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Review Article

Parvovirus B19 Achievements and Challenges

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Parvovirus B19 is a widespread human pathogenic virus, member of the *Erythrovirus* genus in the Parvoviridae family. Infection can be associated with an ample range of pathologies and clinical manifestations, whose characteristics and outcomes depend on the interplay between the pathogenetic potential of the virus, its adaptation to different cellular environments, and the physiological and immune status of the infected individuals. The scope of this review is the advances in knowledge on the biological characteristics of the virus and of virus-host relationships; in particular, the interactions of the virus with different cellular environments in terms of tropism and ability to achieve a productive replicative cycle, or, on the contrary, to establish persistence; the consequences of infection in terms of interference with the cell physiology; the process of recognition of the virus by the innate or adaptive immune system, hence the role of the immune system in controlling the infection or in the development of clinical manifestations. Linked to these issues is the continuous effort to develop better diagnostic algorithms and methods and the need for development of prophylactic and therapeutic options for B19V infections.

1. Introduction

Human parvovirus B19 (B19V) is a human pathogenic virus, member of the *Erythrovirus* genus in the Parvoviridae family. Structural features of parvovirus B19 are common to viruses in the family, and include a genome constituted by a molecule of linear single-stranded DNA of either positive or negative polarity, about 5600 bases in length, encapsidated in isometric virions, and approximately 25 nm in diameter. 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 the pathogenetic potential of the virus, its adaptation to different cellular environments, and the physiological and immune status of the infected individuals.

The virus shows a marked tropism for erythroid progenitor cells in the bone marrow, exerting a cytotoxic effect and causing a block in erythropoiesis that can be manifested as a transient or persistent erythroid aplasia. However, the virus has the capability to infect several different cellular types, as inferred from the detection of viral nucleic acids, and sometimes viral proteins, in diverse tissues, and the pathogenetic potential of the virus is ample. The most common clinical manifestations of infection are erythema infectiosum mainly in children or postinfection arthropathies mainly affecting adults; however the virus has been implicated in a growing spectrum of other different pathologies, among them myocarditis and rheumatic diseases. Of relevance, the virus can be transmitted to the fetus, with possible consequences such as fetal death and/or hydrops fetalis.

The relevant issues in research on B19V and the scope of this review concern the definition of its biological characteristics and of virus-host relationships; in particular, the interactions of the virus with different cellular environments in terms of tropism and ability to achieve a productive replicative cycle or, on the contrary, to establish persistence; the consequences of infection in terms of interference with the cell physiology; the process of recognition of the virus by the innate or adaptive immune system, hence the role of the immune system in controlling the infection or in the development of clinical manifestations. Linked to these issues are the continuous effort to develop better diagnostic

algorithms and methods and the need for development of prophylactic and therapeutic options for B19V infections.

2. The Virus

2.1. Taxonomy. The family Parvoviridae includes viruses with a single-stranded, linear DNA genome and an icosahedral capsid, 20–25 nm in diameter, composed of 60 protein subunits in a $T = 1$ arrangement. Within the family, the subfamily Parvovirinae includes viruses able to infect vertebrate hosts; within the subfamily, the genus *Erythrovirus* includes the human parvovirus B19 (synonym: B19 virus; accepted acronym: B19V) as the type species [1].

Once subdivided broadly in parvoviruses able to achieve autonomous replication and helper-dependent parvoviruses, the taxonomy in the Parvoviridae family has grown in complexity to accommodate the still-increasing number of recognized viruses. In recent years, the introduction of molecular screening techniques has allowed the identification and subsequent characterization of parvoviruses with genetic organization or sequence divergence high enough to propose a new taxonomic and phylogenetic scheme. Within viral species, a subdivision in genotypes has been introduced in many instances to accommodate a broad and unexpected genetic diversity. In the case of B19V, three different genotypes are now recognized as distinct evolutionary lineages, while further distinctions in subtypes are continuously proposed mainly on epidemiological basis.

2.2. The Genome. Initial molecular studies on the genome of viruses that appeared "parvovirus-like" at the electron microscopic observation [2] confirmed the presence of a single-stranded, linear genome, of either positive or negative polarity, that enabled the classification within the Parvoviridae family [3, 4]. Subsequent molecular cloning and sequencing [5–7] led to a first characterization of the genome structure and organization and opened the way to the study of the biological properties of the first human parvovirus of recognized clinical relevance.

Structural features of B19V genome are common to viruses in the family. Linear single-stranded DNA molecules of either positive or negative polarity, 5596 bases in length, and are encapsidated in isometric virions. Molecules of either polarity are encapsidated at the same frequency and can anneal in solution forming linear double-stranded DNA molecules. The viral genome is composed of a unique internal region, containing all the coding sequences, flanked by two repeated, inverted terminal regions. Terminal regions are 383 nt long, the distal 365 forming an imperfect palindrome. As a result of the occurrence of sequence asymmetries within the palindrome, each terminal region can be present in either one of the two alternative sequences, each is the inverted complement of the other (usually referred to as flip/flop). Different combinations of these alternative sequences at both termini can therefore give rise to four different genome isomers. The presence of self-complementary sequences in the terminal regions allows for the single-stranded DNA molecule to adopt terminal loop and stem structures [8]. The presence of mismatched bases within the self-complementary regions necessarily introduces distortions in such double-stranded structures, so that different isomers might differ depending on the distortion introduced. Within terminal regions, the unique internal region, 4830 bases in length, encompasses all open reading frames coding for viral proteins. Two major open reading frames are present, in the left half of the genome coding for the viral nonstructural protein NS and in the right half of the genome coding for the viral capsid proteins VP1 and VP2. Additional minor reading frames are present in the center and right end of the genome, potentially coding for smaller nonstructural proteins (11 kDa, 9 kDa, 7.5 kDa). A schematic representation of B19V genome organization and functional mapping is reported in Figure 1. A more detailed representation of the left terminal region is reported in Figure 2.

2.3. The Virion. B19V was first identified tentatively as a parvovirus on the basis of its dimensions and morphology at the electron microscopy observation. Initial biochemical characterization of virions confirmed properties typical of parvoviruses and a composition of two structural proteins, VP1 and VP2. The larger VP1 protein accounts for about 5% of the virion mass, the smaller, colinear VP2 protein, constitutes the bulk 95% of the virion [9]. Alignment of sequences within capsid protein genes of viruses in the Parvoviridae family and comparison with known molecular structures allowed a first structural prediction of the B19V capsid shell [10]. Thereafter, structural data have been obtained mainly by means of cryoelectron microscopy and crystallographic X-ray diffraction studies on VP2-only B19V capsids, obtained as viral-like particles (VLPs) from recombinant systems such as baculovirus [11–13]. More recently, native virions, either DNA-containing or empty, have been purified and crystallized, and their structure has been compared to that of VP2-only VLPs [14].

The capsid shell is composed of 60 protein subunits; the core structure is formed by the VP common region, forming classical beta-barrel, with eight strands connected by large loops projecting on the outer surface and determining its topography and specific structures at the 5-, 3-, and 2-fold symmetry axes. Similar to other parvoviruses, a cylindrical structure is present at the 5-fold axis, forming a gated channel connecting interior and outer surface of the virion and whose rim is surrounded by a "canyon-like" depression, while typical of B19V is the absence of prominent spikes at the 3-fold axis and a general rounded, smooth surface. When comparing VP2 VLPs with native virions, difference in structures is mainly evident around the 5-fold axis, suggesting that in native virions, either DNA-containing or empty, the cylindrical channel is normally bordered by the N-termini of VP2 proteins.

Due to the submolar frequency of VP1, its N-terminal sequence (VP1 unique, VP1u) has not been determined by crystallography and the exact atomic structure is still unknown. Two possibilities are still debated. It can be exposed, at least partially, on the outer surface of the virion where it may be constantly accessible and recognized by the

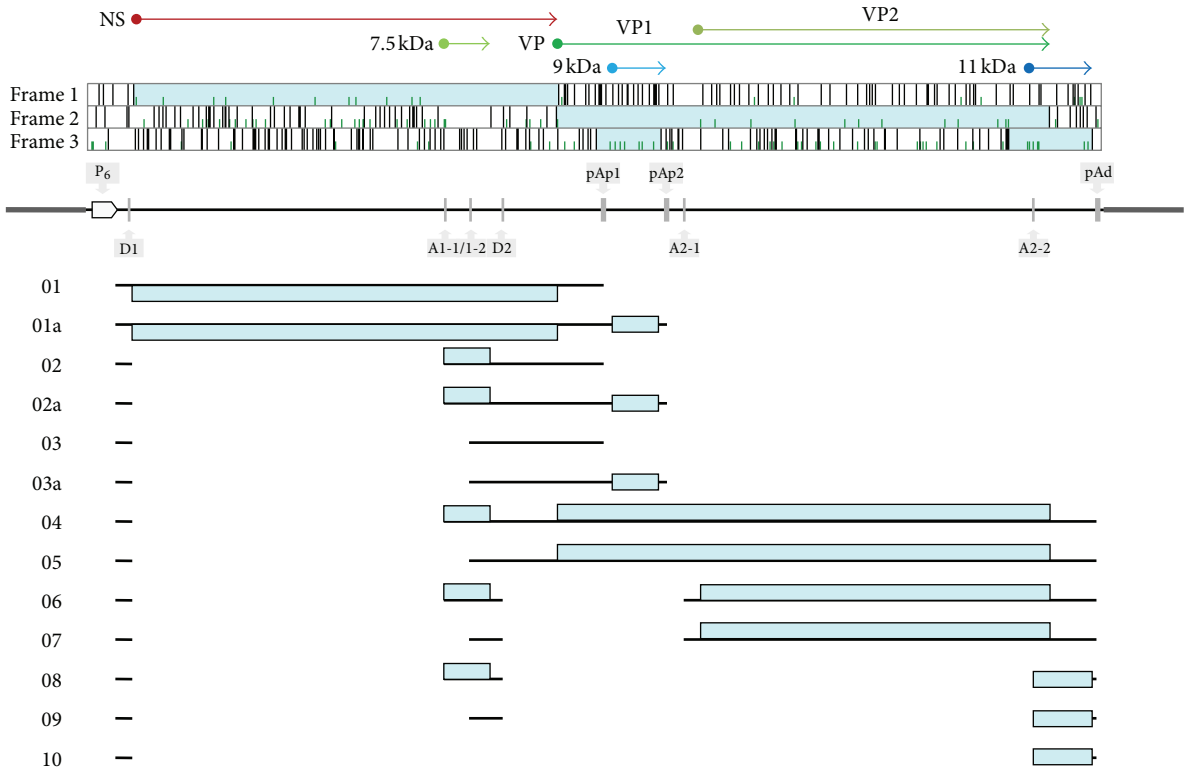

Figure 1: Schematic representation of B19V genome organization and functional mapping. Top: open reading frames identified in the positive strand of genome; arrows indicate the coding regions for viral proteins positioned on the ORF map. Center: genome organization, with distinct representation of the terminal and internal regions and indication of the positions of promoter (P6), splice donor (D1, D2), splice acceptor (A1-1/2, A2-2/2), and cleavage-polyadenylation (pAp1, pAp2, and pAd) sites. Bottom: viral mRNAs species; black boxes indicate the exon composition and light boxes indicate the ORFs contained within mRNAs.

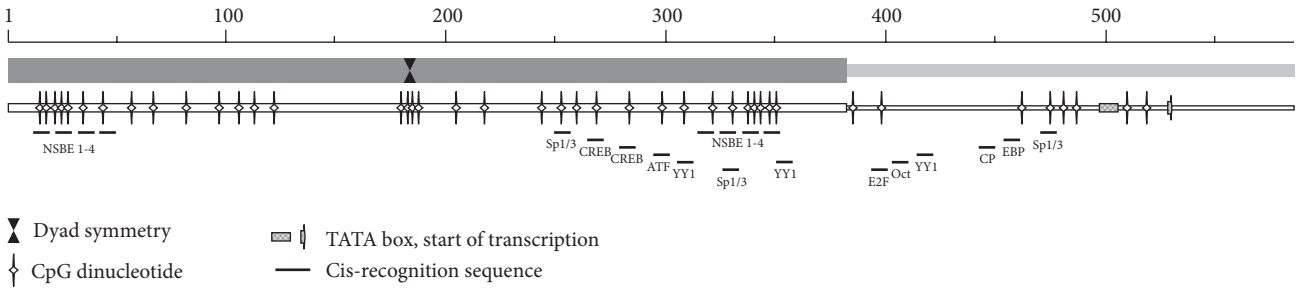

Figure 2: Schematic representation of the left terminal region of B19V and distribution of relevant features. Thick box, left inverted terminal region, and indication of the site of dyad symmetry defining the terminal palindrome. Thin box, contiguous internal unique region. Diamonds highlight the distribution of CpG dinucleotides within sequence. Box and arrow indicate position of TATA box and start of transcription, respectively, as part of the P6 promoter. Black boxes indicate the position of NS protein-binding elements (NSBE 1-4) and a selection of cis-recognition sequences for eukaryotic transcription factors, as indicated.

immune system or, as in other viruses in the family, it can be located internal to the capsid, proximal to the cylinder at the fivefold axis, being exposed at the external surface if virions are damaged or in the first phase of interaction with target cells. As all other viruses in the family, the VP1 unique region possesses an intrinsic PLA(2) phospholipase activity, necessary to maintain viral infectivity [15, 16], so its topographic disposition and dynamic of possible conformational changes might be crucial for determining a productive interaction of virions with target cells.

