Review

Influenza prevention and treatment by passive immunization

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Passive immunity is defined as a particular antigen resistance provided by external antibodies. It can be either naturally or artificially acquired. Natural passive immunization occurs during pregnancy and breast-feeding in mammals and during hatching in birds. Maternal antibodies are passed through the placenta and milk in mammals and through the egg yolk in birds. Artificial passive immunity is acquired by injection of either serum from immunized (or infected) individuals or antibody preparations. Many independent research groups worked on selection, verification and detailed characterization of polyclonal and monoclonal antibodies against the influenza virus. Numerous antibody preparations were tested in a variety of *in vitro* and *in vivo* experiments for their efficacy to neutralize the virus. Here, we describe types of antibodies tested in such experiments and their viral targets, review approaches resulting in identification of broadly neutralizing antibodies and discuss methods used to demonstrate their protective effects. Finally, we shortly discuss the phenomenon of maternal antibody transfer as a way of effective care for young individuals and as an interfering factor in early vaccination.

INTRODUCTION

Antibodies (Abs) are glycoproteins produced by activated B cells. They are able to bind toxic and antigenic proteins of pathogens. Some Abs can directly neutralize pathogens and/or they can evoke a potent antibody-dependent cell-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC). Neutralizing Abs defend organisms (or cells when tested *in vitro*) against pathogens by blocking essential functions of their target antigens. The term "broadly neutralizing Ab" means that such an Ab can recognize and neutralize multiple variants of highly variable antigens, which is particularly important in protection against fast mutating viruses, such as the influenza virus.

Artificial passive immunization relies on administration of pathogen-specific and neutralizing Abs present in the serum of an immunized individual or purified either from such a serum or from any protein expression system. This process leads to curing of the existing infection (therapeutic immunization) and/or to a short-term protection against subsequent infections (protective immunization). The first successful case of artificial passive immunization was recorded for diphtheria therapy in the late 19th century (see: (Schirrmann *et al.*, 2011; Virdi & Depicker, 2013) and references within). Since that time, passive therapy has been used under many conditions, including prevention of tetanus, auto inflammatory disorders and neutralization of snake toxins (Keller & Stiehm, 2000). Increasing knowledge led to huge technological improvement in Ab-based therapy. Direct serum transfusions or Ab filtrates have been replaced by monoclonal antibodies (mAbs), pre-selected in high-throughput screening procedures and carefully characterized by modern techniques (Brekke & Loset, 2003). Passive immunization does not cause long-term protection because of the relatively short Ab half-life. Yet, externally produced Abs can be a source of a quick, specific help to an organism in danger. Accordingly, passive immunization seems to be useful rather in special cases, such as protection of newborns, elderly or immune-compromised patients, than in preventive and broadly conducted treatments.

Reports on successful application of passive immunization include anti-fungal therapy (Bugli *et al.*, 2013) and treatment of neurodegenerative syndromes such as Alzheimer's disease, Parkinson's disease, Huntington's disease or amyotrophic lateral sclerosis (Huang *et al.*, 2013). Furthermore, drugs based on mAbs, such as Infliximab for rheumatoid arthritis and Crohn's disease, Palivizumab for respiratory syncytial virus, Gemtuzumab for relapsed acute myeloid leukemia, have been approved for clinical application and additional ones are being evaluated in clinical trials (Brekke & Loset, 2003). Recently, the concept of application of Abs as therapeutic or prophylactic agents in infectious diseases caused by pathogens such as human immunodeficiency virus or herpes simplex virus was reviewed and the efficacy of topical or systemic delivery of such antibodies discussed (Whaley & Zeitlin, 2013).

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Influenza viruses are constantly evolving in order to escape neutralizing Abs raised by previous infections or vaccinations. Accumulation of amino acid mutations in major surface glycoproteins of the influenza virus (antigenic drift) results in a failure of pre-existing Abs to efficiently recognize those newer, drifted variants. Therefore, identification of Abs able to target the conserved neutralizing epitopes broadly shared among different influenza variants is important if we want to overcome the necessity of regular (seasonal) updates of anti-influenza vaccine composition and the ensuing problems. Studies on passive immunization against influenza viruses gave hope for creation of the pools of properly described broadly neutralizing Abs, ready for every possible change of the antigenic properties of the major influenza antigens. Multiple examples of such Abs and their experimental application in prophylactic and therapeutic treatment of influenza can be found in recent reviews (Clementi *et al.*, 2012; Laursen & Wilson, 2013). In this review we focus on interesting approaches used for identification of neutralizing Abs and on major techniques applied to demonstrate their effectiveness in the virus neutralization. We also discuss the role of natural maternal immunity in passive immunization against influenza.

