic acid amplification techniques have continuously been developed. In particular, standardization and inclusion of competitor or internal controls (71) have been developed for PCR protocols in a continuous effort of accuracy and robustness. Nowadays, real-time quantitative, internally controlled PCR techniques must be considered the standard analytical method for the molecular detection of B19V (15,34) and commercial tests are available. Two main requirements should be met; first, the capability of detection of all genotypes of B19V; then, a calibrated and standardized quantification of viral target. Both of these requirements can take advantage of international standards and can be challenged by international proficiency panels (6). The continuous technical development will certainly lead in the future to novel molecular detection methods and analytical platforms, to improve performances and reduce time and costs (66).

In situ hybridization techniques for the detection of viral nucleic acids (14,62), and immune-histochemical detection of viral proteins by commercially available monoclonal antibodies can be useful as a complement to PCR techniques for investigation of active viral infection in bioptic samples, with the advantage of the identification of target infected cells and allowing discrimination of productive infections from silent persistence of virus.

Immunological diagnosis

Detection of a specific immune response is still considered the standard and most widely used means of laboratory diagnosis of B19V infection. Parallel detection of specific anti-B19 IgM and IgG antibodies is required and interpretation of the combination of results may allow for a presumptive diagnosis of active, recent or past infection.

Antigens used for immunological detection are obtained by means of heterologous recombinant expression systems. Recombinant proteins expressed in prokaryotic systems usually lose their native conformation and are suitable for the detection of immunity against linear epitopes, while recombinant proteins expressed in eukaryotic system can maintain native conformation and can be used to detect immunity against conformational epitopes. In particular, viral capsid proteins assemble as VLPs with antigenic configuration quite similar to that on native virus and are the recognized standard for immunological detection. Enzyme

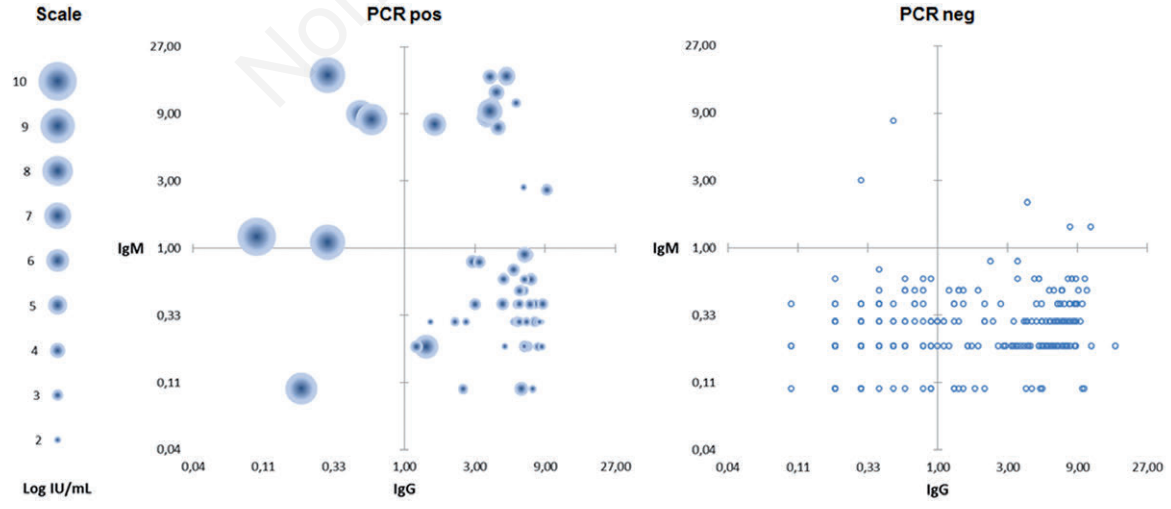

Figure 3. Multiparametric analysis on serum samples, for combined detection of B19V specific IgG and IgM antibodies (EIA, DiaSorin) and B19V DNA (qPCR) [90]. Samples are plotted according to the respective Index Values for both IgG and IgM, in case of positive qPCR the diameter of the bubble is proportional to the Log of viral load (IU/mL) according to scale. The multiparametric approach allows prompt detection and/or confirmation of acute phase infections, as well as identification of chronic infections.

immunoassays, or chemiluminescent immunoassays, can use VLPs composed of VP2 only, or VP2+VP1, or VP2+VP1u expressed in prokaryotic systems, to allow detection of antibodies to conformational VP2 or also VP1u linear epitopes. Line blot assays include an array of conformational and linear antigens, and can be used as a confirmatory assay to dissect the range of antibody response to B19V (44,63). Of limited availability, although potentially useful, are assays to determine IgG avidity, or acute-phase, epitope specific reactivity (32,42).

Surveillance, prophylaxis and therapy

B19V infection is normally considered a benign clinical situation. The virus is widely diffuse and in most cases infection is asymptomatic or unnoticed, with an uneventful clinical course. This situation of course does not account for the totality of cases. Haematological consequences can be relevant, in patients with underlying haematological disorders or in patients with immune system deficits, and the erythroid aplasia can be severe and require transfusion therapy. In otherwise healthy subjects, the development of chronic inflammatory and rheumatic manifestations can be severely impairing. The risk of infection in pregnancy with its possible consequences on the foetus are of major concern.