3. Evolution

The genetic diversity of B19V has always been a subject of interest as a clue to viral evolution, to elucidate possible determinants of virus-cell interaction mechanisms and different

characteristics of virus-host relationships. The continuous development of innovative sequencing techniques has led to a constant growth of sequence data available, so it can be predicted that application of the new generation sequencing techniques will be crucial for a thorough understanding of the dynamics of viral evolution and molecular epidemiology.

So far, the main achievement was the identification of three distinct genotypes of B19V, once thought to be a virus species with very limited sequence diversity. In fact, early sequencing studies reported a uniform picture of sequence variation within B19V isolates, presenting a global degree of homology usually higher than 98% and only minor variations in diversity along the different genomic regions [17]. This picture changed considerably with the first identification of some viral isolates that showed a substantial genetic divergence and distance from previously characterized isolates [18–20].

The species B19V is now formally subdivided into three genotypes, the prototype genotype 1 and the two variant genotypes 2 and 3; isolates belonging to diverse genotypes form clearly separated clusters [21]. At the nucleotide level, considering alignment of consensus sequences obtained from available complete genomes, the genetic distance between clusters is about 10% for genotype 1 and 5-6% for genotypes 2 and 3. At the same level, the genetic distance within clusters is generally lower for genotype 1, normally less than 2%, and higher for genotypes 2 and 3, normally in the range 3–10%. The constant input of new genomic sequences into databases prompts for further subdivisions into subtypes, not formally recognized but useful for molecular epidemiological studies. So, within genotype 1, the majority of isolates are referred to as genotype 1a, while a few isolates from Asia show separate clustering and are referred to as genotype 1b [22]. Within genotype 3, two distinct subtypes are usually referred to as genotypes 3a and 3b [23].

All B19V genotypes appear to cocirculate, but their relative frequency is strikingly different and their spatial and temporal distribution is not uniform [24–28]. The prototype genotype 1a is the major circulating genotype and is present in all geographic areas [27]. The variant genotype 2 is rare but can be sporadically detected in different geographic settings including Europe [29–31]. The variant genotype 3, in its two subtypes, can be detected at higher frequency in western Africa, where it may constitute the prevalent genotype [23], and at lower frequencies in other geographic areas.

A different picture emerges when analyzing and typing viral DNA not from blood, hence from the viremic phase of a productive infection, but viral DNA normally present and persistent within tissues as a result of a past infection, the so-called bioportfolio [32]. In these instances, the frequency of detected viral DNA and the relative abundance of different genotypes would reflect the spread of virus and its epidemiology in the past. These analyses, performed in different population groups and tissue samples, usually led to the detection of all different genotypes confirming the property of B19V to establish a possibly lifelong persistence in tissues. Strikingly, the frequency of genotype 2 detected in tissues is normally much higher than what is normally found for circulating virus [32–34]. In a European setting, the frequency of genotype 2 is highest in elder people and minimal in people born after 1960–1970, when genotype 1 becomes prevalent, therefore suggesting at that date a global replacement of genotype 2 with genotype 1, in a pattern similar to what is described for other animal parvoviruses.

The pattern of evolution of B19V and genetic divergence among genotypes are still under investigation. By inferring the phylogenetic history and evolutionary dynamics of temporally sampled B19V sequences, a high rate of evolutionary change, at approximately 10^{-4} nucleotide substitutions per site per year, was observed [35]. This rate is similar to that of RNA viruses and suggests that high mutation rates, however characteristic of the Parvoviridae family, can lead to a rapid evolutionary dynamics, possibly with formation of viral quasi-species. This model has been further corroborated by a study that used a large VP sequence data set of plasma- and tissue-derived isolates of B19V, to compare the rates of sequence change in exogenous virus populations with those persistently resident in tissues [36]. In this model, plasma-derived B19V showed a substitution rate of 4×10^{-4} and an unconstrained (synonymous) substitution rate of 18×10^{-4} per site per year, a high mutation frequency that may enable rapid adaptive changes that are more commonly ascribed to RNA virus populations. These estimates predict that the last common ancestor for currently circulating genotype 1 variants of B19V existed around 1956 to 1959, fitting well with previous analyses of the B19V bioportfolio that support a cessation of genotype 2 infections and their replacement by genotype 1 infections in the 1960s. In contrast, the evolution of tissue-derived B19V was best modeled when considering slow or absent sequence change during persistence. Hints on a continuous evolutionary process of B19V also come from more recent molecular epidemiological studies utilizing large datasets, suggesting that even within the more recent genotype 1a, a slow and gradual accumulation of point mutations can give way to periodical, dynamic replacement of strains with higher sequence divergence [37, 38].

In conclusion, a high rate of nucleotide substitution, comparable to RNA viruses, may lead to the formation of viral quasi-species and an expansion of the population in the sequence space around master sequences. The rapid spread of infections would then allow for progressive diversification of viral isolates. The observed rate of synonymous versus nonsynonymous substitutions is constantly high, therefore indicating for strong selective pressures on the virus, and this in turn would account for the observed uniformity of the biological behavior and immunogenicity of the diverse genotypes [39].

4. Lifestyle

4.1. Cell Tropism. B19V is characterized by a narrow tropism and restricted replicative ability. The main target cells are erythroid progenitor cells in the bone marrow, that are susceptible and permissive to B19V infection. Viremic sera from the acute phase of infection cause inhibition of erythropoiesis in culture [40]; virus purified from viremic sera specifically inhibits bone-marrow-derived erythroid progenitor cells in a colony formation assay [41] and is detectable in infected erythroid progenitor cells [42].

The virus can be propagated to a limited extent in suspension cultures of human erythroid bone marrow cells stimulated with Epo, in culture systems that allow the study at a molecular level of events associated with the B19V life cycle [43, 44]. Enrichment of erythroid progenitor cells from bone marrow can support a higher viral replication [45]. Erythroid progenitor cells susceptible to B19V infection can be also obtained from peripheral blood [46, 47], from fetal liver [48–50], and from umbilical cord blood [51, 52]. More recently, advances in techniques for generating large numbers of human erythroid progenitor cells (EPCs) ex vivo from circulating peripheral blood hematopoietic stem cells (HSCs) have been exploited [53, 54]. In these systems, it is possible to produce pure populations of CD36+ EPCs expanded and differentiated from CD34+ HSCs that are highly susceptible and permissive to B19V. These cells represent the best available paradigm to study the events in B19V replicative cycle and their effect on primary target cells.

A few continuous cell lines can also support B19V productive infection. These cell lines are mainly of myeloblastoid origin and require Epo both for growth and differentiation and for permissivity to B19V. The megakaryoblastoid cell line UT7/Epo [55], its subclone UT7/EpoS1, and the erythroid cell line Ku812Ep6 [56] are the most widely used. However, all of these systems show limited ability to support viral infection and low viral yield [57]. It should be considered that B19V is not really adapted to grow in cell cultures, and then most studies on B19V life cycle still rely on infection of primary cells or cell lines with native virus obtained from serum of infected patients.

A complementary approach to the study of virus-cell interactions would rely on the generation of complete genomic clones with infectious capability, as it has been possible for other viruses in the family, notably the dependoviruses (adeno-associated viruses, AAVs). Inserts comprising the B19V genome, excised from plasmid vectors and transfected in cells, should be able to maintain their functional competence, complete a full replicative cycle, and generate infectious viral particles, able to start a novel replicative cycle. Such a system might be exploited for performing sequence-structure-function correlation studies, and the capability to generate recombinant viral particles would foster new research activity, for example, in the field of biotechnological applications.

Unfortunately, B19V genome has not been easily domesticated. The first genomic clones obtained possessed either incomplete or rearranged terminal regions, however did not show any functional competence following transfection [5, 7]. AAV-B19V genomic hybrids showed capability to generate transducing viral particles that may share some biological properties with B19V [58, 59]. Finally, complete B19V genomic clones were obtained that showed functional competence and the ability to form novel infectious viral particles [60, 61]. While extremely useful for the study of viral expression profile and function of viral proteins [62], these systems still do not lead to a high yield of infectious viral particles. Further studies and the exploration of novel strategies will be required to achieve this goal.

4.2. Early Events. A main determinant of B19V tropism appears to be its interaction with cellular receptors. A first clue came from the observation of a hemagglutinating activity of B19V [63], similar to other parvoviruses and indicating a possible specific interaction with carbohydrate moieties on the plasma membrane of cells of erythroid lineage. A specific receptor for B19V, present on the plasma membrane of erythroid progenitor cells as well as erythrocytes, was then identified in the glycolipid globoside [64]. Globoside (Globotetraosylceramide, Gb4Cer) is an antigenic determinant within the P blood group system, for which a limited degree of polymorphism is also present in the population. B19V binds to globoside, as measured by thin-layer chromatography. Purified globoside blocks the binding of the virus to erythroid cells and the infectivity of the virus in a hematopoietic colony assay, while target cells can be protected from infection by preincubation with monoclonal antibody to globoside. Furthermore, erythrocytes lacking P antigen cannot be hemagglutinated by B19V.

The central role of globoside in the infectious process was underlined by the demonstration that the rare persons with the p phenotype, lacking globoside on the plasma membrane of erythrocytes and erythroid progenitor cells, were naturally resistant to B19V infection [65]. In in vitro infections, bone marrow cells from donors with the p phenotype maintained normal erythropoiesis despite very high concentrations of virus, and there was no evidence of infection of erythroid progenitor cells by B19V. The demonstration of the resistance of globoside-negative individuals to infection with B19V was the first example of resistance to pathogens linked to genetic polymorphism in the human population. This phenomenon can now be viewed in the context of a complex picture of coevolution of human and pathogens, where antigenic polymorphism may constitute a selective advantage, especially when considering the selective pressure imposed by other microbial parasites, for example, malaria parasites.

The presence of globoside by itself is not sufficient to confer susceptibility to infection [66]. Globoside can be expressed on several cell types, mainly primary cells of erythroid lineage but also other primary cells such as human umbilical vein endothelial cells (HUVEC) and normal human lung fibroblasts (NHLF). Several cell lines show presence of globoside on the plasma membrane. In these cellular systems, globoside can be involved in binding; however the level of expression of globoside does not directly correlate with the efficiency of viral binding. Moreover, despite the presence of globoside, some cell types are resistant to transduction with recombinant B19V vectors.

Coreceptors, involved in virus binding and internalization of B19V particles, have been identified in the $\alpha5\beta1$ integrins. $\alpha5\beta1$ integrins function as receptors for the extracellular matrix component fibronectin, and the ligand-induced activation is involved in numerous cellular functions including anchoring, trafficking, proliferation, and differentiation. These integrins are normally expressed on the surface of susceptible cells, such as erythroid progenitor cells, or can be experimentally induced and confer susceptibility to infection in other cell lines such as K562. $\alpha5\beta1$ integrin coreceptor function can be enhanced either by stabilizing

the high-affinity conformation of β1 integrins or by inducing β1 integrin clustering, suggesting that β1 integrin-mediated signaling might be important for B19V internalization [67]. Integrin function appears to be regulated in a cell type-specific manner through coexpressed integrins, and preferential B19V entry into erythroid progenitor cells is promoted by a robust β1 integrin response that is enhanced through stable preclustering of coexpressed β2 and β3 integrins [68]. In primary human erythroid progenitor cells, the cytoskeleton organization also allows efficient recruitment of β1 integrins by brief pharmacological stimulation of Rap1 GTP loading, significantly increasing B19V internalization [69].

Other molecules may be implicated in the attachment phase of B19V to different cell types. In addition to recognizing globoside as the main receptor, weak interactions of B19V have been shown to occur with other carbohydrate moieties of several tissue-specific membrane glycosphingolipids, thus expanding the spectrum of possible susceptible cells [70]. Although a correlation can be observed between multiple glycosphingolipids expression, B19V capsid binding, and the tissue tropism observed clinically in B19 parvovirus-associated diseases, a functional role has not been demonstrated in instances other than globoside. Finally, the DNA-binding protein Ku80 can possibly act as an alternative coreceptor for B19V [71]. Although described as a nuclear protein, Ku80 is present on the cell surface of CD36+ human bone marrow erythroid cells, CD3 T cells, and CD20 B cells, thus implicating cells of lymphoid lineage as target cells. The possible role of Ku80 in the life cycle and pathogenetic potential of B19V needs to be confirmed by further investigation.