TYPES OF ANTIBODIES AND RECOMBINANT ANTIBODY FRAGMENTS USED FOR EXPERIMENTAL TREATMENT OF INFLUENZA VIRUS

For many years passive immunization relied on sera containing Ab mixtures, however development of molecular techniques allowed for creation of mAbs, which can be precisely characterized and produced under sterile conditions. Different variants of mAbs used in influenza virus challenge experiments demon-

strated protective and therapeutic effects. They include full-length and fragments of conventional mammalian immunoglobulins consisting of heavy and light chains (IgG), chicken yolk immunoglobulins (IgY) and heavy chain-only antibodies from camelides (hcIgG). Schematic structures of these variants are shown in Fig. 1. The full-length Ab consists of constant and variable domains. Each variable fragment (Fv) contains an antigen-interaction site, the only part of the Ab determining its antigenic specificity. It is formed by variable fragments of the heavy and light chain (VH and VL, respectively), each built from four framework regions (FR) and three complementarity determining regions (CDR). The Fv fragment of conventional IgG or IgY functions only when the variable domains are connected by a disulfide bond (which occurs when at least one pair of the constant region such C_H1 and C_L1 is connected to Fv) or connected via the linker sequence with a single-chain variable fragment (scFv). Although scFv can be useful for research purposes, clinical application of such polypeptides is limited because of their reduced stability and shorter half-life in comparison to the full-length Abs. Moreover, soluble scFv might have its specificity altered in comparison to a phage-bound scFv or a scFv converted to mAb (Kaku *et al.*, 2012). The scFv fused to a protein transduction domain (or a cell-penetrating peptide) is called transbody. It can translocate across the cell membrane and can be easily internalized (Heng & Cao, 2005) accessing virus proteins inside the infected cells and targeting internal viral proteins (Poungpair *et al.*, 2010; Mukhtar *et al.*, 2009). An Ab fragment where the VH and VL are connected with parts of constant fragments (C_H1 and C_L1, respectively) is named Fab, while an Ab fragment obtained by cleavage of full-length Ab and containing two Fab fragments connected by a hinge forms an $F(ab')_2$. Obtained from hcIgG single-
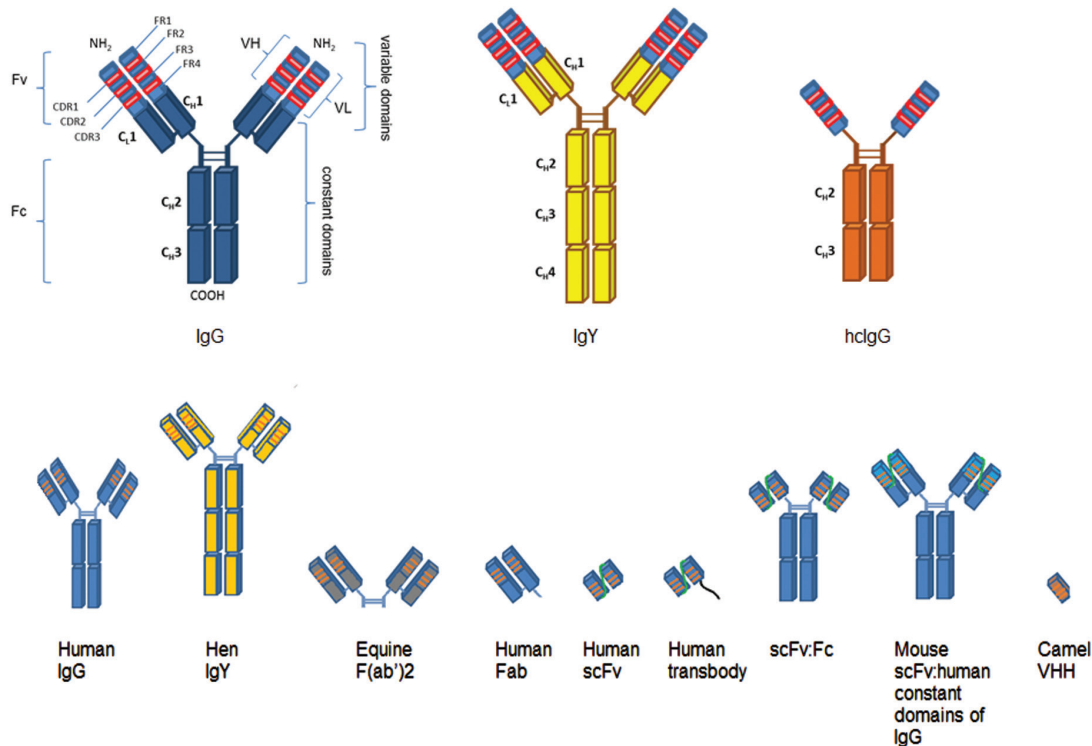