Therefore, not only a prompt and accurate diagnosis of infection is required, but a comprehensive approach including prophylactic, therapeutic and monitoring actions should be considered. Specific actions would include measures for reducing transmission of virus through blood and blood-derived products, use of passive immunization as a therapeutic intervention, and finally the development of a vaccine and specific antiviral drugs.

Screening and blood products safety

The presence of a high-titre early virsemic phase, as well as the delayed clearance of virus from bloodstream and the possible occurrence of low-titre chronic infections, may pose a question regarding the risk of transmission of virus through use of blood, blood components or blood products. Two major factors play a role in determining the clinical outcome of parenteral exposure to the virus, the total amount of virus transfused or infused to recipients, and the immune status and competence of the patients.

Regarding blood transfusions, a threshold level of about 10^7 International Units (IU)/mL seems necessary to obtain infection, as determined by seroconversion and viraemia in recipients (21). The presence of specific antibodies in the donated blood interferes with infectivity, and the presence of previous immunity in recipients seems to be protective. Given both the reported frequencies of high-titre viraemic blood units and seroprevalence rates in the population, the probability of infection by exposure to single blood or blood components units is low (39). Very few case reports describe symptomatic infections, while linked donor-recipient studies indicate that this situation is infrequent and in most cases clinically unapparent. Single donor screenings are therefore unjustified in terms of costs, however in some countries minipool testing has been implemented for post-transfusion surveillance. High risk patients with haematological disorders, immune deficiencies or pregnant women would benefit from the availability of dedicated donations, proven to be specific-Ig positive and free from B19V.

Blood products are manufactured from large pools of donations and in these cases inclusion of high titre viraemic units in the manufacturing pools can be expected with high probability, while dilution during the manufacturing process may not be sufficient to fall below a safety threshold level. To reduce the risk of transmission of virus through blood products, and given the estimated minimal infectious dose of B19V, a safety threshold has been indicated by regulatory offices at 10^4 IU/mL in plasma pools for manufacturing. In the manufacturing process, removal or inactivation steps are normally introduced to reduce the risk of transmission of undetected viruses. B19V is a small virus, resistant to solvent/detergent treatments but only relatively resistant to heat treatment. Heat inactivation, chemical inactivation or physical removal, effective by using nanofiltration devices, should all be considered in the production steps to further increase the safety of blood and blood-derived products (9).

Therapeutic intervention

Therapeutic options for B19V are limited. In specific instances, overt symptomatology may require supportive or symptomatic treatments. Transfusions are required to treat the anaemia in cases of transient aplastic crisis or prolonged anaemia, while in cases of arthralgias, non-steroidal anti-inflammatory drugs may exert beneficial effects. In cases of foetal infections and hydrops, intrauterine transfusions are indicated when the haemoglobin concentration in the foetal circulation falls below a threshold level, with improved survival rates of hydropic foetuses (49).

Passive immunization can be considered as an effective means of reducing the viral load, especially useful in chronic infections that may depend on the inability of the immune system to develop an effective and neutralizing response. IVIG, being prepared from large pools of donors representing the collective immune memory of a population, usually contain high levels of neutralizing anti-B19V antibodies, and can be used with success to reduce the viral load (26,70). However, IVIG may not be normally effective in achieving a complete clearance of the virus, then a beneficial effect is temporary and wanes with decaying of exogenous antibodies, so that in many instances cycles of IVIG administrations need to be repeated. Usually, clearance of infection occurs when the patient own immune system develops a complete mature immune response, so a complementary approach would be to relief known causes of immunodeficiency. As examples, in cases of HIV infection, HAART therapy will allow for reconstitution of the immune function and cure of B19V infection (68); in cases of post-transplant immunosuppression, patients may take advantage of IVIG administration coupled to a change in the immunosuppressive therapeutic scheme (7). It should be noted that no clinical trials have been carried out to determine an optimal therapeutic scheme for the administration of IVIG, so the treatment are still empirical and rely on high dose cycles (2 g/kg). Also, a possible value of prophylactic administration of IVIG to prevent foetal transmission has not been evaluated. Human monoclonal antibodies have been developed but their therapeutic or prophylactic use not evaluated (37). All these limitations prompt for further research and an extensive and controlled study of the efficacy of passive immunization therapeutic or prophylactic schemes.

Concerning documented B19V infection in pregnancy, management is conservative and treatment mainly depends on both the severity of foetal anaemia and the gestational age. If foetal anaemia, assessed by middle cerebral artery peak systolic velocity measurements (MCA-PSV) is severe, intrauterine RBC transfusion (IUT) can be indicated for foetal recovery (58). As foetal thrombocytopenia accompanies frequently anaemia, it is advised

that platelets should be available for transfusion during IUT procedures because of the increased risk of haemorrhage or exsanguination (65). Otherwise, if foetal anaemia is mild, and considering that hydrops can spontaneously resolve, invasive therapy is not recommended and B19V complicated pregnancy is followed-up by serial ultrasound examination and MCV-PSV measurements. The prophylactic administration of IVIG in B19V infected women to prevent foetal transmission has not been yet evaluated.