The interactions of B19V virions with globoside have been further characterized. Visualization of B19V virions complexed in solution with globoside identified the receptor contact area within a depression on the threefold symmetry axis [72]. In a subsequent study [73], neither specific complexes nor specific interaction of B19V with reconstituted plasma membrane could be detected, suggesting the stringency of a definite conformation/distribution of glycolipids on the plasma membrane, for example, within lipid rafts and the presence and a functional role of coreceptors. Therefore, in a multistep model, a first interaction of viral capsids with globoside as a docking molecule might be followed by stronger interactions with integrins, followed by internalization of the virus. The absence of integrins, for example, on erythrocyte plasma membrane would prevent internalization in a nonpermissive cellular environment, while their activation following contact with virus would trigger a downstream cascade of events allowing infection of nucleated cells. This model has been further investigated.

As discussed, there is uncertainty regarding the topographic disposition of the VP1u region. As a possibility, the N-terminal portion of the VP1u region might not be accessible in native capsids; however exposure of capsids to increasing temperatures or low pH might lead to its progressive accessibility to neutralizing antibodies without particle disassembly. The measurement of the VP1u-associated PLA(2) activity of B19V capsids also suggests that this region is normally internal to the capsid shell but becomes exposed in heat- and in low-pH-treated particles [74]. Similarly, a proportion of the capsids can externalize the VP1u-PLA(2) motif upon binding on human red blood cells (RBCs), not causing direct hemolysis but inducing an increased osmotic fragility of the cells by a mechanism involving the PLA(2) activity of the exposed VP1u. In the acute phase of infection, most virions circulating in the blood can be associated with the RBC fraction, not being able to infect susceptible cells but playing a significant pathogenetic role because of the exposure of the immunodominant VP1u region, including its PLA(2) domain [75]. On the other hand, the early exposure of VP1u might facilitate viral internalization and/or uncoating in target cells. In UT7/Epo cells, expression of Gb4Cer and CD49e (integrin α5) is high. B19V colocalizes with Gb4Cer and, to a lesser extent, with CD49e, but only anti-Gb4Cer antibodies can compete with virus attachment. Normally, only a small proportion of cell-bound viruses can be internalized, while the majority detaches from the receptor and acquires a higher cell binding capacity and infectivity. This effect has been attributed to conformational changes in the capsid, triggered by attachment of B19V to cells and leading to the accessibility of the VP1u region. The receptor-mediated exposure of VP1u region is critical for virus internalization, since capsids lacking VP1 can bind to cells but are not internalized [76]. Then, Gb4Cer is not only the primary receptor for B19V attachment, but also the mediator of capsid rearrangements required for the interactions leading to virus internalization. The capacity of the virus to detach from cells and reattach with higher efficiency would enhance the probability of productive infections.

A first insight into the early steps of B19V infection, the path of virions, and mechanisms involved in virus uptake, trafficking, and nuclear import has recently been investigated in UT7/Epo cells [77]. In these, B19V and its receptor Gb4Cer associate with lipid rafts, predominantly of the noncaveolar type. B19V is internalized by clathrin-dependent endocytosis and spreads rapidly throughout the endocytic pathway, reaching the lysosomal compartment within minutes, where a substantial proportion of virus is degraded. Endocytic vesicles are not permeabilized by B19V, indicating a mechanism of endosomal escape without apparent membrane damage. Compounds such as NH_4Cl, which raise endosomal pH, can block the infection by preventing endosomal escape, resulting in a massive accumulation of capsids in the lysosomes. Intact virions do not enter the nucleus, and the amount of viral DNA in the nuclear compartment is too low to be measured over background before the onset of macromolecular synthesis. In contrast, in the presence of chloroquine, the transfer of incoming viruses from late endosomes to lysosomes is prevented, the viral DNA is not degraded, and, even if capsids remain extranuclear, viral DNA is progressively and substantially associated with the nucleus. The effect of chloroquine is to boost B19V infection in different cell types [78], and even nonpermissive cells such as HepG2 [79] can become permissive in its presence.

Novel questions then warrant further investigation, such as the precise role of lipid rafts in the process of virus entry, the mechanism by which B19V escapes from endosomes without detectable permeabilization/damage, and the pathway involved in the nuclear import of viral DNA. In

particular, the exact role of phospholipase activity in the VP1u region [80] required for infectivity needs to be elucidated.

4.3. Macromolecular Synthesis. Once the viral genome is exposed within the nucleus, viral macromolecular synthesis mainly relies on the cellular machinery for transcription of messengers and replication of the viral genome. Typically for the family Parvoviridae, it is assumed that these activities depend on an environment of actively replicating cells and require S-phase specific cellular factors. However, it is now emerging that B19V by itself has the ability to modulate progression through the cell cycle to its own advantage.

Conversion of the incoming, parental single-stranded genome into a double-stranded genome is the first step and a possible key determinant of the replicative cycle [81]. B19V encapsidates single-stranded genomes of either positive or negative sense, and as inverted terminal repeats are identical it is assumed that both strands are functionally equivalent. Terminal regions have an almost perfect palindromic sequence and then possess the ability to fold at a dyad symmetry axis, assuming double-stranded terminal hairpin structures that provide the necessary priming structure for cellular DNA polymerases to synthesize the complementary strand. Accessibility of these terminal structures and their capability to prime synthesis can be regarded as first bottleneck for a productive replicative cycle. Permissive or nonpermissive cellular environments and the outcome towards a productive or nonproductive replicative cycle may depend on the ability of different cells to support, or consent, synthesis of the complementary strand.

Replicative intermediates of B19V have not been thoroughly characterized. ssDNA, monomeric dsDNA, and dimeric dsDNA forms are all detected in productively infected cells [43, 44]. The terminal regions act as origins of replication, and in a dsDNA form they can be present in either closed or extended conformations. It is assumed that, as in other parvoviruses, replication proceeds through cycles of terminal resolution and hairpin-primed strand displacement synthesis, possibly via a rolling hairpin mechanism. The cellular DNA polymerase activity is involved, together with viral NS protein that can bind to cis-recognition elements within the terminal regions and is predicted to have endonuclease and helicase activity, necessary for the terminal resolution process and strand-displacement synthesis [82]. It is not yet known what role, if any, the sequence asymmetries within the palindrome may play in directing or regulating the mechanistic details of the replication process.

Formation of a double-stranded intermediate generates a transcriptional active template. A unique promoter is present and active on the genome, mapped within the internal unique region close to the left inverted terminal region of the genome and usually referred to as P6, directing transcription from the left to the right end of the genome [83, 84]. Mapping of regulatory elements within the promoter shows the presence of a complex regulatory region containing multiple sequences which affect promoter strength, and that the GC-box motif is a major controlling sequence for in vitro transcription [85]. Moreover, the promoter is transactivated by the nonstructural protein NS that, therefore, has a positive feedback effect on the activity of its own promoter [86].

This P6 promoter is broadly active in many cell types, so tropism is unlikely to be regulated at the level of transcriptional initiation, although differences can be measured among different cell types [87, 88]. An investigation of the sequence within the promoter region shows the presence of several cis-recognition elements for transcription factors [89]. For some of these there is experimental evidence of functional involvement: YY1 [90], E4TF1 [91], Oct-1, Sp1, and Sp3 [92, 93] all can bind to elements mapped within the promoter region. The viral NS protein interacts with cellular Sp factors and in this way contributes to transactivation of its own promoter. Finally, activity of the promoter may also be responsive to hypoxic environment [94].

Transcription of the viral genome generates an ensemble of mature transcripts due to a combination of co- and post-transcriptional events. A complex array of viral mRNAs arises from the presence on the pre-mRNA of two splicing donor sites (D1 and D2), each followed by two alternative splice acceptor sites (A1-1/2 and A2-1/2), and of two transcriptional termination sites in the middle and at the right end of the genome (pAp and pAd). Initial mapping showed that at least nine classes of viral mRNAs are produced as a result of the diverse combinations of possible splicing and cleavage events [95]. All viral transcripts share a common, 60 nt long leader sequence at their 5′ end. Then, one class of mRNA is not spliced and terminates in the middle of the genome, containing the left major ORF coding for the NS protein, while all other classes undergo single or double splicing, terminating either in the middle or at the right end of the genome, in this last case encompassing the right major ORF coding for viral capsid proteins [96]. The complement of viral mRNAs is redundant with respect to recognized viral ORFs, and the different viral mRNAs species can be cistronic, monocistronic or, possibly, bicistronic, potentially coding for a rather restricted viral proteome [97].

Precise mapping of splicing junctions and cleavage-polyadenylation sites was first obtained by cloning and sequencing of cDNA libraries obtained from infected erythroid cells [98]. Thereafter, the cis-elements directing processing of precursor mRNA have been actively investigated in several cellular systems. All the spliced B19V mRNA transcripts contain the 60 nt long 5′ leader sequence spliced from the D1 splice site to the central exon, which spans the A1-1/A1-2 to D2 splice site. Further splicing at the D2 donor site is a central step in the control of B19V pre-mRNA processing. The sequence of the central exon contains several exonic splicing enhancers/intronic splicing enhancers (ESEs/ISEs) elements, recognized by SR proteins. Of these, ISE1 and ESE1 elements have been identified within A1-1 and A1-2 sites, ESE2 and ESE3 within A1-2 and D2, and ISE2 downstream of D2 site. Overall, the definition of the B19V central exon will be the consequence of a balanced recognition of cis-elements by SR proteins. SR proteins that bind to ESE1 and ESE2 will promote splicing at the A1-1 and A1-2 acceptor sites, respectively, while SR proteins that bind to ESE3 and ISE2 will facilitate recognition of the D2 donor site. The frequency of recognition events can provide the basis

for regulatory splicing during B19V pre-mRNA processing [99].

A second key factor in the definition of viral mRNAs is the frequency of cleavage-polyadenylation events at the center or right end sites present in the genome. In the center of the genome, two cleavage-polyadenylation sites have been mapped, respectively, pAp1 and pAp2. Processing of pre-mRNAs at the pAp1 site is programmed by a nonconsensus hexanucleotide core motif (AUUAAA). Efficient use of this element requires both downstream and upstream cis-acting elements and is further influenced by a second adjacent nonconsensus motif (AAUAAC). On the contrary, pAp2 shows a canonic hexanucleotide core motif (AAUAAA) [100]. Cleavage at pAp1 will generate the most abundant classes of viral mRNAs, cleavage at pAp2 will occur at tenfold lower frequency and generate alternative mRNAs potentially including an additional small ORF for a 9 kDa protein, while readthrough will generate mRNAs extending into the capsid coding region and coding for VP1 protein. Competitive splicing from D2 to A2-1 or A2-2 sites will regulate the production of mRNAs coding for VP2 or 11 kDa proteins, respectively [101]. All of these events appear to be coordinated and in relation to the replicative process involving the DNA template. In the absence of genome replication, internal polyadenylation of viral mRNAs at pAp sites is favored, while replication of the viral genome enhances readthrough of pAp and the polyadenylation of B19 virus transcripts at the distal site pAd [102, 103]. Therefore, replication of the genome would facilitate the generation of sufficient full-length transcripts that encode the viral capsid proteins and the essential 11-kDa nonstructural protein.

4.4. Expression Profile. Differences in the expression profile of B19V genome may occur during the course of a productive replicative cycle, as a result of an ensemble of coordinated regulatory events, or can be distinctive of the restrictions posed by different cellular environments to a productive replicative cycle.

An early model of B19V genome expression, obtained by Southern and Northern Blot analysis of viral nucleic acids from synchronized, infected UT7/Epo cells, indicated the presence of early and late events involving genome transcription and replication [104]. In particular, RNA transcription appeared as an early event following infection, with viral RNA detected about 6 hours after infection (hpi) and with an earlier appearance of nonstructural protein RNA (6 hpi) compared to capsid protein RNA (24 hpi). In contrast, dimer-replicative intermediate forms of DNA did not appear until more than 16 hpi after infection. More recently, a more precise reevaluation of the expression profile of B19V genome in different cell types was obtained by means of quantitative PCR analysis, first assessing the relative abundance of distinct regions of the viral genome within the mRNA complement [105], and then by using a redundant PCR array to determine the relative frequency of the diverse species of mRNAs [106].