Figure 1. Schematic structure of full-length conventional IgG, chicken IgY, heavy chain-only camelide IgG (hcIgG) and their various recombinant fragments used in passive immunization experiments against influenza virus.
Antibody fragments and domains were labeled only for IgG (see the text for explanation).

domain variable fragments (VHH, also called nano-antibody) are smaller and can penetrate tissues better than conventional Abs (Tillib *et al.*, 2013). Moreover, they can recognize some hidden epitopes that are inaccessible to conventional antibodies. For some applications, especially in treatment of acute infections caused by variable pathogens such as highly pathogenic influenza viruses, polyclonal antibodies (pAbs) might give better results than mAbs. They can be either obtained from immunized subjects or formulated as a mixture of a few known mAbs (Prabakaran *et al.*, 2009; Rinaldi *et al.*, 2014; Simmons *et al.*, 2007; Wallach *et al.*, 2011).

Besides the strategy of passive immunization based on direct delivery of Ab variants, it is worth to mention several approaches based on delivery of the genetic material encoding Ab fragments to the recipient organism. Both, recombinant adenoviral vectors and appropriate plasmids were used as tools for such DNA-based passive immu-nization. Administration of such vaccines resulted in a production of expected antibodies in the recipient cells and in protection against a challenge with influenza virus (Balazs *et al.*, 2013; Tutykhina *et al.*, 2013; Yamazaki *et al.*, 2011).

ANTIBODIES SUITABLE FOR ANTI-INFLUENZA TREATMENT AND THEIR VIRAL TARGETS

Viral neutralization is interpreted as the elimination of viral infectivity. The mechanism of action of neutralizing antibodies depends on their target site (RBS of HA1, fusion peptide etc.) and can rely on disturbing the virion binding to receptors, blocking virion uptake into the cells or precluding fusion of the viral envelope with the host endosome membrane. Neutralizing antibodies targeting various antigens of influenza virus have been described in earlier reviews, such as a review on monoclonal antibodies directed against vari-

Table 1. Examples of broadly neutralizing antibodies with anti-influenza virus activity confirmed *in vivo*.

