Clinical and Developmental Immunology

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Editorial Board

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Editorial

Human Monoclonal Antibodies as a New Class of Antiinfective Compounds

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The concept of using "magic bullets" in the fight against infectious diseases was originally proposed by Paul Ehrlich, one of the founding fathers of immunology and of the basis of antiinfective therapy [1]. These "magic" compounds should have been able to target microbes without harming the infected host. Actually, this concept is the mainstay of antiinfective therapy, as we still know it today. What Dr. Ehrlich did not and could not foresee was the microbial ability of using "magic tricks" against therapeutic compounds, that is, of developing resistance mechanisms. This is true for all groups of pathogens and all classes of antiinfective drugs, making the need of reliable alternatives everyday more compelling [2–5].

Since their first description in 1975 [6], monoclonal antibodies (mAbs) have been depicted as ideal "magic bullets" due to their extremely specific mode of action, associated with an extreme biotechnological versatility [7]. A mAb with potential therapeutic utility should fulfill at least the following three conditions: (i) specific binding to the molecular target by the antigen-binding fragment (Fab) domain, (ii) effective, but controlled, effector functions activated by binding of the constant crystallizable fragment (Fc) region to specific receptors of immune cells, and (iii) good pharmacokinetic characteristics. The first mAbs were exclusively of animal (murine) origin, with dramatic potential drawbacks in terms of high immunogenicity, short half-life, and low capacity of activating Fc-mediated effector functions when administered to patients. These potential and sometimes actual problems were addressed by engineering the constant regions of an antibody molecule leading first to chimeric (originally murine mAbs with a human Fc fragment) and then to humanized (all human mAbs, only keeping the *complementarity determining regions* (CDRs) of the original mouse mAbs) antibodies [8]. In the last two decades, novel techniques allowed the possibility of dissecting directly the human "antibodyome," allowing the selection of fully human mAbs [8]. Different approaches aimed at the regulation of the Fc-mediated effector functions have also been described [9]. Several of these "novel" mAbs are finding or will find their way into the clinics in the next few years [9].

However, almost all of the licensed therapeutic mAbs are directed against nonmicrobial antigens and are used in autoimmune or neoplastic diseases [9]. Several factors may have contributed to such a "minority report" in the use of mAbs as anti-infectious agents, such as the availability of effective drugs or prophylactic strategies, the extreme variability and complexity of most of the surface-expressed microbial antigens, especially in more evolved microbial pathogens, such as bacteria, fungi, and parasites [10, 11].

In this special issue of Clinical and Developmental Immunology, all these aspects are covered by six review articles and four research articles discussing the possible use of human and humanized mAbs against bacterial, viral, and fungal diseases. The different phases of the development of a mAb are discussed, starting from the identification of a potentially effective microbial target and the choice of the potentially most fruitful biotechnological strategy, to importantly the final characterization of each selected mAb. As

an example, in the paper by M. Castelli et al., the use of bioinformatic tools in the definition of a mAb epitope is widely discussed, evidencing their possible important application in the novel field of epitope-based vaccinology [12, 13]. The use of mAbs endowed with different biological activities in the study of novel approaches in the investigation of clinically important "emerging" pathogens is also considered, as in the review article by R. A. Diotti et al. intriguingly proposing a novel mAb-based perspective in the study of JCV-associated progressive multifocal leukoencephalopathy [14].

We are certain that the readers of this special issue will find several interesting points of discussion in the published papers, even if not working on the specific microbiological topics discussed. They will certainly agree with us that it is time to rediscover Ehrlich's "magic" in the use of mAbs as anti-infectious agents and that some effective novel anti-infectious "bullets" may find their way into the clinics very soon.

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Research Article

A Simple Methodology for Conversion of Mouse Monoclonal Antibody to Human-Mouse Chimeric Form

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Passive immunotherapy has mainly been used as a therapy against cancer and inflammatory conditions. Recent studies have shown that monoclonal antibody-(mAb-) based passive immunotherapy is a promising approach to combat virus infection. Specific mouse mAbs can be routinely generated in large amounts with the use of hybridoma technology but these cannot be used for therapy in human beings due to their immunogenicity. Therefore, the development of chimeric and humanized mAbs is important for therapeutic purpose. This is facilitated by a variety of molecular techniques like recombinant DNA technology and the better understanding of the structure and function of antibody. The human-mouse chimeric forms allow detailed analysis of the mechanism of inhibition and the potential for therapeutic applications. Here, a step-by-step description of the conversion process will be described. The commercial availability of the reagents required in each step means that this experimentation can be easily set up in research laboratories.

1. Introduction

Emil von Behring (1854–1917) won the first Nobel Prize in medicine for demonstrating that humoral immunity could be transferred from immunized animals to humans. Using heterologous sera in humans had its own limitations because of immunological reactions to serum proteins, for example, hypersensitivity. With the help of techniques for better purification of antibodies and monoclonal antibody (mAb) engineering, we now have overcome many of these complications and have attained improved specificity. Till recently, the main focus of the use of the recombinant mAb and passive immunotherapy had been for treatment of cancers or inflammatory conditions [1, 2].

MAb-based immunotherapy is becoming important in infectious diseases because of widespread resistance to drugs among pathogens, immunocompromised hosts, and the emergence of new pathogens. For controlling pathogens such as acute cytopathic viruses that can cause fatal damage in infected tissues, the best way is to prevent the disease. As vaccination is not always available or suitable, passive immunotherapy could be used to provide protection in the periods of high-exposure risk [3]. As immunotherapy is a promising approach to combat virus infection, much research efforts have been devoted to the generation and characterisation of virus-neutralizing mAbs [4–6]. In many laboratories, hybridoma clones are derived from mouse or rat B-lymphocytes by fusion with myeloma cell line (e.g., SP2/0, NS0, NS1, Ag8, or P3U1) [7]. One major limitation of using these hybridoma-derived mAbs is that human-anti-mouse or human-anti-rat antibody response can occur as a result of immunogenicity of these mouse or rat antibodies [4]. Therefore, it is important to humanize these antibodies for human therapeutic purposes without impacting their binding affinity towards antigen targets. For example, in virus research, after a mouse mAb is selected for its potent virus neutralizing activity, it will be useful to convert it into human-mouse chimeric form. If the human-mouse chimeric form has similar neutralizing activity; this will be a reason for further development for therapeutic application. Hence, the

technique for converting mouse mAb into human-mouse chimeric form is an emerging research tool.

Chimeric antibody has been successfully produced and tested for specific binding activity in many previous studies [8–12]. For example, chimeric anti-human DR5 MAb (cmDRA6) can bind to DR5 antigen as demonstrated by both ELISA and Western blot [10]. In addition, a human-mouse chimeric antibody generated from mAb against hepatitis E virus (HEV) capsid proteins E2 still maintains binding activity similar to the original mAb as shown by ELISA and Western blot [11]. Since chimeric antibody is expected to be less immunogenic in human, it could be suitable for antibody therapy of viral infections. Indeed, it has been demonstrated that patient, who received chimeric antibody 17-1A, did not show any toxic or allergic reactions and the chimeric antibody appears significantly less immunogenic than its parental murine antibody [13]. The construction of human-mouse chimeric antibody basically involves cloning and ligating of the variable region genes of mouse mAbs into expression vectors, which have heavy- and light-chain immunoglobulin constant regions. A simple methodology for this conversion will be described here in a step-by-step manner.

2. Results and Discussion

The first step is to amplify heavy- and light-chain immunoglobulin variable regions (V_H and V_L) using the polymerase chain reaction (PCR). To obtain first-strand cDNA for PCR reaction, messenger RNA (mRNA) needs to be extracted from hybridoma cells before reverse transcription. The success to obtain a correct sequence for each V_H and V_L mainly depends on the selection of primer set and optimised conditions of PCR reaction. Different primer sets have been developed for amplifying the variable domains [14–17]. Mouse Ig-Primer set is also commercially available. For example, the one from Novagen has been successfully applied in many previous studies (e.g., [18–21]).

In order to determine the sequence of DNA products from PCR, blunt end ligation with a DNA topoisomerase provides an efficient way to clone the DNA into a vector [22]. While the use of polymerase with proofreading activity significantly reduces the chance of mutations being introduced in the PCR amplification step, the sequence alignment of multiple clones will also easily reveal a mismatch that is found in a rare clone. In most of the clones, an identical sequence should be obtained. However, if two or more sets of sequences are obtained, then, it is very likely that the hybridoma cells used for mRNA extraction are heterogeneous, implicating that hybridoma cells need to be further subcloned.

Once the correct V_H and V_L sequences have been obtained, they can be subcloned into vectors containing the constant region of the human heavy and light chain, respectively (Figure 1). These vectors are designed for mammalian expression and can be home-made or commercially available. An example of the latter is the pair of pFUSEss-CHIg-hG1 and pFUSE2ss-CLIg-hk vectors from InvivoGen. Expression of the human-mouse chimeric antibody is achieved by transfecting the vectors into a transfectable mammalian cell line like the Human Embryonic Kidney 293 cells (HEK 293). The secreted antibody can then be purified from the culture supernatant and tested to determine if it still retains the antigen-binding capacity after chimerization. A direct comparison with the mouse monoclonal antibody produced by the hybridoma will reveal if there is any incompatibility between the mouse variable and human constant regions in the mouse-human chimeric antibody.

The previous methodology is summarized in Figure 1 and has been used to convert the mouse mAb 1A9 to its chimeric human-mouse form. mAb 1A9 is an antibody generated in mice against the severe acute respiratory syndrome coronavirus (SARS-CoV) spike protein [23]. As shown in Figure 2, both the mouse 1A9 and chimeric 1A9 are able to bind to spike protein in HEK 293-FT cell lysates that were transfected with plasmid containing the full-length spike gene, pXJ3′-S.

3. Materials and Methods

3.1. Amplification of V_H and V_L Regions

(i) Count and collect up to 5×10^6 hybridoma cells by centrifugation (800 g for 5 minutes). Wash cells twice with PBS and remove the supernatant completely. Store pelleted cells at −20°C if not used immediately.

(ii) Extract RNA from hybridoma cells using RNeasy Mini Kit (QIAGEN). Suspend the cells in 350 μL lysis buffer by vortexing and pass through QIAshredder (QIAGEN) spin column for RNA extraction.

(iii) Produce the first-strand cDNA from the RNA using SuperScript III First-Strand Synthesis kit (Invitrogen). Use 1 μL Novagen mouse 3′ primer (Mouse Ig-Primer set, Novagen) in total of 20 μL reaction volume (Note 1).

(iv) Amplify V_H and V_L regions using Expand High Fidelity PCR System (Roche) (Note 2). Add Novagen 5′ A-B leader primers to final concentration of 10 pmol μL^{-1} and C-G leader primers to 5 pmol μL^{-1}. Optionally, add Novagen 3′ primer to reach final concentration of 5 pmol μL^{-1}.

(v) Run DNA gel electrophoresis on a 2% agarose gel for PCR products. Extract the DNA bands at around 500 bp (Note 3), as determined by GeneRuler 100 bp DNA Ladder (Thermo Scientific), using QIAEX II Gel Extraction Kit (QIAGEN). Elute DNA in nuclease-free water.

3.2. Cloning Potential V_H and V_L into Vector for Sequencing

(i) Ligate potential V_H- and V_L-amplified products after gel extraction into pCR 2.1 vector from TOPO cloning kit (Invitrogen). Transform ligated products into competent TOP10 cells by heat shock method. Add transformed bacteria onto LB agar plates, which contain 100 μg mL^{-1} ampicillin (Sigma) and 40 μL

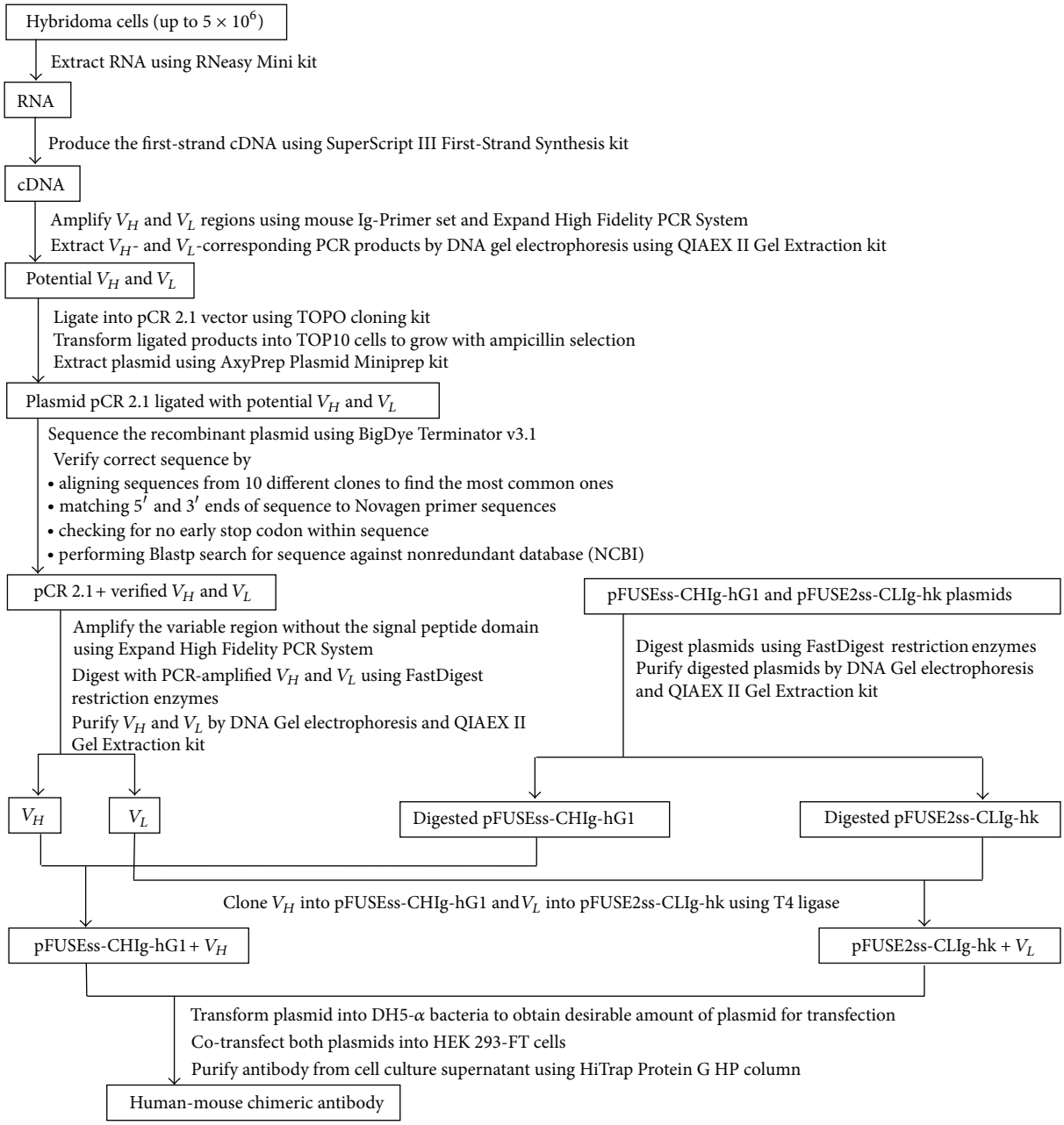

Figure 1: Basic molecular procedure for converting mouse mAbs to human-mouse chimeric forms.

X-gal solution (Thermo Scientific). Incubate plates overnight at 37°C.

(ii) Grow bacteria from at least 10 white or light blue colonies in LB broth containing 100 μg mL^{-1} ampicillin for up to 16 hours at 37°C.

(iii) Collect up to 4 mL bacteria for plasmid extraction using AxyPrep Plasmid Miniprep Kit (Axygen Biosciences). Keep bacteria in LB broth at 4°C for short-term storage (up to 4 weeks).

(iv) Cut recombinant plasmid (5–10 μL) by FastDigest restriction enzyme EcoRI (Thermo Scientific) and run the DNA gel electrophoresis to confirm the presence of DNA insert.

(v) Sequence the recombinant plasmid using BigDye Terminator v3.1 (Applied Biosystems) with M13 forward primer (5′-GTAAAACGACGGCCAG-3′) or M13 reverse primer (5′-CAGGAAACAGCTATGAC-3′).

(vi) Verify correct sequence by (1) aligning sequences from 10 different clones to find the most common ones; (2) matching 5′ and 3′ ends of sequence to Novagen primer sequences; (3) checking for no early stop codon within sequence; (4) performing Blastp

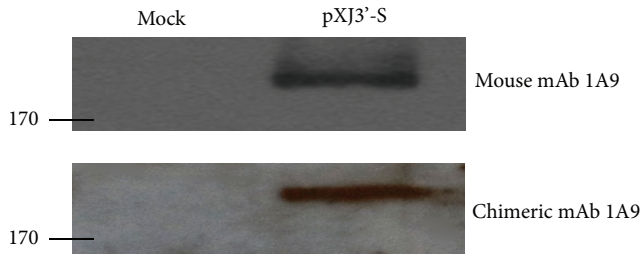

Figure 2: Western blot analysis for the detection of SARS-CoV spike (S) protein (210 kDa). HEK 293-FT cells were transfected with no plasmid (mock) or with plasmid expressing full-length S, pXJ3′-S. Cell lysates were separated in a 7.5% SDS-PAGE gel and detected using mouse mAb 1A9 and human-mouse chimeric mAb 1A9 via Western blot analysis as previously described [23]. Molecular weight markers (in kilodaltons) are indicated on the left.

search for sequence against nonredundant database (NCBI) (Note 4).

3.3. Cloning V_H and V_L into Expression Vector

(i) Transform pFUSEss-CHIg-hG1 and pFUSE2ss-CLIg-hk plasmids (InvivoGen) separately into *E. coli* DH5-α competent cells (Invitrogen) to obtain desirable amount of plasmid for subsequent digestion and ligation steps. Pick up one transformed colony from overnight-incubated plate and grow it in LB broth with appropriate antibiotic (25 μg mL^{-1} zeocin (InvivoGen) or 50 μg mL^{-1} blasticidin S HCl (InvivoGen) up to 16 hours at 37°C. Collect up to 100 mL bacteria culture for plasmid extraction using Midiprep kit (QIAGEN).

(ii) Identify the signal peptide at 5′ end of the V_H and V_L by SignalP (http://www.cbs.dtu.dk/services/SignalP/). Amplify the variable region without the signal peptide domain using Expand High Fidelity PCR System. Forward and reverse primers have 15–20 bp overlap with the appropriate regions within V_H or V_L plus 6 bp at 5′ end as restriction sites (e.g., EcoRI and NheI for V_H, EcoRI and BsiWI for V_L, Note 5) and 2–4 bp before restriction sites to ensure efficient digestion by FastDigest restriction enzymes (Thermo Scientific). It is important to ensure no frameshifting after the V_H or V_L is ligated into pFUSE vectors.

(iii) Digest pFUSEss-CHIg-hG1 and PCR-amplified V_H separately using the same FastDigest restriction enzymes (e.g., EcoRI and NheI) and pFUSE2ss-CLIg-hk plasmids and PCR-amplified V_L separately using the same FastDigest restriction enzymes (e.g., EcoRI and BsiWI).

(iv) Run DNA Gel electrophoresis on 1% agarose gel to purify the digested plasmids or PCR products and extract the DNA by using QIAEX II Gel Extraction Kit. Elute DNA in nuclease-free water.

(v) Clone V_H into pFUSEss-CHIg-hG1 and V_L into pFUSE2ss-CLIg-hk using T4 ligase enzyme (Thermo Scientific). The molar ratio of DNA insert to vector for ligation reaction is 3 : 1 (Note 6).

(vi) Transform the plasmid after ligation step into DH5-α competent cells by heat shock method. Grow pFUSEss-CHIg-hG1 with V_H on LB agar plates containing 25 μg mL^{-1} zeocin and pFUSE2ss-CLIg-hk with V_L on LB agar plates containing 50 μg mL^{-1} blasticidin S HCl overnight at 37°C. Pick up several transformed colonies from plate and grow them in LB broth with appropriate antibiotic (25 μg mL^{-1} zeocin or 50 μg mL^{-1} blasticidin S HCl).

(vii) Collect up to 4 mL bacteria for plasmid extraction using AxyPrep Plasmid Miniprep Kit. Keep bacteria in LB broth at 4°C for short-term storage (2–4 weeks).

(viii) Cut recombinant plasmid (5–10 μL) by FastDigest restriction enzymes and run the DNA gel electrophoresis to confirm the presence of DNA insert.

(ix) Optional step: sequence the DNA insert in the recombinant plasmid using BigDye Terminator v3.1.

(x) Grow bacteria with correct recombinant plasmid in LB broth with appropriate antibiotic (25 μg mL^{-1} zeocin or 50 μg mL^{-1} blasticidin S HCl) up to 16 hours at 37°C. Collect up to 100 mL bacteria culture for plasmid extraction using Midiprep kit.

3.4. Antibody Expression and Purification

(i) Seed 1.5 million of Human Embryonic Kidney 293-FT cells (HEK 293-FT) (Invitrogen) in 6 cm dish (Nunc) with 3 mL transfection media (Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) and 10% fetal bovine serum (Hyclone)) for overnight at 37°C and 5% CO_2 (Note 7).

(ii) Add 2 μg of each recombinant plasmid (pFUSEss-CHIg-hG1 with V_H and pFUSE2ss-CLIg-hk with V_L) and 12 μL Lipofectamine 2000 (Invitrogen) to separate tubes containing 100 μL Opti-Mem (Invitrogen). Vortex briefly and incubate at room temperature (RT) for 5 minutes.

(iii) Spin briefly and mix Opti-Mem with plasmid and Lipofectamine together into the same tube. Incubate for 20 minutes at RT.

(iv) Aspirate cell culture media and gently add 1.5 mL of transfection media with mixture of Lipofectamine and plasmid (Note 8).

(v) Swirl dish gently to mix and incubate for 4–6 hours at 37°C and 5% CO_2.

(vi) Gently aspirate media from the dish. Add 3 mL fresh transfection media and incubate at 37°C and 5% CO_2.

(vii) Collect the cell culture supernatant three times after every 24 hours with replenishment of fresh transfection media each time. Keep cell culture supernatant at 4°C if not used immediately.

(viii) Purify antibody from the cell culture supernatant using HiTrap Protein G HP 1 mL column (GE Healthcare) (Note 9).

(ix) Run the Bradford protein assay (Bio-Rad) with standard BSA to determine protein concentration in each fraction.

(x) Run 3–5 eluted fractions with the highest protein concentration on 12% SDS-PAGE and stain gel with coomassie blue dye to visualise Ig heavy and light chains (approximately 56 kDa and 26 kDa, resp.).

4. Notes

(1) Use MuIgMV_H3'-1 for heavy chain IgM, MuIgGV_H3'-2 for heavy chain IgG, MuIg$\kappa V_L3'$-1 for light chain kappa, and MuIg$\lambda V_L3'$-1 for light chain lambda during first-strand cDNA synthesis. If using Oligo (dT) primer to produce the first-strand cDNA, the Novagen 3′ primer must be added in PCR reaction.

(2) Decrease the annealing temperate by steps of two degrees if no PCR products are found. Increase the annealing temperature by steps of two degrees if many non-specific PCR products are found.

(3) Multiple bands may be present on DNA gel per reaction but purify DNA from band of expected size only (approximately 500 bp). Depending on hybridoma source, two PCR products for V_L region may be obtained since one is from myeloma cells [24].

(4) Alternatively, IgBLAST can be used to verify correct sequence of V_H and V_L (http://www.ncbi.nlm.nih.gov/igblast/index.html).

(5) Restriction sites introduced to forward and reverse primers for amplifying V_H are chosen based on restriction sites of pFUSEss-CHIg-hG1 in the 5′ to 3′ direction, including EcoRI, EcoRV, XhoI, and NheI. Restriction sites introduced to forward and reverse primers for amplifying V_L are chosen based on restriction sites of pFUSE2ss-CLIg-hk in the 5′ to 3′ direction, including EcoRI, AgeI, BstEII, NcoI, and BsiWI. Avoid choosing the restriction site if it is also found within V_H or V_L sequence because short fragment of V_H or V_L after digestion can be ligated into the vector.

(6) Other molar ratios of insert DNA to vector, such as 5 : 1 and 1 : 1, can also be performed at the same time.

(7) After overnight growth, HEK 293-FT cells should be around 80–90% confluent for optimal transfection.

(8) Since the HEK 293-FT cells can be easily dislodged, the media should be gently aspirated and added to the same edge side of the dish.

(9) The following protocol can be alternatively used to purify antibody from cell culture supernatant using HiTrap Protein G HP column:

(i) attach HiTrap Protein G HP 1 mL column to a peristaltic pump and equilibrate by passing through 10–20 mL PBS at flow rate of less than 2 mL per minute,

(ii) filter the culture supernatant through 0.45 μm filter to remove any cell debris and dilute the filtrate with one volume of PBS,

(iii) pass the diluted filtrate and then 10–20 mL PBS through the HiTrap column at flow rate of 1 mL per min,

(iv) elute antibody using elution buffer 0.05 M Glycine-HCl, pH 2.7,

(v) collect and check pH of each 0.5 mL fraction,

(vi) add 2.5 μL of 5 M NaOH for fraction with pH at around 2.7 to return pH to 7-8.

Conflict of Interests

The authors declare that there is no conflict of interests with any financial organization regarding the material discussed in this paper.

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Review Article

Structural and Antigenic Definition of Hepatitis C Virus E2 Glycoprotein Epitopes Targeted by Monoclonal Antibodies

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Hepatitis C virus (HCV) is the major cause of chronic liver disease as well as the major indication for liver transplantation worldwide. Current standard of care is not completely effective, not administrable in grafted patients, and burdened by several side effects. This incomplete effectiveness is mainly due to the high propensity of the virus to continually mutate under the selective pressure exerted by the host immune response as well as currently administered antiviral drugs. The E2 envelope surface glycoprotein of HCV (HCV/E2) is the main target of the host humoral immune response and for this reason one of the major variable viral proteins. However, broadly cross-neutralizing monoclonal antibodies (mAbs) directed against HCV/E2 represent a promising tool for the study of virus-host interplay as well as for the development of effective prophylactic and therapeutic approaches. In the last few years many anti-HCV/E2 mAbs have been evaluated in preclinical and clinical trials as possible candidate antivirals, particularly for administration in pre- and post-transplant settings. In this review we summarize the antigenic and structural characteristics of HCV/E2 determined through the use of anti-HCV/E2 mAbs, which, given the absence of a crystal structure of this glycoprotein, represent currently the best tool available.

1. Introduction: Functions of HCV Glycoproteins

The hepatitis C virus (HCV) glycoproteins E1 and E2 are the most important targets of neutralizing antibodies (Abs). This is a direct consequence of their roles in mediating entry of the virus into susceptible cells in a pH- and clathrin-dependent manner [1–5]. The two genes encoding the HCV glycoproteins lie in the N-terminal part of the HCV genome. The glycoproteins are expressed initially as part of the virus polyprotein, with the mature proteins being released by the action of the host cellular proteinases signal peptidase and signal peptide peptidase [6]. Depending on virus isolate, mature, cleaved E1 protein possesses 192 amino acids and E2 between 363 and 369 amino acids. The glycoproteins form heterodimers through interactions between their transmembrane domains, each chaperoning the folding of the other during synthesis [7, 8]. Amino acid variation in E1 and E2 proteins exceeds 37% among infectious primary isolates, highlighting the extreme genetic diversity that is tolerated in the E1 and E2 genes [9]. The greatest amino acid variation is observed in three hypervariable regions (HVRs) in the E2 protein [10–12]. HVR1 is a 26-27 amino acid region at the extreme N-terminus of E2 and displays the greatest variability in the HCV polyprotein. HVR2 is proximal to the CD81 binding regions of E2, while the intergenotypic variable region (IgVR) lies closer to the transmembrane domain of E2 [12]. Despite this heterogeneity, both proteins possess conserved N- and O-linked glycans, and there is evidence of extensive glycosylation on the surface of both proteins [13, 14]. E2 is the major receptor binding protein, interacting with cell surface molecules CD81, SR-BI, and occludin [15–17]. The interaction between E2 and cell surface receptors has been well described: the binding surface with CD81 is a discontinuous surface encompassing three highly conserved regions of the E2 protein [18–20], while the interaction with SR-BI is believed to be mediated by the N-terminal hypervariable region (HVR1) of the E2 protein [21]. Blockade

of receptor interactions is likely to be the main action of neutralizing Abs.

2. Structure of E1 and E2

There is little direct evidence for the structure and domain architecture of the E1 and E2 glycoproteins. Efforts to crystalize these proteins have so far yielded no accurate structure. As a result, attempts to assign domain structures to these proteins have used a combination of computational models [22] and biochemical analyses [23]. Early studies of the structure of E2 mapped the HCV primary amino acid sequence onto the crystal structure of a prototype *Flavivirus* E protein possessing a type II fusion protein architecture [22]. Recent analyses have mapped the disulphide bridging patterns within the ectodomain of E2, finding patterns consistent with a type II fusion protein [23]. In this study, three domains were defined, relating to the three classical domains of *Flavivirus* glycoproteins, DI, DII, and DIII. In this model DI is discontinuous and features an immunoglobulin fold including the CD81 binding site. DII is predicted to possess a hydrophobic fusion peptide. However, members of the genera *Hepacivirus* and *Flavivirus* are only remotely related. Studies describing the crystal structure of the Pestivirus E2 protein have queried the validity of assigning a type II classification to the HCV glycoprotein [24, 25]. The Pestivirus protein possesses a previously undescribed four domain structure, with four contiguous domains. While this class of structure cannot be assigned to HCV/E2 yet, this data illustrates the possibility that the HCV glycoprotein might belong to an as-yet undescribed class of fusion proteins.

The structure of E1 has been even more intractable. Expressed in the absence of E2, E1 aggregates and does not fold correctly, making structural analysis impossible [26, 27]. This is consistent with reports that E1 might contain the hydrophobic peptide required for envelope fusion [28, 29]. In the absence of direct structural evidence, many studies have investigated the antigenic structure of the E1 and E2 proteins to elucidate its architecture.

3. The Antigenic Structure of HCV/E2 Glycoprotein

There is a wealth of data describing the role of neutralizing Abs in protecting against HCV infection, which has recently been the focus of reviews by Edwards et al. [30] and Fafi-Kremer et al. [31]. Here we will focus on the binding of Abs to specific epitopes in HCV/E2 and the different properties of Abs targeting these epitopes.

Glycoprotein E2 appears to be the more immunogenic of the two HCV glycoproteins [53]. Studies isolating monoclonal antibodies (mAbs) from HCV-infected individuals and experimentally immunized animals have predominantly isolated anti-E2 Abs. Epitopes can be classified as either linear (recognizing linear peptides with contact residues that are only a few residues apart) or conformation sensitive (with contact residues distantly distributed along the primary amino acid sequence but proximal in the three-dimensional space occupied by the protein). Many of the Abs elicited by immunization with recombinant forms of the glycoproteins recognize linear epitopes [32, 54]. In contrast, mAbs isolated from infected humans more often recognize conformation-sensitive epitopes [43, 46, 55] (Tables 1 and 2). Thus the nature of the immunogen is critical to the quality of the Ab response produced. This is also reflected in the neutralizing capacity of mAbs isolated by the two approaches, while many human mAbs possess neutralizing potency [44, 56–58], murine mAbs recognizing linear epitopes have restricted specificity and no neutralizing properties [3].

Initial attempts to map murine mAbs utilized overlapping panels of linear peptides designed from reference isolates [32]. This identified a range of epitopes that were accessible on recombinant proteins and virus-like particles across both E1 and E2 that were immunogenic when animals were immunized with recombinant proteins [54]. With some notable exceptions, mAbs recognizing linear peptides demonstrated restricted patterns of recognition and neutralization. This was particularly notable for the mAbs recognizing epitopes in HVR1 but also for other Abs recognizing linear epitopes [3] (Table 2).

3.1. The HVR1 as a Target for Abs. Following early studies of the Ab response to HCV, the HVR1 was thought to be the major immunodominant neutralization region in the E2 protein. Abs directed to HVR1 were isolated from infected chimpanzees and protected against infection [59]. This was supported by indirect evidence that sequence variability in the HVR1 during chronic infection corresponded to selection of particular HVR1 variants, suggesting that Ab neutralization drives selection of HVR1 quasispecies [60]. HVR1 was subsequently identified to have a direct role in entry, binding to SR-BI and augmenting infectivity [17, 21, 61, 62]. HVR1 also modulates neutralization by Abs targeting the conserved CD81 binding site [63], suggesting that the observed genetic heterogeneity might contribute to persistence in the presence of host broadly neutralizing Abs. HVR1 acts as an immune decoy and prevents generation of a protective Ab response to conserved epitopes in regions essential for E2 function [63]. Mapping has revealed multiple epitopes in the HVR1 region, all of which are restricted in their reactivity. These Abs have different properties. Abs targeting the very N-terminus of E2 have no neutralizing activity [3]. In contrast the rat mAb 9/27 inhibits binding of E2 to SR-BI and potently neutralizes infectivity of genotype 1a strains [3, 64]. This mAb mapped to an epitope in the C-terminal half of the 27-amino acid HVR1 as did other HVR1-specific Abs that neutralized infection [65]. More recently, screening of large panels of mAbs generated by immunization of mice has identified neutralizing Abs with epitopes in the C-terminal end of HVR1 (J6.36, J6.103, and H77.16), which inhibit E2 interaction with SR-BI (Table 1) [38]. These studies highlight not only the potential for anti-HVR1 Abs for preventing infection but also the limitations of restricted reactivity due to sequence variation in primary HCV isolates across the linear epitopes in this region, as recently confirmed in phase 1B clinical trials [66].

Table 1: Schematic representation of the regions targeted by anti-HCV/E2 mAbs. Involved domains (DI, DII and DIII as well as the stem region) of HCV/E2 have been evidenced. In particular, DI has been described to be a discontinuous region containing the CD81 binding site; DII is predicted to possess the fusion peptide and DIII has been recently described to contain antigenic neutralization epitopes and to be involved in heterodimerization with E1.

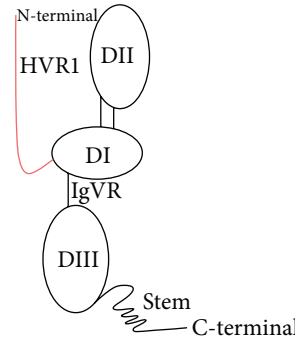
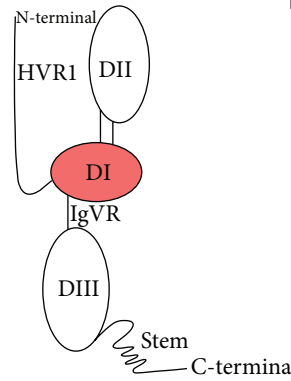
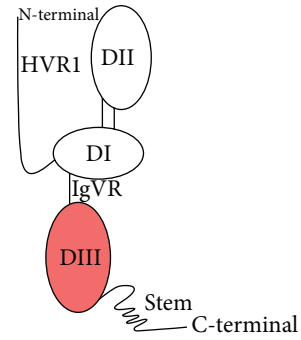
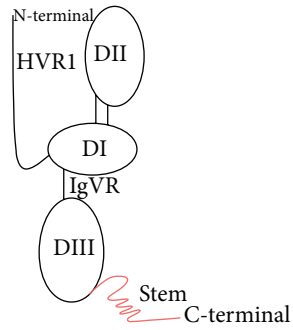
Anti-HCV/E2 mAb	E2 targeted region	E2 domain	Function	Topography	References
9/27, J6.36 J6.103, H77.16, HCV-AB68.	C-terminal of HVR1	HVR1	SR-BI binding		[3, 38, 33]
AP33 (MRCT10.v362), 3/11, HCV1, H77.39, e137, HC33, mu5B3 (hu5B3.v3).	aa 412–423 (epitope I)	DI	CD81 binding		[28, 38, 34, 35, 39, 52, 47, 36]
e20, e137, e509, AR3A, AR3B, AR3D, HC-11, 2/69a, 1/39, 11/20, HC84, #8, #41, #12, #50.	aa 434–446 (epitope II)				[3, 28, 46, 58, 49, 52, 42, 50, 40, 41]
e20, e137, CBH-5, AR3A, AR3B, AR3C, AR3D, HC-1, HC-11, A8, 1:7.	aa 523–535 (Domain B/AR3)				[55, 43, 46, 44 49, 52, 32]
CBH-7, AR2A, AR3B.	aa 540 (Domain C/AR2)				[26, 43, 46]
AR1A, AR3A, AR3C.	aa 538–540 (AR1)				
HC84-27	W616	DIII	E1-E2 heterodimerization		[48]
AR5A, ALP1, ALP98.	aa 639–658 (AR5)				[26, 54, 32]
AR4A	aa 698 (AR4)	Stem			[26]

Table 2: Synopsis of broadly anti-HCV/E2 neutralizing mAbs.

mAb	Origin	Epitope	Genotype breadth of neutralization (HCVpp)	Genotype breadth of neutralization (HCVcc)	*In vivo* tested	Escape generation	References
9/27	Rat	Linear	1a	1a, 2a	No	N.D.	[3, 28, 32]
HCV-AB68	Human	Conformational	1b	N.D.	Yes	Yes	[33]
AP33 (MRCT10.v362)	Murine (humanized)	Linear	1–6	1a, 2a	Yes	Yes	[34–36]
3/11	Rat	Linear	1, 2, 4, 5, 6	1a, 2a	No	N.D.	[3, 28]
2/69a	Rat	Conformational	1a, 2a	1a	No	N.D.	[3, 28, 37]
11/20	Rat	Conformational	1	N.D.	No	N.D.	[3, 28]
1/39	Rat	Conformational	1	N.D.	No	N.D.	
H77.16	Murine	Conformational	N.D.	1a	No	N.D.	
H77.39	Murine	Conformational	N.D.	1a	No	N.D.	[38]
J6.36	Murine	Conformational	N.D.	2a	No	N.D.	
J6.103	Murine	Conformational	N.D.	2a	No	N.D.	
HCV1	Human	Linear	1a, 1b, 2b, 3a, 4a	2a	Yes	Yes	[39]
#8	Murine	Conformational	N.D.	1a	No	N.D.	[40, 41]
HC-1	Human	Conformational	1a, 1b	2a	No	No	[42]
HC-11	Human	Conformational	N.D.	2a	No	Yes	
CBH-5	Human	Conformational	1–6	2a, 2b	No	N.D.	[43, 44]
CBH-7	Human	Conformational	1, 2, 4	2a, 2b	No	N.D.	
A8	Human	Conformational	1–6	2a	No	N.D.	[45]
1:7	Human	Conformational	1–6	2a	No	N.D.	
AR2A	Human	Conformational	1a, 2a, 2b, 4, 5	No	No	N.D.	[46]
AR3A	Human	Conformational	1–6	1–6	Yes	N.D.	[26, 46]
AR3B	Human	Conformational	1a, 1b, 2a, 2b, 4, 5	2a	Yes	N.D.	
AR3C	Human	Conformational	1a, 1b, 2a, 2b, 4, 5	2a	No	N.D.	[46]
AR3D	Human	Conformational	1a, 1b, 2a, 2b, 4, 5	2a	No	N.D.	
AR4A	Human	Conformational	1–6	1–6	Yes	N.D.	[26]
AR5A	Human	Conformational	1a, 1b, 4, 5, 6	1a, 2a, 4, 5, 6	No	ND	
HC33.1	Human	Linear	N.D.	1a–6a	No	N.D.	
HC33.4	Human	Linear	N.D.	1a–6a	No	N.D.	[47]
HC33.8	Human	Linear	N.D.	1a, 2a, 4a	No	N.D.	
HC33.29	Human	Linear	N.D.	1a, 2a, 4a	No	N.D.	
mu5B3 (hu5B3.v3)	Murine (humanized)	Linear	1a, 1b, 2a	2a	No	Yes	[36]
HC84-1	Human	Conformational	1a	1a–6a	No	No	[48]
HC84-27	Human	Conformational	1a	1a–6a	No	No	
e20	Human	Conformational	1a, 1b, 2a, 2b, 4, 5 (N.D. on 3 and 6)	1a, 2a	No	N.D.	[49–51]
e137	Human	Conformational	1a, 1b, 2a, 2b, 4, 5 (N.D. on 3 and 6)	1a, 2a	No	N.D.	[50–52]

ND: not determined.