In permissive cellular environments, such as in bone marrow mononuclear cells, UT7/EpoS1, and KU812Ep6 cells, viral DNA is observed to increase within 48 hpi, rarely exceeding 2 logs with respect to input DNA, viral RNA is already present within 2–6 hpi, its increase preceding that of viral DNA up to 36–48 hpi, and all the different classes of viral RNA are constantly represented in stable relative amounts throughout the infection cycle. In nonpermissive cells, such as TF-1 cells, viral DNA does not increase and only the spliced, proximally cleaved viral mRNAs can be detected in minimal amounts. These data indicate that the B19 virus genome should be considered a single replicative and transcriptional unit, characterized by a two-state expression profile, either silent and nonreplicating or transcriptionally active and replicating [105]. Then, within the single and compact transcriptional unit, the presence and utilization of splicing and cleavage-polyadenylation signals would determine the relative abundance of the different RNA species. Utilization of the processing signals is relatively constant throughout the viral infection cycle, with the only difference emerging as an early modulation in expression profiling of the viral genome, linked to transition from a low-splicing, prevalent pAp cleavage at earlier times after infection to a high-splicing, balanced pAp/pAd cleavage at later times [106], an effect possibly linked to onset of replication of the viral genome [103].

Overall, such data indicate that the genome of B19V can be considered as a single functional unit, whose expression profile can be clearly differentiated depending on the degree of intracellular restriction. In nonpermissive cellular environments the viral genome can be present but is silent, while in permissive cellular environments the genome is active and both full transcription and replication of the viral genome occur, resulting in a productive replicative cycle. The onset of transcription, which is assumed to be on a double-stranded template, generates a complete set of mature transcripts, with a marginal temporal shift between immediate events, the prevalent generation of unspliced and proximally cleaved transcripts, and delayed events, with an increased generation of spliced and distally cleaved transcripts. The generation of a full complement of viral mRNAs appears to occur and be maintained before the onset of template replication, thus blurring a distinction between early and late events, and maximal replication of the viral genome occurs as a final event, in the presence of all species of viral mRNAs.

A further level of regulation of viral expression can be at the epigenetic level [107]. In this respect, CpG DNA methylation is one of the main epigenetic modifications playing a role in the control of gene expression. For DNA viruses whose genome has the ability to integrate in the host genome or to maintain as a latent episome, a general correlation has been found between the extent of DNA methylation and viral quiescence. Within the genome, the inverted terminal regions display all the characteristic signatures of a genomic CpG island, suggesting a possible role of CpG DNA methylation in the regulation of viral genome expression. The effects of DNA methylation on the regulation of viral genome expression were investigated by transfection of either unmethylated or in vitro methylated viral DNA in a model cell line, and results showed that methylation of viral DNA was correlated with lower expression levels of the viral genome. Then, in the course of in vitro infections in

different cellular environments, such as UT7/EpoS1 or U937 cells, it was observed that absence of viral expression and genome replication were both correlated to increasing levels of CpG methylation of viral DNA. Finally, the presence of CpG methylation was documented in viral DNA present in bioptic samples, indicating its occurrence in vivo and suggesting a possible role of this epigenetic modification in the course of natural infections. Viral nucleic acids can be recognized by cellular PRR, such as TLR9. B19V genome lacks typical stimulatory motifs; however its methylation in a typical CpG islands and the presence of an epigenetic level of regulation of viral genome expression, possibly correlated with the silencing of the viral genome and contributing to the maintenance of the virus in tissues, can be relevant to the balance and outcome of the different types of infection associated with parvovirus B19.

4.5. The Proteome. The proteome potentially encoded by B19V appears to be limited [9, 96, 108–110]. A total of six ORFs are distributed on the three positive strand frames, as depicted in Figure 1, and represented in several classes of viral mRNAs. In the left half of the genome, unspliced mRNAs contain a major ORF in frame 1, coding for NS protein. In the right half of the genome, single-spliced mRNAs contain a major ORF in frame 2 encoding for VP1, and double-spliced mRNAs contain a portion of the same ORF encoding for VP2. As both capsid proteins are coded from the same ORF in frame 2, it can be discerned a VP1 unique region (VP1u) and a VP1/VP2 core region. Two ORFs in frame 3 are present in the middle and right end of the genome and have similar topographical relation with respect to spliced transcripts cleaved at the pAp2 and pAd sites, and the potential to code for a 9 kDa and 11 kDa proteins, respectively. A small additional ORF, positioned in frame 2 ahead of the ORF for VP proteins, has the potential to code for 7.5 kDa protein.

However, the RNA complement is only indicative of the potential viral proteome. In fact, posttranscriptional regulation can also occur, influencing the functional profile of B19V in infected cells. An AUG-rich region upstream of VP1 start codon, absent in VP2, can act as a negative regulatory element in the translational control of B19V capsid protein production, leading to a higher relative abundance of VP2 [111]. Strikingly, mRNAs coding for viral capsid proteins might not be translated efficiently in some cellular environments such as UT7/Epo cells [81, 112]. This effect was specifically attributed to the $3'$ UTR of mRNAs coding for the capsid proteins, whose presence represses capsid protein synthesis at the translational level by inhibiting ribosome loading [113]. Although codon optimization of capsid genes was shown to enhance capsid protein synthesis in nonpermissive environments [114], there is the possibility that such mechanisms might be regulated by human miRNAs specifically targeting the viral mRNAs [115].

In the future, a proteomic analysis approach will be required for characterization of the whole viral proteome, definition of posttranslational modifications of viral proteins, investigation of the dynamics and interactions of viral proteins produced in infected cells, and of virus-induced cellular alterations.

4.6. NS Protein. The NS protein is 671 aa protein (calculated Mr 74 kDa), containing an SF3 helicase domain. In nonstructural proteins of small viruses, such as parvovirus, polyomavirus, and papillomavirus, a SF3 helicase domain is usually associated with an origin-binding domain. By pairing such domain with a helicase, the protein binds to the viral origin of replication leading to origin unwinding, then cellular replication proteins are recruited to the origin and the viral DNA is replicated. Several structures of SF3 helicases have been solved, but not that of B19V NS protein. They all possess the same core alpha/beta fold, consisting of a five-stranded parallel beta sheet flanked on both sides by several alpha helices. Finally, the SF3 helicase proteins assemble into a hexameric ring.

B19V NS is a protein with nuclear localization, produced early in the replication of B19V but is detectable throughout a time course of infection. NS protein is not associated with viral particles as other NS proteins of parvoviruses. Within infected cells, B19V NS protein may also be present showing additional forms of lower Mr, but neither posttranslational modifications nor processing has been clearly documented [9, 108]. As discussed, structural and functional predictions indicate the presence of DNA binding, endonuclease, helicase, and transactivating domains. Some of these activities have been documented experimentally.

NS protein is essential for replication of B19V genome [62]. NS operates on terminal structures of B19V DNA replicative intermediates, allowing terminal resolution and strand unwinding, necessary for priming of strand displacement synthesis [82]. It can be assumed that its activity is also necessary for strand unwinding in the packaging phase of replicative cycle. NS transactivates its own promoter [86], boosting viral macromolecular synthesis and promoting viral replication.

Heterologous transactivating activities have been attributed to NS protein, from the HIV LTR [116] to several genes involved in inflammatory responses. Expression of NS protein in heterologous cellular systems, such as K562 cells, can promote production of the inflammatory cytokine interleukin-6 (IL-6), but not the production of other related cytokines, IL-1β, IL-8, or TNF-α. NS-primed IL-6 induction is mediated by a NF-kB binding site in the IL-6 promoter region, strongly implying that NS functions as a transacting transcriptional activator on the IL-6 promoter [117]. In a different system, such as the monocytic cell line U937, expression of a transduced NS gene can induce production of TNF-α mRNA and protein, in a manner associated with NS expression. AP-1 and AP-2 motifs on the TNF-α promoter are responsible for this NS-mediated upregulation [118]. Although differing in the diverse cellular environments, both these mechanisms indicate a potential proinflammatory role of NS protein.

B19V NS protein shows various effects on the cell. Early reports indicated its cytotoxicity [119], that could be abolished by mutating its putative nucleoside triphosphate-binding domain [120]. B19V NS protein was shown to induce apoptosis in UT7/EpoS1 cells as well as K562 cells, in a caspase-3 dependent pathway, separate from the IL-6 activation pathway [121]. In human erythroid progenitors,

CD36+ cells, B19V infection and NS expression both induced DNA fragmentation characteristic of apoptosis, and the commitment of erythroid cells to undergo apoptosis was combined with their accumulation in the G(2) phase of the cell cycle. B19V- and NS-induced apoptosis was inhibited by caspase 3, 6, and 8 inhibitors, and substantial caspase 3, 6, and 8 activities were induced by NS expression. Fas-FasL interaction was not involved in induction of apoptosis in erythroid cells, but these cells were sensitized to apoptosis induced by TNF-α, suggesting a possible connection between the respective apoptotic pathways activated by TNF-α and NS protein in human erythroid cells [122].

4.7. VP Proteins. Two VP proteins constitute the capsid shell, VP1 and VP2. VP1 is a 781 aa protein (calculated Mr 86 kDa), VP2 is a 554 aa protein (calculated Mr 61 kDa). Being derived from the same ORF, the N-terminus of VP1 constitutes the 227 aa long VP1u region, that comprises the viral phospholipase domain. In the VP1/2 common region, these proteins share a beta-sandwich structure consisting of 8 beta-strands in two sheets with a jellyroll fold, and characteristic interactions between the domains of this fold allow the formation of fivefold assemblies that lead to the formation of a $T = 1$ capsid.

Both proteins are produced and accumulate throughout the replicative cycle. The relative abundance of the two protein species is in part regulated by the relative abundance of the respective mRNAs, in part from efficiency of translation initiation [111], and also may depend on effects linked to the 3′UTR region of specific mRNAs [113]. A nonconsensus basic motif in the C-terminal region of VP1/2 mediates transport of capsid proteins to the nucleus, where capsid assembly and genome packaging may occur [123]. As in the case for NS protein, neither posttranslational modifications nor processing has been documented.

Viral capsid proteins produced in eukaryotic expression system have the capability of self-assembly and form viral-like particles (VLP) quite similar to native virions, a property exploited for studies on capsid structure and assembling dynamics. The first systems utilized a genetically engineered cell line [124] or B19-SV40 hybrid vectors and expression in COS-7 cells [97, 125]; however the most widely used heterologous system for the production of viral capsid proteins able to form VLPs soon became the baculovirus expression system in eukaryotic insect cells [126–128].

In the baculovirus expression system, VP2 protein alone is sufficient for formation of VLPs; the percentage of VP1 protein that may become incorporated in VLPs can rise from the normal 5% up to 40% at the expense of efficiency. While VP1 protein alone cannot self-assemble in regular capsids, progressive truncation of VP1u region restores the ability of VP1 to form VLPs with normal morphology [129]. Further truncation of the VP2 protein destroys the ability to form VLPs [130]. In recombinant capsids, most of the VP1 unique region is exposed on the capsid surface [131]. These data may be in accordance with crystallographic comparison of VP2- only VLPs, obtained in an *S. cerevisiae* heterologous expression system, with VP1+VP2 native virions, that suggest externalization of the VP2 N-terminal region and hence possibly of VP1 [14]. Furthermore, our understanding of virion structure needs to be reconciled with other data indicating the presence of a constituent PLA(2) activity in VLPs [80], or that the VP1u region becomes fully accessible and its phospholipase active only following the initial interactions with cell membranes [74].

4.8. The Small Proteins. In B19V genome, additional ORFs are present other than NS and VP proteins. Of these, two are in frame 3, separate from the larger ones, in the center and the right end of the internal unique region, and have the potential to code for a 9 kDa and 11 kDa protein, respectively. Additionally, in frame 2, the same as VP proteins but in a position overlapping with the NS coding sequence, a small ORF has the potential to code for a 7.5 kDa protein. Functional analysis obtained from a complete clone of B19V genome indicated an essential role for the 11 kDa protein to maintain infectivity, in contrast to a dispensable role for the 9 and 7.5 kDa proteins [62].

Of these small nonstructural proteins, the 11 kDa protein is the best characterized [109]. It is translated from the small, double-spliced mRNAs cleaved at the pAd site as a family of proteins, 94–85 aa, due to the presence of multiple start codons. It is a proline-rich protein, presenting three consensus SH3-binding sites, showing mainly cytoplasmic localization. Its essential role in B19V infectivity seems related to posttranscriptional events necessary for obtaining adequate amounts and a correct distribution pattern of capsid proteins. In vitro, interaction has been demonstrated with host cell factors, such as receptor-binding protein 2 (Grb2) [132], and perhaps the role of the 11 kDa protein can broadly act in altering the cellular environment to favor viral replication and maturation. In addition, a role of the 11 kDa protein in inducing apoptosis of EPCs via caspase 10 has been shown [133].