Neutralized group of influenza viruses	Name	Target of Ab	Form of Ab	Mechanism (if known)	Organism, tissue, isolation method	Organisms used to confirm protection	References
Influenza A and B	CR9114	HA2	mAb	blocks the HA pH-induced conformational changes associated with membrane fusion	Human, peripheral B-cells, Phage display	mice	(Dreyfus *et al.*, 2012)
Influenza A group 1 and group 2	PN-SIA28	Stem (HA1 and HA2)	Fab		Human, peripheral B-cells, Human modified hybridoma	not done	(Clementi *et al.*, 2011)
	FI6, FI6v3	fusion peptide (HA2)		inhibits membrane fusion and perhaps HA0 cleavage	Human, peripheral B-cells,	ferrets, mice	(Corti *et al.*, 2011)
	S139/1	non-contiguous conformational epitope near RBS (HA1)	IgG2a	inhibit binding to sialyl-glycan receptors	Mouse, splenocytes, hybridoma	mice	(Yoshida *et al.*, 2009); (Velkov *et al.*, 2013)
	C05	RBS (HA1)	mAb		Human, Bone marrow, Phage display	mice	(Ekiert *et al.*, 2012)
Influenza A group 1	CR6261	Stem (HA1 and HA2),	scFv-phages, full length IgG1	stabilization of pre-fusion state and inhibit membrane fusion	Human, FACS sorted memory B-cells, Phage display	mice	(Throsby *et al.*, 2008); (Velkov *et al.*, 2013)
	D8, F10, A66	HA2	scFv-Fcs*; IgG1**	inhibits membrane fusion	"Non-immune" antibody human phage display library, phage display	mice	(Sui *et al.*, 2009)
	PN-SIA49	stem (HA2)	IgG		Human, peripheral B-cells, Human modified hybridoma	mice	(De Marco *et al.*, 2012)
	C179	stem (HA1)	IgG2a		mouse	mice	(Lipatov *et al.*, 1997; Okuno *et al.*, 1993; Okuno *et al.*, 1994; Smirnov *et al.*, 2000); (Dreyfus *et al.*, 2013)
Influenza A group 2	CR8020	fusion peptide (HA2)	mAb	prevents virion release in the endosome and inhibits the HA pH-induced conformational changes associated with membrane fusion, prevents HA0 cleavage by host protease	Human, FACS sorted memory B-cells, Sorting of immortalized memory B-cells stained with APC-labeled HA	mice	(Ekiert *et al.*, 2011)

	1B12, 1C9	fusion peptide (HA2)			Mouse, splenocytes, hybridoma	mice	(Prabhu et al., 2009a)
	HAb21	conformational epitope (HA1),	IgG1	probably by interfering with viral binding	Mouse, Splenocytes, hybridoma	positive in pseudovirus-based neutralization assay	(Wu et al., 2014)
	3B1	HA1 (2 epitops)	Mouse scFv: human IgG1		Mouse, splenocytes, hybridoma	mice	(Prabhu et al., 2009b)
	FLA21.140,	conformational epitope proximal to the RBS (HA1)			Human, phage display library (Ph.D-12)	not done	(Khurana et al., 2009); (Velkov et al., 2013)
H5N1 cross-clade	3C11, 4C12, 3H12, 3H4,	HA1	mouse			not done, tested only for virus neutralization	(Du et al., 2009)
	9F4,	linear epitope outside RBS (HA1)	IgG2b	blocks the HA pH-induced conformational changes associated with membrane fusion	Mouse, splenocytes, hybridoma	mice	(Oh et al., 2010) (Velkov et al., 2013)
	AFluIgG01	membrane-distal globular head (HA1)	Human FAb: Fc z vectora pAc-L-Fc	inhibits binding to sialyl-glycan receptors and inhibits the HA pH-induced conformational changes associated with membrane fusion	Human, lymphocytes, phage library	mice	(Cao et al., 2012) (Sun et al., 2009) (Velkov et al., 2013)
H1 cross-clade	CH65	RBS (HA)	Vk:CL + VH: CH1-3 of IgG1	reduction of the amount of viral particles released into the supernatant, reduction of HA in the supernatant	Human, plasmablast, screening single sorted plasmablast		(Whittle et al., 2011) (Brandenburg et al., 2013)
H3 subtype	D1-8	conserved and conformational epitope of HA1	scFv		Human, antibody-secreting plasma cell (ASC)	Mice (therapeutic 3dpi)	(Benjamin et al., 2014)
	12D1	HA2	IgG1		Mouse, splenocytes, hybridoma	Mice (theraputic 2dpi)	(Wang et al., 2010)
Influenza B	CR8033		human mAb	preventing virus progeny release	Human, peripheral B-cells, Phage display	Mice, HA1 top of HA	(Dreyfus et al., 2012)
	CR8071	vestigial esterase domain	human mAb			Mice	

ous proteins of influenza virus (Mancini *et al.*, 2011), two papers focused on broadly neutralizing antibodies (Clementi *et al.*, 2012; Laursen & Wilson, 2013), and an excellent review exploring the knowledge on the antigenic sites of H5 HA molecule (Velkov *et al.*, 2013). The most interesting examples of such Abs are provided in Table 1 and some of them (sorted according to the target antigen) are shortly described below.