3.2. Epitopes in the 412–423 Amino Acid Region (Epitope I). Immediately downstream of the HVR1 is a highly conserved region of the HCV genome. Early studies identified murine mAbs that bound to this region in proteins representing genetically diverse HCV strains [28, 32, 54]. This region was established to play an important role in forming complexes of E2 with CD81 [28] and to have a direct role in entry by mediating CD81 binding [19]. Murine mAbs (AP33 and 3/11) that recognized a peptide defined as amino acids 412–423 (also known as epitope I) of the HCV polyprotein were found to efficiently neutralize entry (Table 1) [3, 34]. Although these Abs were originally thought to recognize the same conserved epitope, molecular dissection of their respective contact residues revealed that they recognize overlapping epitopes with different neutralizing potentials [35]. Specifically, AP33 possessed very high neutralizing potency, binding to

contact residues at positions L413, N415, G418, and W420. In contrast, mAb 3/11 formed interactions with residues N415, W420, and H421 and neutralized the same panel of viruses with lower potency [35]. The interaction of AP33 with its epitope was subsequently confirmed by solving the crystal structure of the E2 peptide in complex with AP33 [67, 68]. Other mAbs recognizing this region have more recently been described, including human mAb HCV1 [39], which binds predominantly at positions L413 and W420 [69]. Isolation of mouse Abs using a neutralization screening procedure also isolated a broadly neutralizing mAb, H77.39, that recognized this region [38]. Screening this mAb with a random mutant E2 library resulted in residues N415 and N417 as important in recognition. Indeed, of all 78 anti-E2 mAbs screened in this assay, this was by far the most potent at neutralizing infection. As well as blockading interaction with CD81, H77.39 also inhibited SR-BI binding to E2, suggesting that the proximity of this mAb binding to the HVR1 resulted in two combined modes of neutralizing action. Together these data confirm that discrete, overlapping neutralization epitopes exist in this highly conserved region of E2. Interestingly, all mAbs so far analyzed require W420 as a contact residue. This residue has been described to be critically important in binding to CD81 [19], making up one of the discontinuous regions of the conformation sensitive CD81 binding site [70]. Blockade of the interaction with CD81 is the likely mode of neutralization of these Abs, and it is clear that this region of the protein plays a critical role in the entry pathway of HCV.

Studies of the prevalence of Abs directed to this region in chronically infected individuals have revealed a very low seroprevalence [37, 71], suggesting that this region is not naturally immunogenic. Importantly, while around 2.5% of chronically infected individuals raised an Ab response to the region, less than 1% of infections resulted in an Ab response containing Abs sharing epitope specificity with AP33 [71]. Abs directly purified from those patients with a detectable response to the region spanning 412–423 amino acids were able to broadly and potently neutralize infection, highlighting the potency of Abs to this region. In an alternative approach, Abs purified from hyper-immune globulin by reactivity to a peptide corresponding to the 412–419 amino acid region efficiently neutralized entry, demonstrating that epitopes without the conserved tryptophan at amino acid 420 also contribute to neutralization. Together these studies demonstrate that the Abs to this region have potential for therapeutic administration and vaccine design.

3.3. The Discontinuous CD81 Binding Region as a Target for Abs. While locating linear epitopes recognized by mAbs, such as AP33, is a relatively straightforward process using overlapping peptides, identification of the contact residues of conformation-sensitive Abs targeting the discontinuous CD81 binding site requires a combination of techniques. We and others have mapped a range of Abs directed to the HCV glycoproteins, using a combination of single residue alanine-scanning mutagenesis, mimotope affinity selection from random phage-displayed peptide libraries, mapping using random yeast-display libraries, and competition analysis with mAbs recognizing linear peptides [38, 44–46, 49, 52]. These analyses identified the discontinuous CD81 binding site as a key conserved neutralization determinant. Analysis of the neutralizing potential of Abs against all six major genotypes of HCV highlighted key conserved amino acids that were common to the interaction with CD81 and neutralizing mAbs. One important region of E2 that is involved in this interaction was described to be between amino acids 523 and 535 [19]. Many of the broadly neutralizing human anti-E2 mAbs recognize one of a small number of conserved residues in this region, including G523, Y527, W529, G530, and D535 (Table 1) [44–46, 49, 52]. However, similarly to mAbs directed to the linear region 412–423, different mAbs possess slightly different contact residues. This region is consistent with "Antigenic Domain B" defined by Keck and colleagues and "Antigenic Region 3" (AR3) as defined by Law et al. [46, 72]. This region appears to be immunogenic in natural infection, as human mAbs to overlapping epitopes in this region have been isolated from independent patients, using affinity selection from phage-displayed Ab libraries [46, 55, 73] or transformation of human B cells from HCV infected individuals [44, 72, 74]. Neutralizing murine mAbs to this region has also been isolated following immunization with E2 [38]. Interestingly, there is a third class of Abs targeting this region typified by a human mAb isolated from a phage display library, e137 [52, 75]. This mAb has a unique epitope specificity that overlaps both the 412–423 and the 523–535 neutralizing regions. Key contact residues are T416, W420, W529, G530, and D535, suggesting that the AR3 and the epitope cluster typified by AP33 are proximal on the surface of the E2 protein associated with virions [52]. Another unique epitope overlapping the CD81 binding site has also recently been described. Immunization of an alpaca with recombinant E2 resulted in the production of anti-E2 heavy chain-only Abs (HCAbs) with specificity for the E2 ectodomain [76]. Cloning of the antigen binding domains of these Abs (nanobodies) identified nanobody D03 that possessed epitope specificity for the conserved amino acids N415, G523, and T526. This nanobody was able to both neutralize infectivity and prevent cell-to-cell transmission of HCV. Together these data suggest that the CD81 binding site is a promising target for administration of therapeutic Abs. It is highly conserved [19] and there is an absolute requirement for CD81 binding for entry all strains so far described [5, 9, 21, 77]. This region is also resistant to the emergence of escape mutations [42], indicating that therapy might not suffer from short-term efficacy.

3.4. Other Epitopes in E2. In addition to this major antigenic region overlapping the CD81 binding site, other antigenic domains have also been defined in independent studies by reactivity to human mAbs. A region containing a type-restricted neutralization epitope (AR2) closely overlaps the "Antigenic Domain C," defined by the mAbs CBH-7 and AR2A, centred on an asparagine residue at position 540 [46, 72]. While this epitope region is not extensively characterized, competition assays revealed that this epitope is discrete from those recognized by murine conformation-independent mAbs. The restricted neutralization observed by CBH-7 and AR2A suggests that amino acids positions tolerant to change

are the target of these Abs. From this it can be inferred that these regions of E2 are unlikely to play a key role in the entry cascade and therefore are unlikely to be a good choice to pursue for therapeutic intervention. An additional antigenic region, AR1, has also been identified, with contact residues around V538 and N540. This too overlaps with the epitope of CBH-7 [46] but has no neutralizing activity. It is striking that despite the differences in phenotype between Abs representing different antigenic regions, their epitopes all appear to cluster around a small area of the surface of E2, in the proposed DI [23]. Structural analysis is required to elucidate if this is the case, or whether the common competition with CBH-7 is due to steric hindrance.

Recently two additional antigenic regions have been proposed. Having been well established that the CD81 binding site is the most likely target of neutralizing Abs isolated from human Ab libraries, Giang and colleagues pre-blocked the E2 glycoprotein with known CD81-binding site Abs before selecting a phage-displayed Ab library for affinity to the protein complex [26, 78]. This yielded novel Abs with specificity for epitopes outside the CD81 binding site, binding to regions defined as "Antigenic Region 4 and 5" (AR4 and AR5). Notably, mAb AR4A potently neutralized a range of genetically diverse strains and was able to limit infection *in vivo* [26]. Binding of AR4A and AR5A was dependent on the presence of E1 as well as E2, suggesting that the conformation of the heterodimer is important to their epitopes. Indeed, some mutations in E1 affected binding of both of these Abs. However, distinct residues in E2 were found to be important for each of these Abs, with D698 being essential for AR4A binding, while R639 was essential for mAb AR5A binding (Table 1). These epitopes are located in an otherwise undescribed cluster in a membrane proximal region in E2. However, in common with AR1, AR2 and AR3, AR5A were observed to compete with mAb CBH-7, placing the epitope of this human mAb in a central position overlapping the different antigenic faces of E2. In contrast, AR4A did not compete with CBH-7, emphasizing the unique nature of this Ab epitope.

4. Nonneutralizing/Interfering Anti-HCV/E2 Abs and Topography of Targeted Epitopes

4.1. The Concept of Ab-Mediated Interference. The concept of nonneutralizing/interfering Abs elicitation by pathogens is historically well known. It was first hypothesized by Dulbecco et al. in 1956 analyzing the Ab-mediated neutralization of two animal viruses (Western equine encephalomyelitis and poliomyelitis type 1). In particular, they had observed an inhibition of virus neutralization exerted by some serum samples that probably contained Abs able to inhibit the binding of neutralizing Abs without affecting virus infectivity [79].

In the following years, this phenomenon has acquired a gradually relevant interest giving its possible role in the establishment of chronic viral infections, in which the virus persists notwithstanding the presence of an excess of neutralizing Abs. In particular, very recently, its role is widely debated for HCV and other hypervariable viruses (such as Influenza and HIV) as a further escape mechanism to continually overcome the humoral immune response of the host [80–86].

At present mAbs represent the best available tools to study the role of nonneutralizing/interfering B-cell epitopes, giving their standardized behavior compared to polyclonal preparations (i.e., specific reactivity with individual antigenic determinants) and indeed the ideal probes for studying the spatial relationship or topography of viral targeted epitopes and their role in neutralization [87].

4.2. Role of Nonneutralizing/Interfering Abs in HCV Infection. The hypothesis of the mechanism of Ab-mediated interference in HCV infection was firstly investigated using anti-HCV/E2 mAbs isolated from a chronically HCV-infected patient [88]. In particular, we observed that binding of monoclonal Fabs to HCV/E2 caused conformational changes modifying Fab-binding patterns and reducing, with a negative synergistic effect, Fab-mediated neutralization of binding (NOB) activity to the CD81 receptor. Indeed, our study hypothesized that some Ab clones have the potential to modify HCV/E2 conformation and that, in this state, binding of this glycoprotein to its cellular target is less prone to inhibition by some Ab clones.

More recently, a paper by Zhang et al. finely demonstrated our hypothesis and mapped the HCV/E2 B-cell epitopes involved [89]. In particular, they observed that neutralizing Abs, directed against an E2 region involved in HCV binding to CD81, and in particular encompassing epitope I, could be hindered by the presence of nonneutralizing Abs which bind residues within epitope II, spanning amino acid residues 434–446. Interestingly, they observed that blocking or deletion of these interfering epitope II-specific Abs not only raised the neutralizing titer of HCV-positive sera containing both epitope I- and epitope II-specific Abs but also uncovered a broader cross-genotype neutralizing response.

Recently, we have confirmed the observation of Zhang et al. using anti-HCV/E2 mAbs and analyzing their activity *in vitro* through HCVcc and HCVpp neutralization assays [50]. In particular, it has been found that among the mAbs isolated from a chronically HCV-infected patient, the human mAb e509, recognizing residues within epitope II, was able to interfere with the broadly neutralizing activity of the mouse mAb AP33, which is known to bind the epitope I region. In particular, competition assays suggest that the binding of e509 interferes with AP33 activity by sterically hindering its binding to its epitope or possibly inducing conformational changes on E2 that inhibit AP33 interaction.

On the other hand, the activity of two other broadly cross-neutralizing mAbs, e20 and e137, was not minimally affected by e509. One of the reasons for this lack of interference is probably that e20 and e137 mostly bind residues targeted by neutralizing Abs in another CD81-binding region of HCV/E2 (in particular that encompassing amino acid residues 529–535) outside epitope I and therefore potentially less subjected to the interfering effect of epitope II-directed Abs. Moreover, in contrast to AP33, e20 and in particular e137 also bind residues within the interfering epitope II, but at higher

affinity than e509, thus displacing it from HCV/E2. In fact, as speculated by Duan et al. and also recently confirmed in the work by Keck et al., the neutralization ability appears to be correlated with the overall binding affinity of these mAbs to epitope II [40, 47]. Furthermore, similar to e20 and e137, it has been described that some mAbs which bind the antigenic domain B of HCV/E2 also contain residues located within epitope II (Table 1) [42].

In contrast to these findings, we recently observed that different Abs targeting the region encompassing epitope II not only neutralized HCVpp and HCVcc infection but augmented neutralization mediated by Abs targeting the region encompassing epitope I. In particular, for these studies, the murine and rat mAbs AP33 and 2/69a (with the last one targeting epitope II), as well as human immunoglobulin fractions affinity purified on linear peptides representing distinct HCV/E2 domains clustering within the regions 412–426 and 434–446, were used. Combining these Abs together, we failed to demonstrate any inhibition between these two groups of Abs [37]. These results provided evidence that interference by nonneutralizing Abs, at least to the region encompassing residues 434–446, is not a mechanism for HCV persistence in chronically infected individuals, as it had been originally proposed by Zhang and colleagues.

However, this study confirmed, as previously observed by other groups and by patient sera analysis of binding to epitope I and epitope II, that these two regions are co-immunogenic despite being both recognized by the sera of only a small subset of patients [35, 89]. Indeed, this observation further confirms the known low immunogenicity of the epitope I region compared to epitope II, considering also the less conserved nature of this last epitope [35]. Furthermore, these data confirm the mostly conformational nature of epitope II as previously suggested by our data and by previous structural studies [23, 90]. In fact, as expected, binding assays performed on peptides spanning conformational regions could not completely predict their immunogenicity as Abs directed against conformational epitopes could not be detected. Indeed, we found that, depending on the infected individual, Abs targeting the region encompassing epitope II could differ in phenotype, according to their epitope specificity, or could exhibit a dual phenotype [37].

In accordance with these findings, Keck et al. described anti-HCV/E2 human mAbs binding conformation-sensitive epitopes encompassing also some residues within the 434–446 interfering region. These mAbs are broadly neutralizing and do not lead to viral escape mutants, demonstrating the functional importance of their epitopes. The authors conclude that not all Abs directed against epitope II are interfering and that this activity could be limited to Abs recognizing linear epitopes within it [58].

Furthermore, in a more recent work, Keck et al. isolated human mAbs (named HC33) directed against a peptide spanning epitope I region possessing varying neutralizing activity against HCVcc of genotype 1–6, depending on their affinity for the antigen. Moreover, they observed a unidirectional competition for binding to E2 between HC33 mAbs and human mAbs to the 434–446 amino acid region. In addition, when neutralizing HC33 mAbs were combined with HC-11 or HC84 mAbs (whose epitopes encompass the proposed "Antigenic Domain D" and have contact residues located within the epitope II), they observed antagonistic effect at lower Ab concentrations and synergistic effect at higher Ab concentrations both in neutralization and NOB assays [47].

It is noteworthy that another recent paper supported the possibility of Ab mediated interference in *in vivo* experiments. In particular, treating an HCV chronically infected chimpanzee using HCV1 mAb, the authors observed an interfering effect, probably Ab mediated, of the chimpanzee serum on the neutralizing activity of HCV1. In fact, similar to AP33, HCV1 recognizes the epitope I region of E2 with a comparable affinity [91].

Furthermore, recent clinical trials on HCV genotype 1a-infected patients undergoing liver transplantation evidenced a viral rebound after 28 and 2 days after transplantation in mAb HCV1-treated patients and placebo-treated patients, respectively. The authors observed the occurrence of viral variants within the epitope I region that could determine escape from HCV1 neutralization [92]. However, possible patient-interfering Abs could negatively influence the neutralizing activity of the mAb, but the authors did not investigate on this possible escape mechanism.

4.3. Topography of HCV/E2 Epitope II. As anticipated, differently from the highly conserved nature of epitope I, epitope II is less conserved as it is located within the third hypervariable region of E2 (HVR3), which encompasses also another neutralizing CD81 binding region, suggesting the conformation-sensitive nature of epitope II [18].

In this regard, Duan et al. finely mapped the amino acid residues of epitope II bound by nonneutralizing/interfering Abs. In particular they isolated four mouse mAbs, two of which (#8 and #41) were able to neutralize *in vitro* HCV of genotype 1a, while the other two (#12 and #50) failed to neutralize the virus. Interestingly, #12 mAb could interfere with the neutralizing activity of a chimpanzee polyclonal Ab and of a specific human immunoglobulin preparation both directed against epitope I of HCV/E2. The authors predicted indeed that residues W437, L438, and L441 (located within epitope II) were the common direct contact points for the binding of #8, #41, #12, and #50 mAbs. Interestingly, #12 and #50 binding was more affected by substitution at L441 and F442 than #8 and #41, suggesting that these residues may be more related to the binding of nonneutralizing Abs rather than that of neutralizing Abs (Table 1) [40]. Moreover, in a recent work Deng et al., attempting to better define at the atomic level the fundamental mechanism of Ab-mediated neutralization, reported the crystal structure of the epitope II peptide in complex with mAb #8. In particular, this group found that mAb #8 interacts both with the C-terminal α-helix (contacting aminoacid residues W437 and L438) and the N-terminal loop (contacting aminoacid residues E431 and N434) of epitope II. Indeed the authors speculated that neutralization may be achieved through bifurcated Ab-binding to these two regions, compared to nonneutralizing Abs which simply bind only α-helical structure of epitope II [41].

In this regard, conducting a meta-analysis of the data reported by Duan et al., we can observe that substitutions at W437 and F442 affected also e20, e137, and e509 binding, while substitutions at L438 and L441 affected only e137 and e509 binding, again confirming the speculations of the authors that both L441 and F442 are determinant for the binding of nonneutralizing and interfering Abs [40, 49, 50, 52].

Finally, the predicted recognition of W437 by e20 and e137 does not limit their cross-recognition and cross-neutralization profile of different genotypes/isolates as conversely observed by Duan et al. [40]. Further analysis on the binding of these two cross-neutralizing mAbs to the N-terminal loop of epitope II must be conducted. However, similar to other previously described broadly neutralizing mAbs, the neutralization mechanism of e20 and e137 appear to be mediated by recognition of different discontinuous conformational epitopes involving amino acid regions 412–424, 436–447, and 523–540 of HCV/E2 glycoprotein [42, 52]. In fact, as suggested by Lapierre et al., broadly neutralizing activity of these mAbs is the result of the recognition of structural determinants rather than specific residues of the conformational epitope II [90]. These speculations raise the possibility that residues L441 and F442 are both contact residues only for e509 and thus confirming that the sequence 441-LFY-443 was linked to the non-neutralization of the virus [40].

However, the role (and the existence itself) of interfering Abs in influencing HCV infection is still controversial and, as concluded by the authors themselves, caution must be taken in differentiating neutralizing Abs from nonneutralizing Abs solely on the basis of their residue specificity. Indeed, minor change of residues in the Ab-antigen interface, epitope frameshifting among genotypes/isolates, as well as change in binding affinity may alter recognition capabilities of these Abs and thereby may consequently modulate their activity [40].

Interestingly, according to the putative model for E2 folding, all the three aforementioned regions would lie next to each other on the glycoprotein [23]. Therefore, this structural prediction possibly supports the interfering effect of epitope II-directed Abs. However while this predicted structure is currently the best model available, these conclusions cannot be absolutely asserted. For this purpose, the availability of E1-E2 crystal will certainly accelerate the fine elucidation of the spatial proximities of neutralizing and interfering epitopes on the E1-E2 structure and, consequently, structure-based vaccine progress.

Finally, the low prevalence and the low titer of epitope I-reactive Abs in sera from both chronic and acute resolved infections further support the hypothesis of a conformational masking by adjacent regions such as epitope II [71, 89]. In fact, Zhang et al. originally put forward the idea that once epitope II is bound to an Ab, the site of epitope I becomes masked and can no longer be recognized by specific neutralizing Abs. Indeed, depletion of Abs to epitope II in plasma from a chronically infected HCV patient and vaccinated chimpanzees recovered an otherwise undetectable cross-genotype neutralizing activity [89]. Another possibility is that the initial binding of interfering Abs to the region containing epitope II may induce conformational changes on E2 that inhibit the binding by epitope I-directed Abs, as recently suggested by Lapierre et al. for other anti-HCV/E2 Abs [90].

Thus, the described divergent observations reported above may depend on the different Ab specificities present in the polyclonal preparations used and, probably, also on the different HCV genotypes infecting the considered cohort of patients. Moreover, the different strategies adopted in isolating epitope I- and epitope II-directed Abs could explain the different data obtained. In fact, immunoglobulins purified on peptides representing distinct HCV/E2 regions are obviously directed against linear epitopes; these preparations are certainly different from mAbs cloned using a full-length HCV/E2 glycoprotein, which are more probably directed against conformational epitopes including also residues outside the investigated linear regions.

To summarize, in the HCV field several works support the existence of interfering Ab populations and hypothesize their possible role in HCV persistence, as demonstrated using different Ab preparations such as human plasma-derived immunoglobulin preparations, human mAbs, and sera of animals immunized with recombinant HCV/E2 peptides. The possible mechanism leading to the interference is still controversial, but both direct steric-hindrance and induced antigen conformational changes have been hypothesized. On the other hand, other papers do not confirm these findings, suggesting that the putative interfering epitope II may be targeted by Abs endowed with a broadly neutralizing activity. However other studies suggest that the interfering Abs do exist but that their overall effect may be biased by the presence of neutralizing Abs with different binding features and by the infecting HCV genotype. Future works further investigating the *in vivo* role of these interfering Ab subpopulations in HCV persistence are certainly needed.

5. Conclusions

Although much of the research into neutralizing epitopes has focused on the E2 glycoprotein, some neutralizing determinants have been identified also in E1. Efforts to identify anti-E1 Abs have been hindered by the poor folding of E1 when expressed *in vitro* [93]. Despite this, mAbs directed to E1 192–211 amino acid region have been identified to be cross-reactive with E1 samples representing multiple genotypes and neutralize entry [94], while neutralizing mAbs IGH505 and IGH526 recognize an epitope between the 313–327 amino acid region of E1 and neutralize entry of diverse isolates [95]. However, immunization with E1 alone does not induce a neutralizing response in experimental animals [53]. Although E1 is an interesting target for discovery of neutralizing Abs, the current paucity of Abs directed to this protein limits greater understanding of its antigenic structure.

Furthermore, the escape mechanism utilizing the elicitation of nonneutralizing Ab subpopulations able to enhance the viral infectivity or able to interfere with the activity of neutralizing Abs has further implications in vaccine design as well as in the development of effective therapeutic mAbs [83]. Indeed, many anti-HCV/E2 mAbs recently described in

the literature as well as those currently undergoing clinical trials target regions that could potentially be subjected to the Ab-mediated interfering mechanism [92]. Moreover, other mechanisms of HCV escape from the humoral immune response must be taken into account when considering the potential clinical applications of developing antiviral mAbs such as the lipoprotein and glycan shielding of the viral envelope as well as the recently described glycan shifting consequent to the high mutation rate of the virus [36], the main reason for incomplete effectiveness of currently available therapies as well as the main hurdle for candidate therapeutics under development. Finally, the observed cell-to-cell route of infection for HCV may further limit the breadth of viral entry inhibitors (e.g., anti-E1 and anti-E2 mAbs) in particular in those chronically infected patients undergoing liver transplantation with the consequent augmented risk of graft reinfection and indeed of a more progressive and complicated disease [96]. However, Abs able to neutralize this route of transmission have been recently described [76]. On the other hand, a further therapeutic approach overwhelming these escape mechanisms would consist in the administration of entry inhibitors together with other antiviral compounds targeting different steps of the viral replication cycle such as protease and polymerase inhibitors [97, 98]. In fact, it is well known that in the treatment of chronic diseases caused by hypervariable infectious agents, the administration of single or multiple drugs targeting only limited step of their life cycle leads, in the majority of cases, to the occurrence of escape variants limiting their effectiveness that could be further complicated by an incomplete adherence of the patients to the therapeutic regimen. In this regard, the occurrence of viral variants after treatment of HCV chronic infections with the newly introduced protease inhibitors (Boceprevir and Telaprevir), whose administration is indicated only for genotype 1 infected patients, has been well demonstrated [99]. However, new more effective antiviral drugs targeting the NS3 protein and other viral proteins as well as other cellular targets implicated in the HCV viral life cycle, such as the CD81 receptor, are under development and clinical evaluation [100]. Interestingly, anti-CD81 compounds have shown to limit the cell-to-cell spread of HCV and thus represent valuable candidates for a future combined therapy [101]. Moreover, differently from currently available therapies, the targeting of cellular determinants, such as CD81, may be theoretically beneficial for patients infected with any HCV genotype and could be less subjected to the occurrence of viral variants. Thus, the HCV entry inhibition by antivirals directed against viral and cellular determinants could be an effective and valuable tool, in particular in the course of a post-transplant setting, for which no therapies are currently available.

Authors' Contribution

G. Sautto and A. W. Tarr equally contributed to the paper.

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Review Article

Human Monoclonal Antibody-Based Therapy in the Treatment of Invasive Candidiasis

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Invasive candidiasis (IC) represents the leading fungal infection of humans causing life-threatening disease in immunosuppressed and neutropenic individuals including also the intensive care unit patients. Despite progress in recent years in drugs development for the treatment of IC, morbidity and mortality rates still remain very high. Historically, cell-mediated immunity and innate immunity are considered to be the most important lines of defense against candidiasis. Nevertheless recent evidence demonstrates that antibodies with defined specificities could act with different degrees showing protection against systemic and mucosal candidiasis. Mycograb is a human recombinant monoclonal antibody against heat shock protein 90 (Hsp90) that was revealed to have synergy when combined with fluconazole, caspofungin, and amphotericin B against a broad spectrum of *Candida* species. Furthermore, recent studies have established an important role for Hsp90 in mediating *Candida* resistance to echinocandins, giving to this antibody molecule even more attractive biological properties. In response to the failure of marketing authorization by the CHMP (Committee for Medicinal Products for Human Use) a new formulation of Mycograb, named Mycograb C28Y variant, with an amino acid substitution was developed in recent years. First data on Mycograb C28Y variant indicate that this monoclonal antibody lacked efficacy in a murine candidiasis model.

1. Introduction

Largest scientific effort to develop antibody-(Ab-) based therapies has focused on diseases where the humoral immune system was known to contribute in a crucial way to host defense.

Infectious diseases caused by viruses or by encapsulated bacteria such as pneumococcus and meningococcus have represented the major targets for antibody therapy [1–3].

Despite broad-spectrum of antibiotic therapy has almost completely replaced serum therapy for bacterial diseases, to now, hyperimmune human immunoglobulins are used to treat many viral diseases including those caused by cytomegalovirus respiratory syncytial virus, hepatitis A virus, hepatitis B virus, and others [4, 5], highlighting the concept that antibodies-based therapy remains an effective means of treatment.

The humanized monoclonal Ab (mAb) palivizumab, targeted the RSV F protein, is effective for the prevention of severe respiratory disease in high-risk infants and immunocompromised adults and represents the only one licensed mAb for an infectious disease [6].

The enormous potential offered by monoclonal antibodies as therapeutic agents has been only slightly exploited by the field of infectious diseases, contrary to what happened to areas of medicine like oncology and that of autoimmune diseases where the use of monoclonal antibodies has provided an outstanding contribution to current therapies [7, 8].

Another area where antibodies therapy has definitely brought the leading therapeutic choice is the neutralization of animal venoms [9].

On the other hand, recent works have determined that mAbs could be effective even against microbes, such as fungi or intracellular pathogens, for which the principle studies do not clearly defined a role played by humoral immunity [10].

Macrophage, NK cells, and neutrophils related to cell-mediated immunity and nonspecific cellular immunity are generally believed to be the main protagonists for the primary

defenses against fungi [11]. The importance of cellular defense mechanisms for protection against fungi is supported by the clinical observation that most invasive fungal infections occur in individuals with defective cellular immunity. As a matter of fact, in the field of medical mycology it is generally accepted that cellular immunity is essential for successful host defense against fungi [12].

How long antibodies are actually involved in the defense against fungal infections remains a controversial issue [13]. The literature shows a rather heterogeneous orientation regarding the actual importance of humoral immunity for any of the medically important fungi [14, 15]. Surprisingly, a positive influence of antibody against disseminated fungal disease was first suggested more than 50 years ago [16]. About 15 years later, an interesting work by Pearsall and coworkers again sensitized the scientific attention on the benefic effects of passive serum transfer for murine candidiasis [17].

In recent years several studies have established the potential efficacy of humoral immunity in host protection against *Candida albicans*, and there is a great deal of attention on the benefits that may therefore result from mAb-based therapy against various fungal infections including *Candida* infections [18].

Until today, in the field of clinical mycology, a single mAb able to bind a specific cryptococcal antigen in serum of patients suffering from cryptococcal meningitis has been studied clinically [23].

Candidal diseases are often chronic, difficult to treat, and carrying a high mortality and morbidity despite antifungal therapy. Invasive candidiasis is a promising area for mAb therapy because current therapies are inadequate. Usual treatment for invasive fungal infections consists in monotherapy based on the use of azoles, echinocandins, and the polyene amphotericin B (AMB) or one of its liposomal derivatives. However, the well-known toxicity of antifungal therapy and the emergence of the increasing resistance to these antifungal agents actually represent a potential problem [24, 25]. Considering this scenario, it is reasonable to assume that in the next few years, efforts to increase the antifungal therapies may also be targeted to the field of antibodies-based therapies. Several studies from the first decade of the 80s have focused on the production and characterization of monoclonal antibodies directed against candidal cell surface determinants. After the development of the hybridoma technology [26], many research groups have used *Candida* antigens for the selection of murine mAbs with diverse specificity. Findings related to such *Candida* mAbs have brought interesting observations on the variation of antigen expression by this organism [27, 28].

To date, there are some antibody molecules with more or less demonstrated efficacy in the therapy of systemic candidiasis in animal model, but all of them derived from mice [29–31] with the only exception for Mycograb. Mycograb (NeuTec Pharma, a subsidiary of Novartis AG, Basel, Switzerland) is a human recombinant antibody directed against *Candida* Hsp90 that is essential for yeast viability. This antibody has been designed to work in combination with the best current antifungal therapeutics and entered in a multinational phase III clinical trial. This paper will review Mycograb in its most salient features, with a particular focus for its variant named Mycograb C28Y.

1.1. Mycograb and Its Target Hsp90. The history of Mycograb antibody has distant origins. Its selection as a single chain antibody takes advantage from biotechnologies related to the expression of human antibodies as soluble recombinant proteins in *Escherichia coli* that offers the periplasmic space as an ideal site for the formation of disulfide bridges, essential for the proper folding of the antibody molecules. Mycograb derived from a cDNA coding antiheat shock protein 90 (Hsp90) antibody of patients recovered from invasive candidiasis. It consists of the variable ends of the heavy (VH) and light (VL) chains from one arm of the antibody. These two N-terminal domains are linked together by 2 cross-chain cysteine bonds with a synthetic linker to represent the antigen-binding domains.

Resultant recombinant protein is a polyhistidine-tagged single chain antibody fragment against the immunodominant epitope of *Candida* Hsp90 antigen. Its original name, Efungumab, was converted by NeuTec Pharma in Mycograb. It is expressed in *E. coli* and easily purified by three-step chromatography, filter sterilized and lyophilized [32]. It was developed by NeuTec Pharma, in Manchester, UK, and is actually produced to current good manufacturing practice standards, by 1000 liter batch fermentation of recombinant *E. coli*. The clinical motivation behind the choice of Hsp90 as a target for the generation of an inhibitory antibody was based on the observation that the antibody response in patients with invasive candidiasis, receiving AMB, showed a strict correlation between recovery and antibody titer to the immunodominant Hsp90 [33]. Mycograb mimics this naturally occurring inhibition of Hsp90 and is thus a logical partner in combination therapy. Numerous studies examining the antibody response to *C. albicans* in infected patients and experimentally infected animal have demonstrated divers and specific immunodominant antigens [34].

The molecular chaperone Hsp90 is a key cellular regulator that is critical for setting cellular responses to a wide variety of stressful stimuli, among which, drug-induced stress. The essential role in cell physiological mechanisms makes Hsp90 indispensable for yeast viability.

Hsp90 regulates the stability and function of diverse client proteins [35], like its downstream effector calcineurin, a protein phosphatase that regulates the response to stress. Calcineurin dependent stress responses are required to survive to fungistatic antifungal drugs exposure, like azoles and echinocandins for *C. albicans*. Prejudice Hsp90 or calcineurin function converts antifungal drugs from fungistatic to fungicidal and enhances the efficacy of antifungals in mammalian models of systemic and biofilm fungal infections [36]. Combination therapy with antifungal drugs and Hsp90 inhibitors may therefore provide a powerful strategy to treat life-threatening fungal infections. In addition to its normal cytoplasmic localization, extracellular or membrane bound Hsp90 elicits an immune response providing a link between innate and adaptive immunities [37]. In effect,

Hsp90 represents an important target for protective antibodies in disseminated candidiasis. An antibody response to Hsp90 antigen is significantly more common in patients with deep-seated candidiasis than those with superficial candidiasis [38–40]. In patients with severe, invasive candidiasis, a sustained antibody response to this antigen correlated closely with a good prognosis, whereas lack or falling levels of Ab were associated with fatality [41]. Dissecting this potentially protective antibody response to the level of individual epitopes showed that it was primarily directed against the epitope NKILKVIRKNIVKK. Mice vaccination with an Hsp90-expressing DNA vaccine demonstrated specific humoral immunity associated with protection against invasive candidiasis [42]. Deepening studies on antibody response to specific epitopes of this antigenic target led to the identification of peptides representing the epitopes LKVIRK or DEPAGE derived from the middle domain of Hsp90 able to stimulate a protective immune response against *Candida* infection [43]. Mycograb directly binds the middle domain of Hsp90 inhibiting communication between the terminal domains with client proteins [44]. The antibody is not able to cross the fungal cell wall to enter the fungal cytosol and interact with extracellular or membrane bound Hsp90. The antibody is composed of light and heavy chains without the Fc domain, abrogating the need to recruit the cellular immune system for its function and allowing efficacy even in immunocompromised patients. The antifungal activity of Mycograb can be shown using conventional assays for evaluation of antifungal drugs such as checkerboard and time kill methodologies.

The Fractional Inhibitory Concentration (FIC) is a mathematical expression of the effect of the combination of antimicrobial agents. Checkerboard titration assays result in a number of FIC indices (FICIs). The sum of a number of FICIs divided by the number of indices is designated as average ΣFIC. The synergism-antagonism FICI accepted criteria are traditionally been defined as follow: FICI value of <0.5 was defined as synergistic, a value of >0.5 and <4.0 was defined as indifferent, and a value of >4 was considered antagonistic. Other works reported as synergistic FICI values ranging from 0.5 to 1 [45].

1.2. Mycograb: In Vitro and In Vivo Efficacy. *In vitro* assays developed by Matthews et al. [19], for efficacy evaluation of Mycograb, showed intrinsic antifungal activity against the most important species of *Candida,* including fluconazole sensitive and fluconazole resistant strains of *C. albicans*. The Mycograb MICs were found to be rather high, ranging from 128 to 256 μg/mL. Moreover, Mycograb, even at relatively high concentration (100 μg/mL) demonstrated indifference when administered in combination with fluconazole against all yeasts examined. The FICI values obtained were all >0.5, with the only curious exception of the fluconazole-sensitive strain of *C. albicans* for which there was synergy with a FICI value of 0.34. By contrast, authors demonstrated *in vitro* synergistic action of Mycograb with AMB. Results from checkerboard methods showed a pronounced synergy between AMB and Mycograb against all Candida strains tested, with relatively low concentration of Mycograb of 4 or 8 μg/mL readily achievable in humans. Resultant FICI values varied from 0.09 to 0.31. Table 1 summarizes the *in vitro* interaction of Mycograb with diverse antifungal agents. After such encouraging findings derived from *in vitro* experiments, results from the *in vivo* assessment, in a normal immune mouse model of systemic candidiasis, demonstrated that a single dose of Mycograb of 2 mg/kg in combination with AMB improved the killing of each *Candida* species examined compared with AMB monotherapy [19]. AMB alone cleared the *C. tropicalis* infection but not *C. albicans, C. krusei, C. glabrata*, or *C. parapsilosis* from one or more organs. Mycograb combined with AMB resulted in complete resolution of *C. albicans, C. krusei,* and *C. glabrata,* but for *C. parapsilosis* even though the liver and spleen were cleared, the kidney colony counts were not. Furthermore, statistically significant reduction in the mean organ colony count from the same mouse model of systemic candidiasis was obtained with Mycograb alone, demonstrating an *in vivo* intrinsic antifungal activity of this antibody at a dose of 2 mg/kg. This first preclinical study demonstrated that Mycograb has synergy against a broad range of *Candida* species in combination with AMB *in vitro* and in animal models of invasive candidiasis.

1.3. Mycograb: The Clinical Trials. The first clinical trial of Mycograb [33] in the treatment of fungal infections in combination with antifungal agents involved five patients with invasive candidiasis in an open label tolerance and pharmacokinetic study. A test dose of Mycograb 0.1 mg/kg was given to patients before the start of liposomal AMB (L-AMB) therapy. Subsequently patients received two further doses of Mycograb at 1 mg/kg 8 or 12 hours (h) apart. The highest concentration of Mycograb 30 minutes after the first dose ranged from 1.5 to 4 mg/L, and serum levels were undetectable by 8 h. Despite too limited number of patients involved in the trial, no treatment related adverse events were noted by the investigators.

The most important medical trial in terms of clinical efficacy of Mycograb in the treatment of invasive candidiasis is a double-blind, randomized study conducted by Pachl et al. [46] to determine whether L-AMB plus Mycograb was superior to L-AmB plus placebo in 139 adult patients with invasive candidiasis. Among 139 patients, 117 were included in the modified intention to treat group. Enrollment criteria included one or more positive cultures showing candidiasis from the blood or from a deep normally sterile site but not from respiratory secretions, oropharyngeal specimens, or esophageal specimens. L-AMB was preferred by the research group instead of conventional desoxycholate form for its superior safety profile. A rigorous and sophisticated statistical analysis plan was created prior to unblinding by Hartington Statistics and Data Management (London, UK).

The trial was conducted in 26 institutions across Europe and the United States. Patients were stratified into groups on the basis of *Candida* species *(C. albicans* versus *C.* non-*albicans)* and were randomly assigned to receive either intravenous Mycograb (1 mg/kg) or placebo (saline) every 12 h for

5 days. In addition, each patient was treated, for a minimum of 10 days, with the manufacturer's recommended dose of L-AmB. The Mycograb group included 63% *C. albicans* versus 65% in the placebo group. The two groups were besides well balanced with respect to the APACHE II scores (Acute Physiology and Chronic Health Evaluation II).

For the assessment of the efficacy of combination therapy, both mycological and clinical responses were considered. The primary efficacy endpoint was overall response to treatment on day 10, defined as a complete clinical response with resolution of all signs and symptoms of candidiasis and mycological response with negative cultures.

Secondary endpoints were clinical response at day 10, mycological response at day 10, and rate of mycological clearance of infection and *Candida*-attributable mortality four weeks (day 33) after last administration of Mycograb or placebo. Signs and symptoms of infection as well as culture results were carefully controlled by local investigators. In the meantime, trial's safety, in terms of adverse events to drugs therapy, was monitored by an independent committee.