The expression of the predicted 9 kDa, 81 aa protein has not been actually traced in infected cells. Such protein would be translated from a highly conserved ORF within the small, single-spliced mRNAs cleaved at the pAp2 (but not pAp1) site, that constitute only a minor fraction of viral mRNAs. In heterologous expression system, this protein exerts a transactivating effect on the P6 promoter, comparable to that of NS protein [134]. Finally, a 7.5 kDa, 74 aa protein might be translated from a small ORF present only in mRNAs spliced at the D1/A2-1 sites. Its expression in infected cells has been shown in an early report [110], but it does not exert any demonstrated function.

4.9. Assembly, Maturation, and Egress. The last phases of the viral infectious cycle are poorly characterized. VP proteins are transported to the nucleus, where they can assembly into capsid and incorporate the viral genome, to be then reexported in the cytoplasm. By electron microscopy, empty or full, DNA-containing viral particles can be observed within infected cells, in scattered distribution or paracrystalline arrays [135]. These terminal phases of the replication cycle are probably tightly regulated and may require helper functions,

for example, a role suggested for the viral 11 kDa proteins. As mentioned, the production of VP proteins and the yield of infectious virus can be hampered in some cellular system, so a further level of restriction to a productive cycle may be linked to the efficiency of the packaging, maturation, and egress processes.

4.10. Cell Tropism, Restriction, and Effect on Cells. The distribution of viral receptors and coreceptors, main and alternative, accounts for the tropism of the virus not only towards its main target cells, but also for the interaction of virus with many diverse cell types. On the other hand, replication of the virus appears to be highly restricted in different cell types and even within a cellular population. The identification of the cellular factors involved in restriction or permissivity to viral replication and the definition of what effects the virus may exert on the different cell types as a function of its replicative cycle are major fields of interest.

Characteristics of the viral replicative cycle and the effects of infection on cell physiology have been studied in three main different contexts. First, most studies have been performed on model cell lines. The UT7/EpoS1 cell line has been widely used because of its relative high susceptibility to infection, although the system is basically restrictive and the yield of infectious virus is very low. Second, many studies have recently taken advantage from the capability in obtaining primary cell cultures of defined erythroid lineage, at different stages of differentiation. In these cases, the interaction is probably very similar to what may happen in natural infections. Third, investigation in many instances involved cell types, primary cells, or cell lines, different from standard target cells, either to investigate relationships with diverse cell types that may occur in the course of natural infections or as in vitro systems amenable to experimental manipulation. In these last cases, results should always be considered as possible evidence waiting for validation in a natural context.

4.11. Cellular Permissivity. Almost all cells that can be productively infected by B19V require Epo for growth [43, 55]. Epo is required to promote growth of these permissive cell types, but also the direct involvement and activation of EpoR and activation of downstream signal transduction are necessary to achieve a productive replication [136]. In fact, CD36+ EPCs grown in the absence of Epo do not support B19V replication, in spite of B19V entry, but Epo exposure enables active B19V replication. Jak2 phosphorylation inhibition inhibits phosphorylation of the Epo receptor (EpoR) and abolishes B19V replication in ex vivo expanded erythroid progenitor cells exposed to Epo. Thus, EpoR signaling is required for B19V replication in ex vivo expanded erythroid progenitor cells after initial virus entry, and replication response is dose dependent. These effects may partly account for the restriction of productive B19V infection in human erythroid progenitors.

Hypoxia also promotes replication of B19V [94]. In particular, in cultured EPC, hypoxia promotes replication of the B19V genome, independent of the canonical PHD/HIFα pathway, but dependent on STAT5A and MEK/ERK signaling. Simultaneous upregulation of STAT5A signaling and downregulation of MEK/ERK signaling boost the level of B19V infection in erythroid progenitor cells under normoxia to that in cells under hypoxia. B19V infection of ex vivo expanded erythroid progenitor cells at hypoxia closely mimics native infection of erythroid progenitors in human bone marrow, where hypoxia is physiological; hence the response of virus to hypoxia can be regarded as an adaptation of virus to the environment of its primary target cells [137].

The block to replication of B19V genome in nonpermissive cellular environments can also be overcome by helper functions provided by adenovirus infection. In the UT7/EpoS1 cells, B19V DNA replication can be enhanced by adenovirus infection. In the nonpermissive 293 cells, the replication of transfected B19V DNA and the production of infectious progeny virus were made possible by expression of the adenovirus E2a, E4orf6, and VA RNA genes [82]. In particular, E4orf6 is able to promote B19V DNA replication, resulting in a concomitant increase in VP expression levels, while VA RNA induces VP expression in a replication-independent manner [138].

4.12. Deregulation of the Cell Cycle. In UT7/EpoS1 cells, infection with B19V induces growth arrest with 4N DNA content, indicating G(2)/M arrest. These B19V-infected cells display accumulation of cyclin A, cyclin B1, and phosphorylated cdc2 and show an upregulation in the kinase activity of the cdc2-cyclin B1 complex. Accumulation of cyclin B1 is localized in the cytoplasm, but not in the nucleus, suggesting that B19 virus infection suppresses the nuclear import of cyclin B1, resulting in cell cycle arrest at the G(2) phase and preventing progression to the M phase. The B19 virus-induced G(2)/M arrest may be the critical event in the damage of erythroid progenitor cells seen in patients with B19 virus infection [139]. In the presence of mitotic inhibitors, B19V infection was shown to induce not only G(2) arrest but also G(1) arrest. UV-irradiated B19V still has the ability to induce G(2) arrest but not G(1) arrest; the B19V-induced G(2) arrest is not mediated by NS expression, while expression of NS can induce cell cycle arrest at the G(1) phase [140]. NS expression increases p21/WAF1 expression, a cyclin-dependent kinase inhibitor that induces G(1) arrest, an effect mediated by interaction with Sp1 transcription factor [141]. Thus, also G(1) arrest mediated by NS may be a prerequisite for the apoptotic damage of erythroid progenitor cells upon B19V infection.

In primary human CD36+ EPC culture system, cellular factors that lead to cell cycle arrest after B19V infection have extensively been studied by microarray analysis. It has been shown that B19V exploits the E2F family of transcription factors by downregulating activating E2Fs (E2F1 to E2F3a) and upregulating repressive E2Fs (E2F4 to E2F8). NS protein is a key viral factor responsible for altering E2F1–E2F5 expression, but not E2F6-E2F8 expression. Interaction between NS and E2F4 or E2F5 enhances the nuclear import of these repressive E2Fs and induces stable G2 arrest. NS-induced G2 arrest is independent of p53 activation and leads to increased viral replication. In this model, downregulation of E2F target

genes eventually impairs the erythroid differentiation, while viral DNA replication and RNA transcription are enhanced by activation of G2-related transcription factors and/or DNA repair proteins. This in turn may cause activation of the p53 signal transduction and upregulation of E2F7 and E2F8, as part of a DNA damage response, that may contribute to the of block cell cycle progression [142].

4.13. B19V and Cellular DNA Damage Response. The involvement of the cellular DNA damage response (DDR) machinery in the regulation of B19V replication, and in turn on progression through cell cycle, has been then investigated. B19V infection of EPCs has been shown to induce a broad range of DNA damage responses by phosphorylation of all the upstream kinases of each of three repair pathways: DNA-PKcs, ATM (activated by double-stranded breaks), and ATR (activated by single-stranded breaks). Activated DNA-PKcs, ATM, ATR, and also their downstream substrates and components localize within the B19V replication centers. Virus replication requires ATR and DNA-PKcs signaling, and in particular the ATR-Chk1 pathway is critical to B19V replication [143]. None of the viral proteins acts as initiators of a DDR, that is induced by replication of the B19V dsDNA genome. Moreover, the DDR per se does not arrest the cell cycle at the G(2)/M phase in cells with replicating B19V dsDNA genomes; instead, the B19V NS protein is the key factor in disrupting the cell cycle, via a putative transactivation domain operating through a p53-independent pathway [144].

4.14. Apoptosis and Autophagy. B19V infection of erythroid progenitor cells induces apoptosis, leading to a block in erythropoiesis that is one of the most relevant components of the pathogenetic processes due to the virus [145]. Infected cells show morphological alterations typically associated with apoptotic processes and typical DNA fragmentation [146]. As discussed above, NS protein is responsible for cytotoxicity and is implicated in induction of apoptosis in CD36+ EPCs and UT7/EpoS1 cells [121]. In addition, the nonstructural 11 kDa protein has also been reported as capable of significantly inducing apoptosis in EPCs. Moreover, caspase-10, an initiator caspase of the extrinsic pathway, has been reported as the most active caspase in apoptotic erythroid progenitors induced by 11 kDa and NS as well as during B19V infection [133]. Additionally, mitochondrial autophagy has been described in B19V infected UT7/EpoS1 cells. A significant increase of the protein expression ratio for LC3-II/LC3-I in infected cells and confocal microscopy analyses suggested that B19V infection induced the formation of an intracellular autophagosome, and inhibition of autophagy significantly facilitated B19V infection-mediated cell death. Then, in contrast to B19V-induced apoptosis, B19V-infected cells may improve survival by autophagy mechanisms [147].

4.15. Nonpermissive Systems. In the human monocytic cell line U937, an in vitro infection study demonstrated B19V binding and modest replication with detectable NS mRNA transcription but undetectable levels of VP mRNA transcription, suggesting abortive infection. Levels of B19V DNA and NS mRNA transcription increased in the presence of anti-B19 IgG antibodies, but this effect decreased in the presence of anti-Fc receptor antibodies, showing antibody-dependent enhancement of B19V infection. Antibody-dependent enhancement also caused the increased production of TNF-α. This study showed B19V infection of nonerythroid lineage cells, possibly mediated by antibody-dependent enhancement, leading to abortive infection but a detectable proinflammatory response [148].

In addition to erythroid progenitors, cells of connective and vascular tissue could be involved in the pathogenesis of B19V infection. Primary cultures of human fibroblasts (HF) and human umbilical vein endothelial cells (HUVEC) exposed to B19V can be infected, but only low levels of NS and VP mRNAs might be detected in the absence of any significant increase of B19V DNA levels. Then, HF and HUVEC are normally not permissive for B19V replication, but it is possible that stimulation with different growth factors or cytokines could be required for a B19V productive infection to occur [149]. In an experimental system using a human endothelial cell line, HMEC-1, B19V infection, or NS overexpression produced a significant upregulation in the phosphorylation of STAT3, accompanied by dimerization, nuclear translocation, and DNA binding of pSTAT3, without increased STAT1 activation. The expression levels of the negative regulators of STAT activation, SOCS1 and SOCS3, were not altered but the level of PIAS3 was upregulated in NS-expressing HMEC-1 cells. Analysis of the transcriptional activation of target genes revealed that NS-induced STAT3 signaling was associated with upregulation of genes involved in immune response and downregulation of genes associated with viral defense. The NS-induced upregulation of STAT3/PIAS3 in the absence of STAT1 phosphorylation and the lack of SOCS1/SOCS3 activation may contribute to the mechanisms by which B19V evades the immune response and establishes persistent infection in human endothelial cells [150]. In endothelial cells of different types, infected with B19V or transfected with the B19V genome, subsequent adenovirus infection led to a limited B19V genome replication and synthesis of B19V structural and nonstructural proteins. This effect was mostly mediated at the level of transcription and can be attributed to transactivation by adenovirus E1A and E4orf6, which displayed synergistic effects. Thus the almost complete block in B19V gene expression in endothelial cells can be abrogated by infection with other viruses [151]. On the whole, these studies point to the relevance of endothelial cells for the biology of B19V. Endothelial cells may constitute a diffuse target cell population, where virus can establish a reservoir, and where the cell physiology may have a decisive role in determining the degree of expression of the virus and the balance toward the activation of possible pathogenetic mechanisms.

B19V DNA can be detected in synovial cells and fluid; however already an early report indicated that synoviocytes are nonpermissive to B19V replication [152]. In a following report, B19V DNA detected in the synovial tissues of patients with rheumatoid arthritis was specifically linked to the expression of the VP in synovium with active synovial lesions. In this case, the identified target cells of B19V were

macrophages, follicular dendritic cells, T cells, and B cells, but not synovial lining cells in the synovium. These cells were considered productively infected, as susceptible cells became positive for the expression of viral proteins and more productive for IL-6 and TNF-α when cocultured with RA synovial cells. These data suggested a direct role for B19V in the initiation and perpetuation of RA synovitis, leading to joint lesions [153]. Unfortunately, these data did not find corroboration in subsequent studies.