Anti-Hemagglutinin

Hemagglutinin (HA) is the major surface protein of influenza virus that forms homotrimers and plays a pivotal role in the virus infection cycle. As a major target of neutralizing Abs HA is under high evolutionary pressure, therefore it is the most variable protein of influenza virus. Eighteen different types of HA (H1-H18) are known, however H17 and H18 were found only in American fruit bats (Tong *et al.*, 2013). HA subtypes were classified into two groups, group 1 including H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17 and H18, and group 2 with H3, H4, H7, H10, H14 and H15.

Abs recognizing conserved epitopes within either globular or stem domains of hemagglutinin (HA) might have a neutralizing potential. Most antibodies against the globular domain of HA can be used as valuable diagnostic tools, however their broad therapeutic and prophylactic use is problematic (Mancini *et al.*, 2011). The antigenic drift and the antigenic shift of the HA globular domain result in the limited specificity of antibodies recognizing epitopes located in this domain (Okada *et al.*, 2010). In contrast, the stem domain of HA is highly conserved across multiple HA sub-types, thus it seems to be a more promising target for therapeutic antibodies. On the other hand, immunoglobulins against stem domain of HA represent only a small fraction of Abs raised by natural infections with influenza virus. The reason is poor immunogenicity of this domain which is less exposed on the virus surface than the HA head domain.

MAbs recognizing the globular domain demonstrate some cross-reactivity. For example, human mAb (mAb 5J8) isolated from a healthy person and targeting a conformational epitope adjacent to the shallow pocket of the receptor-binding site (RBS) is capable of neutralizing

a broad spectrum of H1N1 viruses (Krause *et al.*, 2011). Also, human mAb D1-8 that recognizes the proximal epitope but not within RBS, displays a neutralizing activity against diverse H3 viruses (Benjamin *et al.*, 2014). Neutralizing Abs with much broader specificity and reacting with the globular domain of a variety of HA subtypes are quite rare. Such Abs seem to recognize small epitopes formed by the conserved elements of the RBS (Ekiert *et al.*, 2012; Yoshida *et al.*, 2009).

Various mAbs against the stem region of HA recognize distinct epitopes and display varying neutralizing potential and diverse mechanisms of antigen binding. Interestingly, it has been suggested (based on structural studies with three stem-binding mAbs: CR6261, F10 and FI6) that the use of CDR regions from both, VH and VL chains, is characteristic for naturally selected Abs, while the Abs selected by phage-display method bind to HA using only the VH encoded CDR(Corti *et al.*, 2011). The cross-reactivity of neutralizing mAb CR6261 isolated from human peripheral blood mononuclear cells is limited to influenza A group 1 (Throsby *et al.*, 2008). The potent therapeutic activity of CR6261 was demonstrated by 100% survival of mice that received a single mAb dose 4 days post infection (dpi). CR6261 binds via all three CDRs of heavy chain to the conformational epitope (F subdomain) within the stem region (Corti *et al.*, 2011).

The F subdomain is also targeted by mAb FI6. This mAb displays quite a broad spectrum of cross-reactivity; it is able to neutralize influenza A from both, group 1 (H1, H2, H5, H6, H8, and H9) and group 2 (H3, H4, H7, and H10) (Corti *et al.*, 2011). FI6 showed prophylactic efficacy when administered at a dose of 4 mg per kg of body weight and was partially protective at a dose of 2 mg/kg. The therapeutic potential of FI6 was also confirmed in challenge experiments. All mice survived infection with A/Puerto Rico/8/34 (H1N1) when mAb F16 was administered at a dose of 15 mg/kg, 1 or 2 dpi. Binding to the F subdomain of HA increases HA stability, inhibiting the conformational changes necessary for membrane fusion. The FI6 Abs seem to have quite a high potential in medical and diagnostic applications.

Two other **mAbs,** CR8020 and CR9114, act through a similar mechanism of neutralization, namely they inhibit the membrane fusion by blocking pH-induced conformational changes of HA (Dreyfus *et al.*, 2012; Ekiert *et al.*, 2011). The CR8020 Ab potently neutralizes viruses from group 2 of influenza A, namely a wide spectrum of H3N2 and H7N7 and H10N7, while CR9114 has a much broader spectrum of reactivity and can neutralize not only influenza A but also B types of viruses. Interestingly, both antibodies compete with each other in HA binding which suggests that they recognize the same epitope (Dreyfus *et al.*, 2012). Moreover, CR9114 recognizes a nearly identical epitope to that of CR6261 and the mechanism of binding is similar (three CDRs of heavy chain). Therapeutic treatment with 15 mg/kg of CR8020 completely prevented mortality caused by H3N2 up to 2 dpi. Similar dose of CR9114 was sufficient to protect animals from influenza A and B infections. Therapeutic potential of CR8020 has been tested in clinical trials (see below).