Side effects like back pain and vomiting, generalized rash, hypertension, and others revealed that episodes of hypertension occurred more frequently in the Mycograb group (7.4%) than in the placebo group (2.9%) and usually occurred within 2 h after receipt of the first dose of Mycograb.

Authors of the study consider, however, that Mycograb was well tolerated.

A complete overall response by day 10 was obtained for 29 (48%) of 61 patients in the L-AmB group, compared with 47 (84%) of 56 patients in the Mycograb combination therapy group. Moreover, patients who received Mycograb cleared their infections twice as quickly and importantly the Candida-attributable mortality rate decreased from 18% to 4% (>4-fold) among patients receiving Mycograb.

Mycograb in combination with L-AMB produced significant clinical and culture confirmed improvement in outcome of patients with invasive candidiasis.

These very promising results have attracted the attention of the scientific community, and some case reports have been published on the use of antifungal agents in combination with Mycograb for treatment of severe, also pediatric, cases of disseminated *C. albicans* infections [47–49].

1.4. Mycograb and Caspofungin. A study of Hodgetts et al. [20] demonstrated that Mycograb increased the susceptibility of Candida to caspofungin. Echinocandins have recently become popular as an alternative to AMB in the treatment of fungal infection. Caspofungin demonstrated a more conducive safety profile than AMB, with significantly less treatment breaks due to drug toxicity [50].

In the *in vitro* experiments performed in this study, caspofungin and Mycograb concentrations were tested alone and in combination by MIC endpoints and checkerboard titrations (Table 1). Results from susceptibility testing demonstrated that the addition of Mycograb improved the susceptibility to caspofungin of a variety of isolates that represent the most important species causing invasive candidiasis. Wisely, Hodgetts and collaborators tested both endpoints and assessed the combination of caspofungin and Mycograb in the same mouse model used by Matthews et al. in the preclinical assessment of the efficacy of Mycograb to outline its synergy with AMB. Efficacy in mice was measured 48 h after the intravenous injection of the yeasts by the reduction in mean colony counts from kidney, liver, and spleen or the number of negative biopsies as appropriate. The enhanced activity of combination therapy (4 mg/kg caspofungin plus 2 mg/kg Mycograb) compared with monotherapy with 4 mg/kg caspofungin achieved statistical significance against a variety of Candida isolates. Such convincing data support the hypothesis that the addition of Mycograb to caspofungin could improve outcome in a way similar to that seen with L-AMB therapy. Authors of the study rightly point out the involvement of Hsp90 in the mechanisms of Candida resistance to caspofungin [51], assuming a contribution, in the overall treatment success of this combination therapy, and in increasing yeasts susceptibility by Mycograb.

1.5. Mycograb and CHMP. In November 2006, the Committee for Medicinal Products for Human Use (CHMP) adopted a negative opinion, recommending the refusal of the marketing authorization for the medicinal product Mycograb. The motivations were related to quality aspects and safety concerns.

The quality concerns included incoherence in the structure of the compound between manufactured batches, such as the way the molecules of Mycograb may fold or aggregate in the solution for injection and the level of some substances that could stimulate an immune response in patients. Safety concerns were associated with "cytokine release syndrome" a condition that can cause nausea, vomiting, pain, and also hypertension.

In March 2007, following the reexamination, the CHMP removed their concern regarding the cytokine release syndrome and hypertension, as these would be manageable in clinical practice. However, all other concerns due to heterogeneity, including autoaggregation of the mAb, remained, hence, the CHMP confirmed the refusal of the marketing authorization on 20 March 2007.

1.6. Mycograb C28Y Variant. In response to the failure of marketing authorization by the CHMP, Arnold Louie and collaborators [21] attempted to overcome the issues related to the heterogeneities in molecular weight and conformational structure of Mycograb. Authors believed that autoaggregation of the molecule could be due to the presence of a fifth cysteine at position 28 which was unpaired. This inconvenient amino acid was not in the antigen-binding site of the antibody fragment; therefore it was not implicated in the interaction with target Hsp90 and did not contribute to the two disulfide bridges normally present in the molecule. Consequently, a modified form of Mycograb named as Mycograb C28Y variant was developed in which the cysteine (C) at position 28 was changed to a tyrosine (Y).

A polar uncharged r group, with similar physical and chemical properties, characterizes both amino acids. Such amino acid substitution should not modify the antibody

Table 1: Checkerboard assay of FLC (fluconazole), AMB, caspofungin, and Mycograb versus *C. albicans* fluconazole-susceptible (FLC-S) and fluconazole-resistant (FLC-R) strains.

Straints	Agents	MIC (μg/mL) for each agent		FICI	Outcome	References
		Alone	In combination			
C. albicans (FLC-S)	FLC Mycograb	1.56 128	0.4	0.34	Synergy	Matthews et al. 2003 [19]
C. albicans (FLC-R)	FLC Mycograb	50 256	12.5 100	0.64	Indifference	
C. albicans (FLC-S)	AMB Mycograb	1 128	0.03 8	0.09	Synergy	Matthews et al. 2003 [19]
C. albicans (FLC-R)	AMB Mycograb	0.5 256	0.125 4	0.27	Synergy	
C. albicans (FLC-S)	Caspofungin Mycograb	0.125 1024	0.0625 16	0.5	Synergy	Hodgetts et al. 2008 [20]
C. albicans (FLC-R)	Caspofungin Mycograb	0.25 2048	0.125 0.125	0.52	Indifference	

FICI: fractional inhibitory concentration index.

conformational structure, and therefore, new amino acid composition should not interfere with target interaction. Data sets from Novartis on the quality of new formulation has indicated that the Mycograb C28Y variant appeared to be much more stable and homogeneous, with a markedly improved batch-to-batch consistency and an ability to be dissolved in solution as monomeric form equal to 80% of the preparation.

First data on MIC for the Mycograb C28Y variant, as a single agent, was >256 μg/mL. Higher concentrations could not be tested because concentrations of ≥512 μg/mL became turbid.

This turbidity could be due to partial recombinant antibody precipitation as a result of physical and chemical conditions not favorable for those concentrations that have consequently induced aggregation. However, concentrations of 0.5 mg/mL are not particularly high to justify this phenomenon that still regards the largest part of recombinant proteins expressed in *E. coli*.

Moreover, in contrast to the synergistic *in vitro* and *in vivo* interactions that have been demonstrated between Mycograb and AMB by Matthews et al., these new sets of experiments performed by multidose treatment studies in a neutropenic murine model of systemic candidiasis showed indifference between the Mycograb C28Y variant and AMB. The efficacy of Mycograb C28Y variant combined with AMB was not better than AMB monotherapy in clearing *C. albicans* from the kidneys, livers, and spleens of infected mice. Again, in neutropenic mice Mycograb C28Y variant alone had no intrinsic activity against *C. albicans*.

Surprisingly, FICI values describing the synergistic *in vitro* interaction between Mycograb C28Y variant and AMB were similar and consistent with prior data for Mycograb and AMB. Comparison of FICI values for both mAbs is reported in Table 2. The discordance between the synergistic interaction between the Mycograb C28Y variant and AMB observed in the checkerboard assay and lack of the outcomes observed in neutropenic infected mice was not expected. A very detailed discussion of what may be the motivations that could explain the different *in vivo* interactions observed for Mycograb, and the Mycograb C28Y variant is provided in the work of Arnold et al. First point under consideration concerns the compounds themselves as there may be a difference in potency of the Mycograb C28Y variant compared to Mycograb. However, the modification of the structure of a compound is frequently associated with pharmacokinetics changes of the molecule, and pharmacokinetic studies conducted by the group in neutropenic mice have definitively ruled out this hypothesis. With regard to the animal models, original Mycograb formulation was evaluated in normal immune mice and in predominately nonneutropenic human patients, while Mycograb C28Y variant was used alone and in combination with AMB in a neutropenic murine model of systemic candidiasis. Farther, authors opted for a multidose treatment design study, instead of single doses of AMB and Mycograb administered by Matthews et al. to evaluate the potential therapeutic benefit of the Mycograb C28Y variant in order to build upon a phase III human trial which normally used a multidose treatment regimen.

Regarding different animal models, authors reported data by others (data from Novartis) on the efficacy of the Mycograb C28Y variant and AMB in immunocompetent mice. The new Ab formulation provided no benefit over AMB monotherapy. Afterwards the authors discuss the possibility of an interference due to the production of anti-human antibody by mice with normal immune systems in response to receiving multiple injections of this human recombinant Ab fragment. The concentration of the Mycograb C28Y variant in mice serum was not measured at the time the multidose study was conducted as a validated test was not available.

The single-dose and multidose pharmacokinetics of the Mycograb C28Y variant in the neutropenic murine model of systemic candidiasis used by Arnold et al. were similar to those obtained for the original Mycograb formulation, suggesting that the neutropenic mice did not produce anti-human antibody against the Mycograb C28Y variant in response to multiple administrations of this compound.

Table 2: FICI of AMB in combination with Mycograb, Mycograb C28Y variant, and various protein sources.

C. albicans STRAINS	AGENT	FICI	Outcome	References
ATCC 24433	AMB MYC C28Y	0.258	Synergy	Louie et al. 2011 [21]
ATCC 90028	AMB MYC C28Y	0.258	Synergy	Louie et al. 2011 [21]
ATCC 24433 ATCC 90028	AMB MYC C28Y	0.27 ± 0.18	Synergy	Richie et al. 2012 [22]
ATCC 24433 ATCC 90028	AMB Mycograb	0.23 ± 0.12	Synergy	Richie et al. 2012 [22]
ATCC 24433 ATCC 90028	AMB Aurograb	0.14 ± 0.01	Synergy	Richie et al. 2012 [22]
ATCC 24433 ATCC 90028	AMB Human γ-globulin	0.18 ± 0.07	Synergy	Richie et al. 2012 [22]
ATCC 24433 ATCC 90028	AMB Human-serum albumin	0.15 ± 0.05	Synergy	Richie et al. 2012 [22]

FICI: fractional inhibitory concentration index.

Finally, differences in the mouse strains used to evaluate the activities of the original Mycograb formulation and the Mycograb C28Y variant could not explain the difference in activity of the two molecules. It is clear from this investigation that important considerations should be carefully evaluated on the limits of *in vitro* checkerboard assay for the effective estimation of *in vivo* efficacy and synergism with other drugs, of a testing molecule. Retrospectively, previous works missed some important test controls, such as parallel evaluation of *in vitro* antifungal properties of unrelated similar recombinant antibodies.

Work from Richie and collaborators [22] did not miss such relevant *in vitro* controls and at least clarified ambiguous results arising from the checkerboard assay.

In this study, done in a checkerboard design, combinations of AMB and Mycograb C28Y (up to 128 μg/mL) caused a dose-dependent decrease of 2 to 3 dilution steps in the MIC of AMB against two strains of *C. albicans*, in agreement with previous reports.

Unrelated proteins, including 2 murine IgGs, human gamma globulin, bovine serum albumin, and human serum, were tested in parallel as protein controls. All unrelated proteins, with the exception of human serum, at 5 μg/mL, produced a 4-step dilution reduction in the MIC of AMB against *C. albicans*. Human serum showed a paradoxical effect with a 3- to 5-step dilution reduction at concentrations up to 5% and a 1-step dilution reduction at concentrations above 10%.

Results from this study demonstrated that small amounts of serum present in RPMI medium can potentiate the activity of AMB which is attenuated at higher concentrations of serum.

To independently validate their findings, similar experiments were performed by other investigators in a Medical Mycology department belonging to a different University. Microdilution assays were performed in checkerboard methods against two strains of *C. albicans* to evaluate the antimicrobial activity of AMB in combination with various protein sources. This included human gamma globulin and serum albumin, the original Mycograb, Mycograb C28Y, and Aurograb, a similar recombinant antibody fragment designed to bind to an unrelated bacterial target [52]. All proteins tested in combination with AMB recorded a FICI value <0.5, indicating a synergistic relationship (Table 2). Interestingly, the nonspecific, synergistic protein effect was not observed in combination studies with fluconazole or caspofungin, attributing to Hodgetts work an indirect greater validity.

Table 3: *In vitro* and *in vivo* final evaluations of the efficacy of Mycograb and its C28Y variant.

	In vitro		*In vivo*	
	Intrinsic fungicidal activity (MIC)	Combination therapy with AMB (CB)	Intrinsic fungicidal activity (MIC)	Combination therapy with AMB (I.C.)
Original Mycograb	present	synergy	present	effective
Mycograb C28Y Variant	absent	synergy	absent	non effective

(CB): checkerboard assay; (I.C.): invasive candidiasis on a mouse model of systemic infection; (MIC): minimal inhibitory concentration.

2. Conclusion

The growing increase of drug resistance in fungal pathogens compromises the efficacy of the limited number of antifungal drugs available to date. Furthermore, antifungal drugs possess a limit number of distinct targets. Fungi are eukaryotes, and the close evolutionary relationships of these opportunistic pathogens with their human hosts make most treatments toxic to the host or weak in combating infections. The use of drug combinations has emerged as a powerful strategy to enhance antifungal efficacy and abolish drug resistance; even though the impact on the evolution of antifungal resistance

remains largely unexplored and unresolved. Combination therapy has the potential to play down the evolution of drug resistance by more effectively eradicating pathogen populations and by requiring multiple mutations to confer drug resistance. *In vitro* and first clinical data showed that Mycograb owns activity against *Candida* spp. when used alone and synergism when combined with AMB, fluconazole, and caspofungin. Important studies reviewed in this paper suggest that the checkerboard assay does not predict the *in vivo* interaction between Mycograb, its variant C28Y, and AMB. The antifungal potentiation of AMB by Mycograb *in vitro* appears to be a nonspecific effect that can be reproduced by a wide range of unrelated proteins. Therefore, the *in vitro* checkerboard assay cannot replace *in vivo* studies in assessing the interaction of anti-Hsp90 antibody formulations with AMB for the treatment of invasive Candida infections. In this respect, although further confirming data are needed, it looks like that Mycograb may become a new antifungal agent with unique mechanism of action for treatment of invasive candidiasis. Table 3 shows in a very simplified manner the results of the *in vitro* and *in vivo* efficacy of Mycograb and its variant C28Y in combination with AMB. Results from clinical trials on adult patients with invasive candidiasis remain, anyway, very encouraging. Currently, Europe's Committee for Medicinal Products for Human Use (CHMP) refused approval of Mycograb due to a lack of data concerning adverse effects, specifically the cytokine release syndrome characterized by hypertension, nausea, and vomiting which were handled by corticosteroids and antihistamines. CHMP requested more data from a controlled trial to clarify the nature of the cytokine release syndrome. Mycograb has orphan drug status with the Food and Drug Administration (FDA) and is available on a compassionate use basis in Europe. Future directions for Mycograb include adjunctive therapy of cryptococcal meningitis where it has shown synergistic activity with AMB and fluconazole [53]. Identification of protective mAbs against fungi may be useful for both the development of direct Ab-based therapies and isolation and characterization of defined antigens able to elicit protective Ab immunity. The challenge in constructing antibody-based antifungal vaccines is to identify the fungal antigens which elicit protective antibodies response and to develop strategies to direct the antibody response towards the production of effective natural antibodies while avoiding the production of nonprotective or deleterious antibodies [54]. It would be of great impact for medical mycology to take advantage of the available technologies for the selection of human mAbs to strengthen the treatment of invasive fungal infections by combination therapies. Phage display antibody library methodology represents an excellent tool for dissecting the humoral immune response of patients with invasive candidiasis. The enormous advances in the field of proteomics now allow the identification of relevant immunodominant targets closely related to the clinical course of infectious disease [55]. Due to all its multiple antibody specificities, no other biological sample can give as much information as patient's serum about the interaction and fight between host and pathogen. Huge potentialities associated with the use of antibody library on the surface of filamentous phages [56–59] could in part allow to explore such different antibodies specificity by selecting single human monoclonal antibodies against Candida protective antigens.

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Review Article

Peptide-Based Vaccinology: Experimental and Computational Approaches to Target Hypervariable Viruses through the Fine Characterization of Protective Epitopes Recognized by Monoclonal Antibodies and the Identification of T-Cell-Activating Peptides

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Defining immunogenic domains of viral proteins capable of eliciting a protective immune response is crucial in the development of novel epitope-based prophylactic strategies. This is particularly important for the selective targeting of conserved regions shared among hypervariable viruses. Studying postinfection and postimmunization sera, as well as cloning and characterization of monoclonal antibodies (mAbs), still represents the best approach to identify protective epitopes. In particular, a protective mAb directed against conserved regions can play a key role in immunogen design and in human therapy as well. Experimental approaches aiming to characterize protective mAb epitopes or to identify T-cell-activating peptides are often burdened by technical limitations and can require long time to be correctly addressed. Thus, in the last decade many epitope predictive algorithms have been developed. These algorithms are continually evolving, and their use to address the empirical research is widely increasing. Here, we review several strategies based on experimental techniques alone or addressed by *in silico* analysis that are frequently used to predict immunogens to be included in novel epitope-based vaccine approaches. We will list the main strategies aiming to design a new vaccine preparation conferring the protection of a neutralizing mAb combined with an effective cell-mediated response.

1. Introduction

The development of vaccines directed against clinical relevant viral pathogens is perhaps the most important contribution of immunology to public health. Traditional vaccine preparations are based on attenuated or inactivated whole viruses or partially purified viral proteins. These strategies, although effective against a large number of pathogens, present drawbacks due to viral intrinsic characteristics such as poor or null *in vitro* replication and antigenic hypervariability [1].

In order to overcome these issues, quite a number of novel approaches have been developed, one of the most promising focusing on epitope-based vaccine preparation.

The possibility to use minimal structures such as peptides, or a mixture of them, as the main constituent of a vaccinal preparation, presents many advantages. Firstly, peptides can be easily produced *in vitro* reducing production costs and simplifying large-scale vaccine production procedures. Moreover, expression of peptides belonging to viral proteins does not necessarily require *in vitro* pathogens growth, overcoming viral culturing issues.

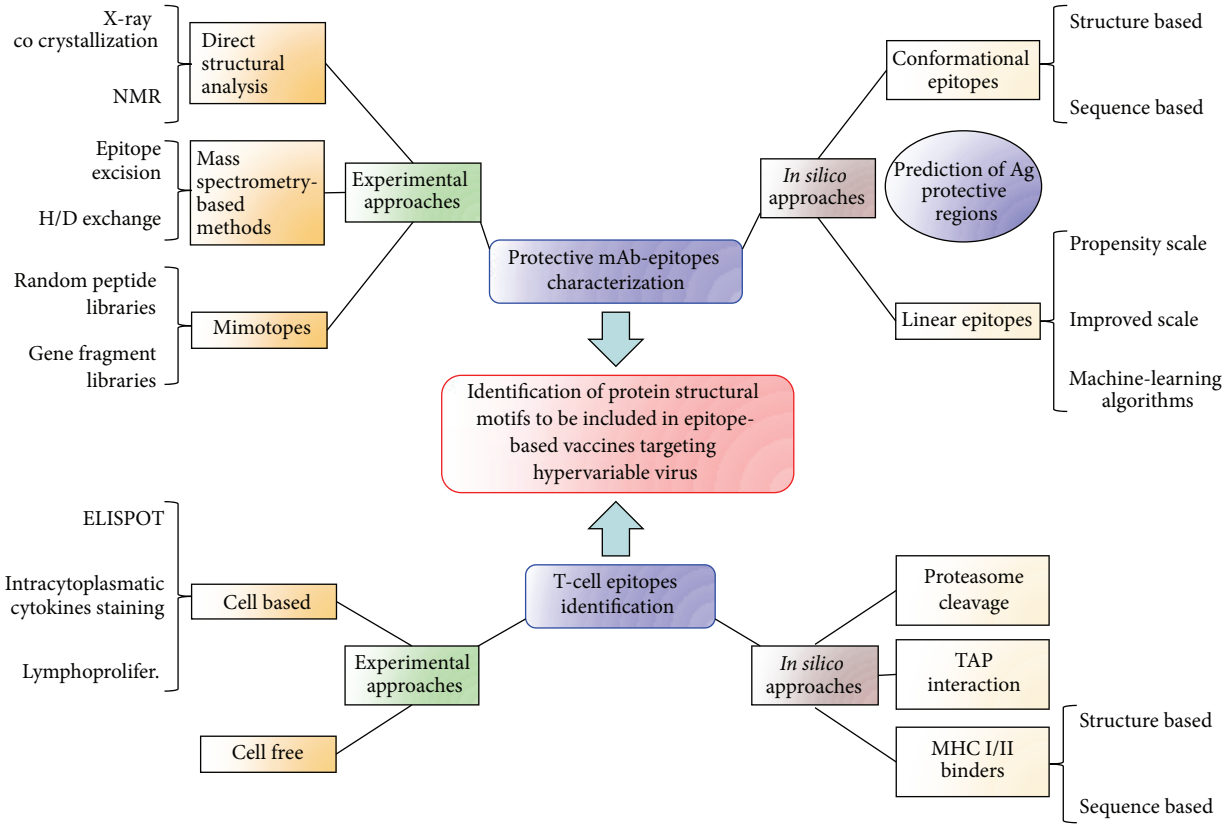

Figure 1: The described approaches to characterize protein structural motifs to be included in new vaccines targeting hypervariable viruses. The synergistic use of techniques combining experimental and *in silico* approaches is also shown.

This strategy also presents safety benefits, zeroing problematic related to back mutations for attenuated viruses and reducing side effects due to possible improper immune response against viral antigenic determinants.

Perhaps the most important aspect of using well-characterized synthetic peptides as immunogens is related to the specific triggering of both humoral and cell-mediated immune responses against a fundamental domain of a viral protein. Moreover, the possibility to remove antigen (Ag) domains activating suppressor mechanisms may elicit only a protective response targeting conserved functional regions shared among hypervariable viruses [2].

Despite these advantages, to date no epitope-based vaccines have been used in clinical practice. This is mainly due to low immunogenicity and difficulties related to the fine identification of protective epitopes and/or properly folded antigen structural motifs to be included in a vaccinal preparation. The latter is fundamental to properly activate an effective immune response. Furthermore, a main goal for a successful epitope-based vaccine approach is the identification of epitopes capable of eliciting both humoral and cell-mediated responses [3, 4].

Different strategies, spanning from antigen presentation techniques to *in silico* design of structural motifs to be included in vaccinal preparations, have been developed in order to overcome these issues. In this paper we review the most promising approaches in peptide-based vaccine setup applicable to hypervariable viruses. In particular we will focus on the methods at the interface between experimental and computational procedures aiming at the prediction of B and T-cell-activating peptides (Figure 1).

2. Selection of B-Cell-Activating Peptides: Immune Humoral Response as a Probe to Identify Crucial Domains

A crucial step in epitope-based vaccine design is the identification of antigens capable of eliciting a protective immune response specific for a pathogen of interest. Depending on the characteristics of the virus to be targeted, humoral and cellular response changes in relevance. As an example, the former plays a crucial role in conferring specific immunity for influenza virus infection. Many researches have been focused on the characterization of protective monoclonal antibodies (mAbs) targeting widely conserved hemagglutinin (HA) regions among different influenza subtypes [5–12]. Considering the clinical potential of mAbs endowed with such peculiar cross-neutralizing activity, their epitope characterization represents a valuable tool to identify functional

and conserved epitopes potentially useful in an epitope-based vaccinal strategy.

Different methods, either exclusively based on experimental approaches or involving the use of *in silico* studies, have been applied to identify regions featuring the aforementioned characteristics. Several of the most frequently used methods are listed and discussed in the following.

2.1. Direct Structural Analysis of mAb/Antigen Complex. Structural resolution of a specific mAb in complex with its target through X-ray crystallography or nuclear magnetic resonance (NMR) is to date the only procedure to obtain interaction information at atomic level [9, 13]. However, considering methods complexity and inability to be applied to certain complexes together with low throughput features, X-ray crystallography and NMR represent useful tools to fully characterize the epitope of a single mAb but are not suitable for mapping all antigenic determinants.

2.2. Mass Spectrometry- (MS-) Based Techniques. The MS based techniques permit to define mAb epitopes at a medium resolution. All the MS-approaches aim at the identification of mAb footprint on the targeted antigen [14, 15]. Different experimental methods involving MS are widely described in the scientific literature. These approaches are mainly based on the protection of mAb binding site on the whole antigen from proteolytic digestion or protein modification (i.e., acetylation or deuterium incorporation), through its bond with the mAb itself [16, 17]. mAb-interacting fragments are subsequently identified through MS and mapped *in silico* on the whole antigen to define epitope sequence and structure. In particular, the computational analysis is generally performed excluding the "nonepitope" antigen regions (Ag unbound regions) followed by the mapping of Ag amino acid residues derived from MS analysis (e.g., not subjected to proteolytic digestions or deuterium incorporation) on the Ag crystal structure.

2.3. Mimotopes. Mimotopes are small peptides able to mimic antigenic conformational structures recognized by an antibody (Ab) paratope. The most frequently used approach to isolate specific mimotopes recognized by a mAb is based on the screening of a random peptide phage display through biopanning techniques [18, 19]. Alternatively, if the antigenic protein can be cloned and expressed from recombinant cDNA, a library composed by antigen fragments can be created and screened for positive binding to mAbs.

Selected peptides are then sequenced, aligned to antigen sequence, and, if available, superimposed to its three-dimensional (3D) structure, allowing the identification of the immunogenic domain. This process often requires the use of specific *in silico* tools, as epitope localization on antigen surface from mimotopes sequences might not be trivial; specific algorithms such as Mimox (http://immunet.cn/mimox/), Pepitope (http://pepitope.tau.ac.il/), and MimoPro (http://informatics.nenu.edu.cn/MimoPro/) are available online [20–22]. They all perform an alignment of provided mimotope sequences to a given PDB structure, returning epitope localization; identification can be done either on a single mimotope sequence or clustering all positive sequences and searching for a consensus patch on the structure. An online database named MimoDB 2.0 (http://immunet.cn/mimodb/) is also available online; it collects from the scientific literature thousands of mimotopes identified from random libraries providing information about identification methods, libraries, and respective protein [23].

Identification of mimotopes is a powerful technique as it easily allows to map many antigenic determinants at the same time using a polyclonal serum or to identify a single mAb epitope at a medium resolution [24, 25]. The canonical 18 mer peptides allow the study of conformational epitopes, as they are long enough to fold into a specific secondary structure. Moreover, it can be efficiently used when antigens 3D structure is not available, returning possible peptides to be used in a peptide-based vaccinal approach disregarding their structure.

2.4. In Silico Prediction of Linear Epitopes: Propensity Scale, Improved Propensity Scale, and Machine-Learning Algorithms. Continuous epitopes include ~10% of all known antibodies epitopes; while they comprise a minority of all epitopes found in nature, many computational methods focus on their mapping [26, 27].

Sequence-based algorithms represent the first attempt to predict B-cell epitopes located on a protein surface without *a priori* immunological data. Most of these algorithms, namely, *propensity scale* (or *amino acid scale-based*) methods, rely upon residues chemical and physical properties based on empirical data (i.e., hydrophilicity, flexibility, solvent accessibility, polarity, and presence of β-turns). Five of the most used amino acid scale-based methods are implemented at the Immune Epitope Database (IEDB) website (http://tools.immuneepitope.org/main/html/bcell_tools.html) [28]. A standard score to evaluate the performance of these methods is the A_{ROC} (Area under the Receiver Operating Curve) value. This value spans from 0 to 1 where a value of 0.5 matches with a random prediction, and 1 represents the ideal performance [29]. None of the methods implemented in IEDB website and listed previously exceeded the A_{ROC} threshold of 0.6 when benchmarked with three standard datasets, pointing out their low reliability in predicting linear epitopes. Only a small improvement in comparison with a random prediction is in fact demonstrated for single propensity scales [30].

Considering the amino acid scale-based methods as a starting point, novel algorithms combining different propensity scales and machine-learning methods have been developed. While the former strategy did not lead to substantial improvements, machine-learning methods have proven their efficacy when tested, exceeding the A_{ROC} threshold value of 0.6. The first generation of these hybrid algorithms comprises, among the others, ABCpred (http://www.imtech.res.in/raghava/abcpred/), a recurrent artificial neural network- (ANN-) based algorithm, and BepiPred (http://www.cbs.dtu.dk/services/BepiPred/), which combines a machine-learning method such as the hidden Markov model (HMM) with two propensity scale methods taking into

account Parker's hydrophilicity and Levitt's secondary structure scales [31–34].

In the last few years several machine-learning algorithms exploiting Support Vector Machine (SVM) have been implemented as well, leading to a progressive prediction improvement in terms of accuracy, sensitivity, and specificity [35, 36].

Recently Lin et al. developed the algorithm BEEPro, an SVM-based learning-machine which uses fourteen physiochemical scales to generate a hybrid propensity scale including antigenicity, hydrophilicity, flexibility, composition, volume, charge transfer and donor capability, hydrogen bond donor capability, and secondary structure features. It is then further combined with an amino acid ratio propensity scale representative of the propensity of each amino acid to be part of an epitope and a position specific scoring matrix (PSSM) which reflects the evolutionary information of a peptide [37].

Considering these parameters, BEEPro, has been trained with the Sollner dataset comprising many non-redundant linear epitopes and proved itself to efficiently predict both linear and conformational epitopes, outperforming other prediction algorithms [38].

2.5. In Silico Prediction of Conformational Epitopes: Structure- and Sequence-Based Algorithms. Conformational epitopes mapping represents a challenging goal in different biological and medical fields. In the last few years many algorithms capable of predicting conformational epitopes have been developed. They can be divided in structure-based and sequence-based algorithms.

Structure-based algorithms work on three-dimensional (3D) proteins structure obtained either through X-ray crystallography or NMR and exploit different spatial parameters as well as amino acids statistics. CEP [39], together with DiscoTope (http://www.cbs.dtu.dk/services/DiscoTope/), is the first web server developed to predict both linear and conformational epitopes; it relies on residues solvent accessibility and defines a linear epitope when at least three consecutive residues satisfy the solvent exposure parameter. Conformational epitopes are then predicted considering linear epitopes whose Cα is closer than 6 Å [39].

DiscoTope is a method oriented to conformational epitopes prediction; the algorithm bases its prediction on the combination of hydrophilicity, amino acids propensity score taken from a dataset of resolved antibody/antigen structures, residues spatial neighborhood, and area of relative solvent accessibility [40]. The 2.0 version of DiscoTope recently implemented includes novel strategies to define the spatial neighborhood and a half-sphere exposure to calculate surface exposure; it has been shown to outperform the majority of previous prediction algorithms [41].

After CEP and DiscoTope, many others machine-learning methods to predict conformational epitopes starting from a 3D structure have been developed; PEPITO (http://pepito.proteomics.ics.uci.edu/), SEPPA (http://lifecenter.sgst.cn/seppa/), EPCES (http://sysbio.unl.edu/EPCES/), and its improved version EPSVR (http://sysbio.unl.edu/EPSVR/) analyze 3D structures and aim at the division of antigens surface in epitopic and nonepitopic patches on the basis of different propensity scores and solvent accessibility; they all rely on training datasets comprising resolved antibody/antigen complexes [42–45].

Moreover, new algorithms try to improve analysis and broaden targets using linear sequences when structures are not available. ElliPro (http://tools.iedb.org/tools/ElliPro/iedb_input) can model proteins of unknown structure aligning their sequence in BLAST and then modeling structures with MODELLER; epitopes search is then performed approximating protein shape to an ellipsoid, calculating every residue protrusion index (PI) and finally clustering neighboring residues based on their PI values [46, 47]. As well as ElliPro, Epitopia (http://epitopia.tau.ac.il/) allows the user to input either antigen structure or sequence; the prediction algorithm calculates an immunogenicity score for each residue through a trained naïve Bayes classifier and clusters them, outputting a probabilistic score for each patch [48].

Despite the effort, none of the structure-based methods reached a high efficiency in terms of accuracy, sensitivity, and specificity. Unsuccessful attempts might be due to many aspects; first of all, the number of antibody/antigen resolved structures is too small to provide a robust statistical sampling of all possible epitopic patches. Moreover, datasets are affected by the low resolution of some structures. Another issue is the lack of consideration of proteins as complexes *in vivo*; during algorithms training, protein patches that are physiologically buried in protein-protein complexes can wrongly be considered as possible epitopes. Other problems come from the definition of an epitope in terms of which residues should be considered as part of it; this involves both the proximity threshold of surface residues to be used and the lack of consideration for buried residues below the epitopic patch. Finally, experimentally not all the possible epitopes of an antigen might have been identified. All these aspects lead to a biased training of the machine-learning algorithms, which in turn cause a prediction far from optimal [49].

Considering efficiency issues and limited available antigens structure, novel sequence-based methods have been developed. The first attempt is represented by the CBTOPE (http://www.imtech.res.in/raghava/cbtope/) algorithm, which reached better results than all structure-based algorithms. A SVM was trained with protein chains belonging to antibodies epitope; each residue was classified as binding or nonbinding and characterized to define residue-specific physiochemical and composition profiles. This strategy allows to define specific epitopic and non-epitopic patterns that are then applied to the local amino acid composition of the antigen; prediction is thus performed without considering the whole protein sequence but searching for epitopic patterns [50].

Recently two more sequence-based algorithms, the aforementioned BEEPro, and the method published by Zhang et al. outperformed CBTOPE results. Results succeeded by these three algorithms are related to the usage, besides many physiochemical properties, of matrices that try to identify specific nonlinear patterns for epitopic and non-epitopic patches.

Considering results achieved by CBTOPE, Zhang et al. tried to explore more potentially relevant sequence-derived features effective for the conformational epitopes prediction. Besides physiochemical characteristics and amino acids propensity to be part of an epitope, residues side chains have been clustered in thirteen classes to compute the propensity for each of them; moreover, a PSSM has been used as in BEEPro to calculate evolutionary conservation. A term representing the secondary structure is included as well. The random forest machine-learning algorithm is then used to classify each query protein patch on the basis of every feature creating an output ensamble and then rank the results. It is interesting to notice that Zhang et al. determined the PSSM to be the most effective feature in predicting epitopes explaining BEEPro performance [37, 49]. CBTOPE, BEEPro and the web server developed by Zhang et al. can provide a satisfactory output that can be used as a good starting point for further experimental evaluation confirming putative epitopes.

3. Identification of T-Cell-Activating Peptides

While moving towards an epitope-based vaccine strategy, both humoral and cell-mediated response have to be taken into account (Figure 1). An effective immunity has indeed to be mediated by the induction of neutralizing antibodies together with the activation of specific cytotoxic CD8 and helper CD4 T lymphocytes. Therefore, as well as with B epitopes, a great effort has been put in the characterization of peptides binding to major histocompatibility complex (MHC) of class I and class II that can be presented to TCRs and in their prediction from antigen sequence/structure [51, 52]. Many experimental techniques involving either cellular of biochemical assays have been developed, but complexity and costs of these methods address the need of reliable *in silico* approaches to reduce and guide them.

Protective T epitopes characterization involves different issues that are related to the complexity of their processing and presentation on MHC I and MHC II; merely screening all possible MHC-binding peptides does not in fact directly correlate to their role in inducing immunity. Physiological pathogen-specific T-cell activation involves in fact several steps, comprising antigen digestion by the proteasome/immunoproteasome, interaction with the transporter associated with antigen processing (TAP) protein for MHC I binding, binding to MHC and TCR recognitions. Efficient T epitopes prediction has to take into account all these aspects; ideal immunogenic peptides thus must be efficiently processed by the immunoproteasome and delivered by TAP into the endoplasmic reticulum to bind to MHC I. Moreover, considering the human leukocyte antigen (HLA) allelic diversity, effective vaccine peptides have to be recognized by haplotypes widely shared among the population [53, 54].

To date many online tools are available to predict cleavage, TAP translocation, and HLA specificity for MHC I and MHC II binding. Several databases reporting binding peptides are available online as well. The synergistic use of these tools can noticeably restrict the number of peptides to be experimentally analyzed. Here we describe *in silico* and *in vitro* approaches, reviewing the most used databases together with structure- and sequence-based prediction methods and experimental procedures used to validate algorithms output.

3.1. In Silico Approaches: Databases. As described previously, protective T epitopes prediction has to take into account different aspects.

A first analysis can be easily done using databases of well-characterized peptides recognized by T cells (Table 1). As an example, the IEDB database (http://www.iedb.org/) collect a large number of peptides already identified, documented in literature, or voluntarily submitted by users. It includes peptides known as MHC binders derived from alloantigens and antigens involved in pathogen infections, allergies, and autoimmune diseases. The database can be easily accessed through a search engine retrieving information about host specificity, HLA restriction, and binding affinity. It also provides analysis and prediction tools that require only antigen primary sequence [28].

Another example of database comprising huge number of peptides characterized and available in the literature is SYFPEITHI (http://www.syfpeithi.de/), which includes as well algorithms calculating binding affinity of a query peptide to a specific MHC type [55, 56].

Other more specific databases are available to date, most notably the HIV-dedicated B- and T-cell epitope database (http://www.hiv.lanl.gov/). As the above-cited databases, besides a search engine that allows the user to look for HIV epitopes specific for CTL or helper T lymphocytes, this database includes a panel of different tools that offer different search options and permit to work with HLA sequences providing graphical distribution of the most frequently targeted regions.

Selecting target HLAs is another crucial step in epitope-based vaccinology, as an effective preparation has to include protective epitopes capable of binding MHCs in the majority of individuals; the IMGT HLA database (http://www.ebi.ac.uk/ipd/imgt/hla/) provides updated information about HLA alleles and polymorphisms with their relative distribution among the population [57].

3.2. In Silico Approaches: Structure-Based Algorithms. Several algorithms are currently used in T-cell epitopes prediction. Considering the increasing importance of *in silico* modeling in predicting protein-protein interaction, here we review the MHC binding prediction tools. MHC-binding predictors can be divided in two main categories relying on structural or sequence analysis; being complex and computationally expensive, few structure-based algorithms are available to date.

Structure-based MHC binding prediction methods can be clustered in three main categories, based on protein threading, homology modeling, or protein-protein docking. Protein-threading methods use a known peptide/MHC complex structure to predict binding features of others peptides to the same MHC; this process involves the substitution of the original peptide with the one to be tested followed by a side

chains orientation optimization [58, 59]. Discrimination of binders from nonbinders is then performed using different scoring schemes.

Homology modeling has been used to predict MHC-binding peptides and potentially represents an improvement of threading methods since it allows to model both novel peptides and homologous MHC starting from a crystallographic structure [60, 61].

Docking techniques differ from protein threading and homology modeling since they do not rely on a template peptide; their aim is in fact to explore all possible query peptide orientations in the binding with MHCs. Many different docking-based approaches have been extensively used, either based on rigid docking evaluation or on molecular dynamics, and Monte Carlo simulations performed to find the best fitting geometry and evaluate binding strength [62, 63]. These techniques allowed to model proteins of unknown structures and, most importantly, to address experimental studies in the comprehension of protective antigen regions involved in the docking but are not suitable to complete antigenic mapping.

3.3. In Silico Approaches: Sequence-Based Algorithms. Sequence-based methods have been far more developed considering their low computational cost and independency from available crystallographic structures. As happened for B cell epitopes prediction algorithms, in the last decade these methods significantly improved and, starting from simple statistical sequence analysis, have moved towards machine-learning methods.

First attempts were based on the evidence that MHC binding pocket presents cavities with specific residues that require a certain degree of complementarity with specific epitope residues, defined as anchor residues; these algorithms thus search for this type of residues in specific positions, giving the highest contribute in MHC/epitope bindings. However, this strategy completely dismisses the contribute of nonanchor residues, resulting in a prediction lacking specificity and sensitivity [64].