Although synoviocytes are nonpermissive to viral replication, exposure to B19V induces an invasive phenotype [154]. A possible mechanism may involve the direct interaction of B19V capsid with their VP1u-associated PLA(2) activity and synoviocytes [155]. Even if B19V does not productively infect human fibroblast-like synoviocytes (HFLSs), it can induce an increase in synoviocyte migration that can be blocked by phospholipase inhibitors. Recombinant proteins with intact VP1u and PLA(2) activity induce cell migration, whereas proteins with mutated VP1u are nonfunctional. The incubation of HFLSs with intact VP1u, but not with mutated VP1u, increases the production of prostaglandin E(2). Expression of cyclooxygenase (COX)-2 mRNA transcripts and COX-2 protein expression were both significantly increased after incubation with intact VP1u. Then, even in the absence of a productive infection of synoviocytes, the interaction of synoviocytes with B19V capsids showing PLA(2) activity may play a pathogenetic role by inducing an inflammatory response in the synovial compartment.

4.16. Virus Persistence. Following in vitro infections, carrier cultures can be established for a limited period of time. For example, in the semipermissive UT7 cells, after a few rounds of productive replication the virus can still be detected for extended periods of time showing a progressive loss of activity [55, 107], and also in nonpermissive systems such as U937 cells the viral DNA can be maintained within cells for some time in the absence of detectable viral activity [107]. These systems may mirror what happens in vivo. In fact, presence of viral DNA has been detected in a wide range of tissues, in normal populations as well and with comparable frequency as from selected group of patients with diverse pathologies.

Bone marrow is the main target organ of B19V, so the virus can normally be detected in bone marrow also in cases of persistent infections with constant low-level viremia; however detection of viral DNA has also been reported in normal subjects without evidence of active viral replication at frequencies of about 60% [156–158]. Viral DNA has been reported in lymphoid tissue, including spleen, lymph nodes, and tonsils [32, 158]. Liver frequently shows the presence of B19V DNA [32, 33, 159], and in the heart the presence of B19V DNA is also a common finding [34], that has been the focus of an ongoing debate on its potential role in the development of cardiomyopathies [160]. Viral DNA is commonly found in synovial tissues [32, 157, 161] and skin [32, 162]. Finally the virus has also been found in brain [158] and testicular tissues [163]. So, the initial picture of a virus capable of acute infections and rapidly cleared by the organism as a consequence of the immune response has given place to the picture of a virus able to establish long-term relationship with human hosts, and the current assumption is that persistence of viral DNA in tissues can be the normal outcome of infections [32].

Relevant issues are what cells can harbor the virus, in what form this viral DNA is present within cells, what can its functional profile be, and what the consequences on the cell physiology are, but most information in these respects is lacking. The presence of viral DNA in tissues is a distinct event from persistence of productive infection, a common clinical finding characterized by ongoing replication in the bone marrow and usually low-level viremia. It is assumed that the viral genome can be maintained in an episomal form, just because the evidence for its integration in the cellular genome has never been reliably reported. However, no data have been reported neither on its possible configuration, so this is only speculative by comparison to what is known for arrangements of other parvoviruses, for example, monomeric versus concatemeric, or linear versus circular, nor on its ability to form chromatin structures. The presence of full-length viral genomes in tissues has been reported [164], but as viral DNA is usually detected by PCR amplification of distinct segments of the viral genome, there is the possibility that in other instances only fragmented, nonfunctional DNA is maintained within tissues.

Within tissues, unless in the presence of a high viral load or expression of mRNAs or proteins at detectable levels, the viral genome is normally considered silent [34]. A possibility is that the virus might establish true latent infections, with the capability of reactivations, but this has only rarely been suggested and not fully documented. Epigenetic levels of regulation may play an important role in the control of viral expression and determining a silencing of persistent viral DNA [107]. Since effects on cell physiology would probably depend on the expression of viral proteins impacting cellular pathways, a silenced genome would not alter a cellular environment by definition. The opposite is probably true, and it is the cellular physiology that would determine a possible expression of a viral genome harbored within the cell.

5. Pathogenesis

5.1. Course of Infection. The course of infection was first investigated in two set of experiments involving human volunteers, who were inoculated and followed with respect to virological, immunological, hematological, and clinical parameters [165, 166]. These studies constituted a framework for the definition of a pathogenic profile of B19V infection, that since then increased in scope and complexity. The virus is now implicated in a wide range of clinical manifestations, that necessarily rely on a complex relationship of virus, its biological characteristics, host, its physiological status, and capacity of immune response.

5.2. Early Events. Following contact via the respiratory route, the virus gains access to the bloodstream. Persistence of the viral DNA has been detected in tonsillary tissues [32, 158], but

it is not known whether tonsils can constitute a true portal of entry, if virus can undergo a first round of replication in tonsils, and to what extent lymphoid vessels are involved in spreading or maintenance of infection. An alternative mechanism of access to vessels could be transcytosis through respiratory epithelia.

Then, in a primary viremic phase that normally is undetected, the virus gains access 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 pathognomonic of B19V infection. These effects are mirrored in the capability of the virus to inhibit the formation of bone-marrow-derived erythroid colonies at the BFU-E and CFU-E stages [40], with susceptibility to infection and cytotoxic effects increasing with increasing erythroid differentiation [145]. The generation of EPCs from peripheral blood and the study of their susceptibility to infection confirmed an increasing susceptibility to virus with erythroid differentiation [53, 54]. Thus, the target cells in bone marrow are CD36+ cells and are mostly susceptible when in the differentiating phase (erythroblasts). 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, and from the dimension and turnover rate of the erythroid compartment. The fact that the more undifferentiated precursors are relatively resistant to infection ensures that the block in erythropoiesis is temporary and can be relieved by a neutralizing immune response. Other cellular types possibly susceptible to infection are the megakaryoblasts, that however constitute a nonpermissive cellular system where the virus may exert a cytotoxic effect in the frame of an abortive infection [167].

In fact, a balance is reached between cell population dynamics and viral replication, considering also possible stresses on the erythropoietic compartments and the immune response [168]. In a normal individual, with physiological erythropoiesis and normal immune response, infection is limited in extent and temporal frame and is controlled by the development of a specific immune system. Production of antibodies with neutralizing activity contributes to the progressive clearance of infection. Levels of hemoglobin only marginally decrease and infection is usually asymptomatic from the hematological point of view. Clearance of infection, determined by detection of virus in the blood, can be relatively rapid, taking usually 3-4 months with constantly decreasing levels, even if very low levels of virus can be detected for years following primary infection [169–171]. The virus can still be detected in the bone marrow of a percentage of individuals [156–158]; however persistence of viral DNA implicates active chronic infection only in the presence of viremia.

Infection becomes manifest as pure red cell aplasia (PRCA) and anemia when preexisting alterations in the erythropoiesis process, or defects in the immune response, alter the balance between viral replication and cellular turnover [168, 172]. In case of stressed and expanded erythropoietic compartment, in situations where the number of erythroid progenitors and their replication rate are expanded because of a reduced lifespan of erythrocytes, or increased need, infection by B19V can lead to an acute episode of profound anemia, that presents as the classical aplastic crisis in patients with underlying hematological disorders. The range of situations favorable to the development of acute B19-induced PRCA and anemia can be very ample, from hemoglobinopathies to thalassemia, from enzymatic defects to iron deficiencies, to coinfection with other viruses, microorganisms, or parasites.

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 situations can be typical of congenital or acquired immunodeficiencies, such as HIV infection [173], in cases of malignancies [174], in the course of chemotherapy [175], or in the course of immunosuppressive treatments, such as in bone marrow or solid organ transplant recipients [176–178]. Depression of erythropoiesis can be manifest, with anemia of different grade, but also compensated and unapparent. A general correlation may be present between viremic levels and anemia, but a clinical threshold has not been univocally defined, as very high-level viremia may be compensated by active erythropoiesis. On the other hand, the definition of inability to mount a neutralizing immune response must take into account that many normal and asymptomatic individuals support low-level replication of the virus. The distinctions between a normal course and active chronic infections are smooth, and again 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, in a mutual adaptation.

5.3. Late Events. Bone marrow supports a productive infection and leads to release of progeny virus in the blood, leading to a secondary viremia that is the mark of active infection and that in the acute phase of infection, before the development of an effective immune response, can reach exceedingly high viremic levels (10^{12} virus/mL) before a progressive clearance. The secondary viremic phase leads to the systemic distribution of the virus and preludes to possible late clinical manifestations of infection. The two classical manifestations of B19V infection are erythema infectiosum, typical of children [179, 180], and arthropathies, typical of adult patients [181, 182]. Since the initial reports, the range of clinical manifestations associated with 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 of viral components and activities in pathological tissues, or closely associated clinical and epidemiological parameters.

When investigating 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 PLA(2). The interplay with the immune systems, by its innate and adaptive recognition mechanisms, may lead to the development of immunopathological mechanisms, or autoimmune processes that also have been described [183, 184].

In the systemic phase of infection, cells of mesodermic origin are mainly involved [70], and of these 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 and, normally nonpermissive [151], can be a site of persistence of the viral genome, as it happens for many other viruses. In some cases, however, markers of viral activity have been precisely localized to epithelial 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.

5.4. Cardiomyopathies. In recent years, B19V gained interest as a cardiotropic virus. In a sort of wave, B19V has been detected at ever-increasing frequencies in endomyocardial tissues, replacing other cardiotropic viruses as the most prevalent virus detected in the heart [185–187]. This is probably linked to a growing interest in the virus and also to the development of high-sensitivity and quantitative PCR detection methods; however the data may also reflect a true epidemiological phenomenon although not readily explainable given the relative uniformity of viral isolates.

B19V has been directly involved as an etiologic agent in acute myocarditis both in pediatric [188–192] and adult populations [193–195]. 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 [193, 196]. 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, also when compared to that of classical cardiotropic viruses such as enteroviruses, and a marked cardiotropism should be mentioned as one of the main characteristics of B19V. As a possibility, given adequate tissue sampling and sensitive detection methods, the presence of B19V DNA in endomyocardial bioptic samples might be expected in every individual previously infected by B19V. Then, it is not straightforward to assign a pathogenetic role to B19V on the basis of the distribution of prevalence of B19V DNA within selected groups of patients or controls [160]. In particular, the definition of a possible role of B19V in the development of chronic cardiomyopathies and cardiac dysfunction is a matter of ongoing 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 [197–205], but on the opposite, a lack of significant clinical association has been supported by other groups [206–212].

A positive association and an etiopathogenetic role of B19V in the development of chronic cardiomyopathies have been proposed on the basis of significant higher frequencies of detection in patient versus control groups, or on 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. The focal point in a pathogenetic mechanism would be the ability of the virus to induce endothelial dysfunction within cardiac tissue, which in turn would trigger inflammatory processes leading to the development of chronic cardiomyopathies. In this scenario, endothelial dysfunction might be triggered by viral-dependent mechanisms and be a consequence of viral replication and/or expression of viral proteins, for example, NS and/or VP1. In alternative, endothelial dysfunction may be a consequence of the response to the presence of virus through innate recognition mechanisms. More studies will be necessarily required to investigate possible pathogenetic mechanisms at the cellular level and associate these to what has been observed at the clinical and epidemiological levels.

5.5. Arthritis and Rheumatic Diseases. B19V has been recognized as an agent responsible for arthropathies since the early studies [181, 182]. Accumulated evidence and case series have shown that B19V can cause acute arthritis or arthralgias and occasionally chronic arthropathies. Arthritis can develop in children, at lower frequencies (<10%), accompanying or following the classical erythema, and is usually asymmetric and pauciarticular. Arthritis and arthralgias are more common clinical manifestations in adults, in many cases presenting without concurrent symptoms, more frequent in females than males, respectively, presenting in approximately 60% or 30% of documented infections. In adults, the typical onset is acute, symmetrical, and involving mainly the small joints of hands and feet. Chronicization has been reported for children but is more frequent in adult patients, up to 20% of cases in affected women. Chronic infections are usually self-limiting and do not lead to joint erosions and damage; however in some cases they meet clinical diagnostic criteria for rheumatoid arthritis, can be erosive, and followed by the development of rheumatoid factor [213].

The association of B19V infection and the actual development of chronic destructive arthropathies, such as

juvenile idiopathic arthritis and rheumatoid arthritis, has been the subject of continuous interest and conflicting results. A pathogenetic role for B19V has also been proposed for other rheumatic diseases, such as, among others, chronic fatigue syndrome/fibromyalgia, systemic sclerosis, vasculitis, and systemic lupus erythematosus. Suggestions of the involvement of B19V in the pathogenesis of rheumatic diseases may come either from a parallelism in the pathogenetic mechanisms or from clinical and laboratory findings in selected groups of patients, but in most cases the association is controversial at the epidemiological level and not corroborated by strong virological evidence [214].