As described by other researchers, 1C9 mAb binds to an antigenically conserved linear epitope GLFGAIAGF, situated in the HA2 fusion peptide (Prabhu *et al.*, 2009). Experiments with the use of a mouse model confirmed 100% protection against H5N1 viruses from clade 1 and

2 when administered 1 dpi, and 50% protection when administered 3 dpi. These antibodies were also effective as a prophylactic therapy when administered 1 day before challenge.

Mechanisms of HA-targeted influenza virus neutralization by several previously described neutralizing anti-HA antibodies binding to known epitopes, were recently investigated by using live cell imaging and infecting viruses (Brandenburg *et al.*, 2013). Results of this study allowed to distinguish four physiologically relevant mechanisms, by which these antibodies interfere with the pivotal functions of HA, namely receptor binding, membrane fusion, HA0 cleavage and egress of newly synthesized viruses. The HA head domain-binding Abs either interfere with attachment of the influenza virus to its cellular receptor (e.g. mAb CR8033) or inhibit viral egress (e.g. mAb CR8071) resulting in reduction of the amount of viral particles released from the infected cells. Antibodies against the stem (e.g. mAbs CR6261, CR8020) overlap with the fusion peptide and inhibit membrane fusion. Alternatively, they can bind close to the cleavage site and prevent cleavage of HA0 into HA1 and HA2. As a consequence, conformational changes of HA, which are essential for infection, are inhibited. This mechanism of virus neutralization (inhibiting the membrane fusion) does not decrease the amounts of viral particles released from the cell but causes the released particles to be non-infectious.

Anti-Neuraminidase

Neuraminidase (NA) is the second most abundant influenza glycoprotein that is localized in the external viral membrane, and it exists as a homotetramer. It is less variable than HA, but it also has a tendency to undergo antigenic shift and antigenic drift. Anti-NA Abs do not neutralize the virus infectivity but they limit the infection by impairing the efficient release of the virus from infected cells, which hampers virus shedding, thus reducing severity of the illness (Mancini *et al.*, 2011). Four antigenic sites were identified on NA. Some anti-NA Abs inhibit the sialidase activity of NA and, in consequence, block viral release from the host cell (Gamblin & Skehel, 2010). They can also reinforce anti-influenza virus response by evoking ADCC (Mozdzanowska *et al.*, 1999).

Two broadly cross-reactive NA-specific mAbs (1H5 and 3H10) interacting with conserved conformational epitope were recently demonstrated to possess a prophylactic efficacy in mice (Wan *et al.*, 2013). Interesting results were also reported for mAb targeting the highly conserved linear epitope of NA (ILRTQESE/SC located between amino acids 222-230). These Abs were able to inhibit all nine types of NA *in vitro* and provided a hetero-subtypic protection in mice challenged with mouse-adapted H1N1 and H3N2, representing group 1 and 2 viruses, respectively (Doyle *et al.*, 2013). In contrast, regular administration of other anti-NA mAb, 2B9, to mice and ferrets resulted only in 50% protection in both animal species against A/Vietnam/1203/04(H5N1) virus (Shoji *et al.*, 2011).

Anti-M2 protein

The M2 protein is located in viral envelope and forms a homotetrameric proton-selective channel, active in acidic pH (Iwatsuki-Horimoto *et al.*, 2006). Extracellular part of M2 (M2e) is strongly conserved among all influenza A viruses. Therefore, despite its poor immunogenic

ability and absence of anti-M2e Abs during a natural infection, it is a promising candidate for broadly-reactive Abs (Mancini *et al.*, 2011). Anti-M2e mAbs do not have neutralization activities but they can reduce virus replication by ADCC and CDC (Wang *et al.*, 2008). Human M2-specific mAb TCN-032 had therapeutic activity against H5N1 *in vivo* and significantly reduced mortality in case of mice challenge with H1N1 (Grandea *et al.*, 2010). Binding of Ab to the highly conserved N-terminal part of M2e also seems to restrict the virus escape. Therapeutic potential of TCN-032 is being tested in clinical trials (see below).