From a simple search of specific residues, new algorithms moved towards a binding matrix-based strategy that takes into account residue frequencies at each epitope position; scoring matrices are built on the sequences of experimentally known binders and comprise information about position-specific frequencies and binding affinity. Binding matrices algorithms return more reliable results, and some of them, such as SYFPEITHI (http://www.syfpeithi.de/Scripts/MHCServer.dll/EpitopePrediction.htm) and BIMAS (http://www-bimas.cit.nih.gov/), are still used and are part of many prediction servers [56, 65]. An improvement of binding matrices algorithms is represented by the stabilized matrix method (SMM); Peters and Sette optimized a standard matrix algorithm strategy including a new score for heavy nonbinders peptides and a regularization technique to minimize the distance between predicted scores and experimental binding affinities contained in the training dataset [66]. The combination of this SMM with a pair coefficient that calculate a score for peptide residue pairs is included in the IEDB database and, together with ANN algorithms, showed the best prediction results in a broad comparative evaluation of MHC I binders predictors [67–69].

Novel algorithms evolved and adopted machine-learning approaches such as ANNs, HMMs, and SVMs; these algorithms have the advantage to perform predictions handling nonlinear data. ANN algorithms are some of the best predictors; they represent epitopes features as amino acid descriptors and perform complex pattern recognition after being trained with a dataset of epitopic and nonepitopic peptides. Their main drawback is the capability to predict epitopes only when query peptides and the training dataset are of the same length. Considering MHC II epitopes length variability, an alignment of peptides contained in the dataset to search for a pattern in the sequence core of defined length is necessary [70].

To date there are tens of online tools to predict MHC I and MHC II epitopes; considering the lack of standardization in dataset, the heterogeneity in output features and a highly variable performance of the same algorithm depending on the HLA type, defining the most reliable predictor, is not trivial. Lin et al. defined a standard benchmark protocol for both MHC I and MHC II predictors and tested the performance of the most used algorithms [68, 70]. The first conclusion describes a lower prediction accuracy (measured as A_{ROC}) for MHC II algorithms than for MHC I that is explained by the increased biological complexity in terms of peptide length. Among the others, they identify the ANN and SMM algorithms embedded in the IEDB website together with NetMHC (http://www.cbs.dtu.dk/services/NetMHC/) ANN as the best predictors for MHC I epitopes [66, 71, 72]. For MHC II epitopes, the ANN algorithm Net-MHCIIpan (http://www.cbs.dtu.dk/services/NetMHCIIpan/), the SMM IEDB and PROPRED (http://www.imtech.res.in/raghava/propred/) outperformed the other methods [73, 74].

Although MHC binding prediction algorithms have reached high performances, they do not take into account the biological processes involved in epitopes production; predicted epitopes might not in fact be produced from antigen degradation [75, 76]. Many strategies exploiting sequence-based and machine-learning algorithms have been developed to predict antigen cleavage from the proteasome/ immunoproteasome and TAP interactions. These tools are available either as stand-alone online servers or integrated with other algorithms to provide a complete prediction from the whole antigen to single epitopes. Furthermore, many of them are embedded in online databases.

Among the others, the ANN algorithm NetChop-3.0 (http://www.cbs.dtu.dk/services/NetChop/) seems to be the best predictor for proteasome cleavage; it is part of the online server NetCTL (http://www.cbs.dtu.dk/services/NetCTL/) for complete prediction [77, 78]. The whole suite is also part of the IEDB analysis tools. Another processing prediction algorithm is FragPredict, which predict both antigen cleavage searching and TAP binding; it uses a statistical analysis to search for amino acid motifs characterizing proteolytic sites [79, 80]. FragPredict is part of the MAPPP server (http://www.mpiib-berlin.mpg.de/MAPPP/), which takes positive peptides and further analyzes them for MHC

Table 1: Examples of the most commonly used databases and sequence-based algorithms for T-cell epitopes prediction.

Databases	Link	Algorithms used (cited ones)
Immune Epitope Database (IEDB)	http://www.iedb.org/	Stabilized Matrix Method-NetMHC-NetMHCIIpan-NetChop
SYFPEITHI	http://www.syfpeithi.de/	SYFPEITHI
HIV Molecular Immunology Database	http://www.hiv.lanl.gov/	
IMGT/HLA Database	http://www.ebi.ac.uk/ipd/imgt/hla/	
Sequence-based algorithms	**Link**	**Brief description**
SYFPEITHI	http://www.syfpeithi.de/Scripts/MHCServer.dll/EpitopePrediction.htm	Use of anchor residues Score based on frequency in natural ligands
BIMAS	http://www-bimas.cit.nih.gov/molbio/hla_bind/	MHC I epitopes predictor Use of coefficient tables of dissociation halftime
Stabilized Matrix Method	http://tools.immuneepitope.org/main/html/tcell_tools.html	*Peters and Sette, 2005* Score system for nonbinders Use of training datasets
NetMHC	http://cbs.dtu.dk/services/NetMHC/	Artificial neural network MHC I epitopes predictor Trained with 57 human HLA
NetMHCIIpan	http://cbs.dtu.dk/services/NetMHCIIpan/	Artificial neural network MHC II epitopes predictor Analyze >500 HLA-DR alleles
PROPRED	http://www.imtech.res.in/raghava/propred/	Use of quantitative matrices derived from the literature MHC II epitopes predictor
NetChop	http://cbs.dtu.dk/services/NetChop/	Artificial neural network Proteasome cleavage predictor Part of NetCTL server
FragPredict	http://www.mpiib-berlin.mpg.de/MAPPP/expertquery.html	Proteasomal cleavage sites and proteolytic fragments predictor Part of MAPPP server

binding through the BIMAS and SYFPEITHI algorithms [81] (Table 1).

3.4. In Vitro Approaches: Cell-Based Methods. Experimental techniques for T-cell epitopes mapping can be roughly divided in two main groups defined as cell based and cell free.

Cell-based techniques mainly involve the screening of synthetic peptides on T-cell population to evaluate binding specificity. The aforementioned computational methods play a fundamental role to focus the analysis on a selected cohort of peptides, reducing the number of potential ligands to be tested. Hereafter, we review the most common approaches used to date [82].

A broadly used cell-based approach is the enzyme linked immunospot assay (ELISPOT) [83]; it evaluates T-cell cytokines secretion levels (generally IFN-γ) after antigen recognition. In details, lymphocytes are incubated on plates coated with anticytokines Abs with different peptides to be tested. Produced cytokines are captured and secretory activity is then evaluated immunochemically. The advantages derived from this technique mainly consist in its high resolution (single-cell) and high throughput results that can be further improved by the use of dedicated scanners allowing the scaling-up of the technique.

Other cell-based assays are based on flow cytometry techniques that allow the selection of activated T cells. A widely used approach involves the culture of T cells in copresence of putative epitopes and a secretion inhibitor [84]. Activated cells are then sorted through after intracellular staining of retained cytokines with labeled Abs; different cytokines can be simultaneously evaluated using specific fluorescent-labeled antibodies. The most important limitation of this technique consists in the requirement of high quality sorting facilities.

Lymphoproliferation assays rely as well on cytometric relevation; they consist in the uptake of the CFSE dye from T cells before activation [85]. After incubation with different peptides, antigen stimulation is evaluated through dye dilution caused by activated T-cell proliferation.

The use of cell-based techniques presents several advantages, most notably the possibility to test the putative T cell-activating peptides directly against target cells. The main drawback consists in the need to be addressed by preliminary computational studies to reduce time and resources expense.

3.5. In Vitro Approaches: Cell-Free Methods. Many cell-free methods have been developed to identify a definite antigen region potentially able to stimulate an effective T-cell response. Here, we briefly review one of the most promising approaches adopted in this research field [86]. It consists in recreating the antigen-processing compartment through the proteolytic digestion of an antigen of interest. The whole antigen is incubated with adequate soluble MHC molecules and proteases (mainly cathepsins and exopeptidases). Digested peptides specifically recognized by MHC molecules are bound and eluted after immunoprecipitating the complex, and T epitopes can then be analyzed by MS to identify immunogenic protein domains. The most important advantage of this assay relies on the direct employment of the whole antigen present on the pathogen to be targeted and on the simulation of its protelytic digestion into immunogenic peptides. The use of the entire antigen can permit, in fact, the identification of antigen-derived peptides that can be omitted during a synthetic peptide library design and/or during the *in silico* evaluation of the peptides to be assayed [87]. Moreover, the use of mass spectrometry methods allows the recognition of peptide posttranslational modifications that can affect the binding.

4. Discussion

Several approaches combining the use of computational analysis with laboratory techniques have been widely described in the scientific literature [88–93]. Here we take influenza virus as an example of hypervariable pathogen that requires the development of novel vaccinal strategies to elicit a broad immune response. Two studies are reported as examples of B-cell epitope characterization and T-cell-activating peptides identification through the combination of computational and experimental approaches.

First example regards the epitope characterization of PN-SIA28, a mAb endowed with potent neutralizing activity against highly phylogenetically divergent isolates of Influenza A virus and directed against a conserved region of the surface glycoprotein hemagglutinin. PN-SIA28 has been characterized through different experimental and *in silico* approaches [94–96]. In particular, Clementi et al. employed techniques such as random peptide library screening, alanine scanning on HA, and *in vitro* generation of escape viral variant under mAb selective pressure. The experimental derived data have been then analyzed through freely available bioinformatics tools, allowing the identification of the putative epitope recognized by PN-SIA 28. More in details, the analysis of mimotopes sequences selected through the peptide panning technique has been performed using *Pepitope,* a freely available online server. It allowed the identification of putative PN-SIA28 epitope through the superimposition of panning-selected peptide structural motifs on HA crystal structures. Epitope preliminary prediction has been confirmed and extended by experimental approaches such as alanine scanning.

As previously described, T-cell epitopes prediction requires the use of databases and bioinformatic tools to address experimental studies. Predictive algorithms are employed to significantly reduce the number of putative peptides to be tested against T cells. As an example, Wang et al. used the NetCTL server, which rely on ANN-based algorithms to predict proteasomal cleavage, interaction propensity to TAP and MHC bindings to obtain a limited number of putative HLA-binding peptides derived from influenza A proteins [97]. The binding-dependent T-cell activation of *in silico* identified peptides has been then evaluated through cell-based techniques such as ELISPOT and intracellular cytokines staining. This integrated study identified 13 peptides highly conserved among the H5N1 Influenza subtype able to elicit a T cells-mediated immune response. Later on, the same research group used an almost identical approach to extend their analysis to protein domains less conserved but more protective [98]. Considering both researches, Wang et al. characterized 30 peptides capable of elicit a cellular immune response that require *in vivo* studies to verify their protective activity. These combined approaches are largely used to target different hypervariable viruses [99, 100] and have been extensively used as well to study nonviral pathogens [101–104].

5. Conclusions

Hypervariable viruses still represent a major world health threat. The identification of conserved protein domains, shared among the different viruses and able to elicit a protective immune response, opens new perspectives in the development of epitope-based vaccines. In particular, the discovery of protective mAbs, able to target these broadly shared protein motifs, permits to work on the identification of peptides able to mimic these epitopes, and hopefully, to elicit an immune response similarly protective. Moreover, the possibility to identify peptides able to elicit an effective T-cell response against these viruses can enormously implement the efficacy of a new vaccine formulation able to elicit both T- and B-cell protective responses (Figure 1). Here, we reviewed different strategies based on experimental techniques and aimed to reach this main "goal" through the use of "*in silico*" strategies allowing to address and analyze the empirical obtained data and reducing experimental time and costs by improving identification efficacy.

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Research Article

Increased Risk of RSV Infection in Children with Down's Syndrome: Clinical Implementation of Prophylaxis in the European Union

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Prospective cohort studies show that Down's syndrome (DS) is an independent risk factor for hospitalization for RSV bronchiolitis. It is unknown whether this observation has been translated into specific management for DS children. The primary goal was to assess the knowledge of healthcare providers in the European Union about RSV infection in DS children and to determine whether it influenced the implementation of prophylaxis. DS caregivers were surveyed using a standardized questionnaire, and country-specific guidelines were obtained. Fifty-three caregivers participated. Thirty-nine (86.7%) had knowledge of the increased risk of severe RSV infection in DS children, and 30 (71.4%) graded that it was important to have a statement on the use of RSV prophylaxis in existing guidelines. Twenty-eight participants had a local DS guideline; hard copies of twelve unique guidelines were obtained. Only one (8.3%) contained a statement on RSV prophylaxis for DS, and five considered such a statement for the next version. *Conclusion*. Most pediatricians had knowledge that DS children have an increased risk of severe RSV infection. Despite the lack of a specific RSV prophylaxis trial in DS, they felt that a statement on RSV prophylaxis in DS guidelines was important, but this was rarely present in current guidelines.

1. Introduction

Children with Down's syndrome (DS) suffer more often and more severely from respiratory tract infections. Respiratory syncytial virus (RSV) is a common virus that everybody encounters, which can cause severe infection in high-risk infants. In 2007, studies showed that DS itself is an independent risk factor for severe RSV infection and hospitalization (OR 12.6) [1]. This was confirmed in 2009 and in 2012 [2, 3]. In addition, children with DS have a significantly longer length of hospital stay [4, 5]; they require more frequent mechanical ventilation [4, 5] and sustain a higher mortality rate [5]. RSV cannot be cured and can only be prevented. RSV management includes education of parents on how to prevent infection, the implementation of good hand hygiene, and/or the monthly administration of palivizumab (a monoclonal antibody against the RSV-F protein) during the RSV season [6]. This humanized monoclonal antibody neutralizes the virus as it binds to the antigenic site of the F-fusion protein of RSV. The fusion protein neutralized both RSV serotypes A and B. Palivizumab has become the mainstay for infants with other risk factors for severe RSV bronchiolitis, such as congenital heart disease, chronic lung disease, and prematurity. In those infants, palivizumab has proven to reduce hospitalization rates by 39%–78% [7]. In these populations, the adoption of RSV prophylaxis varies from 25%–100% [8].

The routine use of prophylaxis for children with DS who have additional risk factors for RSV hospitalization is unknown. In addition, it is not known whether the recent knowledge regarding the higher risk of severe RSV bronchiolitis in children with DS has influenced the approach to RSV prophylaxis. In order to determine whether the

emerging scientific evidence has impacted RSV prophylaxis in DS infants, both direct and indirect influences merit investigation. An indirect influence is defined as any change in knowledge, awareness, or attitude. Direct influence is any change in behavior, which can include changes in clinical practice, both observed and self-reported. The primary goal of this study was to assess the knowledge of healthcare providers in the European Union (EU) about RSV infection in DS children with and without additional risk factors and to determine whether it has influenced the implementation of RSV prophylaxis. The information will hopefully provide a solid foundation for future research on this topic.

2. Materials and Methods

2.1. Questionnaire for DS Caregivers. Of the 27 countries in the EU, 25 with more than 1 million inhabitants were selected. Included countries were the following: Austria, Belgium, Bulgaria, Cyprus, the Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, The Netherlands, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, and the United Kingdom. The target number of participants per country was based on the estimated number of children with DS in that particular country. The estimated number of pediatricians needed in the EU was 68. The sample size was derived based on the combined total of the estimate per country using the population, the birth rate, and the incidence of DS (Table 1).

Local DS patient organizations in the included countries were asked to provide contact information of pediatricians to participate in the questionnaire. Additional participants were recruited using snowballing (every participant was asked to provide the contact details of two pediatricians specialized in the care of infants with DS). If this resulted in insufficient participations, more potential participants were identified, first, by conducting web-based searches for pediatricians who published articles on DS, RSV, or general pediatric topics and for location for DS clinics preferentially and large hospitals and second, by asking contacts in the countries to identify potential participants. Contact was made using standardized call scripts and electronic mails. Participation was voluntary and non-anonymous.

Indirect knowledge was defined as the percentage of DS caregivers that responded affirmatively to the question "Were you aware that RSV bronchiolitis occurs more often in children with Down's syndrome?" The attitude of DS caregivers regarding the implementation of RSV prophylaxis was determined using a Likert scale that ranged from "very important" (A) to "I do not know" (F) by asking the respective individuals how they felt about the inclusion of a statement on RSV prophylaxis in DS in current guidelines issued by country-specific pediatric advisory bodies. The percentage of DS caregivers answering "very important" was considered a positive attitude. Participants were asked whether they have a local DS guideline, whether this guideline contains a statement on RSV management, and, if not, whether such a statement is being considered for the next version of the guideline. When there was a statement in the guideline about RSV prophylaxis, participants were asked whether and how prophylaxis is reimbursed. Participants were asked which children with DS qualify for RSV prophylaxis locally and whether risk factors are included in the decision to administer RSV prophylaxis. In addition, all participants were asked to provide an estimation of the percentage of children with DS in different subgroups receiving RSV prophylaxis. The familiarity with DS was assessed by asking the participants to estimate the number of children with DS they take care of every month, the incidence of DS in their country and the RSV hospitalization rate for the different subgroups of children with DS.

2.2. Down's Syndrome Guidelines. Direct knowledge was defined as the percentage of existing guidelines with a statement on RSV prophylaxis. The local guidelines were obtained via pediatricians, DS patient organizations, and websites. In the obtained local DS guidelines, we evaluated whether the guideline contained the word *RSV* and the word *palivizumab* or *Synagis* or *monoclonal antibody*. We assessed whether the guideline stated that RSV infection is found more often in children with DS and whether the guideline stated that children with DS suffer more severely from RSV infections. We also determined whether the guideline contained a statement on which subgroups of children with DS should receive RSV prophylaxis. Statements were assessed for clarity and the availability of supporting information about RSV infections in children with DS.

2.3. Statistical Analysis. A database was set up in Excel and SPSS 20. Characteristics of the participants and the outcomes were summarized, and results were analyzed using frequencies and proportions. The indirect influence of attitude is portrayed as a radar figure which shows the proportion of the different opinions relative to each other. An overview of the results for the direct influence of existing knowledge is provided in a heat map. Results were analyzed using the Chi-square test or ANOVA where appropriate. Significance was set at a P value of ≤ 0.05. Participants with missing data were excluded from analyses involving the respective variable.

3. Results

3.1. Questionnaire for DS Caregivers. The participation rate was 77.9% (53/68; Table 1). There were no participants from Latvia, Bulgaria, and Cyprus. Eight participants (15.1%) were linked to a DS patient organization. Most participants were pediatricians ($n = 41$; 78.8%), two participants were not in the medical field (3.8%), and two were nurses. The mean number of children with DS regularly taken care of within the local hospitals was 171 (SD 288, range 0–1200).

All participants had knowledge of RSV bronchiolitis, six (13.3%) were not aware of the increased risk in DS patients, and the majority ($n = 39$, 86.7%) were aware that RSV bronchiolitis occurs more often in children with DS (Figure 1). The source of this knowledge was almost always the scientific literature ($n = 32$; 82.1%). Data were not available from eight participants. The majority ($n = 30$, 71.4%) of participants reported that a statement on RSV prophylaxis in DS guidelines is important or very important, whereas only three (7.1%) reported this to be unimportant; seven

Table 1: Number of pediatricians needed in the European Union.

Country	Population[1]	Birth rate 2011[2]	Incidence DS[3]	Participations
Austria	8.2	9	15,66	1
Belgium	10.4	10	16	3
Bulgaria	7.1	9	12,29	0
Cyprus	1.1	0*	17,12**	0
The Czech Republic	10.2	9	18,94	1
Denmark	5.6	10	16,92	2
Estonia	1.3	10	17,12**	1
Finland	5.3	10	24,53	2
France	65.3	12	29,42	7
Germany	81.5	8	26,31	6
Greece	10.8	9	17,12**	1
Hungary	9.9	10	13,32	1
Ireland	4.7	16	26,82	3
Italy	61.0	9	16,28	5
Latvia	2.2	10	17,12**	0
Lithuania	3.5	9	17,12**	1
The Netherlands	16.8	10	15,19	1
Poland	38.4	10	15,06	0
Portugal	10.8	10	7,61	4
Romania	21.9	10	17,12**	1
Slovakia	5.5	10	17,12**	1
Slovenia	2.0	9	17,12**	1
Spain	46.8	11	27,2	2
Sweden	9.1	10	29,3	2
The United Kingdom	62.9	12	28,09	7
Total				53

[1]Population in millions (http://www.europa-nu.nl/).
[2]Births per 1000 populations (http://www.indexmundi.com/).
[3]Incidence per 10.000 births based on average prevalence measured between 1980 and 2009 (http://www.eurocat-network.eu/).
*Birth rate in Cyprus is not published.
**DS incidence is not published; average of all in the database was utilized.

participants were neutral (16.7%), and one participant did not know whether such a statement was relevant (2.4%) (Figure 2). Twelve participants did not provide responses to this question.

Five participants (11.1%) stated that they have a local DS guideline with a statement on RSV prophylaxis, and according to five participants such a statement is being considered for the next version of the guideline. Three participants responded that they never use palivizumab for children with DS (5.6%), and two used palivizumab for all children with DS (3.8%). Thirty one (58.5%) and 25 (47.2%) of the participants supported the implementation of RSV prophylaxis in DS patients with congenital heart disease or prematurity, respectively. Participants responded most commonly that DS children with CHD and those born prematurely (<32-week gestational age) received RSV prophylaxis *regularly* (defined as >10% receiving prophylaxis) ($n = 21$, 39.6%; $n = 18$, 34.0%; Figure 4). Preventative hygiene and education is promoted by only 11 (20.8%) of the participants.

3.2. DS Guidelines. Not all guidelines were obtained, mostly because the guideline was not available or not in English. A copy was obtained of twelve unique guidelines. Guidelines were obtained from Belgium, Germany, Ireland, and The Netherlands, and two distinct ones were obtained from the group representing Italy, Spain, the United Kingdom, and Portugal. The guidelines and publication dates varied from 2001 to 2011. The presence of a DS statement was confirmed in only one guideline ('The Netherlands'; Figure 3); this was also the only guideline that contained the words RSV and palivizumab or Synagis or monoclonal antibody.

3.3. Factors Associated with Implementation. Participants in the questionnaire were asked to provide their best estimate of the incidence of RSV hospitalization. Hospitalization rates did not significantly influence implementation of a guideline for RSV prophylaxis. An estimated incidence of DS of >1 : 800 live births was arbitrarily considered a high incidence of DS. A high incidence of DS was associated with having

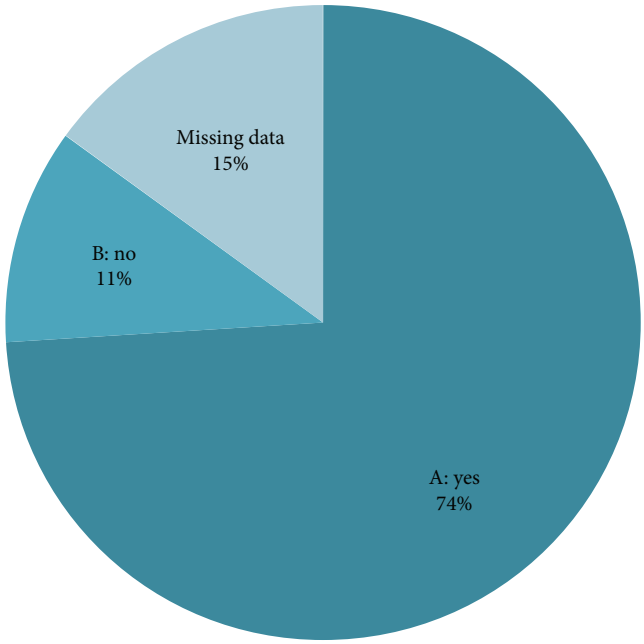

Figure 1: Responses to the following question: Were you aware that RSV bronchiolitis is found more often in children with DS? (A) indicates the answer yes, I was aware. (B) indicates the answer no, but I have heard of the disease. (Missing data) indicates the percentage of participants who did not answer this question.

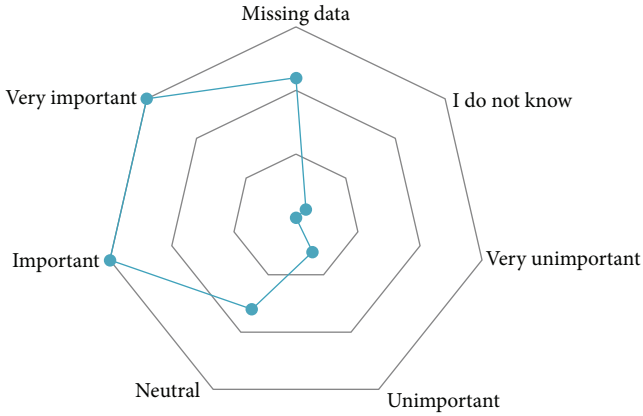

Figure 2: Responses to the following question: How do you feel about a statement on RSV palivizumab use in guidelines/protocols for management of children with DS? The responses are shown in relation to each other.

a DS guideline ($n = 5$, 83.8% compared with $n = 22$, 59.5%).

4. Discussion

Children with Down's syndrome have a very high risk of severe RSV bronchiolitis. Implementation of this knowledge in the care for these children, however, is unknown. We found a statement on the use of RSV prophylaxis in only one (8.3%) of the twelve guidelines. According to five DS caregivers who

Figure 3: RSV statement in guidelines. Heat map according to the responses in the questionnaire about the presence of a guideline and the presence of a statement in the guideline.

participated in the questionnaire, such a statement on the use of RSV prophylaxis is being considered for the next version of the local guideline. Overall, there was a high awareness of the increased risk of RSV infection for children with DS among DS caregivers, and the majority of participants felt that a statement on RSV prophylaxis in DS medical guidelines was very important.

This is the first study to investigate the breadth of knowledge regarding RSV infection among DS healthcare providers and the impact of their knowledge on the implementation of RSV-specific prophylaxis in the countries of the European Union. In this survey, only 2 to 21 (3.8%–39.6%) of the participants estimated that RSV prophylaxis is given *regularly* to children with DS for a defined indication. The lower rate of prophylaxis might explain why the hospitalization rate is estimated higher, most commonly >10%, compared with the expected rate of 1%-2% with palivizumab use. The gauged hospitalization rate by the caregivers for healthy DS children closely aligns with the literature with 5%–10% being the most common answer. Paes et al. [9] observed a 4-fold proportional increase, from 2006–2010, in the number of patients receiving palivizumab for off-label underlying medical disorders of which the largest increase was evidenced in the DS group that comprised 20.3% of the entire cohort [9]. Although the study did not distinguish DS infants with or without risk factors, more recently Zachariah et al. confirmed that DS infants independent of risk factors are at higher risk for hospitalization following RSV lower respiratory tract illness [3]. Our study also shows that palivizumab is being used "off-label" in the EU for children with DS without additional risk factors. Two (3.8%) of the participants estimated that

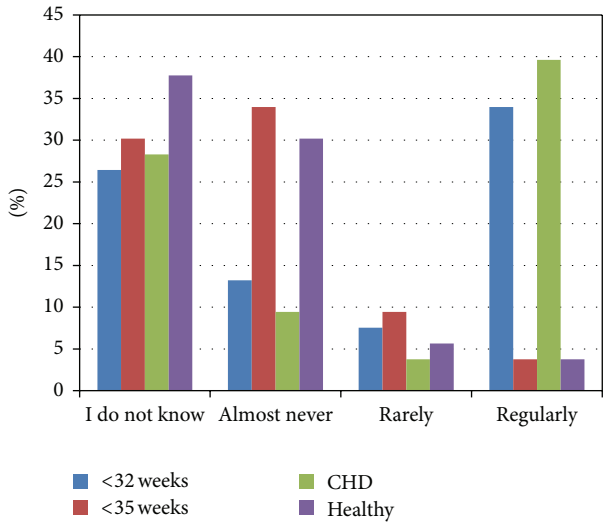

Figure 4: Answers to the following question: Can you give an estimation of the percentage of children in your country/state receiving RSV prophylaxis among the following groups? Answer options are given as follows: I do not know, almost never (0%–5%), rarely (5%–10%), regularly (>10%); <32 weeks: children with DS born prematurely gestational age <32 weeks (missing data: $n = 10$, 18.9%); <35 weeks: children with DS born prematurely gestational age <35 weeks (missing data: $n = 12$, 22.6%), CHD: children with DS and congenital heart disease (missing data: $n = 10$, 18.9%); healthy: otherwise healthy children with DS (missing data: $n = 12$, 22.6%).

>10% of the healthy children with DS receive RSV prophylaxis.

Principles governing the lack of a specific RSV management guideline for children with DS (direct influence) cannot be determined from this research. Anderson et al. determined that lack of understanding of the severity of RSV infection is a major obstacle for successful implementation of RSV prophylaxis [10]. In our study, the participants did show an awareness of the increased risk of severe RSV infection in children with DS. Since the indirect influence appears positive and indirect and direct influences are not significantly associated, additional factors must impact the implementation of prophylaxis. One factor may be patient agreement to receive prophylaxis and ongoing commitment to complete the course of injections throughout the RSV season. Compliance with palivizumab is improved with home-based administration compared with office-based administration [8]. Anderson et al. [10] also identified that pediatricians wanted more educational materials about RSV disease for both personal and family edification and felt that parent reminders might improve compliance and the implementation of a successful program. Warren et al. [11] optimized RSV prophylaxis using a provincial approval system and reached 100% of eligible children with congenital heart disease. Our research does not address potential patient factors impairing the implementation of prophylaxis, and we did not investigate how cost of the monoclonal antibody may impede the adoption of prophylaxis across the EU.

Strengths and limitations of this study merit further discussion. First, conducting a patient-based study to determine reasons that may govern the implementation of prophylaxis was not feasible. Second, a nonvalidated questionnaire was used. Third, the proportion of valid responses in the questionnaire was lower for participants with an RSV statement in their local guideline. Fourth, not all available guidelines were collected, and proportionally more English than non-English guidelines were obtained. However, participants from 22 different countries took part in this research. Fifth, both the direct and the indirect influences have been addressed using the questionnaires and complementary local guidelines. Several questions had a considerable proportion of missing data. Although this is a methodological limitation, it also provides additional information regarding which questions were most difficult to answer.

RSV bronchiolitis is a major health issue in children with Down's syndrome, as it accounts for 17.6% [5] of all DS admissions to hospital (compared with 7%–9% overall [12]). The incidence of hospitalization for RSV infection in children with DS in large cohorts is 9.9%–17.6% [1, 5], which is higher than the hospitalization rate (1%) in the normal population. Moreover, it has recently been shown that palivizumab treatment reduces the wheezing days in otherwise healthy-late preterm infants [13]. This might be of particular interest for children with DS, as they have an increased risk of recurrent wheezing, although the role of RSV in recurrent wheezing in DS is not yet established [14].

Despite this knowledge, RSV management has not been integrated into the care of children with DS. The results of this study can be considered a baseline for future research on this topic. The results may be instrumental in designing and implementing a prophylaxis program aiming to improve the care of children with DS through the prevention of RSV bronchiolitis.

Five years after the first publication on the risk of severe RSV bronchiolitis in children with DS, the majority of the participating DS caregivers in the EU are aware of the increased risk of severe RSV bronchiolitis for children with DS. Despite the absence of a randomized placebo-controlled trial, in general the implementation of specific RSV management for this group was deemed to be very important. Nevertheless, virtually no guidelines in the EU have a statement on the management of RSV bronchiolitis in this high-risk population.

Abbreviations

DS: Down's syndrome
EU: European Union
RSV: Respiratory syncytial virus.

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Review Article

JC Polyomavirus (JCV) and Monoclonal Antibodies: Friends or Potential Foes?

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Progressive multifocal leukoencephalopathy (PML) is a demyelinating disease of the central nervous system (CNS), observed in immunodeficient patients and caused by JC virus ((JCV), also called JC polyomavirus (JCPyV)). After the HIV pandemic and the introduction of immunomodulatory therapy, the PML incidence significantly increased. The correlation between the use of natalizumab, a drug used in multiple sclerosis (MS), and the PML development of particular relevance. The high incidence of PML in natalizumab-treated patients has highlighted the importance of two factors: the need of PML risk stratification among natalizumab-treated patients and the need of effective therapeutic options. In this review, we discuss these two needs under the light of the major viral models of PML etiopathogenesis.

1. Progressive Multifocal Leukoencephalopathy (PML) and JC Polyomavirus (JCV): An Epidemiological Overview

1.1. The Emergence of PML in the Monoclonal Antibody Era. Progressive multifocal leukoencephalopathy (PML) is a demyelinating disease of the central nervous system (CNS) usually observed in immunodeficient patients. The first case was described in 1958 [1], and the detection of inclusion bodies in the nuclei of damaged oligodendrocytes suggested a possible viral cause. The etiological agent of PML was isolated in 1971 and named JC virus ((JCV), also called JC polyomavirus, (JCPyV)), after the initials of the studied patient [2, 3].

After the HIV spread, the PML incidence has increased 50-fold compared to previous years and 80% of PML cases are represented by HIV-positive patients [4]. Since the advent of antiretroviral therapy, the incidence of PML in AIDS patients is still estimated to be 0.07/100 persons/year, and it has not decreased as significantly as other opportunistic infections [5–8].

In the very last years, PML has become a growing concern in other categories of patients, and its incidence remains high. The new cases of PML are associated to the use of novel immunomodulatory therapies in patients affected by several diseases, such as multiple sclerosis (MS), Crohn's disease, non-Hodgkin's lymphoma, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and autoimmune hematological disorders [9, 10]. The incidence of PML in patients under immunomodulatory therapy depends on the drug used and on the treated disease. For example, the risk of PML during rituximab administration, an anti-CD20 humanized monoclonal antibody (mAb), has been estimated to be approximately 1/4000 when used in SLE patients and 1/25000 when used in RA [11]. An even higher incidence (1/500) was observed in psoriatic patients treated with efalizumab, a humanized mAb against a T lymphocytes adhesion molecule, and as a consequence efalizumab was voluntarily withdrawn from the market [12].

1.2. PML and Natalizumab: The Evidence. In the literature, the association between natalizumab administration and PML has been widely reported and described. Natalizumab

is an IgG4/κ humanized mAb, which interferes with the interaction between *very late antigen-4* (VLA-4), expressed on leukocytes, and *vascular adhesion molecule-1* VCAM-1 expressed on endothelial cells, thus preventing leukocyte extravasation in inflamed sites [13]. Natalizumab is generally well tolerated, but due to its correlation with PML, it was approved with a restricted distribution format in 2006. In particular, the risk of PML development during natalizumab treatment is very high, and it has been evaluated to be as high as 3.85 per 1000 patients [14], and survival rate is 70% (natalizumab-associated PML has improved survival rate compared with PML in other populations) [15].

Natalizumab is used in several autoimmune diseases but, in particular, for the MS treatment. MS is a chronic inflammatory autoimmune disease of the CNS affecting more than 2.5 million people worldwide, characterized by chronic leukocyte infiltration [16]. Most patients suffer from a relapsing-remitting course that is characterized by about one and two episodes of neurological deficits per year, that often tend to resolve, at least partly, after days to months [17, 18]. Natalizumab reduced the rate of clinical relapse at one year by 68% and the risk of sustained progression of disability by 42–54% over 2 years, turning out to be the most effective drug in MS treatment. Its efficacy in MS is probably correlated to its capacity of blocking leukocyte infiltration into the inflamed plaques within CNS [19].

On the other hand, the pathogenesis of PML in patients receiving natalizumab is complex, and it is not clear whether it is caused by a local (within CNS) or peripheral reactivation of JCV leading to a massive crossing of the blood-brain barrier (BBB) by free or B cells shuttled viral particles. To date, three main molecular mechanisms have been proposed. According to some authors, the blockage of VLA-4 by natalizumab may prevent the entry of JCV-specific cytotoxic T cells into the brain, necessary for the control of latent JCV within infected oligodendrocytes (the viral life cycle will be better explained in what follows) [20]. Another proposed possibility is that natalizumab may inhibit the VLA-4-dependent retention of lymphocytes in bone marrow and spleen (both sites of JCV latency), thus leading to an increase of JCV-infected peripheral leukocytes and to a possible increase of the peripheral JC viral load capable of crossing the BBB (this late aspect has not been confirmed, to date) [21]. Another suggested mechanism is the natalizumab-induced expression of factors involved in B-cell differentiation, such as transcription factor Spi-B, that has been shown to increase JCV transcription, thus probably leading to an increased viral load, at least *in vitro* (Figure 1) [22].

1.3. PML and Natalizumab: The Need of Risk Stratification. Natalizumab was first approved by Food and Drug Administration (FDA) for MS treatment in 2004. Due to the first confirmed cases of PML, its commercialization was suspended in March 2005. In March 2006, the advisory committees of FDA voted in favor of the return of natalizumab in the market as monotherapy in MS with a black box warning about PML. For this reason, it would be very important to have a reliable strategy to quantify the risk of PML in patients with MS [19]. Recently, Bloomgren et al. proposed a clinical flowchart based on three different risk factors, all associated to an increased risk of PML: positive status with respect to anti-JC virus antibodies, prior use of immunosuppressants and increasing duration of natalizumab treatment (prolonged natalizumab treatment likely increased PML risk, but some studies found no evidence of JCV reactivation in natalizumab-treated MS patients of 18 month follow-up study). The reason of this delay is not well characterized [23, 24]. Although important, these kind of studies are far from conclusive especially considering that up to 65% of healthy patients are seropositive to JCV. Other parameters are therefore necessary for a better risk stratification. Recently, a possible risk stratification based on the level of neutralizing activity of the anti-JCV humoral response has been proposed [25]. Overall, a better comprehension of the physiopathology of JCV and of PML would surely help in the identification of a panel of risk factors for a better stratification profile.

2. The Virus: JC Virus

2.1. Viral Genome and Structure. JCV is a member of the Polyomaviridae family, *Orthopolyomavirus* genus [26]. JCV and BK virus ((BKV), which causes a severe nephropathy in kidney transplant recipients) were the first two human polyomaviruses identified, both detected in 1971 [27]. JCV, like all polyomaviruses, is a nonenveloped, icosahedral, and small (≈40 nm) virus with a closed circular double stranded DNA genome [28]. The genome is approximately 5130 bp-long, although single isolate can be differ in length, due to alteration in their noncoding regions [29]. The genome can be divided in three different parts: (i) a noncoding control region (NCCR), (ii) early coding region sequences that are transcribed counterclockwise from NCCR, and (iii) late coding region sequences that are transcribed clockwise from NCCR [27].

The NCCR lies between the early and late coding regions and contains the origin of replication, the TATA box, the T antigen binding sites, the cellular transcription factor-binding sites, a bidirectional promoter, and an enhancer for transcription of early and late genes. NCCR is thought to be the main determinant of the viral tropism. Importantly, modifications on the NCCR region are associated with an increase of viral transcription and replication in patients with PML [30–32].

The early coding region spans 2.4 kb, and it encodes five proteins: the large T antigen, the small t antigen, and three different splice variants [33]. The large T antigen is a 688 amino acids nonstructural, multifunctional protein that regulates the switch from early to late viral proteins transcription and the replication of the viral genome. This protein interacts with a number of cellular proteins (such as retinoblastoma protein and p53), and its role in cancerogenesis is being investigated [34]. The small t antigen and the other T antigen variants are produced by cellular splicing of the large T antigen RNA, and these proteins perform multiple functions and may contribute to PML progression [35].