In vitro, synoviocytes are nonpermissive to B19V [152], although they can respond to infection and assume an invasive phenotype [154] suggesting a parallel role in the pathological processes within synovia. Synovial tissues commonly harbor B19V DNA, and its mere presence is not correlated with any distinct pathological process [157, 161]. Therefore, any pathogenetic mechanisms linked to B19V would require the expression of viral genes, such as the NS protein with its transactivating activity or the VP1 protein with its phospholipase activity, capable of activating and maintaining an inflammatory response targeted to the synovial tissue. A possible similar mechanism, involving macrophages, dendritic follicular cells, and lymphocytes rather than synovial cells, has been reported in tissues from rheumatoid arthritis patients [153] but awaits confirmation and further investigation. The involvement of lymphocytes, possibly productively infected by B19V, has also been reported in a case series of chronic inflammatory joint diseases [215] and can be a rare and unexpected finding in atypical clinical situations characterized by chronic inflammation [216].

A common concurrent phenomenon is the production of autoantibodies in the course of infection, that can also act as a possible trigger to the induction of autoimmune diseases. In the acute phase of infection, heterologous, cross-reactive, and self-reactive antibodies can normally be produced. These can be confounding factors in the diagnosis of disease but normally do not play a substantial role in the pathogenetic process. In some cases, autoantibodies may persist and function as cofactors in the establishment of autoimmune processes [217–219].

5.6. Intrauterine Infection and Fetal Damage. A relevant property of B19V is its ability to cross the placental barrier and infect the fetus. This characteristic was recognized early in the studies on B19V [220], and since then many studies have been dedicated to assess the risk of intrauterine infection in pregnant women, the extent of consequences for the fetus, and the involved pathogenetic mechanisms.

The viral receptor, globoside, is present on the villous trophoblast layer of human placenta and its expression levels, highest in the first trimester, progressively decrease until vanishing in the third trimester. This temporal change broadly correlates with what is observed on the frequency of transmission of infection to the fetus [221]. Trophoblasts are not permissive to the virus but may bind viral capsid via the globoside receptor, hence their role in facilitating transcytosis of virus to the fetal circulation [222]. Both inflammatory [223] and apoptotic [224] aspects have been observed in placental tissues in case of B19 infection, probably contributing to fetal damage. Endothelial placental cells can be productively infected, facilitating the establishment of fetal infection and contributing to placental damage [225]. When in the fetal 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 [226, 227] as well as in the amniotic fluid [228, 229]. The block in fetal erythropoiesis can be severe, because of the physiologically expanded erythropoietic compartment combined to an immature immune response, and lead to fetal anemia, tissue hypoxia, possible development of nonimmune hydrops, and/or possible fetal death [230, 231]. Fetal cardiomyocytes, contrary to adult, are reported to possess the viral receptor and be susceptible to infection; therefore, direct infection of these cells may induce fetal myocarditis that will contribute to fetal damage [232, 233].

The natural course of fetal infection is affected by several factors. First of all, 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 fetus. Therefore, intrauterine infections should be confined to the case of primary infections in nonimmune 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 fetus will also depend on its developmental stage, depending on the rate of expansion of its erythroid compartment and maturity of fetal immune response. As a consequence, infections occurring at earlier stages carry a higher risk of leading to fetal deaths, while the development of hydrops is more frequent in the central part of pregnancy. Hydrops may lead to fetal death, or the fetus may more frequently recover without persistent damage. In the third trimester, transmission is more difficult and fetuses are relatively resistant, so the overall risk of fetal damage decreases to background values [234]. Some case series also reported a high frequency of detection of B19 DNA in late intrauterine deaths [235–237], a finding not confirmed in other case series, that did not show such correlations and indicated that late intrauterine fetal death is a rare event [238]. Finally, the infected fetus may show presence of virus at birth [239, 240]. Congenital infections have been sporadically associated with neonatal anemia or anomalies [241], while their possible consequences on the neurological development are currently investigated [242–244].

6. Immune Response

6.1. Innate Immunity. The role of innate immunity in contrasting B19V infection has not been investigated in detail. In general, like other viruses, B19V might be recognized through its PAMPs by cellular PRRs, but what component of the virus may act as PAMPs needs to be defined. The viral genome is devoid of stimulatory sequences; however its terminal regions are GC rich and can possibly be recognized by

receptors such as TLR9. The CpG-ODN 2006 is a TLR9 ligand with phosphodiester backbone that selectively inhibits burst-forming unit-erythroid growth and induces accumulation of cells in S and G(2)/M phases and an increase in cell size and frequency of apoptotic cells, features similar to those observed in erythroid progenitors infected with human parvovirus B19V. A consensus sequence also located in the P6-promoter region of B19V can inhibit erythroid growth in a sequence-specific manner and downregulate expression of the erythropoietin receptor, effects also induced by B19V DNA. Although TLR9 mRNAs were not upregulated by stimulation with ODN 2006, these results hint at possible diverse mechanisms of inhibition of erythropoiesis [245]. A recent study investigated the variation in expression of defensins and toll-like receptor in COS-7 cells transfected with different expression vectors, producing EGFP-fused NS and VP proteins. In this system, NS was shown to play a major role in inducing both short- and long-term upregulation of defensins and TLR9, with some effects also played by VP2 protein. The different effects of NS and VP2 on the stimulation of defensins and TLRs could provide a clue in understanding the roles of B19V on innate immunity [246].

6.2. Adaptive Immunity, Antibodies. Antibodies are the hallmark of the adaptive immune response to B19V. In naïve individuals, B19V specific antibodies is produced early after infection and are assumed to be able to neutralize viral infectivity and progressively lead to clearance of infection. IgM are produced first and can usually last about 3–6 months following infection, soon followed by production of IgG that is assumed to be long-lasting. IgA can also be detected in body fluids.

Viral capsid proteins are the major antigens recognized by the immune system and inducing the production of antibodies with neutralizing activity. From an antigenic point of view, viral capsid proteins show epitopes on the VP common region or on the VP1u region. Epitopes on the common region are mainly conformational and have been mapped in several regions dispersed on the capsid surface, while epitopes on the VP1u region are mainly linear and have been mapped close to N-terminus [247–253]. The development of the immune response typically shows the recognition of an acute-phase epitope, followed by the development of higher-avidity antibodies [254, 255]. A mature and effective immune response most frequently shows the presence of antibodies-directed anti-VP2 conformational and VP1u linear epitopes [255–260].

Antibodies to NS protein are also produced as a response to B19V infection. These can be detected in a subpopulation of individuals showing antibodies to capsid proteins, in frequencies that have been reported to vary depending on the population groups. Antibodies to NS proteins are probably produced as a result of prolonged antigenic stimulation, but their presence has not been conclusively linked to any particular course of infection or clinical condition [261–268].

The immune reactivity to B19V proteins was further investigated by evaluating sera from healthy B19 IgG-positive individuals for antibody reactivity against linear peptides spanning the NS protein, or representative of selected antigenic regions within the capsid proteins. By this approach, three novel antigenic regions on the NS protein were identified, and three major antigenic determinants in the VP1u and VP common region have been mapped [269]. The development of a neutralizing activity is typical of a mature and effective immune response, while antibodies with incomplete neutralizing activity are typical of persistent infections. The presence of antibodies directed against viral capsid proteins is assumed to protect from secondary infections and this may pose the rationale for the development of a vaccine.

Then, B cell enzyme-linked immunospot assay was used to evaluate B19-specific B cell memory in volunteer donors. Result showed that a B cell memory is maintained against conformational epitopes of VP2 and is absent against linear epitopes of VP2. Individuals seronegative for IgG against the unique region of VP1 have detectable B cell memory, with the potential to mount a humoral response on reexposure to B19. The finding that B cell memory is established and maintained against conformational epitopes of VP2 and against linear epitopes of VP1 but not against linear epitopes of VP2 is in accord with what observed by testing serum reactivity against diverse B19 antigens [270].

6.3. Adaptive Immunity, Cellular. T-cell-mediated immune responses to B19V infection were first shown by measuring the proliferative responses of peripheral blood mononuclear cells following in vitro stimulation by recombinant VP1, VP2, and NS proteins. Results indicated the presence of B19V-specific cellular immunity directed against the capsid proteins VP1 and VP2, presented to CD4+ T cells by HLA class II molecules [271]. T-cell proliferation as a response to stimulation by B19V VLP antigen was then confirmed and measured in patients with recent as well as remote B19V infection, showing that B19V-specific, HLA class II-restricted CD4+ cell responses were detectable, most vigorous among the recently infected patients but not confined within the acute phase [272].

Following a different approach for the detection of epitope-specific cell-mediated immunity, a peptide library was designed to span the whole coding sequences of B19V NS protein, and peptides used to detect specific CD8+ T-cell proliferative responses and cytolytic activity following in vitro stimulation. By this approach, a single HLA-B35-restricted, CD8+ T-lymphocyte epitope from the NS protein was identified, its functional relevance also confirmed in ex vivo experiments, using an IFN-γ Elispot assay. This epitope was strongly immunogenic in B19V-seropositive donors, suggesting persistent antigen stimulation in normal individuals [273].

In an even more extensive approach to map T-cell epitopes, a total of 210 overlapping peptides were synthesized, covering the nonstructural protein NS, the VP1 unique region, and the common VP1/VP2 region of the structural proteins. These peptides were used in in vitro cytotoxicity and ex vivo stimulation assays to assess for their functional relevance, while HLA restrictions were first estimated in silico and then experimentally confirmed. A series of CD8+ T-cell

epitopes could be identified, nine in the NS protein and one in the VP common region. Broad CD8+ T-cell responses to these epitopes were observed in acutely infected individuals and maintained or even increased over many months after the resolution of acute disease. Then, CD8+ T cells appear to play a prominent role in the control of B19V infection [274]. A discrepancy was observed in persistently infected individuals, where a comparatively higher reactivity was observed against viral capsid protein epitopes [275].

A central role of CD8+ cells was confirmed by definition of the extent and phenotype of B19-specific CD8+ T-cell responses, during and after acute adult infection, studied using HLA-restricted, specific peptide multimeric complexes. Results indicated the presence of sustained responses, increasing in magnitude over the first year after infection despite resolution of clinical symptoms and control of viremia, with T-cell populations specific for individual epitopes comprising up to 4% of CD8+ T cells. These cells possessed strong effector function and intact proliferative capacity. B19-specific cytotoxic T lymphocytes were also detectable in individuals tested many years after infection, at frequencies typically lower than 0.5% of CD8+ T-cells, which showed a mature phenotype [276]. Furthermore, a strong and selective CD8+ response was seen in a group of acutely infected patients, showing HLA restriction, immune dominance of a single viral epitope, and focused usage of the T-cell receptor. A highly focused T-cell response may aid in the rapid identification and elimination of even low levels of virus and might be crucial for the effective control of viral infection [277].

The role of T helper cells in the immune response to B19V was first investigated by using recombinant VLPs, containing the two structural proteins VP1 and VP2 (VP1/2 capsids) or VP2 alone (VP2 capsids), and additionally the VP1u region expressed in a prokaryotic system. The ability of these antigens to stimulate Th cells to proliferate and to secrete IFN-γ and IL-10 was measured in recently and remotely B19V-infected subjects. Similar proliferation, IFN-γ, and IL-10 responses were found with the VP1/2 and VP2 capsid antigens. B19-specific IFN-γ responses were generally stronger than IL-10 responses in both recent and remote infection; patients with relapsed or persisting symptoms showed strikingly lower IL-10 responses. VP1u-specific IFN-γ responses were very strong among the recently infected subjects but absent among the remotely infected subjects, a finding contrasting with the documented persistence of anti-VP1u antibodies [278, 279].

Overlapping peptides and IFN-γ immunospot assays were also used to investigate the magnitude and extent of the CD4+ T-cell response to the capsid proteins. Again, responses in acutely infected individuals were compared to those in remotely infected individuals. Acutely infected individuals were found to make broad CD4+ responses to several peptide pools, with the strongest responses toward peptides from the VP common region and a decrease in magnitude correlating with the time postacute infection. By in vitro stimulation assays, an ample CD4+ specific response was also detected in the remotely infected individuals, in this case putting in evidence a single dominant epitope in the VP common region. Phenotypic analysis of the B19-specific CD4+ cells indicated that CD4 cells were mainly of the central memory phenotype, thus diverging from the evolution seen in CD8 cells. This difference may also be relevant to the definition of the pathogenetic mechanisms of B19V infection, so that activation of CD4 cells in the acute phase, however functional to the development of antibodies, may also contribute to the clinical manifestations, or even to the development of immunopathological or autoimmune processes, while development of a CD8 immune response would on the other hand limit viral replication, therefore reducing CD4 activation, and eventually contribute to the control of viral persistence [280].