Vaccine development

The development of a vaccine for B19V has been a problematical endeavour. A vaccine would be useful and probably effective, as B19V is a virus adapted exclusively to human host, transmitted by direct interpersonal contact and effectively neutralized by the immune response. The aim to the development and introduction of a vaccine would mainly be to protect at-risk populations, such as patients with underlying haematological disorders. In particular, women of childbearing age would have benefits from vaccine protection, chiefly in terms of foetal safety. In this case, vaccination would reduce the needs for screening, surveillance, follow-up and would avoid a small but definite number of foetal losses. On the other hand, infection is in most cases subclinical, clinical consequences are mild and self-limiting, or on the opposite with a tendency to chronicisation even in the presence of immune response, so the real utility of a general vaccine might be questioned.

Preliminary work on vaccine development has been conducted. Main immunogenic determinants are considered the viral capsid proteins, with their VP2 conformational and VP1u linear epitopes. Viral capsid proteins expressed in eukaryotic heterologous systems will retain original structure and form VLPs that are antigenically similar to native virions. Therefore, for vaccine, VLPs can be produced and assembled by VP2 protein only, or can be enriched in VP1 to include neutralizing epitopes encoded in the VP1u region (5). Such VLPs are immunogenic in the animal experimental model as well as in humans. Phase I studies on initial VLPs produced in the recombinant baculovirus system showed immunogenicity and relative safety in humans (4), while phase II studies showed a remarkable reactogenicity (8), that lead to termination of the trial. VLPs devoid of the VP1u-associated viral phospholipase activity and produced in a yeast system recently led to the development of an alternative, apparently efficient and safer vaccine candidate (24).

Antiviral drugs development

A rationale for the development of a specific antiviral therapy would be in the treatment of chronic infections in immunosuppressed patients, or for reducing the inflammatory aspects of acute or chronic infections, or possibly for prophylaxis in selected cases. Although the virus and the virus-cell system potentially offer several targets for an antiviral therapy, only few studies have until now been reported in this field. Viral NS protein, or the phospholipase domain in the VP1u region are both required for viral infectivity and responsible of pathogenetic effects, so they constitute potentially relevant targets. Specific inhibition of their activity would probably impair the capacity of the virus to replicate, as well its cytotoxicity and proinflammatory activity. The development of pharmaceutical biotechnology tools and of accurate quantitative methods to evaluate reduction in viral infectivity are required to lead research in this field.

The absence of the complete characterization of B19V proteins and related molecular mechanisms makes difficult a rational drug design strategy, directing the on-going efforts in the search for compounds inhibiting B19V replication towards a drug repositioning approach. The anti-B19V activity of the antiviral drug cidofovir (CDV) has been demonstrated, widening the antiviral spectrum of a compound already known to be effective against all families of dsDNA viruses (12,13). CDV exerts a potent inhibitory effect on B19V in the myeloblastoid cell line UT7/EpoS1, but a relevant inhibitory activity in primary human EPCs only when extendedly exposed to high doses, thus limiting its use *in vivo*. This evidence, however, supports a drug discovery process by identifying new clinical uses for existing approved drugs. In this frame, a further study demonstrated the inhibitory activity of hydroxyurea (HU), the only disease-modifying therapy approved for sickle cell disease (SCD), towards B19V replication (11). Results demonstrated that HU strongly inhibits B19V replication at concentrations lower than those affecting cellular DNA replication and viability and, most importantly, at levels measured in plasma samples of SCD patients undergoing HU therapy. Of note, it has been recently observed that SCD paediatric patients, the main population at risk of B19V-induced transient aplastic crisis (TAC), under HU treatment and in course of B19V infection experienced in TAC episode attenuation of symptoms with improved hematological indices compared to untreated children. Hence, in vitro research lends support for clinical observations, implying a possible double target use of HU (38).

Conclusions

As a final comment to this review, B19V is a virus that can offer continuous matter of interest to basic and clinical virologists for many reasons. The pattern of genetic evolution, its peculiar properties and functional profile, the characteristics of its narrow tropism and restricted replication, its complex relationship with the host and its ample pathogenetic potential are all topics that are far from a comprehensive understanding.

B19V is underestimated from a clinical perspective. Its wide circulation and prevalent benign and self-limiting clinical course generally lead to a diminished appreciation of its pathogenetic potential. In this review, only selected clinical aspects have been discussed, but B19V is a possible etiological agent in a wider ensemble of diseases, encompassing practically all organs and systems. An extended awareness and definition of the actual pathogenetic role of B19V in the human diseases, the development of better diagnostic methods and algorithms, the development of prophylactic and therapeutic options will continue to be relevant issues, worth of efforts by the scientific community.

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