The late region spans 2.3 kb and encodes four different proteins: agnoprotein, VP1, VP2, and VP3 (Table 1). The

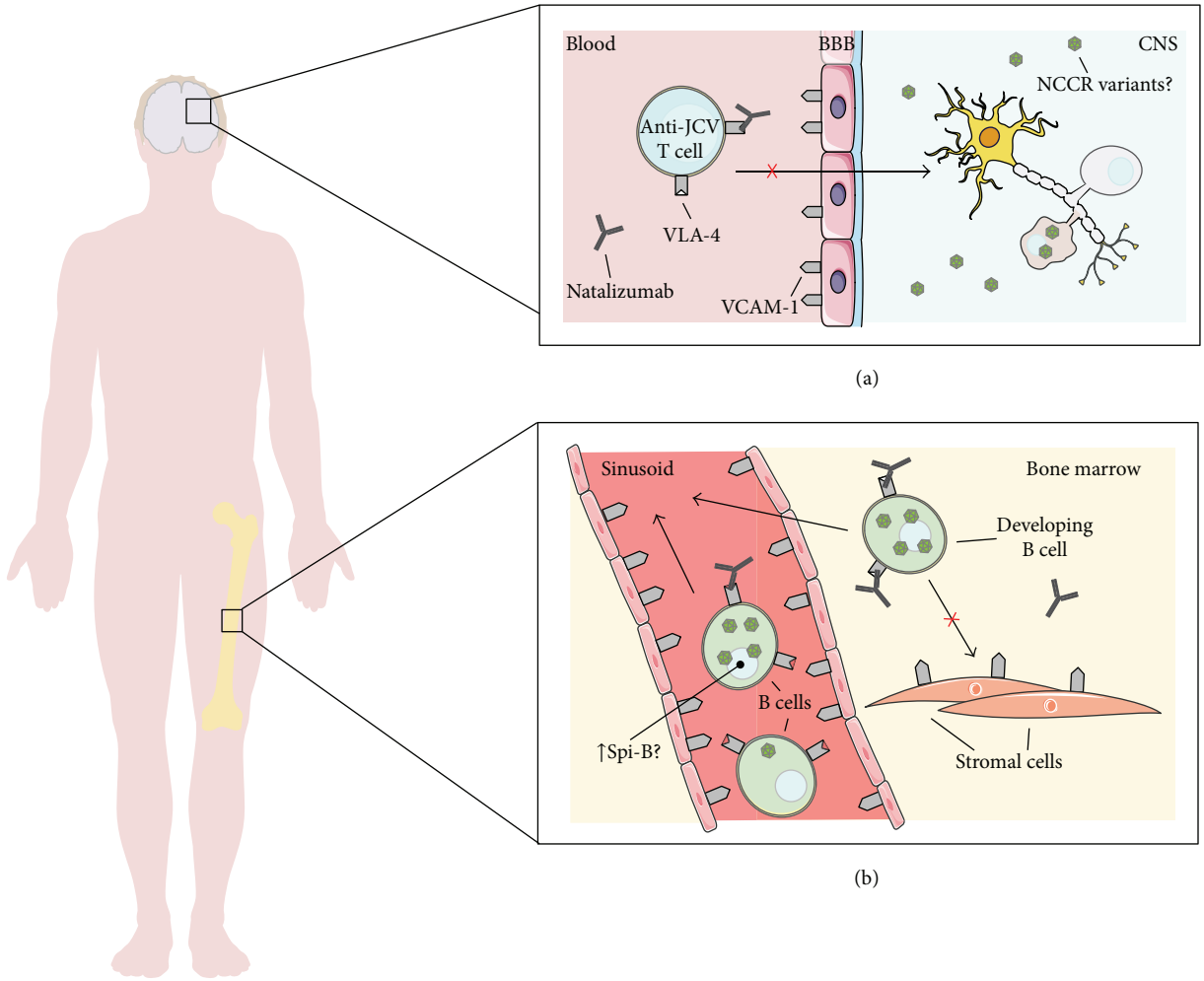

Figure 1: Development of progressive multifocal leukoencephalopathy during natalizumab treatment. This figure summarizes the three hypotheses on how natalizumab may lead to PML. (a) Natalizumab may prevent the entry of JCV-specific cytotoxic T cells into the brain, necessary for the control of latent JCV within infected oligodendrocytes. (b) Natalizumab inhibits the VLA-4-dependent homing and retentions of lymphocytes in bone marrow (sites of JCV latency), thus leading to an increase of JCV-infected peripheral leukocytes. Finally, another possible factor is the natalizumab-induced expression of Spi-B, a transcription factor that has been shown to increase JCV transcription.

smallest protein is the agnoprotein, and it has been proposed to harbor functions during the late phase of infection as follows: interacts with large T antigen to control viral DNA replication and acts as a viroporin facilitating viral release from cells [36, 37]. The other three proteins are structural proteins: VP1 is the major capsid protein and allows the binding and entry into target cells; the VP2 and VP3 are assumed to function during escape from membranous structures and viral nuclear entry as described in SV40 [38–41]. JCV capsids are predicted to contain 360 molecules of VP1 arranged in 72 pentameric subunit, with each VP1 pentamer associated to a single VP2 or VP3 molecule to form the individual capsomeres [42] (Figure 2). Sequencing studies on VP1 from PML patients have shown characteristic mutations not evidenced in JCV isolates from healthy subjects. In particular, the mutations in positions L55, K60, N265, S267, and S269 are all limited to isolates from PML patients and cluster in close proximity to the receptor binding site. According to various authors, some of these mutations could alter the binding specificity of the virus from that dependent on sialic acid to that specific to other sugar moiety [43, 44]. PML-specific mutations are characteristic, but these are not present in all isolates from PML (statistical analysis of JCV sequences demonstrate, that 52% of PML patients carry JCV with one of these mutation, in VP1), and this suggests that VP1 mutations are not the only possible mechanisms leading to PML development.

Furthermore, several JCV VP1 loop-specific polymorphisms (restricted to four positions 74, 75, 117, and 128) have been described to be associated with favorable prognosis for PML [45].

2.2. Viral Lifecycle. The replicative cycle of JCV can be divided into two phases: early and late stages. The early stage begins with the initial interaction of the viral protein, VP1,

Table 1: Nonstructural and structural viral protein.

Protein	Molecular weight	No. of amino acids	Function
Early protein (transcribed counterclockwise from NCCR)			
Large T antigen	79,305	688	Nonstructural protein. role in viral replication and transcription, interaction with host protein and probably in cancerogenesis
Small t antigen	20,236	172	Viral replication
Splice variants called T'_{135}, T'_{136}, and T'_{165}			Viral DNA and cancerogenesis
Late protein (transcribed clockwise from NCCR)			
VP1	39,606	354	Major capsid protein, role in cellular binding and entry functions, and interaction with host receptors; it mediates hemagglutination
VP2	37,366	344	Minor capsid protein, assumed role on escape from membranous structures and nuclear import
VP3	25,743	225	Minor capsid protein assumed role on escape from membranous structures and nuclear import
Agnoprotein	8,081	71	The smaller protein that facilities capsid assembly. It is proposed as a viroporin

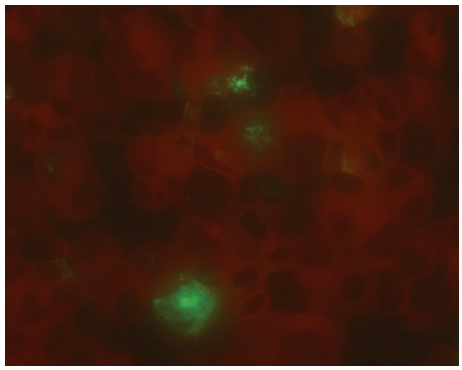

Figure 2: Immunofluorescence staining of COS7 infected by JCV (Mad4), five day after infection. The cells were stained with anti-VP1 monoclonal antibody (green-stained cells) and counterstained with Evans blue (red-stained cells).

with the surface of the host cell and continues until the onset of viral DNA replication. The late phase includes all the events that lead to the release of viral progeny. In both stages, viral and host proteins are essential for the complete viral lifecycle.

The early stage is initiated by adsorption of the virions to the cells surface. *In vitro* studies have demonstrated that JCV requires N-linked glycoproteins containing terminal α(2,6)-linked sialic acid to successfully bind human cells [46]. Recently, LSTc pentasaccharide has been described as functional receptors motif for this interaction [47]. Other studies have also evidenced the importance of the serotonin receptor $5HT_{2A}$ ($5HT_{2A}R$) in viral entry [48]. However it is important to remember that JCV is able to bind different cell lines without producing viral progeny, evidencing the importance of intracellular cell type-specific factors in determining permissiveness to JCV replication [49, 50]. For these reasons, the tropism of JCV turns out to be very narrow; in fact, the virus productively infects stromal cells in tonsillar tissues, some B cells, CD34+ hematopoietic cells, oligodendrocytes, and astrocytes of the human brain.

Similarly, to other DNA viruses, JCV penetrates into the cytoplasm by clathrin-dependent endocytosis [51, 52]. Once inside the cell, JCV is transported through the cytosol to the nucleus by the endosomes, traffics to the endoplasmatic reticulum, and subsequently binds to nuclear pore complexes. The nucleus is the site of viral replication and viral assembly. As with the other members of the family, JCV lifecycle exhibits a fine temporal regulation and is particularly slow (DNA replication is undetectable for some days) [53]. Initially, upon entering the nucleus, transcription of the early viral genes occurs (large T and small t antigens, proteins required for the viral DNA replication), followed by viral DNA replication. After the complete DNA replication, the late viral genes (VP1, VP2, VP3, and agnoprotein) are transcribed [27]. Transcription is regulated by cell-specific factors, while DNA replication is most likely regulated by species-specific factors, and for this reason, JCV has a limited host replication tropism; thus, *in vitro* cell transfection with JCV DNA results in the infection of only those cell types known to allow infection *in vivo* (as tonsillar stromal cells, B cells, and CD34+ hemopoietic cells). In particular, only certain cells have the necessary protein to allow complete viral lifecycle, for example, the nuclear transcription factor NF-1X has been described as a cell-specific regulator of JCV transcription, and this protein is expressed at higher concentration in human brain cells than in other human cells or in nonhuman brain cells [54].

Expression of the viral structural proteins leads to the assembly of the viral capsid. The newly packaged virion

progeny is thought to be released by lysis of the host cell, although electron microscopy observations report secretion of virions from the plasma membrane of intact cells. It remains to be determined whether cell lysis or intracellular vesicular transport is the preferred pathway for the release of JCV progeny virions [55].

2.3. JCV Reactivation: The Clinical Picture and the Laboratory Diagnosis. After infecting the host, the virus can persist in at least two forms: nonpathogenic and a pathogenic forms. In particular, nonpathogenic form is most frequently found in urine, and its NCCR is not rearranged (named "archetype"), while the pathogenic form is principally detected in brain of PML patients, and its NCCR rearrangements (named "prototype") include deletions and duplications of specific sequence elements (however, it is important to remember that postmortem studies have shown the presence of the prototype also in the brain of non-PML subjects) [56].

The pathogenesis of PML is due to the infection of oligodendrocytes by JCV [2, 3]. It is still widely discussed whether PML derives from JCV reactivation within oligodendrocytes infected during the initial phases of the infection or from its peripheral reactivation with a novel massive crossing of the BBB. As a consequence, oligodendrocytes undergo cytolytic destruction that results in loss of myelin, thus leading to the appearance of foci of demyelination, which initially are microscopic and asymmetrically distributed in space. As the disease progresses, the areas of demyelination enlarge and these foci may coalesce, making them visible on gross examination in cut sections of the brain. In addition to the oligodendroglial pathology, greatly hypertrophic giant pleomorphic astrocytes may be observed in areas of demyelination in 80% of cases [57]. The progression of the disease is usually very rapid and leads to death in less than one year from diagnosis, although it was observed that some PML patients can survive for many years [58].

Since PML involves the subcortical white matter, the lesions may manifest as a wide variety of neurological disturbances. The three characteristic symptoms at onset and during disease progression are (i) visual deficit, (ii) motor impairment, and (iii) change in mentation. The most common sign (35–45%) is visual deficit, while motor weakness is the initial sign in 25 to 33% of cases, and approximately one-third of patients shows a change in mentation, as personality change, difficulty with memory, emotional lability, and dementia. Other common symptoms are headache, vertigo, seizure, sensory deficit, parkinsonism, aphasia, and neglect syndrome [59, 60].

The expanding spectrum of iatrogenic conditions favouring PML and the frequent occurrence of atypical cases explain the importance of definitive clinic-radiological and laboratory diagnosis. Diagnostic tests investigating PML include neuroimaging, electroencephalography, component analysis of celebrospinal fluid (CSF), biopsy, and PCR. In a patient with PML, a computerized tomography (CT) scan shows nonenhancing, subcortical hypodensities, that correspond to areas of demyelination. Magnetic resonance imaging (MRI) is superior to CT scanning, in fact it shows not only the number but also the extent of the lesions. Electroencephalography (EEG) is both insensitive and nonspecific for PML, but it may corroborate the presence of a lesion seen on neuroimaging. In particular, EEG shows focal slowing corresponding to white matter lesions and generalized slowing with advancing disease. CSF findings are nonspecific, with most patients demonstrating a normal profile, but in some patients elevation in protein or an increased cell count could be present. All the above may give a strong suspicion of PML, but the confirmatory test is the demonstration of the presence of JCV DNA in CSF or brain by molecular methods [61]. In this test the specificity and the sensitivity are very important; in fact, the former has to be 100%, without cross-reaction with other polyomaviruses, and the later has to be able to detect even very few copies of viral DNA [62].

A problem that may be encountered with serological analysis is related to the high homology between polyomaviruses (e.g., BK virus display 75% homology with JCV) and in serum may be present cross-reactive antibodies that may give a false positive. Since about 65% of the healthy population is positive for JCV-specific antibodies, the fact that a patient does not present these antibodies could be considered as exclusion diagnosis for PML [27].

3. The Role of the Immune System: Friend or Foe?

3.1. Role of Immune System in Viral Pathway. The initial infection with JCV is thought to occur in tonsillar tissue after inhalation, although transmission of the virus through ingestion of contaminated food or water has also been suggested. Tonsillar lymphocytes infected with JCV carry virions to the kidney and bone marrow, the primary sites of viral latency; though several studies examined non-PML, normal brain tissue has suggested that the virus might enter or persist in the brain causing a latent infection that might reactivate in case of immune suppression, leading to a productive infection in oligodendrocytes. Another suggested model through which the lymphocytes may contribute to the dissemination of the infection is a sort of JCV association with the cell membrane without internalization; this could explain why viral DNA, but not RNA, is often detected in lymphocytes [49, 63].

The dissemination to the brain remains to be fully elucidated, even if a hematogenous route of infection of the CNS has been suggested, following a possible "Trojan horse" mechanism for the BBB crossing. This hypothesis is supported by the presence of infected B lymphocytes in multiple PML brain tissue samples [64]. Other studies have shown that JCV may also infect microvascular endothelial cells through infected lymphocytes, and thereby possibly cross the BBB as free virus [62, 64, 65].

Once JCV, as free virus or associated to B cells, crosses the BBB (Figure 3), at least three other events must occur so that the PML develops: (i) the host immune system must be compromised or altered, (ii) the viral NCCR must acquire changes that increase viral transcription and replication, and (iii) DNA binding factors that bind to recombined NCCR sequence motif must be present or upregulated in infected

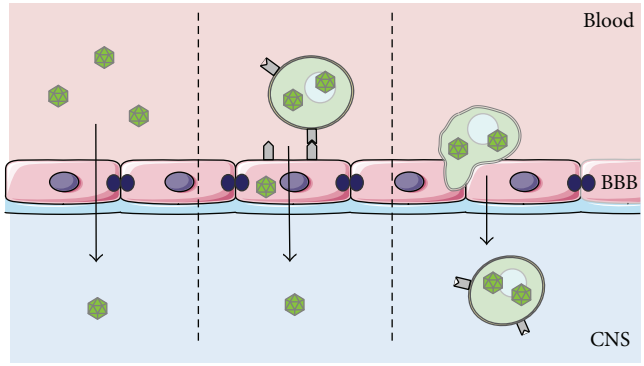

Figure 3: Mechanisms of PML pathogenesis. The necessary condition for the PML developments is that oligodendrocytes are infected by JCV. In this figure the three ways through which the virus could cross the BBB are represented: (a) as free virus or (b) the infection of the endothelial cells of the barrier by JCV-infected B cells (c) using the B cells as "a Trojan horse."

hematopoietic cells, B cells, or glial cells. Only when all these conditions are present, PML develops.

3.2. Immune Control of JCV. Although JCV is widespread in the population, only few people develop the disease, and this occurs only in the presence of underlying changes to the immune system. In fact, before the HIV pandemic and the introduction of immunomodulatory therapies, the PML was very rare, indicating that the infection is well controlled by the immune system. Epidemiological studies of PML in HIV-positive patients show that in pre-HAART era, the incidence of PML varied from 0.3 to 8%, but after the diffusion of antiviral treatment, its incidence decreased (from 0.7 cases/100 personyear to 0.07 cases/100 personyear) [66]. These data suggest that the cellular branch of the immune system probably plays the principal role in the control of the infection. In particular, in PML patients it was observed that the number and the functionality of CD4+ cells are reduced after stimulation with JCV antigen compared to normal control [67]. As a matter of fact, several studies have implicated an impairment of the T-cell response in the PML development, and in particular, an effective cytotoxic T lymphocyte response specific to the viral capsid protein has been associated with greater control of JCV and longer PML survival rates [68–71].

Moreover, a recent study shows differences in JCV-specific T-cell response during natalizumab treatment and in natalizumab-associated PML. In particular, it was observed that in patients treated with natalizumab, the magnitude and the quality of JCV-specific T-cell response did not change from the healthy patients, while in patients with natalizumab-associated PML, JCV-specific T cells were not measurable or JCV-specific T cells were dominated by IL-10 (human cytokine synthesis inhibitory factor) production, giving further evidence of the role of T-cell response in PML development [72].

On the contrary, the role of the humoral response has not been well defined yet. In fact, PML patients have substantial antibody titer direct against viral capsid protein before and during disease [73], and in particular it has been demonstrated that anti-JCV IgG are synthesized intrathecally but this was not associated with an improvement in their clinical outcome [74]. Also, the virus seems to have adapted to replicate and disseminate through B cells and their progenitors. However, when the human immune response against viruses that are causing persistent and latent infection has been dissected with modern tools allowing unprecedented accuracy [75, 76], it has been shown not only that the single antibody clones are endowed with a very different neutralizing activity [77–79] and that effective antibodies are very rare [80] but also that a part of the response can also have a biological activity not necessarily beneficial for the host [81–84]. Considering these aspects, it could be very important to better investigate this crucial aspect of the virus-host interplay, by identifying the role played by selected antibody clones capable of effectively neutralizing the viral particles, in case of peripheral reactivation.

4. Treatment of PML: Old and New Strategies

4.1. Immune Reconstitution Inflammatory Syndrome (IRIS). Since the etiopathological origin of PML is associated with compromised or altered host immune response, the immune reconstitution is actually considered the best strategy for an improved outcome. In particular, the immune reconstitution is based on the reduction of HIV load through HAART in the case of HIV-positive PML patients and on the elimination or reduction of the immunomodulatory drug in the case of immunomodulatory-treated PML patients [10, 85]. In this second group of patients, there are two strategies that can be adopted: the plasma exchange and the administration of intravenous immunoglobulins. In particular, plasma exchange has been safely and successfully used to eliminate free unbound natalizumab [86–88]. The second therapeutic option is the administration of intravenous immunoglobulins, in the hope that they may somehow limit the binding of the immunomodulatory drug to its target [89, 90].

However, the immune reconstitution may have very serious consequences; in fact it, can be associated with increasing inflammation and a clinical deterioration called immune reconstitution inflammatory syndrome (IRIS) [91]. In fact, when the immune system is reconstituted, fully functional and activated T cells regain access to the CNS compartment, initiating a strong inflammation within the brain, as a side effect of the massive destruction of virus-infected cells. IRIS is usually associated with an increasing CD4+ cell count and an exaggerated reaction of CD8+ T cells, especially in HIV-positive patients [92–95]. Inflammation can be visualized by contrast-enhancing lesions on MRI due to the open BBB. IRIS can lead to a rapid deterioration of the patient's clinical state and death in 30–50% of cases [96]. An effective therapeutic treatment for IRIS does not exist, even if an immunomodulatory therapy able to attenuate the T cells response is suggested [94].

4.2. Antiviral Drugs. Currently, no anti-JCV-specific drugs are available; a number of treatment options targeting different stages in the viral life cycle have been proposed.

The use of drugs potentially interfering with the viral entry has been suggested. A treatment for the inhibition of viral entry includes blocking access to $5HT_{2A}R$ by antibodies or by serotonin receptor agonists (chlorpromazine and clozapine). Some authors described that monoclonal antibodies to $5HT_{2A}R$ blocked infections of glial cells by JCV, while chlorpromazine inhibits clathrin-dependent endocytosis and in combination of clozapine can block the glial infection. However, these drugs have serious side effects and toxicity issues. Recently, newer antipsychotics (ziprasidone, risperidone, and olanzapine) have been shown to reduce JCV infection up to 10 fold in an *in vitro* system, but further studies are warranted to determine efficacy in PML treatment *in vivo*, either alone or in combination therapy with other drugs [51, 97].

Many broad-spectrum nucleoside analog chemotherapeutics (including cytosine arabinoside, Ara-C, cidofovir, and CDV) that target DNA replication have been used to inhibit JCV replication without much success. CDV is an acyclic nucleotide phosphonate analog of deoxycytosine monophosphate; due to its inhibitory action on DNA polymerase, its effect on JCV has been tested with contradictory results. The low efficacy of CDV may reflect poor penetration, and severe side effects have been reported [98, 99]. Recently, a lipid derivative of CVD (a hexadecycloxypropyl lipid conjugate of CVD, called CMX001) was found to reduce JCV replication, with no significant toxicity in cell cultures derived from human fetal brain, suggesting that it could be a promising candidate for the treatment of PML [100]. Ara-C is another nucleoside analog, and it was effective in decreasing viral replication in cultured human neuroglial cells. Limitations of this drug could be its short half-life, poor ability to cross the BBB, and bone marrow toxicity [90].

Other drugs that inhibit viral DNA replication act on DNA topoisomerase. Topotecan blocked JCV DNA replication with no effect on host transcription and translation, but other studies should be conducted before using the drug in patients [101].

As described, all tested therapeutic options show no significant impact on survival or neurological improvement. The ineffectiveness of these molecules can be explained by many factors, such as the low ability to cross BBB, the variability of the viral structures, and the extreme complexity of the JCV/host interplay.

5. Discussion and Future Perspectives

In the literature, PML was considered a rare disease, and, before the HIV pandemic and the availability of immunomodulatory drugs, it was only associated with neoplasms impairing the immune system, such as chronic lymphocytic leukemia or Hodgkin's lymphoma. In the last two decades, the incidence of PML has begun to increase exponentially. In patients treated with immunomodulatory therapy, the incidence of PML depends on the drug used in therapy and on the treated disease. Of particular interest is the PML incidence in patients who receive natalizumab (approximately 1/500), a very effective drug mainly used in multiple sclerosis patients. This is a completely new scenario, especially if compared with HIV patients that were, in the past, almost irreversibly condemned to a death due to other, non-JCV-related, opportunistic infections. Indeed, although potentially at high risk, natalizumab is still being marketed for its high benefits in the MS treatment. These data lead to two very important considerations: the first regarding the management of the patient who is subjected to immunomodulatory therapy, and the second the treatment to be adopted in case of PML development.

The management of the patients is based on the identification of patients who truly are at high risk of developing the disease after immunomodulatory therapy. Recently, three risk factors have been proposed: presence in the serum of anti-JCV antibodies, prior use of immunosuppressants, and duration or natalizumab treatment. Unfortunately, this analysis shows limitations based primarily on the inadequacy of information on the pathophysiology of the disease and of the JCV biology. These are also the reasons why, to date, there is no specific and effective therapy for the treatment of PML. New therapies have been proposed and some of these appear to be very promising. In particular, many recent works have focused on the possible role that the main viral structural protein (VP1) involved in viral entry may have in the PML etiopathogenesis, suggesting it as a potential drug target. On the diagnostic side, it was recently described that mutations on VP1 are associated with PML, but it remains to be determined whether these changes influence PML risk. On the therapeutic side, a recent study by Balduzzi et al. [102] reported that the generation *in vitro* of JCV-specific CD8+ T cells using 15-mer peptides derived from VP1 and large T antigen and its clinical use in an allogenic hematopoietic stem cells recipients with PML may be important in the control of PML. Although not reported in the previously mentioned study, it is important to remember that the risk of IRIS is mainly related to an exaggerated CD8+ T cell response [95]. Furthermore, studies on the combination of low-dose chlorpromazine and neutralizing antibodies showed their possible use in prophylactic and therapeutic treatment of PML [103].

Other immunological paths are therefore to be considered and could not only allow a better comprehension of JCV-host interplay but, hopefully, also a better clinical management of PML patients, similarly to what has already happened for other persistent and latent viral infections. In particular, the clarification of the role played by the humoral response in controlling JCV dissemination to CNS and, more generally, a better understanding of the molecular features of this crucial aspect of the virus-host interplay can be crucial for opening new vistas in this field. It is intriguing that in the era of monoclonal antibody-related opportunistic infections, antiviral compound of the same class of drug could be potentially useful. In particular, the anti-JCV human humoral response should be dissected and studied. The possible activity of human neutralizing monoclonal antibodies directed against conserved regions of VP1 should be considered, as

already happened for other viral infections where the role of the humoral response was for too long considered negligible or too limited in breadth [75, 80, 83, 104–110], since even if the overall contribution of the antibodies to the host-virus interplay can appear not too relevant, the role played by selected antibody subpopulations could all the same be of great importance.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Respiratory-Related Hospitalizations following Prophylaxis in the Canadian Registry for Palivizumab (2005–2012) Compared to Other International Registries

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Respiratory syncytial virus (RSV) infection occurs commonly in infants aged ≤2 years, and severe infection results in hospitalization with accompanying morbidity and mortality. Palivizumab has been available for prophylaxis for the past 15 years. Prospective data on patients who received palivizumab from 2005 to 2012 has been assembled in the Canadian registry (CARESS) to document utilization, compliance, and health outcomes in both hospital and community settings. Long-term data is necessary to evaluate the impact of palivizumab on the incidence of RSV infections, minimize healthcare resources, and identify which infant subpopulations are receiving prophylaxis. A database search was also conducted for similar information from published registries, and hospitalization rates were compared to results from randomized clinical trials (RCTs).Overall hospitalization rates (percent; range) for respiratory-related illnesses and RSV-specific infection in infants who meet standard indications for prophylaxis were 6.6 (3.3–7.7) and 1.55 (0.3–2.06), respectively, in CARESS, which closely aligns with registry data from 4 other countries, despite the former comprising the largest cohort of complex patients internationally. Overall RSV-related hospitalization rates were lower across registries compared to equivalent patients in RCTs. Registry data provides valuable information regarding real-world experience with palivizumab, while facilitating the genesis of new research themes.

1. Introduction

Respiratory syncytial virus (RSV) continues to play a dominant role among the spectrum of viruses causing acute lower respiratory infection and subsequent hospitalization in infants and young children [1–6]. The burden of illness with accompanying morbidity, mortality, and associated healthcare costs is equally significant both within the community and world-wide [7–13].

Palivizumab, a humanized monoclonal antibody that targets the A antigenic site of the F-protein of RSV for the prevention of disease in high-risk children, demonstrates both neutralizing and fusion-inhibitory activity [14]. It was licensed in the USA by the Food and Drug Administration (FDA) in 1998 and subsequently by the European Medicines Evaluation Agency (EMEA) in 1999. Since its release, two major randomized, double-blind, placebo controlled trials [15, 16] and several follow-up studies [17, 18] have established the safety and efficacy of palivizumab in premature infants aged < 6 months who are ≤35 weeks gestational age and in children <2 years with hemodynamically significant congenital heart (HSCHD) or chronic lung disease (CLD).

Over several years, international registries have closely monitored patients who have received RSV prophylaxis, in order to determine utilization and compliance relative to country-specific or national pediatric guidelines and position statements [19–22]. The Canadian Registry for the evaluation of palivizumab (CARESS) was initiated in 2005, with

the principal objective of documenting usage, compliance, and health outcomes of infants receiving RSV prophylaxis in both hospital and community settings during the annual RSV seasons. The registry tracks data on patient demographics, annual indications for prophylaxis, incidence of RSV infections, rates of hospitalization for respiratory-related and RSV-related illnesses with respective lengths of hospital stay, risk factors that govern time to hospitalization, acquired morbidities following hospital admission, and safety and compliance with palivizumab.

The primary objective of this report is to document hospitalizations for respiratory illnesses (RIH) and RSV-specific infection (RSVH) within CARESS that spans the 2005–2012 RSV seasons and compare our results with published data from similar international registries and published randomized clinical trials (RCTs).

2. Material and Methods

Infants who received at least one dose of palivizumab during any RSV season from 2005 to 2012 were eligible for inclusion in CARESS, if they had at least one of the following risk-factors: prematurity (≤35 completed weeks gestational age [GA]) without underlying medical disorders, CLD, HSCHD, or other "off-label" provincially approved medical conditions such as Down syndrome, congenital airway anomalies, immunodeficiency, or neuromuscular disorders. Preterm infants, 33–35 completed weeks GA, qualify for palivizumab only if they are considered at moderate (score 49–64) to high (score 65–100) risk for severe RSV infection and hospitalization based on a validated, Canadian risk-scoring model [23]. Children were excluded if a parent or legal guardian could not communicate in either English or French. Additionally, infants had to be recruited after their first injection of palivizumab and preferentially before receiving their third injection.

Subjects were enrolled by the local physician investigator and/or research nurse, which included providing the parent or legal guardian with an information package and consent form for review. Once consent was obtained, the research nurse completed an enrolment form to collect baseline data on patient demographics, prior medical history, neonatal course, and details of palivizumab administration. Following study initiation, the research nurse at the local site contacted the parent or legal guardian either in person or by telephone monthly, until the end of the RSV season, obtaining data on palivizumab administration, changes in baseline information, and specific facts regarding possible respiratory infections since the last contact. In the event of a hospitalization, and with parental consent, the relevant hospital records were reviewed by the site's research nurse for detailed information on patient diagnosis, reason for hospitalization, length of stay, days on respiratory support and/or intubation, and RSV specimen type and diagnostic test, as reported in the discharge summary. Collected data was logged into a central website.

Compliance was evaluated by two methods: actual number of doses prescribed versus expected number of doses for the duration of the RSV season and interdose interval. Palivizumab clinics currently administer about 5 monthly injections of palivizumab at 30 ± 5 day intervals, based on pharmacokinetic evidence from RCTs [15, 16, 24–26]. For expected number of doses, the number was calculated assuming monthly injections from the first dose to the end of the RSV season. The criterion for the start and end of the RSV season was defined by the previous study conducted by the Pediatric Investigators Collaborative Network on Infections in Canada [27]. For number of days between injections, 30 ± 5 day intervals were considered acceptable (i.e., as being within compliance). However, an interval of 20 ± 4 days between the first and second injections, likely results in higher trough levels after the first dose, offering better protection against the virus [28]. Therefore, an interval of 16–35 days between the first and second injections was considered compliant.

Comparative data from international registries was obtained through a search of Web of Science, PubMed, Medline, CINAHL, Cochrane, DARE, and OVID databases, using the key words "registry" AND "RSV" OR "respiratory syncytial virus" AND "newborn" OR "neonatal" OR "infant-newborn" AND "infant" AND "prophylaxis" OR "palivizumab". All identified reports were compiled based on the aforementioned criteria and were further checked for references regarding additional pertinent studies, and a nucleus of key articles was derived for analyses (Figure 1).

3. Statistical Analysis

CARESS data were examined using standard descriptive methods. Data was entered into SPSS v20.0 (SPSS Inc, Chicago, Illinois) for analysis. The primary endpoint of this observational study was hospitalization. The RIH rate was defined as the number of children hospitalized for a respiratory-related illness/total number of children who received palivizumab. The RSVH rate was calculated using the formula: RIH × the number of RSV-positive children/the number of children with a respiratory illness tested. The characteristics of hospitalized versus nonhospitalized patients were evaluated to identify potential risk factors for respiratory illness-related hospitalization. The statistical tests used to determine these factors included Student's t-test and analysis of variance (ANOVA) for continuous variables and Pearson's chi-square (χ^2) test for nominal variables. An ANOVA was also applied in place of Student's t-test when more than two groups were assessed. A P value of <0.05 was considered statistically significant.

To determine any factors that may affect time to RSVH, a Cox proportional hazards analysis was conducted using a backwards conditional method. Patients were followed for up to 30 days after their final injection. Results are presented in terms of hazard ratios (HR), with 95% confidence intervals (95% CI).

4. Results

A total of 13,310 patients were recruited across 32 sites since the 2005-2006 RSV season. The proportions of patients recruited for the CARESS study are representative of the population proportions found by Statistics Canada in their

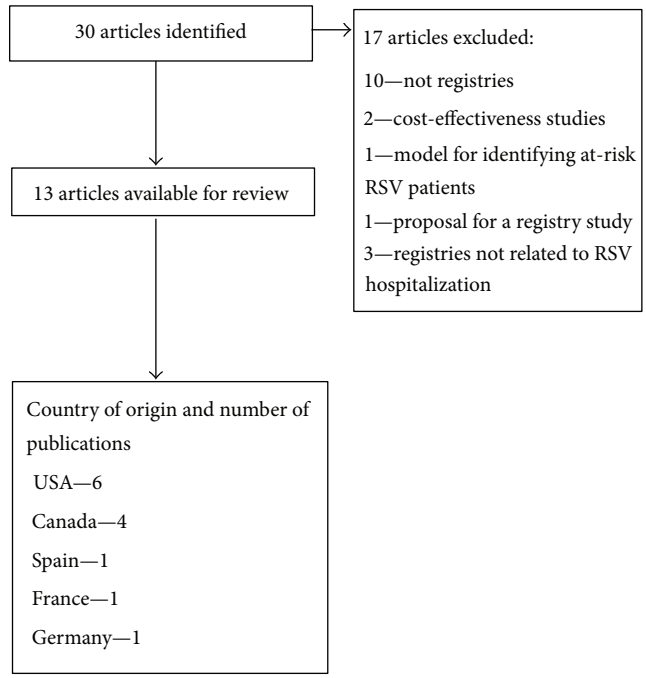


Figure 1: Flow chart of assembled articles from the scientific literature.

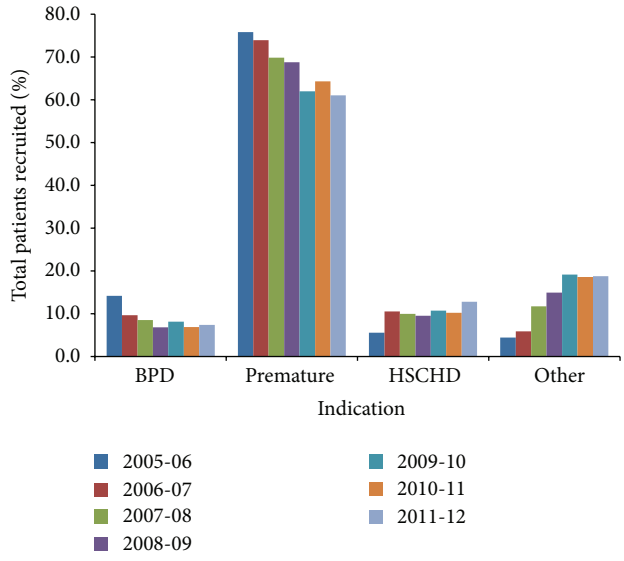


Figure 2: Palivizumab indications across seasons. Indications are subcategorized into chronic lung disease (BPD/CLD, $n = 1048$), premature ($n = 8751$), hemodynamically significant congenital heart disease (HSCHD, $n = 1414$), and "other" ($n = 2097$). The "other" group comprises infants with serious underlying medical disorders.

latest quarterly demographic estimates [29]. Over the 7 years, 65.7% of patients prophylaxed with palivizumab were premature, 7.9% had CLD only and were not classified in any other category, 10.6% had HSCHD, and 15.8% had "other" serious medical disorders. Figure 2 shows the distribution of patients based on indication for prophylaxis from 2005 to 2012. Table 1 compares demographics across the indications. There were statistically significant differences between the groups in percent Caucasians, mean birth weight, enrolment and gestational age, daycare attendance, family history of atopy, multiple births, household smoking and more than 2 smokers in the home, siblings, siblings in daycare, and >5 people in the household. A *post hoc* analysis was conducted using the Tukey test to determine which indications contributed to the statistical significance. Birth weight across all indications was statistically significantly different from the "other" subcategory ($P < 0.05$). With regard to enrolment weight, the premature group was significantly different from the other 3 indications (CLD, HSCHD, and "other"; $P < 0.005$), while the HSCHD and "other" groups were similarly significantly different ($P < 0.05$). For gestational age, with the exception of the CLD and premature groups ($P = 0.953$), the indications were all significantly different from each other ($P < 0.05$). Over the seven RSV seasons encompassed by CARESS, there has been a 4.3-fold increase in the percentage of patients recruited that have been prophylaxed for serious underlying medical disorders, from 4.4% in the 2005-2006 RSV season to 18.8% in the 2011-2012 RSV season. Within the "other" category, there has also been a change in the distribution of recruitment in each subcategory (Table 2). More than >50% of the patients comprise the miscellaneous subcategory, which suggests that overall greater numbers of patients are receiving "off-label" palivizumab because of their illness severity.

More than 50% of the patients received respiratory support (59.4%) and oxygen therapy (52.6%) during the neonatal period. The average ± standard deviation duration of respiratory support was 23.3 ± 35.8 days, and the average duration of oxygen therapy was 37.5 ± 64.9 days. The average length of hospital stay after birth was 50.6 ± 80.8 days. Not surprisingly, significantly higher percentages of premature

Table 1: Cumulative patient demographics by prophylaxis indication (2005–2012).

	Premature $N = 8751$	CLD $N = 1048$	HSCHD $N = 1414$	Other $N = 2097$	Total $N = 13310$	P value
Male, *n* (%)	4996 (57.1)	601 (57.3)	777 (55.0)	1161 (55.4)	7535 (56.6)	0.280
Caucasian, *n* (%)	6031 (68.9)	750 (71.6)	1047 (74.0)	1538 (73.3)	9366 (70.4)	<0.0005
Daycare attendance, *n* (%)	107 (1.2)	58 (5.5)	117 (8.3)	196 (9.3)	478 (3.6)	<0.0005
Atopy in the family, *n* (%)	3390 (38.7)	471 (44.9)	610 (43.1)	920 (43.9)	5391 (40.5)	<0.0005
Mean enrolment age (mo ± SD)	3.3 ± 3.1	10.3 ± 7.3	8.7 ± 7.3	10.0 ± 8.9	5.5 ± 6.2	<0.0005
Mean gestational age (wk ± SD)	30.9 ± 3.3	30.5 ± 5.8	38.1 ± 10.6	35.6 ± 5.7	32.4 ± 5.8	<0.0005
Mean birth weight (g ± SD)	1590 ± 632	1628 ± 1132	3048 ± 1154	2583 ± 1390	1902 ± 1043	<0.0005
Mean enrolment weight (g ± SD)	4017 ± 2167	7047 ± 2510	6092 ± 4423	7258 ± 3257	5082 ± 3087	<0.0005
Multiple births, *n* (%)	3140 (35.9)	250 (23.9)	89 (6.3)	340 (16.2)	3819 (28.7)	<0.0005
Mother that smokes, *n* (%)	1259 (14.4)	163 (15.6)	197 (13.9)	260 (12.4)	1879 (14.1)	0.067
Mother smoked during pregnancy, *n* (%)	1193 (13.6)	151 (14.4)	184 (13.0)	252 (12.0)	1780 (13.4)	0.188
Smoking in the household, *n* (%)	2471 (28.2)	276 (26.3)	335 (23.7)	465 (22.2)	3547 (26.6)	<0.0005
≥2 smokers in the home, *n* (%)	979 (11.2)	101 (9.6)	138 (9.8)	198 (9.4)	1416 (10.6)	0.042
Siblings, *n* (%)	5384 (61.5)	658 (62.8)	791 (55.9)	1364 (65.0)	8197 (61.6)	<0.0005
Siblings in daycare, *n* (%)	1588 (18.1)	153 (14.6)	242 (17.1)	451 (21.5)	2434 (18.3)	<0.0005
≥5 people in the household, *n* (%)	2509 (28.7)	238 (22.7)	299 (21.1)	505 (24.1)	3551 (26.7)	<0.0005

HSCHD: hemodynamically significant congenital heart disease; CLD: chronic lung disease.