Characterization of the proinflammatory and T helper (Th)1/Th2 cytokine responses during acute infection or persistent infection was then carried out in acutely and persistently infected subjects. An initial peak of proinflammatory cytokines (IL-1β, TNF-α, IL-6 and IL-8) was found at onset of acute B19 infection. Induction of the Th1 type of cytokines IL-2, IL-12, and IL-15 was already seen in the early phase and was sustained in many patients during the follow-up period. In contrast, cytokines associated with a Th2 type of immune response (IL-4, IL-5 and IL-10) as well as an IFN-γ response remained low during the observation time. Despite the lack of Th2 cytokines the patients developed normal B19-specific IgG levels, so antibodies may have been induced by a low-grade IL-6 response as well as other cytokines, for example, TGF-β [281].

On the whole, the B19V specific T-cell responses are peculiar, combining characteristics typical of viruses capable of lytic infections as well as capable of establishing a persistent infection, with the requirement for a continuous surveillance by the immune system. This concept strengthens our view of B19V as a virus capable of establishing a long-term relationship with its host, whose outcome depends on the interplay and balance between the pathogenetic potential of the virus and the control mechanisms operated by the immune system. Finally, the dissection of the immune response, with its HLA restriction, TCR selection and epitope-specific recognition, definition of the different phenotypes, and activation profiles of the cells involved, will be crucial not only to our knowledge of the course of infection, but also for development of therapeutic and prophylactic options, such as the development of a B19V vaccine.

7. Epidemiology and Transmission

Parvovirus B19 is a human pathogenic virus widely and worldwide diffuse in the population. Epidemiology, based on seroprevalence studies, indicates that the virus is actively circulating in all areas of the world, albeit with some regional differences. Complementary information on virus prevalence comes from screening on blood donors by means of nucleic acid detection techniques.

A comprehensive study was conducted on seroprevalence in five different European countries in a five-year time frame [282]. In this study, definition of seroprevalence in the different class ages allowed for analysis of the force

of infection, and, when considering demographic data of the different countries, led to an estimate of the risk of acquiring infection per class age. This model assumed the lifelong persistence of antibodies as a correlate with protective immunity, but it can be further refined by considering a more complex pattern of serological response to virus, assuming that protective immunity, determined by antibody presence, can wane and allow reinfections [283]. This pattern fits well to experimental data and can be representative of the most temperate areas in the world. Within this general pattern, regional differences may occur, with some geographic areas reporting lower seroprevalence. In addition, areas of very low seroprevalence have been reported in cases of isolated communities, but in these cases periodical epidemics may raise these values abruptly. The three genotypes constitute a single serotype, so epidemiology usually cannot distinguish areas with different circulation of genotypes [39]. However, in western Africa, where genotype 3 is most prevalent, a confounding effect with respect to a clear separation of nonreactive to reactive populations has been attributed the prevalent circulation of genotype 3 [284].

Data on the prevalence of infection in the general adult population also come from surveys on blood donors, by means of nucleic acid detection techniques [285]. Data have accumulated in the years, reporting values differing widely in dependence of the sensitivity of the technique used and the epidemiological context [286]. More recent data on large populations over several years, obtained by sensitive quantitative PCR techniques, indicate prevalence rates in the range 1%–0.01% of viremic donors [24, 287–291], with higher frequencies to be expected in the younger age groups and in epidemic periods. The more recently developed molecular techniques ensure the detection of all genotypes, also allowing for their discrimination [292, 293]. The prevalent genotype is genotype 1, that accounts for almost the totality of circulating virus in temperate areas. Genotype 3 is prevalent in western Africa but is now increasingly reported in many areas of the world. Genotype 2 is considered an ancient genotype, frequently detected as persistent in tissues of elder individuals but very rarely detected as circulating virus.

The main route of transmission of the virus is the respiratory route. The virus can be detected in the saliva in the acute phase of infection and has been experimentally transmitted via nasal inoculation. Circulation of the virus is higher in the spring/early summer, and epidemic cycles are reported to occur every 4-5 years. Close contacts, such in households or schools, favor transmission with attack rates as high as 50% [180]. A concern arises with respect to transmission in hospitals, when staff or susceptible patients can be exposed to source-infected individuals [294, 295]. Due to the viremic phase in the course of infection, the virus can be present in blood at high titers, then posing a risk of iatrogenic transmission of the virus via blood, blood components, and blood products [286].

As discussed, the virus can be transmitted transplacentally from mother to fetus, then B19V should always be included in the assessment of risk of fetal infections. An overall assessment first needs to evaluate the risk of acquiring infection by pregnant women, by considering the fraction of the susceptible population and its distribution per class ages, the force of infection for the respective ages, the demographic curve, and relative distribution of pregnancies [282]. Then, an estimated rate of transmission of the virus to fetus has been reported varying in the range 25–50%, while an overall risk of fetal death has been reported to be lower than 10% [234, 296]. Although approximate, these calculations will lead to an overall estimate of the total number of fetal losses due to B19V infection in a given epidemiological setting, necessarily taking into account variations due to geographical and temporal fluctuations.

Several studies have been carried out to obtain an experimental evaluation of the impact of B19V infection on prenatal pathologies, although with discordant results [297–301]. A major problem arises with the heterogeneity of the diagnostic procedures employed by different groups and, therefore, of the criteria used to include subjects in the study groups. Inclusion criteria have been chosen depending on maternal symptoms, known risk of exposure, or fetal abnormalities, or postmortem analysis in cases of fetal deaths. Diagnostic methods have used a wide combination of immunological and direct molecular detection methods. Most studies have been performed by retrospective collection of data, while only a few have been based on prospective evaluation of population cohorts. Large population-based studies to determine the burden of prenatal infections have been carried out in a few settings [302–305]. In these cases, the incidence of reported cases of B19V infection has usually been exceedingly low. While possibly reflecting true epidemiological situations, these results should be considered indicative of a lower estimate, due to inherent limits in inclusion and diagnostic criteria.

8. Diagnosis of Infection

8.1. Analytical Profile. A clinical diagnosis of B19V infections can only be suspected. Infections can be asymptomatic or present with unspecific symptoms, while the typical symptoms attributed to B19 infection can be the results of other infections. Then a laboratory diagnosis is required in the presence of a specific or generic request.

The fact that the virus is not adapted to grow in cell cultures promoted from the very beginning the development of direct molecular methods for the detection of B19V in clinical specimens. The diagnostic approach can now rely on the detection of specific viral components, viral genome, or proteins, that is a complement to the more traditional approach to the 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, in the same sample, of specific antibodies and viral DNA in the viremic phase. Viral DNA, or proteins, can be detected also from bone marrow aspirates, or bioptic samples. In case of suspected fetal infections, amniotic fluid is suitable for the detection of viral DNA, while in case of fetal death analysis of fetal or placental tissues may allow to indicate viral etiology [306].

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 [307].

8.2. Molecular Methods. 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 [308–314]. In particular, standardization and inclusion of competitor or internal controls [315–317] 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 [318–321]. 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 [293]. 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.

Finally, in situ hybridization techniques for the detection of viral nucleic acids [322–327], and immunohistochemical detection of viral proteins [328, 329], can be useful as a complement to PCR techniques for investigation of 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.

8.3. Immunological Methods. 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 carried out and interpretation of the combination of results may allow for a presumptive diagnosis of active, recent, or past infection.

Historically, at the beginning of the studies on B19V, immunological assays were established using native virus as antigens [330, 331], but very early on this limitation was overcome and the antigens used for immunological detection have been obtained by means of heterologous recombinant expression systems. Recombinant proteins expressed in prokaryotic systems usually lose their native conformation and have been used as 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 immunoassays, or recently developed 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. Western blot, or better line blot assays, will 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 [332, 333]. In this kind of assay, also NS can be used as antigen to detect the presence of specific antibodies, whose correlation with clinical course is however still controversial. Of limited availability, although potentially useful, are assays to determine IgG avidity or acute-phase ETS reactivity [334, 335].

9. Prophylactic and Therapeutic Options

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. Hematological consequences can be relevant, in patients with underlying hematological 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 fetus is 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.

9.1. Screening and Blood Products' Safety. The presence in the course of infection of a high-titer early viremic phase in absence of specific symptoms, as well as the delayed clearance of virus from bloodstream and possible occurrence of low-titer 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 the first issue, some studies indicate possible viral concentration levels necessary for transmission

of infection. In the absence of specific Ig in both donor and recipient, a threshold level of about 10^7 International Units (IU) per mL seems necessary to obtain infection, as determined by seroconversion and viremia in recipients [285]. 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-titer viremic blood units and seroprevalence rates in the population, the probability of infection by exposure to single blood or blood components units is low [336, 337]. Very few case reports describe symptomatic infections, while linked donor-recipient studies indicate that this situation is infrequent and in most cases clinically irrelevant [338]. Single-donor screenings are therefore unjustified in terms of costs; however, high-risk patients with hematological disorders, immune deficiencies or pregnant women would benefit from the availability of dedicated donations, proven to be free from B19V.

A completely different situation is present for blood products, that are manufactured from large pools of donations. In these cases, inclusion of high-titer viremic units in the manufacturing pools can be expected with high probability, and dilution during the manufacturing process may not be sufficient to fall below a safety threshold level. In fact, B19V DNA could be detected at high frequencies in most blood-derived products before introduction of screening and measures to exclude contaminated donations. High rates of transmission of infection, as inferred from higher seroprevalence or viremia rates, were detected in at-risk recipients groups, mainly hemophiliac and/or immunodeficient patients [339].

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. Quantitative molecular detection methods, accurately calibrated and able to detect all genotypes, are or should now be used to detect above-threshold contamination of manufacturing pools, trace, and withdraw the contaminating donations in an effort to implement blood products safety. 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; in particular B19V susceptibility to heat inactivation is dependent upon the physical conditions and composition of medium. Heat inactivation, chemical inactivation, or physical removal of virus should all be considered in the production steps to further increase the safety of blood and blood-derived products [286].

9.2. Therapeutic Intervention. Therapeutic options for B19V are limited. Only rarely, acute-phase symptomatology may require symptomatic treatments. Transfusions are required to treat the anemia in cases of transient aplastic crisis or prolonged anemia, while in cases of arthralgias, nonsteroidal anti-inflammatory drugs may exert beneficial effects. In cases of fetal infections and hydrops, intrauterine transfusions are indicated when the hemoglobin concentration in the fetal circulation falls below a threshold level, and case series report improved survival rates of hydropic fetuses [231, 340].

A different scenario is present in the course of chronic infections, that may depend on the inability of the immune system to develop an effective and neutralizing response. In this case, passive immunization can be considered as an effective means of reducing the viral load. IVIG preparations, 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 [341]. 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 have to be repeated [342]. Usually, clearance of infection occurs when the patient's 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 probably allow for reconstitution of the immune function and cure of B19V infection [343]; in cases of posttransplant immunosuppression, patients may take advantage of IVIG administration coupled with a change in the immunosuppressive therapeutic scheme [344].

As a final remark, it should be noted that in the case of B19V no clinical trials have been carried out to determine an optimal therapeutic scheme for the administration of IVIG, so the treatment is still empirical. Also, a possible value of prophylactic administration of IVIG to prevent fetal transmission has not been evaluated. Human monoclonal antibodies have been developed but their therapeutic or prophylactic use has not been evaluated [345]. All of these limitations prompt for an extensive and controlled study of the efficacy of passive immunization therapeutic or prophylactic schemes.

9.3. Vaccine Development. The development of a vaccine for B19V has been a problematical endeavor. In theory, a vaccine would be useful and effective, as B19V is a virus adapted exclusively to human host, transmitted by direct interpersonal contact and effectively neutralized by the immune response. 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 chronicization even in the presence of immune response, so the real utility of a general vaccine might be questioned.

A rationale for the development and introduction of a vaccine would mainly be to protect at-risk populations, such as patients with underlying hematological disorders. In particular, women of childbearing age would have benefits from vaccine protection, chiefly in terms of fetal safety. In this case, vaccination would reduce the needs for screening, surveillance, and followup and would avoid a small but definite number of fetal losses.

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 [346]. These VLPs are immunogenic in the animal experimental model. Phase I studies showed their immunogenicity and relative safety in humans [347]; however phase II studies showed a remarkable reactogenicity [348]. These results still prompt for the development of efficient and safer vaccines.

9.4. Antiviral Drugs Development. No antiviral therapy has been developed or evaluated for B19V. 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, no studies have yet 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 as the cytotoxicity and proinflammatory activity of the virus. The development of pharmaceutical biotechnology tools and of accurate quantitative methods to evaluate reduction in viral infectivity will be required to lead research in this field.

10. Conclusions

As a final comment to this review, B19V is a virus that can offer continuous matter of interest to 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. The lack of efficient adaptation to in vitro cellular cultures and the absence of animal models have limited classical virological studies and made studies on B19V dependent on molecular biology. In this case too, the difficulties in obtaining efficient recombinant systems have impaired a thorough understanding of the viral lifecycle and virus-host interactions.

B19V is also 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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