Table 2: Changing patient profiles in the subcategory "other" over 6 RSV seasons*.

Indication, *n* (%)	RSV Season					
	2006-2007 $N = 72$	2007-2008 $N = 169$	2008-2009 $N = 298$	2009-2010 $N = 462$	2010-2011 $N = 511$	2011-2012 $N = 538$
Down syndrome	2 (2.8)	47 (27.8)	62 (20.8)	81 (17.5)	107 (20.9)	150 (27.9)
Cystic fibrosis	13 (18.1)	19 (11.2)	28 (9.4)	55 (11.9)	54 (10.6)	52 (9.7)
Congenital airway anomaly	16 (22.2)	46 (27.2)	50 (16.8)	66 (14.3)	91 (17.8)	75 (13.9)
Miscellaneous	41 (56.9)	57 (33.7)	158 (53)	260 (56.3)	259 (50.7)	261 (48.5)
Pulmonary disorders	13 (18.1)	12 (7.1)	65 (21.8)	90 (19.5)	89 (17.4)	73 (13.6)
Neuromuscular impairment	12 (16.7)	11 (6.5)	15 (5.0)	40 (8.7)	40 (7.8)	35 (6.5)
Immunocompromised	1 (1.4)	3 (1.8)	4 (1.3)	9 (1.9)	9 (1.8)	24 (4.5)
Cardiac disease ≥ 2 yr	0 (0.0)	7 (4.1)	7 (2.3)	7 (1.5)	10 (2.0)	11 (2.0)
Multisystem anomalies	5 (6.9)	6 (3.6)	32 (10.7)	15 (3.2)	30 (5.9)	41 (7.6)
Various medical disorders	10 (13.9)	18 (10.7)	35 (11.7)	99 (21.4)	81 (15.9)	77 (14.3)

*The 2005-2006 season was excluded as this information was not collected sequentially in all the subcategories for that year.

and CLD patients received respiratory support than HSCHD and "other" indications (63.8% and 76.1% versus 40.2% and 45.9%). Compared to HSCHD and "other" indications significantly higher percentages of subjects in the CLD group received oxygen therapy (84.7% versus 44.8–52.6%) and had documented necrotizing enterocolitis (6.4% versus 2.0–3.3%), sepsis (30.9% versus 8.9–14.8%), and surgery for patent ductus arteriosus (19.9% versus 3.6–6.6%).

4.1. Palivizumab Utilization. Overall, patients received 98.2% ± 32.1% of their expected injections. Using inter-dose intervals, overall, 73.2% of infants were compliant. The 2006-07 season had a lower percentage of compliant subjects compared to other years (60.9% versus 67.8%–79.8%, $P < 0.00005$).

4.2. Hospitalizations for Respiratory Illness-Related Events. Of the 13,310 patients that have been enrolled into the CARESS study, 875 patients had a total of 1,022 hospitalizations for a respiratory illness, giving a hospitalization rate of 6.6%. Patients were hospitalized for a range from 0 to 6 episodes per season. The average length of hospital stay was 8.8 ± 17.2 days with an average of 1.9 ± 8.9 days in intensive care. There may be an emerging trend towards higher hospitalization rates, with a low of 3.3% (2005-2006) and a high of 7.7% (2010-11) but with some variation (Figure 3).

Reviewing hospitalizations by indication (Table 3), a lower proportion of hospitalized versus nonhospitalized patients were premature (43.9% versus 67.3%, $P < 0.0005$) with a higher proportion ≤ 28 completed weeks GA (16.6% versus 14.5%). Conversely, there were a significantly higher

Table 3: Hospitalized versus nonhospitalized patients for respiratory-related illness based on indication.

Indication	Hospitalized (%) $N = 875$	Not hospitalized (%) $N = 12435$	P value (χ^2)
Premature	384 (43.9)	8367 (67.3)	<0.0005
≤28 weeks GA	145 (16.6)	1805 (14.5)	0.101
29–32 weeks GA	175 (20.0)	4645 (37.4)	<0.0005
33–35 weeks GA	64 (7.3)	1902 (15.3)	<0.0005
CLD	128 (14.6)	920 (7.4)	<0.0005
HSCHD	146 (16.7)	1268 (10.2)	<0.0005
Other	217 (24.8)	1880 (15.1)	<0.0005
Neuromuscular disorders	28 (3.2)	125 (1.0)	<0.0005
Airway anomaly	45 (5.1)	299 (2.4)	<0.0005
Cystic fibrosis	9 (1.0)	222 (1.8)	0.115
Down syndrome	38 (4.3)	411 (3.3)	0.096
Pulmonary	37 (4.2)	309 (2.5)	0.003
Cardiac ≥ 2 years	4 (0.5)	38 (0.3)	0.355
Immunocompromised	9 (1.0)	41 (0.3)	0.005
Multisystem anomalies	18 (2.1)	113 (0.9)	0.004
Various medical disorders	29 (3.3)	322 (2.6)	0.188

HSCHD: hemodynamically significant congenital heart disease; CLD: chronic lung disease.

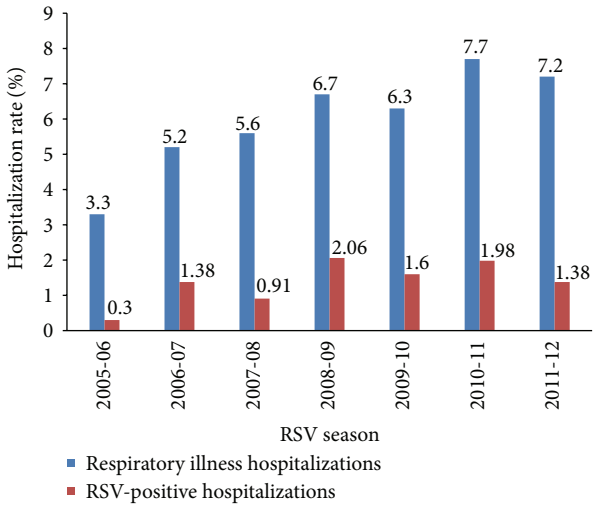


Figure 3: Hospitalizations for respiratory-related illness and RSV-positive infection (2005–2012).

proportion of hospitalized patients in each of the other indication groups than nonhospitalized patients apart from cystic fibrosis. However, no significant differences were found between hospitalized and nonhospitalized patients in terms of the proportion that had Down syndrome ($P = 0.096$), cystic fibrosis ($P = 0.115$), cardiac problems ($P = 0.355$) and various medical disorders ($P = 0.188$).

Comparing demographic information between hospitalized and nonhospitalized patients, a greater proportion attended daycare (5.8% versus 3.4%, $P < 0.0005$) and had siblings that attended daycare (26.2% versus 17.7%, $P < 0.0005$). Hospitalized infants also had more exposure to smoking, specifically, having a mother that smoked (19.1% versus 13.8%, $P < 0.0005$), a mother that smoked during pregnancy (18.4% versus 13.0%, $P < 0.0005$), smokers at home (32.2% versus 26.3%, $P < 0.0005$), and more than 2 smokers at home (13.6% versus 10.4%, $P = 0.004$). While a greater proportion of hospitalized infants had siblings (71.8% versus 60.9%, $P < 0.0005$), a lower proportion were from multiple births (20.8% versus 29.2%, $P < 0.0005$). A greater proportion of hospitalized infants also had a history of atopy in their immediate family (47.1% versus 40.0%, $P < 0.0005$).

Analyzing hospitalizations in terms of palivizumab compliance showed no significant difference between hospitalized and nonhospitalized patients in terms of compliance by expected number of injections (63.8% versus 66.2%, $P = 0.149$). However, based on interdose intervals, a significantly lower proportion of hospitalized patients were compliant with treatment (67.8% versus 73.6%, $P < 0.0005$). On average, hospitalized infants received a statistically greater number of injections than nonhospitalized infants (4.4 versus 4.2, $P < 0.0005$). There was no significant difference between the two groups in terms of the number of days between infants' first and second injections (28.8 versus 28.0 days, $P = 0.149$).

4.3. RSV-Related Hospitalizations. Of the 13,310 patients enrolled, 875 patients had a total of 1,022 RIHs. Among these, 847 RSV diagnostic tests were performed on 733 patients, predominantly using a nasal swab (30.6%) or a nasal aspirate (61.4%) and 28 (3.3%) were unreported. Of the 847 tests conducted, 177 (20.9%) were found to be positive in 173 patients. The RSV-positive hospitalization rate was 1.55% ([875/13310] × [173/733]).

On review of the 7 RSV seasons, the RSV-positive hospitalization rate (Figure 3) has fluctuated from 0.30% (2005-2006) to 2.06% (2008-2009). With regard to demographic data, a greater proportion of infants hospitalized with RSV

Table 4: Respiratory illness (RIH) and RSV-related hospitalization (RSVH) rates and morbidities encountered during hospital stay according to indication.

	Prematurity	CLD	HSCHD	Other	*P* value
RIH					
RIH rate	4.4%	12.2%	10.3%	10.3%	<0.0005
Length of stay (mean ± SD)	7.9 ± 14.8	9.9 ± 25.0	8.7 ± 10.9	9.8 ± 18.3	0.469
Length of ICU stay (mean ± SD)	1.5 ± 4.8	1.7 ± 6.9	2.3 ± 5.7	2.5 ± 14.7	0.494
Days of intubation (mean ± SD)	0.7 ± 3.0	1.2 ± 6.6	0.9 ± 3.4	1.2 ± 8.9	0.643
Days of respiratory support (mean ± SD)	1.4 ± 4.8	2.4 ± 7.3	1.8 ± 5.2	3.1 ± 14.9	0.117
RSVH					
RSVH rate	1.36%	1.64%	2.05%	2.03%	<0.0005
Length of stay (mean ± SD)	7.7 ± 10.3	10.1 ± 11.4	7.2 ± 7.7	7.2 ± 5.8	0.776
Length of ICU stay (mean ± SD)	1.2 ± 2.6	3.1 ± 11.8	2.6 ± 5.0	1.6 ± 4.0	0.346
Days of intubation (mean ± SD)	0.6 ± 2.0	3.1 ± 11.8	1.6 ± 3.3	1.1 ± 3.3	0.180
Days of respiratory support (mean ± SD)	1.2 ± 2.7	3.6 ± 11.7	2.1 ± 4.7	2.1 ± 4.3	0.294

CLD: chronic lung disease; HSCHD: hemodynamically significant congenital heart disease; ICU: intensive care unit.

infections had siblings (80.3% versus 61.3%, $P < 0.0005$), attended daycare (8.7% versus 3.5%, $P = 0.001$), and had siblings that attended daycare (32.4% versus 18.1%, $P < 0.0005$). A greater proportion also had a history of atopy in their immediate family (50.9% versus 40.4%, $P = 0.006$) were more likely to have been exposed to smoking, either by having a mother that smoked (20.2% versus 14.0%, $P = 0.024$), a mother that smoked during pregnancy (19.7% versus 13.3%, $P = 0.021$), or smokers in the household (37.6% versus 26.5%, $P = 0.001$).

Table 4 shows the RIH and RSVH rates by indication with encountered morbidities during hospitalization. The RIH and RSVH rates ranged between 4.4%–12% and 1.36%–2.05%, respectively, across the groups and were statistically different (both $P < 0.0005$).

The average length of hospital and ICU (mean ± SD) stay for the total group was 8.8 ± 17.2 and 1.9 ± 8.9 days, respectively.

4.4. Cox Proportional Hazards Regression. To determine factors that may affect time to RSVH, a Cox proportional hazards regression was conducted. The overall model was significant ($\chi^2 = 65.847$, df = 5, $P < 0.0005$) and showed that having siblings (Figure 4(a)) (HR = 2.1, 95% CI 1.4–3.3, $P < 0.0005$), smokers in the household (Figure 4(b)) (HR = 1.8, 95% CI 1.3–2.5, $P < 0.0005$), >5 individuals in the household (Figure 4(c)); HR = 1.7, 95% CI 1.3–2.4, $P = 0.001$), attending daycare (Figure 4(d)) (HR = 2.3, 95% CI 1.3–4.0, $P = 0.004$), and number of injections received (HR = 0.9, 95% CI 0.8–1.0, $P = 0.032$) were significant predictors of hospitalization. Other possible risk factors such as gestational age ($P = 0.233$), history of atopy ($P = 0.081$), gender ($P = 0.776$), being part of a multiple birth ($P = 0.845$), and compliance with treatment ($P = 0.538$) were not significant predictors. Interestingly, the hazard ratios also increased with increasing number of risk factors experienced by any infant (Figure 5). Infants with all 4 risk factors were 9.5 times more likely to be hospitalized with an RSV infection than those that had none of the risk factors.

Table 5 compares the data derived from international registries [28, 30–41], in 5 countries (USA, Canada, Germany, France, and Spain) accumulated from 2002 to 2012. Table 6 outlines the RI and RSVH rates in various subpopulations of infants drawn from the respective registries versus the existing RCTs. Overall RIH rates for preterm infants <35 weeks GA and CLD patients ranged from 2.6% to 14.9% across studies while the corresponding RSVH rates inclusive of HSCHD were 0.2%–9.0%. In the RCTs, the RSVH rates for the same subgroups ranged from 1.8% to 7.9%. There was only one cystic fibrosis registry that found an adjusted HR for RSVH of 2.4 (95% CI; 0.8–6.6).

5. Discussion

Cumulatively, a total of 13,310 patients have been enrolled in the CARESS study, with 56.6% of the population being male, 70.4% Caucasian, and the majority were premature (≤35 completed weeks GA; 65.7%). The CARESS registry is the largest, comprehensive database of infants who have most currently received palivizumab (2005–2012) compared to other international registries that have published data from 1998 to 2007 (Table 5). Through the seven seasons of CARESS, there has been a steady increase in the percentage of patients that were given palivizumab prophylaxis for reasons that are not specifically indicated by Health Canada. This increase perhaps reflects emerging scientific data and an increased awareness of the potential morbidities and associated mortality with medical conditions such as neuromuscular disorders, Down syndrome, congenital airway and pulmonary abnormalities, immunocompromise, and cystic fibrosis [10–12, 42–49].

The 13,310 patients recruited into the CARESS study were given a total of 55,523 injections of palivizumab. More than half the patients received at least 4 injections per season, with an overall average of 4.2 ± 1.5 injections per infant. Compliance was 73.2% using interdose interval, with patients receiving 98.2% ± 32.1% of their expected injections.

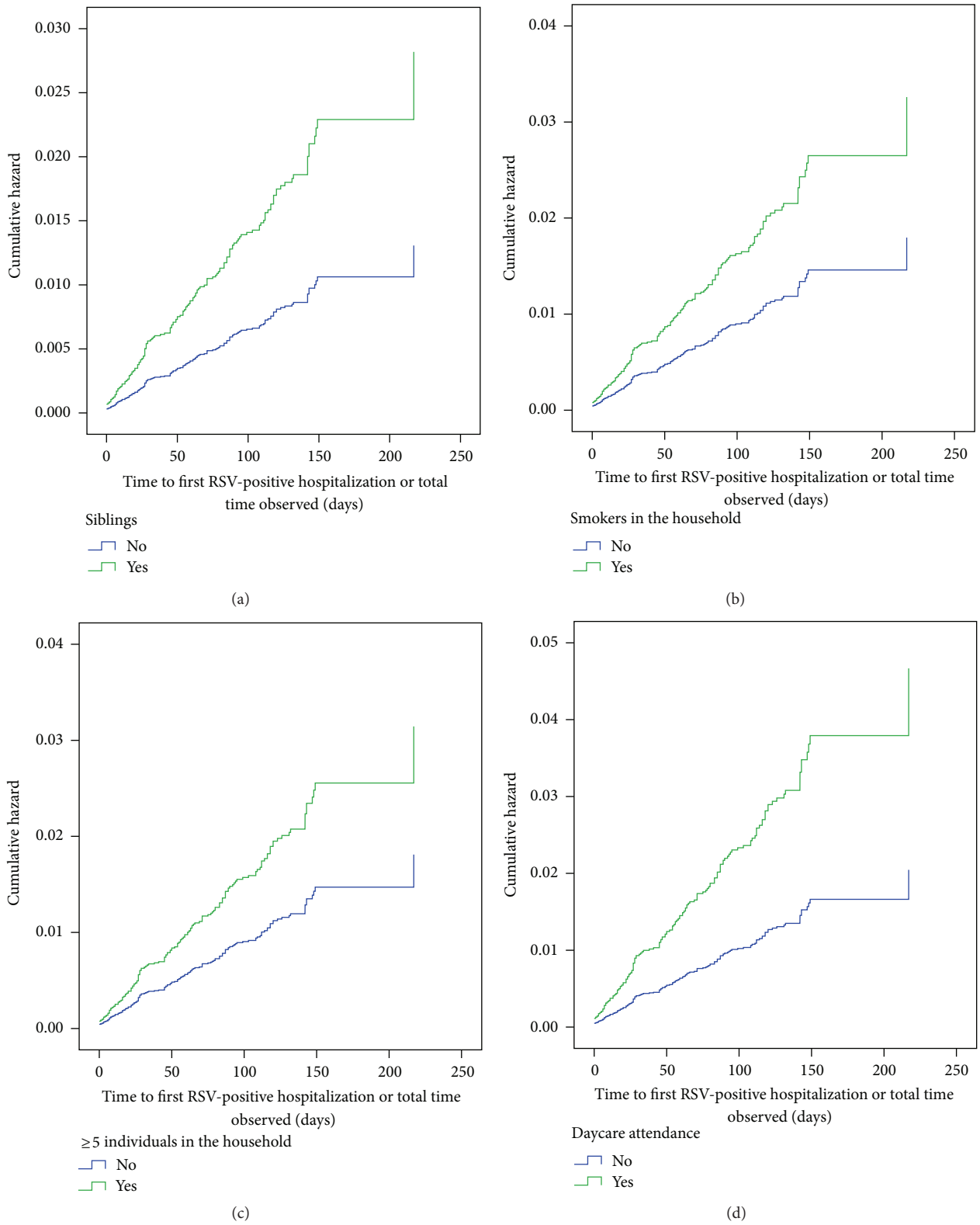
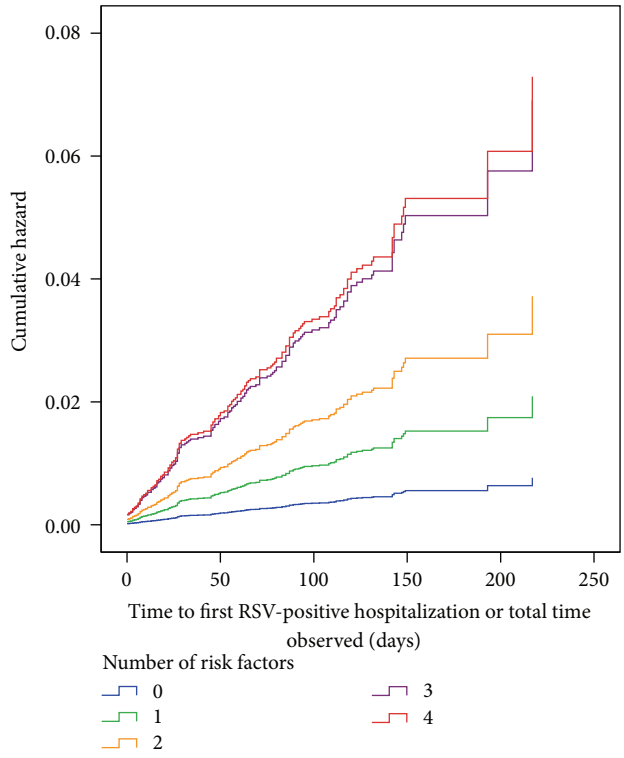


Figure 4: Hazard curves for the Cox proportional regression. (a) Comparing infants with siblings (green line) and those without (blue line). (b) Comparing infants with smokers in the household (green line) and those without (blue line). (c) Comparing infants with ≥5 individuals in the household (green line) and those with ≤5 members (blue line). (d) Comparing infants attending daycare (green line) versus nonattendees (blue line).



Number of risk factors	HR (95% CI)
0	Baseline
1	2.7 (1.5–5.1)
2	4.9 (2.6–9.0)
3	9.0 (4.6–17.6)
4	9.5 (1.2–73.4)

FIGURE 5: Hazard curves for the Cox proportional regression based on the number of risk factors per infant. Risk factors included were siblings, smokers in the household, ≥5 individuals in the household, and attending daycare.

875 patients were hospitalized a total of 1022 times for RIs within the CARESS registry, resulting in a hospitalization rate of 6.6%. Palivizumab was designed to preferentially target RSV subtypes A and B and reduce related hospitalizations. Singleton et al. [50] described the outcomes of 335 high-risk, Alaska Native palivizumab recipients from 1998 to 2001. RSV hospitalizations occurred in 20.6% (69/335), and 26.9% were admitted with respiratory illnesses during the same period, confirming the selective effect of palivizumab against RSV. Across the registries, the RIH rates for prophylaxed premature infants and those with CLD ranged from 2.6% to 14.9% [32, 34, 38, 41], the highest being in infants with CLD (10.5%–14.9%) [34, 41]. Paes et al. [31], previously documented that following RSV prophylaxis, infants with complex medical disorders when compared to a healthy cohort ≤35 weeks GA had an increased risk of RIH (HR = 2.0, 95% CI 1.5–2.5, $P < 0.0005$) but not RSVH. Moreover the RIH rates varied from 3.4% in infants with cystic fibrosis to 17.9% for those with neuromuscular impairments. This substantiates the fact that children with serious, underlying conditions remain prone to sever illness with a broad spectrum of viral infections apart from RSV.

The cumulative CARESS (2005–2012) RSVH rate was 1.55%, and this is within the range of other palivizumab outcome registries (1.3%–8.1%) [28, 33, 37–39, 41]. The upper limit of 8.1% was found in the French registry [41], where the prevalence of CLD (81%) was significantly higher compared to the other described cohorts. In general, the registries reported lower RSVH rates compared to the RCTs; CLD (1.31%–5.8%) versus 7.9%, infants <32 weeks (1.5%–4.5%) versus 5.8%, infants 32–35 weeks (0.2%–1.6%) versus 2.0%, and HSCHD (1.99%) versus 5.3%, respectively. The US outcomes registry [37] documented steadily declining RSVH rates from 2000 (2.9%) to 2004 (0.7%) for all subjects, and 9.1% ($n = 1{,}123$) had congenital airway anomalies or severe neuromuscular impairments. However, the CARESS database comprises 2,097 (15.8%) patients with a spectrum of serious underlying medical disorders who have received palivizumab, and these infants are likely to have higher breakthrough RSVH rates following immunization, despite optimal adherence to dosing schedules [15, 16, 31, 36]. Apart from striving to achieve 100% compliance, to further reduce RSVH, another potential strategy that can be adopted is a home-based prophylaxis program [36]. However, operationalizing this concept is administratively demanding and needs to be proven as cost-effective.

Infants with cystic fibrosis like patients with bronchopulmonary dysplasia (CLD) may develop severe, acute illness with RSV. In CF, synergy between virus and bacteria may lead to repetitive bacterial exacerbations [51]. While the majority of pediatric advisory bodies have universally approved RSV prophylaxis for CLD, there remains active debate as to whether CF patients merit palivizumab [43, 45, 52, 53]. The US CF foundation [54, 55] recommends that RSV prophylaxis should be considered for CF patients based on estimated net benefit which was graded as moderate and was derived from the limited number of existing uncontrolled studies. In the absence of a completed RCT in this population [42], the only available CF registry data [28] also suggests a potential benefit for prophylaxis, but more conclusive evidence from larger studies is awaited.

The Cox proportional hazards analysis found that patients with siblings, those attending daycare, and who have either smokers or ≥5 people in their household were at higher risk of an RSV-positive hospitalization, with hazard ratios increasing concurrently with an incremental increase in the number of risk factors. These factors have a well-established association with severe RSV lower respiratory tract infection [56, 57], and similar findings were identified in several of the registries [33, 35–40]. Interestingly, in CARESS, compliance with treatment based on the expected number of injections rather than interdose intervals was not found to be a predictor of time to first RSV-positive hospitalization. This result is identical to what was found in the Palivizumab Outcomes Registry [37] suggesting that perhaps more stringent control of the timing of individual doses is perhaps more beneficial in reducing RSVH. The number of RSVHs is also likely dependent on

Table 5: Patient populations and outcomes of RSV prophylaxis across published registries.

Author/year/country	Study design	Characteristics of patients—*n*	Overall RSV hospitalization rate	Comments
Winterstein et al. [30]/2012/USA	National CF registry. Case control (1999–2006)	1,974 CF patients aged 0–2 yr over 2,875 patient seasons. Treated (PZ) compared to nontreated group	32 RSV-related hospitalizations. Adjusted HR for RSVH: 0.57 (95% CI; 0.2–1.6) Adjusted incidence rate: PZ, 2.4 (95% CI 0.8–6.6)	Low event rate. Only serious RSV illness was captured. Potential confounders
Paes et al. [31]/2012/Canada	CARESS registry-prospective, observational (2006–2010)	All treated (PZ). 4,880 infants ≤35 wk gestational age (GA) (group 1) compared to 952 with spectrum of medical disorders (MD; group 2)	RSVH: 1.3% versus 2.4% ($P = 0.003$) but ranged from 0.78% to 11.8% based on MD type. Hazard for RSVH was similar in both groups	Higher severity of illness in group 2. No control group. Study patients included only those approved for (PZ)
Paes et al. [32]/2012/Canada	CARESS registry-prospective, observational (2006–2011)	All treated (PZ). 5,183 ≤ 32 wk GA (group 1) versus 1,471 33–35 wk GA (group 2)	RSVH rates were similar (1.5% (Group 1) versus 1.4% (Group 2); $P = 0.3$). Hazard for RSVH was similar in both groups	No control group. Only moderate-high risk 33–35 wk infants were evaluated.
Mitchell et al. [34]/2011/Canada	CARESS registry-prospective, observational (2005–2009)	All treated (PZ). Total 5,286 infants: 3,741 (premature); 449 (CLD); 508 (HSCHD); 588 (other MD)	Overall RSVH was 1.38%: premature (1.12%); BPD (1.31%); HSCHD (1.99%); MD (2.78%)	No control group. Only patients approved for (PZ) were studied.
Simon et al. [33]/2011/Germany	German registry-observational (2002–2007)	All treated (PZ). 10,686 enrolled: evaluable patients 6,967 (<33 wk GA); 1500 (33–35 wk GA); 481 (>35 wk GA)	RSVH in worst-case scenario—2.5%	No control group. Not all patients were included and RSV tested. Possible missing data.
Frogel et al. [35]/2008/USA	Palivizumab outcomes registry-prospective, observational (2000–2004)	All treated (PZ). Total 19,548 infants, 19,474 with followup. PZ administered at home (1,226) versus clinic/office (17,641): 7,517 (<32 wk GA); 9,061 (32–35 wk GA); 2,285 (>35 wk GA)	RSVH: Home: 0.4%; clinic: 1.2%, $P = 0.0139$. Highest rate found in patients with mixed settings (5.8%)	No control group. Potential confounders were identified.
Cohen et al. [36]/2008/USA	Palivizumab outcomes registry-prospective, observational (2000–2004)	All treated (PZ). Total 19,548 infants. 1067 (acyanotic), 428 (cyanotic) CHD. 32.3% had HSCHD (485/1500). 468 (<32 wk GA); 327 (32–35 wk GA); 705 (>35 wk GA). Of these 448 also had CLD and 5 had CF.	Overall RSVH: 1.9% (all CHD); 1.6% (acyanotic); 2.6% (cyanotic)	No control group. 67.7% did not have HSCHD. Event rate possibly was underestimated.
Frogel et al. [37]/2008/USA	Palivizumab outcomes registry-prospective, observational (2000–2004)	All treated (PZ): Total 19,548 patients, 19,474 with follow-up. 7826 (<32 wk GA); 9,317 (32–35 wk GA); 2400 (>35 wk GA). Of these 4,349 (CLD); 1500 (CHD); 91 (CF)	Overall RSVH was 1.3%. <32 wk GA: 1.84; 32–35 wks GA: 0.83; >35 wk GA: 1.13. RSVH decreased in each subgroup from 2000 to 2004	No control group. Possible underestimation of event rate.
Parnes et al. [28]/2003/USA	Palivizumab outcomes registry-prospective, observational (2000-2001)	All treated (PZ). Total 2,116 infants: 986 (<32 wk GA); 957 (32–35 wk GA); 172 (>35 wk GA). Of these 500 (CLD); 102 (CHD); 12 (CF)	Overall RSVH: 2.9%. RSVH rate: 2.1% (prematurity without CLD); 4.3% (CHD); 5.8% (CLD)	No control group. 97% followup was achieved. 6% of infants >35 wk had no CLD

Table 5: Continued.

Author/year/country	Study design	Characteristics of patients—*n*	Overall RSV hospitalization rate	Comments
Romero [38]/2003/USA	Palivizumab outcomes registry-prospective, observational (1998–2002)	All treated (PZ). Total 4,669 infants (1998–2000) and 5,091 (2001-2002). Data for 2000-2001 [9]	Overall RSVH 2.3%; (1998-1999), 2.4% (1999-2000), 1.5% (2001-2002)	No control group
Pedraz et al. [39]/2003/Spain	Spanish registry (IRIS) case control (1998–2002)	Untreated (n = 1583; 1998–2000) versus treated (PZ; n = 1919; 2000–2002)	RSVH: control (13.25%); PZ (3.95%)	
Oh et al. [40]/2002/Canada	Canadian Therapeutic Products Program-prospective, observational (1999-2000)	All treated (PZ). Total 444/480 evaluable infants: Premature (345); CLD (40); both CLD and prematurity (68); others (27)	Overall RSVH (2.4%). RSVH in premature infants (1.6%); CLD (6.0%)	No control group. All patients were not tested for RSV and sampling method was not specified.
Lacaze-Masmonteil et al. [41]/2002/France	French Drug agency-prospective, observational study (1999-2000)	All treated (PZ). Total 516 infants (499 evaluable); 258 (<28 wk GA), 182 (29–32 wk GA), 31 (33–35 wk GA), 28 (>35 wk GA).	Overall RSVH (8.1%)	High proportion of infants had CLD (81%) which possibly influenced RSVH.

CF: cystic fibrosis; CHD: congenital heart disease; CLD: chronic lung disease; HR: hazard ratio; HSCHD: hemodynamically significant congenital heart disease; PZ: palivizumab; RSVH: respiratory syncytial virus-related hospitalization.

Table 6: RSV hospitalization rates within published registries based on specific subpopulations compared to published randomized clinical trials.

Author	Specific subpopulation	*RI hospitalization rate †RSV hospitalization rate	Length of hospital stay in days	RCT	Comments
Parnes et al. [28] Winterstein et al. [30] Paes et al. [31]	Cystic fibrosis	†0.0% (n = 12) [28]; *26.2 and †3.9/1000 season months [30]; *3.4% and †1.14% (n = 117) [31]	Undefined [30, 31, 42]	At 6 months follow up RSVH (1.08% [1/92 PB]) [42]	Incomplete RCT [42]-PB (n = 92); placebo (n = 94)
Parnes et al. [28] Mitchell et al. [34] Frogel et al. [37] Romero [38] Pedraz et al. [39] Oh et al. [40] Lacaze-Masmonteil et al. [41]	Chronic lung disease (CLD)	†5.8% (n = 482) [28]; *10.5% and †1.31% (n = 449) [34]; †2.40% (n = 4,329) [37]; †4.0% (n = NS) [38]; †3.9% (n = NS) [38]; †5.5% (n = 217, P < 0.007) [39]; †3.3% (n = 35) [40]; *14.9% (n = 77); †9.0% (n = 400) [41]	Undefined [15, 28, 34, 37–41] Overall total days/100 children for treated (PB) patients (≤35 wk GA + CLD): 36.4 (PB) versus 62.6 (Placebo) [15]	n = 762. RSVH: 7.9% (PB) versus 12.8% (placebo)-39% reduction [15]	Prevalence of CLD very high compared to other registries (81%) [41]
Parnes et al. [28] Paes et al. [32] Mitchell et al. [34] Frogel et al. [37] Romero [38] Pedraz et al. [39] Lacaze-Masmonteil et al. [41]	All Infants <32 wk GA [15, 38] Infants ≤32 wk without CLD [28, 32, 34, 37, 39, 41]	†4.5% (n = 949) [28]; *4.7% and †1.5% (n = 5,183) [32]; †1.84% (n = 7786) [37]; *10.5% and †2.8% (n = 1,056) [38]; †3.2% (n = 1,446) [38]; †≤28 wk: (1.34%, n = 1,704) [34], †5.4% (n = 739, P < 0.001) [39]; †29–32 wk: 1.25%, (n = 1449) [34]; †2.5% (n = 1170, P < 0.0000) [39]; †7.72% (n = 440) [41]	Mean (±SD): 6.7 ± 5.4 [32]; <28 wk (6 ± 2.6) [38], 28–31 wk (14.8 ± 22.8) [38]; ≤32 wk (median 6 [IQR 4–9]) [39] Undefined [15, 28, 34, 37, 41]	n = 1,111. RSVH: 5.8% (PB) versus 11.0% (placebo)-47% reduction (P = 0.003) [15]	IMpact trial [15] Romero [38] reported on two US outcomes registry cohorts (1998-1999) and (1999-2000). The 2000-2001 cohort is reported by Parnes et al. [28]
Parnes et al. [28]	All infants without CLD [15]	†2.1% (n = 1,444) [28]	Undefined [15, 28]	n = 740. RSVH: 1.8% (PB) versus 8.1% (placebo)-78% reduction [15]	IMpact trial [15]
Frogel et al. [37] Romero [38]	Premature infants 32–35 wk GA [15, 37, 38]	†0.83% (n = 9294) [37];*2.6% (n = 1096) and †1.5% (n = 548) [38]; †1.3% (n = 972) [38]	Undefined [15, 37] Mean (±SD): 4.9 ± 3.6 [38]	n = 373. RSVH: 2.0% (PB) versus 9.8% (placebo)-80% reduction (P = 0.002) [15]	IMpact trial [15]
Paes et al. [32] Mitchell et al. [34] Frogel et al. [37]	Premature infants 32–35 wk GA without CLD [15, 28, 37] Infants 33–35 wk GA without CLD [32, 34]	†1.6% (n = 936) [28]; *3.7% and †1.4% (n = 1,471) [32]; †0.2% (n = 588) [34]; †0.83% (n = 9,294) [37]	Mean (±SD): 5.2 ± 5.0 [32]; Undefined [34, 37]	n = 335. RSVH: 1.8% (PB) versus 10.0% (placebo)-82% reduction [15]	IMpact trial [15]
Parnes et al. [28] Frogel et al. [37]	Infants >35 wk GA	†0.6% (n = 164) [28]; †1.13% (n = 2390) [37]	Undefined [15, 28, 37]		
Mitchell et al. [34]	Hemodynamically significant congenital heart disease (HSCHD) [16, 34]	HSCHD: †1.99% (n = 508) [34]	Undefined [33] Total days/100 children: 57.4 (PB) versus 129.0 (Placebo; 56% reduction, P = 0.003) [16]	n = 1287: 682 (cyanotic), 605 (other). Overall RSVH: 5.3% (PB) versus 9.7% (placebo)-45% reduction. RSVH for cyanotic group: 5.6% (PB) versus 7.9% (placebo)-29% reduction (P = 0.285). RSVH for "acyanotic group": 5.0% (PB) versus 11.8% (placebo)-58% reduction (P = 0.003) [16]	Feltes et al. [16]. All patients had hemodynamically significant CHD. Study was not powered for subgroup analyses. Incidence of serious adverse events was lower in the treatment arm: 55.4% (PB) versus 63.1% (placebo; P = 0.005)

CHD: congenital heart disease; CLD: chronic lung disease; GA: gestational age; HSCHD: hemodynamically significant congenital heart disease; NS: not specified; PB: palivizumab; RI: respiratory related hospitalization; RSVH: respiratory syncytial related hospitalization.

the pharmacokinetics of palivizumab [15, 16, 26], with the highest frequency occurring between the first and second injection (range 31%–46%) with steadily declining rates to approximately 10% between the 3rd and 4th dose [28, 37].

There are perhaps several reasons that may account for the decreasing rates of RSVH seen in the Palivizumab Outcomes Registry [37] compared to the current CARESS study and older studies such as COMPOSS (1999-2000) [40], Romero (1998–2002) [38], and the IMpact-RSV trial (1996-1997) [15]. Since the Palivizumab Outcomes Registry, which spanned the 2000–2004 RSV seasons, encompasses data that is, more recent, the lower RSVH rate may reflect changes in the health system, such as preventative education initiatives targeted at patients, improved compliance, variability in RSV epidemiology, and hospital admission criteria. The fact that the CARESS registry did not show a similar trend in RSVH rates may be explained by the increasing percentages of patients being tested for RSV with more precise diagnostic tests such as polymerase chain reaction and the steady increase in the prophylaxis of patients with complex disorders who are more likely to be hospitalized with RSV.

There are several limitations of this data that deserve mentioning. Registries are handicapped by the absence of a control arm which would help to more clearly delineate the true impact of RSV prophylaxis as documented in the RCTs [15, 16]. Though the majority of assembled patients are similar because enrollment is founded on evidence-based local or national pediatric prophylaxis guidelines, variations do exist based on country-specific approval of populations such as 33–35 weeks GA infants and those patients with "off-label" medical, disorders for example, Down syndrome, neuromuscular impairments, and cystic fibrosis. However, the variance also facilitates new research endeavors especially in patients with complex medical disorders. RIH and RSVH detection rates are additionally influenced by the type of samples collected, the number and type of tests conducted, and the formula used for the standardization and reporting of results which were not always stated. Lastly, the changing demographic profile with varying levels of risk that contribute to RSV infection as in the Inuit population in the Canadian Arctic [58], term Alaska Native infants from the Yukon Delta [59], and the Aboriginal children in central Australia [60], combined with fluctuating epidemiological patterns of disease, may influence both RI and RSVH rates.

6. Conclusions

Over the past 15 years, palivizumab has been proven to be highly effective in decreasing RSVH rates, predominantly in children aged <2 years. The cumulative RIH and RSVH rates from 2005 to 2012 in the CARESS registry were 6.6% and 1.55%, respectively, and these incidences align closely with the data from 5 international registries across 13 publications in the scientific literature. Overall RSVH rates from the registries, which reflect everyday use of palivizumab in clinical practice for the key subpopulations of prematurity, CLD, and HSCHD, are lower than in the two randomized trials. The CARESS database also indicates that over the seven RSV seasons there is a growing trend to prophylax patients with other serious medical conditions from 4.4% in 2005-2006 to 18.8% in 2011-2012. This 4.3-fold increase indicates that pediatricians are strongly advocating for protection against serious RSV infection and possible sequelae in extremely high-risk patients. However, more evidence from well-conducted clinical trials is necessary before this strategy becomes standard of care for these infants.

Conflict of Interests

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Review Article

The Use of Humanized Monoclonal Antibodies for the Prevention of Respiratory Syncytial Virus Infection

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Monoclonal antibodies are widely used both in infants and in adults for several indications. Humanized monoclonal antibodies (palivizumab) have been used for many years for the prevention of respiratory syncytial virus infection in pediatric populations (preterm infants, infants with chronic lung disease or congenital heart disease) at high risk of severe and potentially lethal course of the infection. This drug was reported to be safe, well tolerated and effective to decrease the hospitalization rate and mortality in these groups of infants by several clinical trials. In the present paper we report the development and the current use of monoclonal antibodies for prophylaxis against respiratory syncytial virus.

1. Introduction

Respiratory syncytial virus (RSV) was discovered in 1956 and it was classified as a member of *Pneumovirus* genus and Paramyxoviridae family. It has an RNA genome which is enveloped, negative-sense single-stranded, and nonsegmented; it is composed of 10 genes encoding for 11 proteins. The envelope is formed by 4 proteins in a lipid bilayer: the two glycosylated surface G and F (fusion) proteins, the M (matrix) protein, and the SH (small hydrophobic) protein.

The G and F proteins play a key role in the pathogenesis of the infection since the G protein determines the adhesion to the cells of the respiratory epithelium, while the F protein is responsible for the entry of the virus in the cells and determines the insertion of viral RNA in the cell which is responsible for the formation of syncytia [1].

Two subtypes of RSV, A and B, are different for the G protein structure; RSV A and B coexist during every RSV epidemic season and the subtype A seems to be associated with more severe infections [2, 3]. Neutralizing antibodies against G protein are subtype specific, while antibodies against F protein neutralize both subtypes and could be more useful for active and passive immunization.

RSV is responsible for respiratory tract infections that could lead to severe respiratory failure and death in infants, especially in those born extremely preterm or affected by some chronic conditions. RSV is a worldwide infection whose specific antibodies are detected in 87% of 18-month-old infants [4] and virtually in all infants older than 3 years.

RSV is one of the major causes of lower respiratory tract infections (LRTI) during infancy with high rates of hospitalization and mortality during the first years of life [5, 6].

The widespread diffusion of the virus and the high interhuman diffusion lead to large epidemics in infants younger than 5 years with a strong economic impact due to the increase of pediatric visits, emergency room accesses, and hospitalizations [7].

Moreover, it was observed that RSV-associated mortality during the first year of life was ninefold higher than influenza-associated mortality [8].

The number of worldwide episodes of RSV infections [9] in children younger than 5 years was estimated to be over 33 million in 2005. During the same year, the number of hospitalizations for severe acute LRTI was estimated to be 3.4 (2.8–4.3) million among young children, with a mortality rate up to 66,000–199,000/year for children <5 years. Ninety-nine percent of all deaths were recorded in developing countries [10], where the disease-specific mortality is nearly 7%. In the developed countries the mortality is far lower (0.5–2%),

Table 1

Study	Study design	Population	Risk factors
PICNIC	Prospective	Infants hospitalized for RSV infections	(i) Underlying disease (CHD, CLD, immunodeficiency, multiple congenital malformations). (ii) Postnatal age <6 weeks. (iii) GA 33–35 weeks.
FLIP	Prospective case-control study	Preterm born at 33–35 weeks' GA (risk factors for RSV-related hospitalization)	(i) Chronologic age ≤ 10 weeks at the beginning of RSV epidemic season. (ii) Breastfeeding ≤ 2 months. (iii) ≥1 school-age siblings. (iv) ≥4 residents or visitors at home. (v) Family history of wheezing.
Multicenter Italian birth cohort	Multicenter prospective cohort study	Infants born at 33 weeks' GA or more	(i) GA 33 + 0–37 + 6 weeks (ii) No breastfeeding. (iii) Presence of siblings. (iv) Maternal smoking. (v) Family history of atopy or wheezing.

and severe RSV infections are mostly observed in high-risk infants [4].

The incidence of RSV-related hospitalization in the USA increased from 22.2% in 1980 to 47% in 1996 during the first year of life and from 5.4% to 16.4% at any age [11]. The estimated rate of hospitalization in infants younger than 1 year was 92/1000 infants with congenital heart disease (CHD) and 388/1000 infants with chronic lung disease (CLD). According to gestational age (GA), the estimated hospitalization rate was 70/1000 infants born before 28 weeks' GA, 66/1000 infants born at 29–32 weeks' GA, 57/1000 infants born at 33–36 weeks' GA and 30/1000 healthy infants born at term.

Pediatric patients at high risk of RSV morbidity and mortality include preterm infants, especially those with CLD, infants with CHD, neuromuscular diseases, cystic fibrosis, and congenital or acquired immunodeficiency [12, 13].

Considering the great impact of RSV infections on children health, several studies were conducted to identify risk factors and to develop an effective tool for specific prophylaxis in high-risk infants.

Prematurity is one of most important risk factors for severe RSV infections in young infants because of their immature immune response and their incomplete development of the lungs and the airways. Low GA is one of the most relevant risk factors for RSV-related hospitalization during the first months of life: the yearly hospitalization rate is lower in term infants (4.4%) than in infants born before 28 weeks' GA (9.4%) [14].

Multicenter studies were conducted to analyze RSV epidemiology in large cohorts of term and preterm infants; a higher risk for RSV infections was reported among preterm infants, including also late preterm infants (33–35 weeks GA); these studies also evaluated the risk factors for RSV-related infection and hospitalization in preterm infants [15–22]. The knowledge of the epidemiology of RSV infection and its risk factors is extremely useful to improve the use of the prophylaxis with palivizumab.

A multicenter study conducted in Italy [17] reported a higher rate of RSV infections in infants with GA ≤ 35 weeks than in infants born at term.

Risk factors for severe RSV infections in late preterm were also investigated in the PICNIC study (Pediatric Investigators Collaborative Network on Infections in Canada) by Wang et al. [15] (1995) and in the FLIP study [16] which investigated risk factors that most likely may lead to development of RSV-related respiratory infection and subsequent hospital admission among premature infants born 33–35 weeks' GA.

Moreover, a multicenter cohort Italian study started in November 2009 enrolled newborns born at GA ≥ 33 weeks: preliminary data showed that infants of the lower GA group (33 weeks + 0 days–34 weeks + 6 days) were at a slight higher risk of hospitalization for LRTI during the first year of life [18].

Risk factors for RSV hospitalization reported in these three studies are summarized in Table 1.

CLD is a chronic pulmonary disease which may affect premature infants characterized by oxygen requirement after 28 days of age [23]. The pathogenesis of CLD is multifactorial and is related to prenatal (chorioamnionitis, intrauterine growth restriction) and postnatal (ventilator-induced lung injury, oxidative stress, infections, steroids, pulmonary fluids overload, and nutritional deficits) factors which interfere with lung development before and after preterm birth.

The RSV-related hospitalization rate in infants younger than 6 months with CLD is 56.2/100 children/year [5]. The severity of RSV infections in this population is related to the reduction of lung volume and the airways hyperreactivity, deformation, and inflammation.

In our experience [24], infants with CHD waiting for surgical repair are at high risk of nosocomial RSV infections (9.8%).

Other congenital malformations are associated to a more severe course of RSV LRTI; in a birth cohort study conducted in Colorado from 1997 to 2004, the risk for RSV-related hospitalization was higher in infants <2 years with

spina bifida, agenesis, hypoplasia or dysplasia of the lung, cleft palate alone, and biliary atresia [25].

Congenital or acquired immunodeficiency may also increase the risk of RSV related hospitalization.

Moreover, Luján-Zilbermann et al. [26] reported that the risk for severe respiratory virus infections increased after hematopoietic stem cells transplantation; 14% of the infections detected in this cohort were determined by RSV.

Neuromuscular diseases are also associated with severe respiratory infections, especially in the presence of technology dependence and respiratory support [27]; in a prospective multicenter study conducted in Germany during 6 consecutive RSV seasons (1999–2005), 4.7% of infants with infection had a clinically relevant neuromuscular disease associated to increased risk for seizures (15.1% versus 1.6%), need of mechanical ventilation (9.6% versus 1.9%), and death (5.5% versus 0.2%) [28]. These data were confirmed by Resch et al. [13], who reported that RSV LRTIs are frequently more severe in infants with neuromuscular diseases, because of low pulmonary capacity, coexisting gastrooesophageal reflux and muscle weakness; these conditions lead to impaired cough reflex with increased risk of aspiration and atelectasis.

The prevention of RSV infections in infants is extremely important to decrease the great amount of complications and hospitalization in young infants. For this reason, pharmacological research during the last 20 years aimed at the development of a safe, well-tolerated, and effective drug and led to the current use of monoclonal antibodies.

2. The "History" of the RSV Prophylaxis

The development of a safe, effective, and well-tolerated drug for RSV prophylaxis in high-risk infants has been studied for many years through clinical trials. Many authors reviewed the "history" of the development of the drug used for specific prophylaxis [29–32].

Passive prophylaxis was at first introduced in the 1990s with standard intravenous immunoglobulin (IGIV) after studies developed in cotton rats [33]; neutralizing antibody titer was detected, and it determined virus reduction mainly in the lower airways. After these results in animal models, clinical trials based on the administration of IGIV to high-risk infants monthly during the RSV season were started [34, 35] and reported no statistically significant decrease in the severity of RSV infection, no major adverse events, and a slight decrease in the length of hospital stay. This lack of efficacy could be explained by the insufficient anti-RSV antibody concentration in standard immunoglobulin; for these reasons, the evaluation of the efficacy of a hyperimmune RSV polyclonal globulin (RSV-IVIG) was the subject of two multicenter randomized controlled trials [36, 37]. These studies evaluated the response to five doses of RSV-IGIV administered monthly to preterm infants during the epidemic season and reported a 41–63% decrease in hospital admissions. The use of this drug was invalidated by some drawbacks [38]: the need of an intravenous access, large fluid infusion (15 mL/kg), supply shortages, high protein load (750 mg/kg), theoretical risk of transmission of blood-borne infections, and possible interference with pediatric vaccines.

The effectiveness of RSV-IGIV to prevent RSV infections requiring hospitalization in children with cardiovascular disease was analyzed in a randomized controlled trial enrolling 416 children younger than 4 years with CHD or cardiomyopathy [39]. Monthly RSV-IGIV infusion (750 mg/kg) during the RSV season did not reduce the number of RSV-related hospitalizations, even if it slightly reduced the number of hospital admissions for any respiratory tract infection. The clinical trials investigating the safety and effectiveness of RSV-IGIV were interrupted because of the high incidence of sudden cyanotic adverse events and the worsening of the outcome following cardiac surgery [39, 40]. The use of RSV-IGIV was completely withdrawn in 2003.

2.1. The Use of Monoclonal Antibodies for RSV Prophylaxis. Beyond the environmental prevention used to limit the virus diffusion especially in hospitalized patients, nowadays the prophylaxis of RSV-related hospital admissions is based on the administration of specific monoclonal antibodies to the infants at high risk.

At first, a murine monoclonal anti-RSV antibody (mAb) was developed; it was an IgA intended for topical nasal administration [41]. However, the clinical study did not overcome phase III.

The mAbs neutralizing G surface glycoprotein are not enough effective because of the variability of this protein between the two viral subtypes A and B. F protein has less heterogeneity, and it is stable in different seasons and in different geographic areas [42]; thus it has become the ideal target for the two specific mAbs: SB 209763 and palivizumab.

SB 209763 is an IgG1 specific antibody against the C epitope of the F protein. Its effectiveness is unclear because a large, multicenter, placebo-controlled clinical trial did not report a significant reduction in the number of RSV-related hospital admission after the monthly 10 mg/kg dose administered to 800 European and American children [43].

Palivizumab is an IgG1 antibody specific for a different epitope (A) of F protein that was introduced in the United States in 1998; it is currently the only approved monoclonal antibody used for RSV prophylaxis [44, 45]. It is composed of two sequences, a human one (95%) and a murine one (5%).

The mAbs have the same properties of a human IgG1, with a long half-life (28 days). It is free from the risk of transmission of blood-borne pathogens and it can be produced in large batch lots that provide sufficient supply.

Beeler and Van Wyke Coelingh in 1989 [44] evaluated in a murine model the biological properties of the different epitopes of the RSV F glycoprotein and reported that sites A and C were involved in viral fusion activity. Epitope A and epitope C were reported to be less variable than epitope B and for this reason their neutralizing activity against glycoprotein F is more stable. Moreover, epitope A appeared to be involved in the viral fusion which leads to the formation of syncytia; subsequently, mAbs-neutralizing epitope A contrasts syncytia formation.

Palivizumab reduces RSV replication [46] through the inhibition of the virus fusion with the lung endothelial cells; it has no effect on viral attachment and interaction with target cells and it does not reduce the viral budding.

The biological characteristics of palivizumab were analyzed in cotton rat models [47]. The rats received an injection of antibodies and on the following day they received RSV subtype A and B intranasally. The reduction of the replication of both viral subtypes was greater than 99% after an IV dose of 2.5 mg/kg, obtaining a titer of serum antibodies near 25–30 μg/mL. This serum concentration was considered to be a protective value for human receiving palivizumab to prevent RSV infections.

Several trials were also conducted to determine if a noninhibitory concentration of antibodies could promote viral replication or virus-mediated respiratory disorders; this hypothesis was derived from previous studies conducted in the 1960s [48]. It was reported that the animal lung tissue grew a single viral plaque after the administration of a very low dose of palivizumab (0.0032 mg/kg), and the immunized animals were completely resistant to infection after the clearance of the drug [47].

The possibility of failure of palivizumab efficacy due to genetic variation of the A epitope of the F protein was excluded by surveillance studies [49, 50].

The pharmacokinetic characteristics of palivizumab after a single intramuscular (IM) 15 mg/kg injection were analyzed with the aim of defining the prophylaxis schedule [51, 52]; the mean half-life was 20–30 days with highly variable serum concentration 30 days after each dose.

The effect of the drug on RSV detection in the airways was investigated in hospitalized children with severe RSV disease after an intravenous (IV) dose [53]. The RSV antigen concentration decreased only in the lower respiratory airways, confirming that the reduction of virus concentration by 99% in the nasopharynx requires an antibody titer 10-fold higher than the neutralizing titer needed in the lungs [54].

A study conducted to investigate pharmacokinetic properties of palivizumab in infants <2 years without CHD receiving 15 mg/kg of palivizumab IM monthly reported a half-life of approximately 20 days and a mean serum antibody titer (mean ± standard error, SE) of 37 ± 1.2 μg/mL after the first dose, 57 ± 2.4 μg/mL after the second dose, 68 ± 2.9 μg/mL after the third dose, and 72 ± 1.7 μg/mL after the fourth dose. Serum antibody concentration in infant with CHD was not different after the first and the fourth doses [51].

Children who received palivizumab in the previous epidemic season had mean concentrations 60.7 ± 2.4 μg/mL after the first administration and 86.2 ± 4.2 μg/mL after the fourth administration in the second season of prophylaxis [55].

Another study administered palivizumab with the same schedule to 139 infants younger than 2 years with haemodynamically significant CHD and reported a serum concentration of 98 ± 52 μg/mL before cardiac bypass and of 41 ± 33 μg/mL after bypass; this 58% decrease had no defined clinical consequences [56]. Pharmacokinetic characteristics of palivizumab were also determined in a cohort of Japanese adults [57]. A phase I safety trial was conducted in six Japanese and six overseas adults who received 3 mg/kg IM, 3 mg/kg IV, 10 mg/kg IV, and 15 mg/kg IV of palivizumab. The IV infusion was preferred because of the large volume of fluid in the dose for adult patients; the pharmacokinetic properties were similar for both IM and IV administrations [58]. Maximum concentrations, area under the curve (AUC), half-life, and clearance were similar in the two groups of adult volunteers; no adverse events were registered.

Subsequently a phase II trial was conducted enrolling 31 Japanese children (19 preterm and 13 with CLD) with the previous prophylaxis schedule; mean serum titer through levels and AUC was not different in both Japanese and overseas infants [57]. No adverse events occurred; a case of mild RSV upper respiratory tract infection (URTI) not requiring hospitalization was reported.

These pharmacokinetics trials guided the development of current indications for palivizumab use.

The monthly schedule of palivizumab administration should be continued also if an RSV infection occurs; the prophylaxis should start before the beginning of the RSV season (from November to April in the northern hemisphere) [59].

Since the antibody serum titer is lower after cardiac bypass, patients undergoing this procedure should receive an injection of palivizumab as soon as possible after surgery.

The monthly dose should be injected by aseptic technique in the anterolateral area of the thigh, avoiding the gluteal area because of the risk of injury of the sciatic nerve. Volumes over 1 mL should be administered in divided doses.

The efficacy and safety of palivizumab were investigated in two randomized, double-blind, placebo-controlled trials: the Impact-RSV trial [55] and another study conducted in children with haemodynamically significant CHD [56].

The Impact trial was performed during one RSV season in 1998 and enrolled a cohort of 1502 infants younger than 2 years with CLD or infants born before 36 weeks' GA and aged less than 6 months. The cohort was divided as follows: 1002 randomized children received 15 mg/kg palivizumab at monthly intervals, and 500 received placebo during the RSV season; all children were followedup for 150 days after the enrollment. Primary endpoints were an RSV-positive respiratory illness requiring hospital admission or a moderate respiratory illness in infants who had had a positive RSV test during a previous hospital stay; the number of RSV-related hospitalizations decreased by 55% in the overall palivizumab group, with a reduction in days of hospitalization; the number of days requiring mechanical ventilation, days of hospitalization for other causes, and the incidence of otitis media did not differ between the two groups. This reduction was greater in preterm infants (78%; 95% CI 66–90%) than in infants with CLD (39%; 95% CI 20–58%), who often require hospitalization also for mild respiratory disease. The reported adverse events were not different between the two groups and included injection site reactions (2.7% in palivizumab group versus 1.8% in placebo group), rash (0.9% versus 0.2%), fever (2.8% versus 3%), and nervousness (2.6% versus 2.5%). The mortality rate was 0.4% in the palivizumab group and 1% in the placebo group; none of the deaths was related to palivizumab administration.

The mortality during RSV-related hospitalization was 6.7% in studies that included deaths for causes not related to the infection [15, 20].

The efficacy and safety of palivizumab in two seasons were analyzed in 88 infants of the Impact study [55]. The prevalence of anti-palivizumab antibodies (titer > 1/40) was

observed in one subject and did not determine serious adverse events.

A randomized, double-blind, placebo-controlled multicenter trial enrolled 1287 children aged ≤2 years with haemodynamically significant CHD [56] who received monthly 15 mg/kg doses of palivizumab for five months. This trial reported a reduction of the incidence of hospital admissions (5.3% versus 9.7%), length of hospitalization for RSV infection, and total hospital days with requiring of supplemental oxygen. The study cohort included both cyanotic and noncyanotic CHD; the reduction of hospital admission was 29% in the cyanotic group and 58% in the noncyanotic group.

The incidence of adverse events (fever, injection site reactions, conjunctivitis, and cyanosis) was not different in the palivizumab group and in the placebo group and never led to drug discontinuation. Mortality was not different in the two groups and no deaths were related to palivizumab; moreover, the drug did not affect the management of the CHD.

A recent review [60] stated that prophylaxis with palivizumab is effective to prevent hospitalization for RSV bronchiolitis in infants with CHD, since an effective vaccine is not yet available. Pharmacoeconomic studies were also reviewed; the cost-effectiveness of palivizumab's use in infants with CHD was confirmed.

Palivizumab was also reported to reduce by 80% the risk of recurrent wheezing in children aged 2–5 years and born prematurely, with a 68% decrease in infants without family history of asthma and 80% in infants without atopic background, but this protective effect was not observed in infants with an atopic family [61]. This result could be explained by the fact that RSV-related recurrent wheezing is not mediated by an atopic response.

Moreover, a prospective Italian study comparing 154 palivizumab recipients to 71 palivizumab nonrecipients [62] observed a decrease in hospital admissions after palivizumab administration in infants <6 months at the beginning of their first RSV season.

The treatment of bronchiolitis is essentially based on respiratory support, adequate fluids and nutrition supply, and therapy of respiratory symptoms, since an etiologic therapy does not exist. The safety and effectiveness of palivizumab in children hospitalized for acute RSV infections were investigated in a phase I/II, randomized, multicenter, placebo-controlled, double-blind trial that reported the absence of differences in clinical outcomes between palivizumab and placebo group; no adverse events requiring trial interruption were observed [63].

Another drug of the group of monoclonal antibodies (motavizumab, Medi-524 MedImmune) is a molecule that differs from palivizumab in 13 amino acid residuals; both mAbs are specific for the site A of the F protein of respiratory syncytial virus. The neutralizing effect was measured both in vitro and in animal models and found to be higher for motavizumab [64–66].

Large trials examined clinical effects of this drug for RSV prophylaxis. A double-blind, randomized, phase 2 trial [67] compared properties of palivizumab and motavizumab injected sequentially to 260 infants younger than 2 years with major risk factors for severe RSV infections who received 15 mg/kg palivizumab or motavizumab IM for 5 doses at monthly intervals. Adverse events had the same incidence in all the study groups; mortality was not related to the drug administration. Mean drug serum titer did not differ in the study groups.

A multicenter phase 3 trial involving more than 300 centers [68] analyzed the safety and efficacy of motavizumab in 6635 preterm infants younger than 6 months or younger than 24 months with CLD. The infants received 5 monthly 15 mg/kg doses of palivizumab or motavizumab and were involved in a 5 months followup. Motavizumab was reported not to be inferior to palivizumab in the decrease of RSV-related hospital admissions and RSV LRTI in nonhospitalized patients; the reported adverse events were comparable for the two antibodies.

Safety, immunogenicity, and pharmacokinetics of motavizumab were determined in a randomized, open-label phase I-II study enrolling 217 high-risk children; motavizumab characteristics were comparable to palivizumab [69].

In January 2008 MedImmune submitted to the Food and Drug Administration the request of a biologic license application for motavizumab, but the advisory committee refused to endorse the request for the use of motavizumab in the prophylaxis of RSV infections in the high-risk pediatric population because it had the same safety, efficacy, and tolerability of palivizumab, which has been in use since the 1990s.

Since palivizumab is a safe, well-tolerated, effective but expensive drug, its use is regulated by national and international guidelines based on pharmacoeconomic studies.

The use of the prophylaxis also in late preterm infants has been a matter of concern for many years [70, 71], since this group is at risk of frequent respiratory infections for the immaturity of the respiratory and the immune system; however, the American Academy of Pediatrics guidelines recommend the use of palivizumab only in the first three months of life, in the presence of risk factors for severe disease, in infants born at 32–34 weeks and 6 days, on daycare attendance, and with 1 or more siblings or other infants <5 years old living in the same house.

The prophylaxis was observed to be useful especially in late preterm infants with >1 risk factors for RSV LRTI (birth weight, chronological age, presence and age of siblings, and daycare attendance) [72].

A prospective, multicenter Italian study [17] including 1232 infants in the first 2 years of life confirmed these data and reported in addition an increase of risk for severe RSV LRTI associated with tobacco smoke exposure.

Clinical trials are ongoing in order to establish the cost-effectiveness of prophylaxis in this population and to improve the existing guidelines for prophylaxis schedule with the support of epidemiological data.

A cost-utility analysis was made in 4 cohorts of preterm infants [73]; palivizumab was observed to reduce costs and improve QALY (quality-adjusted life year) in preterm infants <32 weeks. Moreover, it was demonstrated to be cost-effective in infants with 32–34 weeks' GA with the risk factors described by the American Academy of Pediatrics in 2009

and in infants with 32–35 weeks' GA with 2 or more risk factors of the report published in 2006.

The positive impact of prophylaxis with palivizumab on healthcare expense was also confirmed by two retrospective analyses made in Austria [74] and in Spain [75].

A pharmacoeconomic study could also improve the guidelines in use for the prophylaxis of RSV infections in infants with other risk factors than prematurity [76].

Palivizumab cost-effectiveness was confirmed in infants with neuromuscular diseases, congenital diaphragmatic hernia, but it is still controversial in infants with other diseases (Down syndrome, cystic fibrosis).

3. Conclusions

RSV has a worldwide diffusion and it is responsible for a large amount of hospital admissions in young infants, with a subsequent strong impact on infants' health and healthcare costs. RSV infections may lead to respiratory failure and death, especially in infants with chronic pathological conditions. An etiologic treatment for RSV bronchiolitis does not exist at the present, while the use of palivizumab for prophylaxis of RSV infections in high-risk infants is recommended since it is well tolerated and effective in the reduction of RSV-related hospitalizations. Palivizumab is the only monoclonal antibody currently used for anti-infectious purpose.

The extension of prophylaxis with palivizumab to late preterm infants (GA 33–35 weeks) is still a matter of concern since this drug is too expensive to be used for the entire population of late preterm infants. The cost-effectiveness of the use of palivizumab in the late preterm has been analyzed by several studies to identify environmental or individual risk factors for severe RSV infection. The use of risk scores derived from this study is helpful to detect the subjects for whom the administration of palivizumab could be effective to reduce RSV-related mortality and morbidity.

These positive results subsequent to the use of palivizumab to prevent an infectious disease could encourage the use of monoclonal antibodies for prevention and treatment of other infectious diseases in infants and adults.

Abbreviations

RSV: Respiratory syncytial virus
CHD: Congenital heart disease
CLD: Chronic lung disease
LRTI: Lower respiratory tract infection
GA: Gestational age
IVIG: Intravenous immunoglobulin
mAbs: Monoclonal antibodies
IV: Intravenous
IM: Intramuscular
SE: Standard error
AUC: Area under the curve
URTI: Upper respiratory tract infection
QALY: Quality-adjusted life year.

Conflict of Interests

Dr. Lanari has consulted Abbott S.r.l. The authors have no conflict of interests to disclose.

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Review Article

Multiantibody Strategies for HIV

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Vaccination strategies depend entirely on the appropriate responsiveness of our immune system against particular antigens. For this active immunization to be truly effective, neutralizing antibodies (nAbs) need to efficiently counter the infectivity or propagation of the pathogen. Some viruses, including HIV, are able to take advantage of this immune response in order to evade nAbs. This review focuses on viral immune evasion strategies that result directly from a robust immune response to infection or vaccination. A rationale for multi-Ab therapy to circumvent this phenomenon is discussed. Progress in the formulation, production, and regulatory approval of monoclonal antibodies (mAbs) is presented.

1. Introduction

The persistence of HIV as a global epidemic has revealed our limited understanding of how immune barriers function to protect humans against disease [1]. Soon after the recognition of the HIV virus as the causative agent of AIDS, the prediction was made that a vaccine would be available for testing within two years [2]. In the intervening 30 years, the inability to create an effective protective or therapeutic vaccine can be attributed to a number of characteristics of HIV. Some of these characteristics naturally result in evasion from immune surveillance and are also utilized by other viruses [3–5]. Viral evasion in general can involve accumulation of point mutations on immune-dominant regions of surface proteins, glycosylation of functionally pivotal residues (the glycan shield) or their association with host serum components (e.g., lipoproteins) in order to mask them from the immune system, and cell-to-cell transmission. In addition, molecular mimicry, whereby the expression of proteins structurally similar to host defense proteins, can lead to viral persistence [6–9]. These strategies can in turn result in further damaging effects. The secondary consequences of molecular mimicry range from viral-induced autoimmune disease to chronic immune stimulation, for example, HCV-induced cryoglobulinemia.

In the particular case of HIV, immune evasion results from a variety of additional strategies. The incredible sequence diversity within each HIV subtype as well as within individuals during the course of active infection represents an enormous challenge to the immune system. Furthermore, HIV attacks the very cells that are needed to mount an effective and coordinated immune response. The destruction of $CD4^+$ T cells can further facilitate viral replication [10]. Additional evasion strategies involve downregulation of MHC molecules [11–13], establishment of latent viral genomes that can result in production of infectious virus perhaps years later [14], as well as very high mutation rates of the viral genome resulting in infectious viruses that the immune response does not recognize [1, 15].

Evasion strategies that result directly from a robust immune response include neutralization interference by nonneutralizing antibodies (non-nAbs) [3], a potential for enhancement of viral infectivity due to the presence of anti-viral Abs [16], and the propensity of our memory immune system to become overly influenced by the earliest immune response after infection or vaccination. The uncertainties in the development of robust active immunization strategies for viruses such as HIV provide the rationale for passive immunization strategies that employ multiple mAbs as a basis for both protective and therapeutic clinical modalities against a variety of viral infections.

2. Interfering Nonneutralizing Abs (Non-nAbs)

The problem of non-nAb interference has been investigated in a number of viruses and represents a viral evasion strategy

that needs to be addressed if the development of new vaccines is to be successful. This type of evasion strategy also suggests that passive immunization may be an alternative. In the case of HCV, broadly crossneutralizing Abs (bnAbs) are most effective when directed against highly conserved and functionally critical epitopes (e.g., the CD81-binding site) among different genotypes [17–27]. However the binding of these HCV bnAbs may be inhibited by the presence of non-nAbs that bind proximal to the critical residues [28–34]. This hypothesis is still controversial [26] but recent experiments support the existence of interfering Ab populations [35].

In the case of influenza, humoral immunity resulting in the inactivation of the receptor-binding site on HA appears to be the main mechanism of influenza neutralization [36–39]. In addition, bnAbs often inhibit the fusion of the viral envelope with the endocytic vesicle membrane [20, 40–44]. Non-nAbs, if produced in sufficient abundance, may provide a basis for viral escape from the bnAbs [45–48]. Overall, the experimental results suggest that non-nAbs that bind to epitopes of HA may interfere with the binding of nAbs to proximal neutralization epitopes.

Further evidence that prevalent non-nAbs can result in viral escape is found in severe acute respiratory syndrome coronavirus (SARS-CoV). Vaccine strategies, directed to preventing infection, have used the SARS-S viral glycoprotein as a target [49]. This strategy has proven to be problematic since vaccination for coronavirus may result in excessive and sometimes uncontrolled cellular immune responses contributing to the severity of the disease [50]. In the case of SARS-CoV, it has been reported that a nonneutralizing mAb can disrupt the neutralizing activity of mAbs that inhibit infection *in vitro* [51, 52]. Overall, the results suggest that a cocktail of nmAbs binding to different epitopes may be a valid clinical approach [53].

The cocktail strategy may be especially relevant in the case of HIV where cytotoxic T lymphocytes and neutralizing Abs have long been known to select for immune escape mutations during the course of infection [54–57]. In addition, inactivation of bnAbs by non-nAbs has been reported [58–61]. This antagonism has been proposed to be due to steric hindrance [62]. In contrast, the observation of additive reactivity involving non-nmAbs and nmAbs suggests that multi-mAb combinations can support HIV inactivation irrespective of the individual mAb neutralizing potency [59]. In all probability, however, a cocktail approach to passive immunotherapy for HIV will need to involve highly crossneutralizing mAbs [63, 64] whose affinity and epitope locations can overcome the inhibitory effects of interfering non-nAbs.

3. Evasion Resulting from "Original Antigenic Sin"

The human immune system has evolved to respond very quickly and effectively to infectious challenges long after the primary infection has been resolved [65–68]. This immune memory is essentially a quick response capability that avoids the much slower process of the original immune reaction that ultimately gives rise to affinity maturation and an antibody repertoire. With memory, the antibody repertoire can be brought to bear in a matter of days, rather than weeks and months [65–68]. Whereas this rapid response can be essential to preventing repeated infections, it does however have some drawbacks that have provided the opportunity for certain viruses to continually establish successful infections. This susceptibility has to do with the characteristics of the initial immune response and the subsequent inability of the memory response to adequately broaden the repertoire of antibodies in the face of an infection by a similar or mutated strain. In essence, the diversity of a secondary immune response can be compromised by the dominance of the original immune response [69–71].

The first description of this phenomenon was published 60 years ago and was referred to as "original antigenic sin" (OAS) [72]. After an influenza virus infection, antibody produced after re-infection or vaccination with a related strain of virus is apparently still directed against the first strain that resulted in an immune response [73]. In other words, there was a recall of the first influenza virus experienced. This phenomenon, in which the immune system commits itself to the viral variant initially present and continues to make antibodies against the image of this virus even when contemporaneous virus has effectively shed this image, has been observed after infection by a number of viruses [36, 37, 74]. What stops the immune system from continually producing high-affinity neutralizing antibodies against emergent viral variants is not entirely clear.

One potential consequence of OAS is simply a lack of an adequate immune response to mutated virus. In addition, OAS presents a risk of the elicitation of Abs that could potentially enhance disease severity by enhancing viral infection. A prime example where this mechanism has been invoked is dengue virus. In the case of dengue, Abs derived from an initial immune response may act as agents that exacerbate disease by increasing the cellular uptake of viruses, resulting in higher viremia, a phenomenon termed antibody-dependent enhancement (ADE) [38]. While ADE has been the leading theory to explain the observation of increased risk of severe disease upon a secondary infection from a heterologous serotype, recent studies in humans have called into question ADE as the principal mechanism of increased disease risk [39, 75, 76]. Additionally, modifications to antibody Fc regions that disrupt antibody interaction with Fcγ receptors have been shown to be effective strategies in preventing ADE-mediated lethal disease in a mouse model [77].

In spite of the apparent drawbacks of OAS, it has been shown that individuals can mount immune responses to an HIV infection that have all the hallmarks of an OAS response and nonetheless manage to generate bnAbs that coevolve with the mutating virus. A recent study followed this evolution in a single infected individual over a three year period [78]. In spite of the propensity for matured bnAbs to maintain neutralizing activity against the founder virus, potential viral escape mutations in the vicinity of the bnAb epitope were nonetheless neutralized due to bnAbs gaining neutralization breadth during affinity maturation.

OAS therefore is a complex immune response that can result in production of effective neutralizing Abs in some cases.

4. Repertoire Freeze and Anti-Idiotypes

One explanation for OAS is that early induction of Ag-specific B cells and consequent free Abs are able to recognize viral escape mutants with sufficient affinity to successfully compete for viral antigens and minimize the effectiveness of naïve B cells encountering the viral escape [79]. Since these previously activated B cells and antigen-specific Abs are far more abundant than the naïve B cells, they can be selected to undergo somatic hypermutation and affinity maturation that, in some cases, can drive viral escape. The benefit of this phenomenon has been proposed to reside in an adaptive immune response that limits ineffective or even pathological antibodies along a narrow idiotypic axis, hence conserving idiotypic space for functional antibody responses [74].

It has been observed that those Abs derived from early infection very often carry a common idiotype, termed 1F7, that has been proposed as a potential target for therapeutic anti-idiotypic suppression [74, 79]. Whereas suppression of the 1F7-bearing population can allow for a higher titre of Abs capable of neutralizing the autologous contemporaneous viruses, some evidence suggests that bnAbs can develop within the 1F7 repertoire. It has been suggested that the continual selection of the 1F7-idiotype Abs may in fact drive the V region mutations that are the hallmark of HIV bnMAbs. Six well-characterized bnMAbs (b12, VRC01, 2F5, 4E10, 2G12, and Z13e1), and perhaps others, all express the 1F7 idiotype. In addition, the 1F7 idiotype has been found in Abs derived from other chronic infections such as HCV and SIV [74].

Some potential methods for avoiding OAS have been described [37]. These include masking gp120 epitopes [80, 81], using cytokines [82], and suppressing dominant B and T cell clones [80, 83].

5. Broadly Neutralizing Antibodies

The importance of conserved epitopes that are crucial to viral infection or propagation cannot be overstated. As targets of an immune response, conserved epitopes are the foundation of an antibody repertoire containing broadly neutralizing Abs. This is true for the immune response to variety of viruses. The immune response to influenza, for example, has provided insights into the difficulty of devising effective vaccine strategies [84]. This is because in influenza, as in other viruses, the best bnMAb candidates for use in therapy and prophylaxis are not directed against the major antigenic sites. Anti-influenza mAbs with broad-range neutralization activity against highly divergent isolates are generally able to interfere with the viral fusion process in the endosomic vesicle by targeting conserved epitopes at that site. These bnMAbs are poorly induced by infection or vaccination as is the case with HIV and other viral infections. The bnMAbs against influenza and other viruses have been isolated by phage display techniques [41, 85, 86] or directly from human peripheral B cells [20, 44, 87].

Although a robust initial immune response to HIV infection is a hallmark of the disease, only about 20% of infected individuals mount an immune response that contains bnAbs. In addition, neutralizing immune responses rarely contain neutralizing antibodies against all the HIV clades. Broadly neutralizing anti-HIV mAbs are rare but there has been impressive recent progress, utilizing new mAb discovery technologies that have produced a variety of bnMAbs (Table 1) [87–94]. To date, there are approximately 50 bnMAbs that represent an essential arsenal of anti-infectious agents against HIV.

The hope that a single bnMAb will ultimately be found that will not readily select for escape mutations has persisted since the beginning of HIV antibody discovery [95]. The proposition that infectious diseases including HIV can be managed by the use of a cocktail of mAbs was suggested over ten years ago [96]. Clearly, for HIV, a cocktail of bnMAbs would stand a better chance of avoiding selection and providing protection and therapy [3]. The remainder of this paper will focus on HIV and the use of the broadly neutralizing anti-HIV mAbs that have been developed to date.

6. The Effectiveness of Multi-mAb Therapy

Progress towards establishing the effectiveness of a multi-mAb approach compared to single-mAb strategies has recently been reported [97, 98]. In one report [97], in order to evaluate the therapeutic potential of multiple broadly neutralizing antibodies on established HIV-1 infection, groups of humanized mice were infected with CCR5-tropic HIV-1 isolates (HIV-1_{YU2}). Humanized mice were used in order to minimize production of anti-human antibodies.

Mice were first treated using antibody monotherapy that evaluated five different broadly neutralizing antibodies. These antibodies were selected based on their neutralizing activity as well as the breadth of clades that could effectively be neutralized *in vitro*. In addition, each mAb targeted different epitopes. The serum half-lives of these mAbs ranged up to 6.3 days. In general, using monotherapy, viremia rebounded after 14–16 days with the concomitant appearance of gp120 mutations that allowed viral escape from mAb selection. Monotherapy therefore selected for viral escapes by mutation of antibody-targeted epitopes. The ability of a trimix and a penta-mix of bnMAbs to alter the course of infection was then evaluated. In contrast to monotherapy and the trimix, all of the pentamix-treated mice remained below baseline viral loads during the entire treatment course. Prolonged control of the infection was observed with the pentamix primarily due to the long serum half-life of the injected antibodies [99]. The efficacy of these antibody-based drugs may be further enhanced with modifications that extend half-life several folds [100].

Similar experiments in humanized mice and humans where multiple mAbs were evaluated for therapeutic efficacy against established infections did not reveal a significant benefit to the combination bnMAb approach [101–103]. In those experiments, the broadly neutralizing antibodies (b12,

Table 1: Broadly neutralizing monoclonal antibodies (bnMAbs) against HIV.

Epitope	bnMAb	Discovery method	Median or range of IC_{50} values ($\mu g\ mL^{-1}$)	References
MPER[1]	2F5	EBV tfm[2]	3.8–7.8 [132]	[133]
	4E10	EBV tfm[2]	3.4 [134]	[88]
	10E8, 7H6	Neutralization assays[6]	0.3–1.5 [135]	[135]
	Z13e1	Phage display	—	[57]
V1V2[3]	PG9	Neutralization assays[6]	0.1–9.4 [134]	[89]
	PG16	Neutralization assays[6]	0.1–7.6 [136]	[89]
	CH01–04	EBV tfm[2]	0.02–4.9 (CH04) [137]	[137]
	PGT141–145	Neutralization assays[6]	0.2–2.1 [134]	[134]
V3[4]	2G12	EBV tfm[2]	2.4 [a]	[91]
	PGT121–123	Neutralization assays[6]	0.03–0.05 [134]	[134]
	PGT125–131	Neutralization assays[6]	0.02–0.5 [134]	[134]
	PGT135–137	Neutralization assays[6]	0.2–7.8 [134]	[134]
	HGN194	B cell immort[10]	0.1–3.7 [138]	[138]
CD4 bs[5]	b12	Phage display	2.8 [134]	[92]
	HJ16	B cell immort[10]	0.01–9.8 [138]	[138]
	VRC01–03	RSC3[7]	0.3 (VRC01 [134])	[139]
	NIH45-46	gp120, 140 probes[8]	0.06–1.9 [140, 141]	[140]
	3BNC55, 60, 62, 117	gp120, 140 probes[8]	0.01–1.4 (BNC117 [141])	[140]
	12A12, 21, 30	gp120, 140 probes[8]	0.08–2.6 (12A12 [141])	[140]
	VRC-PGV04, 4b	RSC3[7], pyrosequencing[9]	0.2 (PGV04 [134])	[139]
	8ANC37, 131, 134	gp120, 140 probes[8]	0.06–6.3 (131 [141])	[140]
	1B2530	gp120, 140 probes[8]	0.06–9.8 [141]	[140]
	1NC3, 7, 9	gp120, 140 probes[8]	0.02–1.2 (INC9[141])	[140]

[1]Membrane-proximal external region of gp41.
[2]EBV transformation of B cells.
[3]V1V2 site on gp120.
[4]Glycan V3 site on gp120.
[5]CD4 binding site on GP120.
[6]Neutralization assays of B cells from infected donors.
[7]Resurfaced stabilized core 3 probe.
[8]Somatic mutation primers, gp120 and gp140 probes.
[9]454 pyrosequencing to characterize additional VRC01-like antibodies from HIV-1—infected individuals.
[10]Efficient B cell immortalization and high throughput screening.

2G12, and 2F5 in mice; 2G12, 2F5, and 4E10 in humans) were less potent than VRC01 or the bnMAbs used in the Klein et. al. study [97]. This difference in potency as well as the inclusion of two additional mAbs to make a penta-mix may account for the different results.

The mutli-mAb approach is similar to the combination therapies involving antiretroviral, antimicrobial, and anticancer agents since circumventing the selective pressure necessarily involves the simultaneous appearance of multiple mutations. Antibody therapy for HIV also offers the advantage of being able to specifically neutralize the virus, and can recruit other components of the immune system resulting in viral clearance from infected cells by eliciting effector functions [104]. Moreover, immune complexes from bnMAbs may augment native immunity and have far longer half-lives than antiretroviral drugs [105].

7. Multi-mAb Prevention of Transmission

A multi-mAb microbicide has demonstrated 100% efficacy in a humanized mouse model [106]. Broadly neutralizing HIV antibodies 2F5, 2G12, and 4E10 manufactured in mammalian cells and combined as MabGel have completed early clinical trials as a vaginal microbicide [107]. A Nicotiana-manufactured (see Section 9 below) multi-mAb consisting of VRC01-N, 10E8-N, and HSV8-N as an HSV/HIV microbicide is currently in development (Mapp Biopharmaceutical, 2013). Nicotiana-manufactured 2G12 mAb that was vaginally delivered has completed a small clinical trial; no product-related adverse events were reported (Julian Ma, personal communication).

Since intracellular virus would be better protected than free virus from adverse effects of antiviral factors in the

genital environment such as antiviral antibodies [108], and cell-cell transmission enables HIV-1 to evade inhibition by potent CD4bs directed antibodies [109], anti-cell mAbs [110, 111] will be an important component of a multi-mAb microbicide.

8. Regulatory Challenges of Multi-mAb Therapeutics

The regulatory and manufacturing challenges of a multi-mAb strategy have until recently been assumed to be nearly insurmountable. However both the regulatory and manufacturing procedures have been shown to be amenable to straightforward approaches involving FDA guidance and technological advances that have allowed for reproducible batch-to-batch potency as well as genetic stability and consistency [112]. A Phase 1 clinical trial has been performed with a three-mAb cocktail for botulinum toxin being developed by Xoma [113], and Phase 2 trials have been performed by Symphogen involving a 25-mAb and a two-mAb cocktail [114] and by Crucell (two-mAb rabies cocktail [115]).

In one recent report [112], product-specific methods addressing the polyclonality of a multi-mAb product were focused at the genetic level using a T-RFLP methodology, as well as at the protein level using CIEX- and MS-based methodologies to verify the consistency of manufactured batches. At the level of antigen reactivity, methods have been established to verify the potency of each antibody contained in each batch of the product. In December 2010, FDA published a draft Guidance for Industry entitled "Co-development of Two or More Unmarketed Investigational Drugs for use in Combination" (http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htm). The recommendations in this, and an earlier draft guidance (FDA Points to Consider, February 28, 1997), may direct the development of recombinant antibody mixtures for multidisease products (e.g., HSV/HIV microbicides). New and cost-efficient cell banking and manufacturing concepts for multi-mAb products have been described [112, 115–121], and it has been demonstrated that a complex mAb composition containing 25 antibodies can be manufactured in a highly consistent manner in a scaled-up production process. This single-batch manufacturing concept represents a relatively simple approach to the production of complex mixtures of antibodies with an integrated high flexibility with respect to number of antibodies and design of composition.

9. Alternative Production Systems

Given the enormity of the HIV problem as well as the cost sensitivity inherent in the economic environments where HIV therapies are most urgently needed, alternatives to the mammalian cell culture technology might be appropriate. In the past, cost of production for life-threatening antibody-based drugs has not been a significant factor in determining the price of any particular drug [122]. In the case of HIV however the shear size of the unmet need may be beyond the current worldwide manufacturing capability of animal-cell-based production [122].

The cell culture system reported by Frandsen et al. [112] employed a recombination target site for integration of each individual mAb into the same genomic site thereby minimizing genomic position effects caused by the expression cassettes [123]. Each of these mammalian production cell lines is expected to be similar with regard to growth and production characteristics. Other production systems however obviate the need for uniform genetic integration events since no genomic integration is involved in generating the antibody producing cells. For example, using a transient plant system, expression of each mAb can result from the infection of plant cells by *Agrobacterium tumefaciens* [124]. This infection is performed after introducing several provectors into the *Agrobacterium* that can deliver the viral components and the foreign genes to plant cells. In this sense, *Agrobacterium* is the vehicle for primary infection and systemic movement in the plant, whereas the ultimately recombined, functional viral replicon provides cell-to-cell spread, amplification, and high expression. None of the provectors contain plant-selectable markers (e.g., kanamycin resistance), and they are not selected for genome integration and expression (a process that can consume years). Instead, the *Agrobacterium*-delivered provectors are engineered with specific recombinase sites that, when codelivered into the cell with their counterpart enzyme (phage C31 integrase), recombine efficiently *in planta*, forming the completed viral replicon. The mixing and codelivery of multiple *Agrobacterium*-based vectors, each containing a separate component of the viral replicon, is a fast and efficient method for expressing a wide range of proteins combining different elements. The combinatorial and iterative nature of antibody research is well matched to such an approach [124].

Unlike traditional transgenic plant production of mAbs which requires from months to years for scale-up (Table 2), the transient expression technology has proved not only versatile, but capable of rapid, high-yielding production of a variety of proteins [125]. Its ability to rapidly produce gram quantities of mAb within 10 days (from vector delivery to purified mAb) is exceptional in biopharmaceutical manufacturing. Dozens of mAbs to multiple pathogens have been produced in this fashion, and to date, all have been similar to those produced in mammalian cell culture when analyzed by a variety of *in vitro* and *in vivo* assays. In economic terms, the costs of manufacturing of mAbs for preclinical development using traditional mammalian cell culture (e.g., CHO or NS0) can be cost-prohibitive—cGMP—production of a mAb from CHO or NS0 as a contract manufacturer would cost a minimum of $5 M [122]. In contrast, production in the plant transient system under GMP has been estimated to require approximately one-sixth of that cost. It is also anticipated that significant cost-savings in the final commercial product will be realized where it is estimated that the drug substance at commercial scale will cost less than $50/g [126].

Glycosylation has historically been the only practical difference between mAbs produced in mammalian cell culture and in plant tissue [127]. Because of the potential for plant glycans to affect pharmacokinetics as well as immunogenicity

Table 2: Transient plant technology: the advantage of RAMP*.

Expression system	Time to mg of mAb	Time to g of mAb
Mammalian cell culture	2–6 months	3–12 months
Transgenic animals	>12 months	>12 months
Transgenic plants	12 months	>24 months
RAMP	14 days	14–20 days

*adapted from Hiatt and Pauly, 2006 [124].

in humans, a transgenic *Nicotiana benthamiana* line with xylosyltransferase and fucosyltransferase activity effectively knockedout has been frequently used. The resulting glycans in the double-knockout are more homogeneous than current FDA-approved mAbs produced in mammalian cell culture. The 2G12 mAb (Table 1), when produced in the double-knockout plants to yield glycans without xylose or fucose, showed significantly enhanced binding to FcγRIIIa and mediated higher antiviral activity [128]. It is noteworthy that although non-fucosylated mAbs are rare in CHO- and NS0-derived mAb products in comparison to the plant-produced mAb, a large fraction (~30%) of human serum IgG is non-fucosylated [129]. It is particularly relevant for *in vivo* studies that plant-derived mAbs have serum pharmacokinetics identical to those of mAbs produced in mammalian cell culture [130].

10. Summary

Viruses can escape the mammalian immune system by a variety of methods. The evasion methods that derive directly from the characteristic of our immune response include interfering non-nAbs, antibody-dependent enhancement of infection, and an attenuation of the immune response resulting in a limited diversity of Abs to mutated virus. There is a compelling rationale for multi-mAb products that can serve as both preventive and therapeutic drugs for HIV in particular and potentially for a variety of other infections that have proven to be recalcitrant to vaccine development. The availability of numerous broadly neutralizing mAbs for HIV provides the impetus for determining the most appropriate mAb combinations. In the future, multi-Ab candidates for HIV (and other viruses) may use a transformative strategy of epitope delineation based on neutralization fingerprints for screening sera or characterizing antibody specificities induced upon infection or vaccination [131]. In addition, new scalable production systems as well as a favorable regulatory environment may enable multi-mAb products for infectious diseases to be commercialized.

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Research Article

A Human/Murine Chimeric Fab Antibody Neutralizes Anthrax Lethal Toxin *In Vitro*

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Human anthrax infection caused by exposure to *Bacillus anthracis* cannot always be treated by antibiotics. This is mostly because of the effect of the remaining anthrax toxin in the body. Lethal factor (LF) is a component of lethal toxin (LeTx), which is the major virulence of anthrax toxin. A murine IgG monoclonal antibody (mAb) against LF with blocking activity (coded LF8) was produced in a previous study. In this report, a human/murine chimeric Fab mAb (coded LF8-Fab) was developed from LF8 by inserting murine variable regions into human constant regions using antibody engineering to reduce the incompatibility of the murine antibody for human use. The LF8-Fab expressed in *Escherichia coli* could specifically identify LF with an affinity of 3.46×10^7 L/mol and could neutralize LeTx with an EC_{50} of 85 μg/mL. Even after LeTx challenge at various time points, the LF8-Fab demonstrated protection of J774A.1 cells *in vitro*. The results suggest that the LF8-Fab might be further characterized and potentially be used for clinical applications against anthrax infection.

1. Introduction

Anthrax infection is caused by *Bacillus anthracis* which primarily affects livestock but can spread to humans [1]. It is known that anthrax spores have the potential use as a weapon of bioterrorism. The anthrax attacks of 2001 heightened awareness concerning the treatment of anthrax exposure [2]. One of the current clinical treatments for anthrax is to use antibiotics which are effective but limited [3]. This is mainly because of the effect of the remaining anthrax toxin in the body, which cannot be eliminated by antibiotics. Anthrax toxin consists of three protein components: protective antigen (PA), lethal factor (LF), and edema factor (EF). PA combining with LF or EF constitutes lethal toxin (LeTx) or edema toxin (EdTx), respectively [4]. The 83 kDa form of PA (PA83) binds either of two known receptors on the surface of mammalian cells: anthrax toxin receptor 1 (ATXR1)/tumor endothelial marker 8 (TEM8) or anthrax toxin receptor 2 (ATXR2)/capillary morphogenesis protein 2 (CMG2) [5]. Then, PA83 is cleaved by a furin-like protease, producing PA20 and PA63. The latter oligomerizes to a heptamer and forms a pre-pore to bind LF and/or EF. The complex is internalized into cells by receptor-mediated endocytosis, and LF and/or EF are released to cytosol under acid conditions [6]. LF is the major virulent factor which is responsible for shock and death. LF is a zinc-dependent protease which can cleave several members of mitogen-activated protein kinase kinase (MAPKK) family causing lysis of macrophages [7]. In addition, LF offers *Bacillus anthracis* an efficient mechanism to evade the host immune responses by inhibiting interferon regulatory factor 3 (IRF3) activation by lipopolysaccharide and subsequent cytokine production through bacterial

membrane components [8]. EF is a calcium-calmodulin-dependent adenylate cyclase which causes local edema [9].

Recent studies of antitoxin treatments have focused on three aspects: vaccines [10], monoclonal antibodies (mAbs), and other inhibitors, such as dominant-negative mutants of PA [11], soluble receptors [12], and noncatalytic domains of LF and EF [13]. Many neutralizing mAbs against PA have been developed and utilized in clinical trials [14], as PA shares the common part of LeTx and EdTx. However, the neutralization effect may become invalid against mutant strains of *Bacillus anthracis* [15]. Hence, EF and LF mAbs are alternative options to be used alone or in combination with PA mAb [16]. Murine mAbs may have some limitations to be used in humans directly because of the human anti-mouse antibody (HAMA) response [17]. It is necessary to develop mAbs with low immunogenicity including human, humanized, and chimeric mAbs. Human mAbs are generated by technologies of phage display library, transgenic mouse, EBV immortalized human B cell, and human-human hybridoma [18]. Humanized and chimeric mAbs, produced by genetic engineering, have the original target specificity of the murine precursor. Compared to the time-consuming and laborious mutations in development of humanized mAb, chimeric mAb is prepared by recombining of whole murine variable regions, not only CDRs, with human constant regions. Furthermore, in contrast to the repeating administration of the mAb against tumor, the dosage of the anti-infective mAb is not so frequent. Sometimes only a single dose is necessary before or after the exposure to the microorganism [19]. In this situation, chimeric mAb may have as fewer side effects as humanized and human mAbs.

In a previous study, we reported the production of a neutralizing murine mAb (coded LF8) against LF that blocks LeTx formation [20]. In this study, we develop a human/murine chimeric Fab mAb (coded LF8-Fab) which was generated by antibody engineering using LF8 variable regions combined with human constant regions. The LF8-Fab could bind LF specifically and protect J774A.1 cells against LeTx challenge *in vitro* under prophylactic and postexposure conditions. Our results suggest that this chimeric LF8-Fab mAb might be further characterized and potentially be used for clinical treatment of anthrax infection.

2. Materials and Methods

2.1. Murine LF8 and LeTx. Murine mAb against anthrax lethal factor (LF8) was developed and purified in our lab, as described previously [20]. Briefly, BALB/c mice were immunized with purified LF protein, and spleen cells were fused with P3X63AF8/653 myeloma cells using standard protocol. The LF8 was screened by ELISA, immune precipitation, Western blotting, and gel mobility-shifting assay. This murine mAb could inhibit LeTx both *in vitro* and *in vivo*. Purified LeTx (PA and LF) was produced in Dr. Nick Duesbery's lab at Van Andel Research Institute [21].

2.2. Construction of Expression Vector. Vectors pComb3XSS and pComb3XTT were kindly provided by the Barbas laboratory (Scripps Research Institute, USA). Total RNA was extracted from the LF8 hybridoma cells by the TRIzol reagent, and cDNA was synthesized using SuperScript II Reverse Transcriptase according to the manufacturer's protocols. The prokaryotic vector was constructed by cloning LF8-Fab into pComb3XSS following three rounds of PCR amplification as described previously [22]. First, murine variable regions of the heavy chain (V_H) and the light chain (V_L) were amplified by PCR using cDNA of LF8 as template. GAPDH severed as the internal control of RT-PCR using the primers (Forward: 5′-CCCTTCATTGACCTCAAC-3′ and Backward: TTCACACCCATCACAAAC) [23]. Human constant regions of both the heavy chain domain 1 (C_H1) and the light chain (C_L) were amplified by PCR using vector pComb3XTT as the template. Second, equimolar quantities of V_H and C_H1 PCR products were used in the overlap PCR to create the heavy chain Fd (Fd), while equimolar quantities of V_L and C_L PCR products were used to create the light chain. Third, equimolar quantities of Fd and light chain PCR products were used in the overlap PCR to create the Fab DNA. Then, LF8-Fab DNA was cloned into pComb3XSS following single digestion of restriction endonuclease *Sfi* I. The recombinant vector pComb3X/LF8-Fab was sequenced using an ABI 3700-capillary electrophoresis DNA sequencer. Sequences were further analyzed using the VBASE2 database (http://www.vbase2.org/).

2.3. Expression and Purification of Human/Murine Chimeric LF8-Fab. The recombinant vector pComb3X/LF8-Fab was transformed into competent *E. coli* HB2151. The LF8-Fab expression was induced by 1 mM isopropyl-β-D-thiogalactoside (IPTG) overnight at 30°C. Individual clones were identified by Western blotting using goat antihuman IgG (Fab-specific) and IRDye 800CW donkey antigoat IgG in an Odyssey infrared image system (LI-COR Biosciences, Lincoln, NE). Soluble LF8-Fab in the cell lysate was purified by immobilized metal affinity chromatography (IMAC) using a 5 mL His-trap HP column, followed by ion exchange chromatography (IEC) using a 5 mL Q sepharose column as described previously [24]. The purity of the LF8-Fab was examined by SDS-PAGE (10%) with coomassie blue staining.

2.4. Affinity Calculation of the LF8-Fab. The affinity of the LF8-Fab was calculated by noncompetitive ELISA [25]. Ninety-six-well EIA plates were coated overnight at 4°C with LF at two different concentrations, 4 μg/mL and 2 μg/mL. After the plate was blocked, serial concentrations of the LF8-Fab were added (4 replicated wells for each concentration) as the primary antibody. The alkaline phosphatase- (AP-) conjugated antihuman Fab severed as the secondary antibody. The absorbance was read at 405 nm after color development. The concentration of the LF8-Fab and the absorbance at 405 nm were plotted to two hyperbolic curves by GraphPad Prism software version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Affinity constant (K_{aff}) was calculated using SPSS statistical software version 13.0 (SPSS Inc., Chicago, IL, USA) by equation $K_{aff} = 1/(2[Ab']t - [Ab]t)$, where $[Ab']t$ was the free mAb concentration at the OD_{50} of 2 μg/mL coated antigen, while $[Ab]t$ was the free mAb concentration at the OD_{50} of 4 μg/mL coated antigen.

2.5. In Vitro LeTx Neutralization Assay. The LeTx *in vitro* neutralization assay was performed as described previously [26]. Murine macrophage J774A.1 cells, cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS, were seeded in 96-well plates (2×10^4 cells in 100 μL medium per well), 12 h prior to the assay. The LF8-Fab was serially diluted and mixed with LeTx (0.5 μg/mL of PA plus 0.1 μg/mL of LF). The mixture was applied to cells (3 replicated wells for each dilution). Untreated cells and cells with only LeTx acted as controls. Plate was incubated for 3 h at 37°C. Cell viability was determined using a CellTiter 96 AQ_{ueous} assay. The concentration of the LF8-Fab and the percentage of viability were plotted to a hyperbolic curve by GraphPad Prism software. SPSS statistical software was used to calculate the 50% effective concentration (EC_{50}).

2.6. In Vitro LeTx Challenge Study. To determine the protection effect of the LF8-Fab under prophylactic and postexposure conditions, cells were treated with either LeTx prior to the LF8-Fab or the converse at various time points (–120, –60, –30, –15, –5, 0, +5, +15, +30, +60, +75, +90, +105, and +120 min). Minus (–) means mAb treatment prior to LeTx, while plus (+) means LeTx prior to mAb treatment. The percentage of cell viability was calculated in the same way as above.

3. Results

3.1. Construction of Expression Vector. Total RNA was extracted from the LF8 hybridoma cells, and cDNA was synthesized. As expected, murine V_H, V_L, and human C_H1 products were about 350 bp, while human C_L product was about 400 bp. GAPDH severing as the internal control of RT-PCR was also amplified. Then, chimeric heavy chain Fd (about 750 bp), light chain (about 800 bp), and Fab (about 1500 bp) products were consequently achieved (Figure 1(a)). The recombinant vector pComb3X/LF8-Fab was constructed successfully according to sequencing analysis and could be recut by *Sfi* I (Figure 1(b)), indicating the integrity of the vector. The complementary determining region (CDR) and framework region (FR) of V_H and V_L were determined by VBASE2 database (Figure 2). The V_H sequence was one member of the Igh-V15 VH15 family, while the V_L sequence belonged to IGKV4/5 subgroup.

3.2. Expression and Purification of Human/Murine Chimeric LF8-Fab. Recombinant vector pComb3X/LF8-Fab was transfected into competent *E. coli* HB2151. The LF8-Fab expression was induced by 1 mM IPTG overnight at 30°C and then identified by Western blotting in the Odyssey infrared image system. Both heavy chain Fd and light chain were expressed as the expected sizes (Figure 3(a)). The theoretical pI was 7.92, calculated by the Compute pI/Mw tool at ExPASy (http://web.expasy.org/compute_pi/). The optimized IEC eluent was start buffer plus 150 mM NaCl. SDS-PAGE (10%) followed by Coomassie Blue staining showed that heavy chain Fd and light chain were equally expressed, and the purity was above 95% (Figure 3(b)). The output level was about 1 mg purified protein from 1 L *E. coli* culture by BCA protein assay.

3.3. Affinity Calculation of the LF8-Fab. The concentration of the LF8-Fab and the absorbance at 405 nm were plotted to two hyperbolic curves using GraphPad Prism software (Figure 4). The LF8-Fab could identify LF (either at 4 μg/mL or 2 μg/mL) in a dose-dependent manner. Using SPSS statistical software, [Ab′]t was 20.5 nM and [Ab]t was 12.1 nM. According to the equation $K_{aff} = 1/(2[Ab']t - [Ab]t)$, the K_{aff} of the LF8-Fab was 3.46×10^7 L/mol.

3.4. In Vitro LeTx Neutralization Assay. The *in vitro* neutralization assay showed that the LF8-Fab could protect cells against LeTx in a dose-dependent manner and could offer 100% protection at a concentration of 5 μM (Figure 5(a)). In contrast, the irrelevant Fab could not protect cells in the presence of LeTx. The EC_{50} of the LF8-Fab was 85 μg/mL, according to the calculation of SPSS statistical software.

3.5. In Vitro LeTx Challenge Study. In order to determine the protection effect under prophylactic and postexposure conditions, the LF8-Fab was chosen at a concentration of 100% protection (5 μM). LeTx and the LF8-Fab were added to cells at different time points. The results of cell viability indicate that the LF8-Fab could completely neutralize LeTx and protect cells if the mAb treatment was prior to LeTx addition. As for the converse situation when LeTx challenge was prior to the mAb treatment, protection effect declined in a time-dependent manner and had 37.8% protection at +30 min and 26.8% protection at +60 min (Figure 5(b)).

4. Discussion

In the present study, we have produced a human/mouse chimeric anti-LF Fab (LF8-Fab) in *Escherichia coli* which could specifically identify LF with an affinity of 3.46×10^7 L/mol and could neutralize LeTx *in vitro* with an EC_{50} of 85 μg/mL. Even after LeTx challenge prior to the mAb treatment, the LF8-Fab demonstrated protection of J774A.1 cells *in vitro*.

Some of anti-LF mAbs (5/9) listed in Table 1 are murine ones. The others include human, chimpanzee, and chimpanzee/human chimeric mAbs. Only one is Fab, while the rest mAbs are all IgGs. Chimeric mAb, like LF8-Fab, keeps a balance between murine mAb which has high affinity and human mAb which has low immunogenicity. Undoubtedly, human mAbs are the most desirable source for clinical application in human body, while molecular modifications of murine mAbs, including the chimeric and humanized mAbs, can reduce the immunogenicity and can retain the similar affinity and stability of murine mAbs as well. Compared to humanized mAb, generation of chimeric one is relatively time and labor saving. Chimeric mAb with two isoforms, Fab and IgG, has equivalent therapeutic effect and less side effect as humanized mAb, especially in the treatment of infection diseases. Fab mAb, consisting of light chain and heavy chain Fd, lacks Fc region which is not necessary for antibody

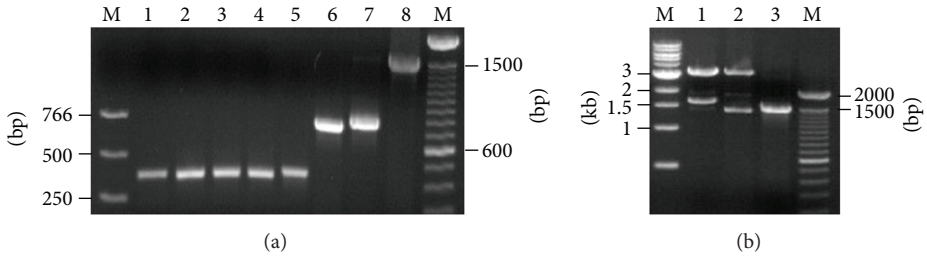

Figure 1: Construction of expression vector. (a) PCR products from three-round amplification. M, DNA marker; lane 1, GAPDH as internal control; lane 2, V_H; lane 3, V_L; lane 4, C_H1; lane 5, C_L; lane 6, heavy chain Fd; lane 7, light chain; lane 8, Fab. (b) Recombinant vector pComb3X/LF8-Fab recut by *Sfi* I. M, DNA marker; lane 1, pComb3XSS cut by *Sfi* I; lane 2, pComb3X/LF8-Fab recut by *Sfi* I; lane 3, PCR product of Fab.

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E  V  Q  L  Q  Q  S  G  A  E  L  V  K  P  G  A  S  V  K  L
GAG GTT CAG CTG CAG CAG TCT GGG GCA GAG CTT GTG AAG CCA GGG GCC TCA GTC AAG TTG   1–60

S  C  T  A  S  G  F  N  I  K  D  S  Y  M  H  W  V  K  Q  R
TCC TGC ACA GCT TCT GGC TTC AAC ATT AAA GAC TCC TAT ATG CAC TGG GTG AAG CAG AGG   61–120
                   CDR1

P  E  Q  G  L  E  W  I  G  R  I  D  P  A  N  G  N  T  K  Y
CCT GAA CAG GGC CTG GAG TGG ATT GGA AGG ATT GAT CCT GCG AAT GGT AAT ACT AAA TAT   121–180
                                CDR2

D  P  K  F  Q  G  K  A  T  I  T  V  D  T  S  S  N  T  A  Y
GAC CCG AAG TTC CAG GGC AAG GCC ACT ATA ACA GTA GAC ACA TCC TCC AAC ACA GCC TAC   181–240

L  Q  L  S  S  L  T  S  E  D  T  A  V  Y  Y  C  T  R  L  D
CTG CAG CTC AGC AGC CTG ACA TCT GAG GAC ACT GCC GTC TAT TAC TGT ACT AGA TTG GAC   241–300
                                                    CDR3

Y  W  G  Q  G  T  T  L  T  V  S
TAC TGG GGC CAA GGC ACC ACT CTC ACC GTC TCT                                       301–333
```

(a)

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E  N  V  L  T  Q  S  P  A  I  M  S  A  S  L  G  E  K  V  T
GAA AAT GTG CTC ACC CAG TCT CCA GCA ATC ATG TCT GCA TCT CTA GGG GAG AAG GTC ACC   1–60

M  S  C  R  A  S  S  S  V  N  Y  M  Y  W  Y  Q  Q  K  S  D
ATG AGC TGC AGG GCC AGC TCA AGT GTA AAT TAC ATG TAC TGG TAC CAG CAG AAG TCA GAT   61–120
                         CDR1

A  S  P  K  L  W  I  Y  S  T  S  N  L  A  P  G  V  P  A  R
GCC TCC CCC AAA CTA TGG ATT TAT TCC ACA TCC AAC CTG GCT CCT GGA GTC CCA GCT CGC   121–180
                               CDR2

F  S  G  S  G  S  G  N  S  Y  S  L  T  I  S  S  M  E  G  E
TTC AGT GGC AGT GGG TCT GGG AAC TCT TAT TCT CTC ACA ATC AGC AGC ATG GAG GGT GAA   181–240

D  A  A  T  Y  Y  C  Q  Q  F  T  S  S  P  S  A  L  T  F  G
GAT GCT GCC ACT TAT TAC TGC CAG CAG TTT ACT AGT TCC CCA TCC GCG CTC ACG TTC GGT   241–300
                              CDR3

A  G  T  K  L  E  I  K
GCT GGG ACC AAG TTG GAA ATC AAA                                                   301–324
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(b)

Figure 2: Nucleotide and deduced amino acid sequences of V_H and V_L. The CDRs are underlined based on the analysis of VBASE2 database. (a) Nucleotide sequence of V_H and deduced amino acid sequence of V_H. (b) Nucleotide sequence of V_L and deduced amino acid sequence of V_L.

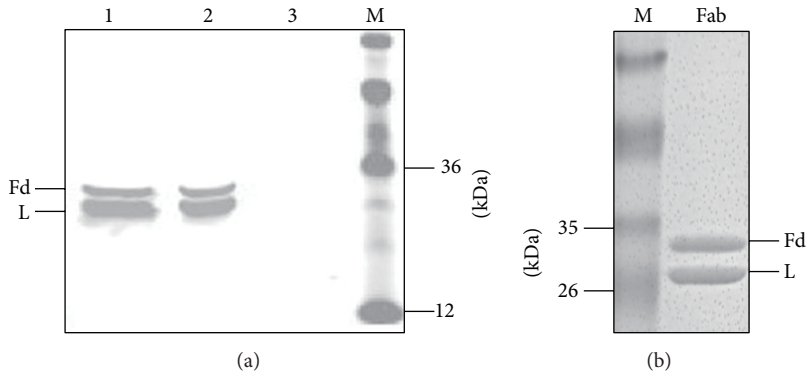

Figure 3: Expression and purification of the LF8-Fab. (a) A goat antihuman Fab and IRDye 800CW donkey antigoat IgG were used to detect Fab expression in Western blotting. Both heavy chain Fd (Fd) and light chain (L) were expressed. Lane 1, supernatant of sonicated lysate; lane 2, pellet of sonicated lysate; lane 3, lysate of untranfected *E. coli* HB2151 as negative control; lane 4, protein marker. (b) Coomassie blue staining showed that heavy chain Fd (Fd) and light chain (L) of the LF8-Fab were equally expressed after the purification of IMAC and IEC.

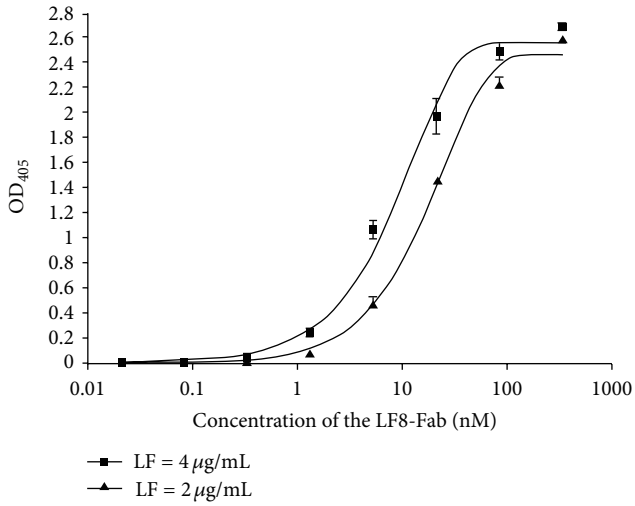

Figure 4: Affinity calculation of the LF8-Fab. Affinity was measured by noncompetitive ELISA. The concentration of the LF8-Fab and the absorbance at 405 nm were plotted to two hyperbolic curves using GraphPad Prism software. The LF8-Fab could identify LF in a dose-dependent manner with an affinity constant of 3.46×10^7 L/mol according to the calculation of SPSS statistical software.

Table 1: List of neutralizing mAbs against anthrax LF.

Authors	Source	Isoform	Reference
Little et al. (1990)	Murine	IgG1	[27]
Zhao et al. (2003)	Murine	IgG	[20]
Lim et al. (2005)	Murine	IgG1	[8]
Albrecht et al. (2007)	Human	IgG1	[28]
Staats et al. (2007)	Murine	IgG1	[29]
Chen et al. (2009)	Chimpanzee	Fab	[15]
Chen et al. (2009)	Chimpanzee/human	IgG1	[15]
Kulshreshtha and Bhatnagar (2011)	Murine	IgG2b	[30]
vor dem Esche et al. (2011)	Human	IgG1	[31]

LF: lethal factor.

binding. Moreover, Fc region may bind to Fc receptors (FcRs) on the surface of certain cells, including leukocytes, epithelial cells, endothelial cells, and hepatocytes [32]. Thus, Fc region may attenuate the specificity of IgG mAb and increase the dosage of antibody. Fab mAb has the advantage of eliminating nonspecific binding between Fc region and FcRs. Moreover, Fab mAb may penetrate tissues more efficiently, as it has smaller size than IgG mAb (only 1/3 the size of IgG mAb) [33].

VBASE2 is used to analyze sequences of V_H and V_L of LF8-Fab. This is an integrative database of germ-line variable genes from the immunoglobulin loci of human and mouse, while all variable gene sequences are extracted from the EMBL-Bank [34]. It is often used to analyze variable regions of antibody sequences with intact FR4 information. According to this database, CDRs and FRs of V_H and V_L were determined. The V_H sequence was a member of the IGH15 family, while the V_L sequence belonged to IGKV4/5 subgroup. Sequences were also examined in the V-QUEST tool at IMGT database (http://www.imgt.org/), and similar results were achieved. The only differentia was that the V_H sequence belonged to the IGH14 family by IMGT. This might be caused by the criterion of different databases. The pComb3X vectors (GenBank accession number AF268281) are phagemids for phage display and antibody expression [35]. In this study, pComb3XTT vector was employed as PCR template to amplify human C_H1 and C_L. The other vector, pComb3XSS, was used for Fab expression. The light chain and heavy chain Fd were expressed, respectively, and assembled in the periplasm of bacterium, and soluble Fab mAb was obtained. A number of techniques have used to purify Fab mAb, including antigen affinity, IMAC, IEC, protein L affinity, gel filtration, and so on [36]. As vector pComb3XSS has the His6 tag, so IMAC was carried out first. However, the relatively low purity of LF8-Fab of IMAC (about 60%) was mainly caused by the endogenous histidine residues in *E. coli* HB2151. So, it was necessary to make a secondary

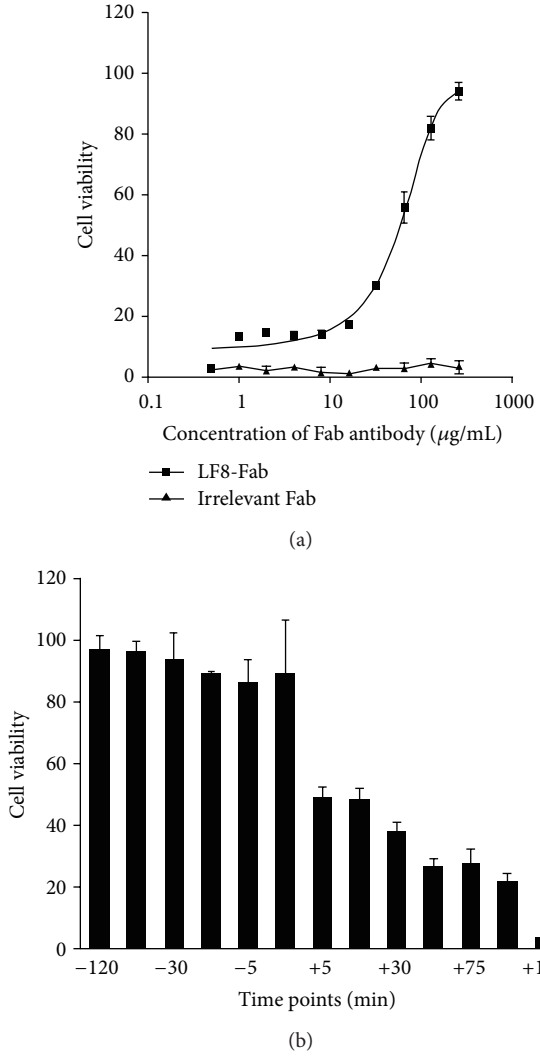

Figure 5: *In vitro* protection of J774A.1 cells against LeTx challenge. (a) *In vitro* neutralization assay showed that LF8-Fab could protect cells against LeTx in a dose-dependent manner, with an EC_{50} of 85 μg/mL according to calculation of SPSS statistical software. The percentage of viability was demonstrated by GraphPad Prism software. (b) *In vitro* LeTx challenge study to test neutralization effect of LF8-Fab before or after LeTx exposure of J774A.1 cells at different time points. Minus (−) means LF8-Fab prior to LeTx, while plus (+) means LeTx prior to LF8-Fab. LF8-Fab could completely neutralize LeTx and protect cells as long as the treatment was prior to LeTx addition. As for the converse situation, protection effect declined in a time-dependent manner.

purification of IEC by Q sepharose column. According to the preliminary experiment of small scale, the optimized IEC eluent was start buffer plus 150 mM NaCl. This was consistent with the calculation by the Compute pI/Mw tool at ExPASy. With the combination of IMAC and IEC, the purity rose to 95% and could be used in further experiments.

Several methods have been established to measure affinity of mAbs, such as surface plasmon resonance (SPR) [37] and quartz crystal microbalance (QCM) [38]. Here we utilized a simple while reliable method based on noncompetitive ELISA, which utilizes the dose-response curve to calculate an affinity constant. This method is based upon the Law of Mass Action, using total antibody concentration added per well rather than the bound-to-free antigen ratio. It compares the OD_{50} of two sigmoid curves of antibody serial dilutions on a plate coated with the same antigen at two different concentrations. It is generally believed that the Fab (and other monovalents) fragment displays a relatively low affinity than the divalent constructs, such as the F $(ab')_2$ and the full-length immunoglobulin [39]. Compared to the affinity of the LF8 (data not shown), LF8-Fab revealed a moderate affinity against LF.

In *in vitro* assay, the molar ratio of PA and LF consisting of LeTx is the key to LeTx challenge. According to other studies [40] and our preliminary test, 0.5 μg/mL of PA to 0.1 μg/mL of LF is an appropriate ratio of LeTx. Under these concentrations, cell viability of J774A.1 cells dropped to zero in less than 2 h. Hence, employing LeTx 120 min prior to mAb treatment was the last time point under postexposure condition. Neutralization activities *in vitro* demonstrated that the LF8-Fab could protect J774A.1 cells well against LeTx challenge. When mixed with LeTx, LF8-Fab could protect cells against LeTx in a dose-dependent manner and offer 100% protection at a concentration of 5 μM. And then this concentration (5 μM) of the LF8-Fab was used to determine the protection effect under prophylactic and postexposure conditions. LeTx and the LF8-Fab were added to cells at different time points. As expected, the LF8-Fab could completely neutralize LeTx and protect J774A.1 cells if the mAb treatment was prior to LeTx challenge. On the reverse situation when the mAb treatment was posterior to exposure to LeTx, the LF8-Fab could partly retrieve the cells in a time-dependent manner. There was about 37.8% protection at +30 min and 26.8% protection at +60 min after exposure.

Further study will focus on several aspects of this mAb. First, the ability of the LF8-Fab to protect mice against LeTx challenge will be evaluated under both prophylactic and postexposure conditions. Second, the affinity needs to be improved by affinity maturation. Third, this mAb will be characterized in detail (i.e., specificity, toxicity studies, autoantigen testing, etc.). Last, epitope mapping and structure-function analysis of the murine LF8 mAb have been performed. Generally, it is believed that conversion from murine mAb to chimeric one may not change the interaction of antigen and antibody. However, the LF8-Fab lacks Fc region, compared to the LF8. So, mAb epitope analysis of the LF8-Fab is still worth investigating. The epitope mapping of Fab-LF interaction will bring out a better understanding of the neutralization mechanism of the LF8-Fab.

In summary, we report herein the development of a human/murine chimeric Fab mAb, the LF8-Fab, to reduce murine immunogenicity. The LF8-Fab can identify LF specifically with moderate affinity and can neutralize LeTx and protect the macrophage cells *in vitro*. Thus, we believe that LF8-Fab might be further characterized (i.e., specificity, toxicity studies, autoantigen testing, etc.) and potentially be used alone or in combination with other neutralizing mAbs for medical therapy of anthrax infection.

Conflict of Interests

All authors have declared that there is no financial conflict of interests in regard to this work.

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