It is worth to note that mAb D005 had prophylactic and therapeutic activities *in vivo* in mice infected with mouse-adapted virus A/Puerto Rico/8/1934 (H1N1), when administered as either human scFv fused to the mouse Fc-γ2c domain or as human IgG1ϰ antibody (Beerli *et al.*, 2009).

Anti-Internal proteins

Other proteins of influenza virus, because of their internal character, do not interact directly with Abs and do not induce production of virus neutralizing Abs. However, they might increase viral clearance by mechanisms such as those reported for anti-NP induction of CDC (Yewdell *et al.*, 1981) or by increase of the T cell response (Thomas *et al.*, 2006). It was also demonstrated that MDCK cells expressing anti-PB2 mAb 143/3 became resistant to H5N3 infection, probably by blocking viral transcription (Fujimoto *et al.*, 2013). It is worth to mention that PEN-HuScFv, an anti-M1 scFv fused to a penetration peptide from *Drosophila*, was able to inhibit binding of M1 (a highly conserved protein, involved in viral replication) to RNA and reduced the amount of viral particles released from the infected cell (Poungpair *et al.*, 2009).

SELECTION OF ANTIBODIES USEFUL IN ANTI-INFLUENZA TREATMENT

The choice of the isolation method is crucial for properties of the resulting Abs. Three very important decisions to be considered during the design stage of such experiments include: (i) a type of donor(s) species or cells, (ii) an organ used as a source of Ab or its gene and (iii) a selection technique (Fig. 2).

Type of donor and source of antibody and its gene

Although genetic manipulation permits for large variations among the selected Abs, every species has some preferences in codon usage or type of synthesized Abs, thus decision concerning donor species can be crucial to final results. Because the mice immune system is well described, the first monoclonal antibodies were obtained in this species. Still, many of therapeutic mAbs are of murine origin. Unfortunately, repeated administration of murine antibodies can cause human anti-mouse antibody reaction, thus their usage in clinical applications is limited (Schirrmann *et al.*, 2011). However, some of them are accepted by FDA (U.S. Food and Drug Administration) (Brekke & Loset, 2003). In order to minimize the side effects of murine Abs, chimeric and humanized Abs
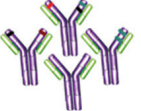
Monoclonal Ab	Donor	Ab source	Selection technique
	Mouse	Splenocytes	Hybridoma
	Human	Bone marrow RNA / PBMC (unsorted, lymphocytes, B-cells – mature, memory, plasmablast or IgG+)	- Hybridoma and other methods related to supernatant screening - Display on phage, mammalian cell or yeast two-hybrid display - Sorting of EBVi B-cells with labeled HA
	Chicken / Camel	Splenocytes / PBMC	Phage display

Polyclonal Ab	Donor	Ab source	Purification technique
	Horse / Sheep / Chicken	Serum / Milk / Eggs	Antibody purification
	Ferret / Guinea pig	Serum	Serum purification

Figure 2. Scheme of the methods used for anti-influenza antibody isolation. PBMC, peripheral blood mononuclear cells, EBVi, Epstain Barr virus immortalized; HA, hemagglutinin.

were created and approved. Moreover, transchromosomic mice engineered to produce solely human-type Abs are already in use (Wang *et al.*, 2008). If a mouse donor is to be used, splenocytes and hybridoma selection techniques are generally applied (Wu *et al.*, 2014; Yoshida *et al.*, 2009; Zebedee & Lamb, 1988).

Modern technologies allow researchers to isolate human mAbs from bone marrow (Ekiert *et al.*, 2012; Kashyap *et al.*, 2008) or from peripheral B-cells (Beerli *et al.*, 2009; Dreyfus *et al.*, 2012; Thathaisong *et al.*, 2008). Promising results concerning influenza virus neutralization were obtained for several other donor species, including horse (Herbreteau *et al.*, 2014), chicken (Pitaksajjakul *et al.*, 2010; Wallach *et al.*, 2011), camel (Tillib *et al.*, 2013); sheep (Rinaldi *et al.*, 2014; Simmons *et al.*, 2007); ferret (Fang *et al.*, 2012) and guinea pig (Howard *et al.*, 2011).

The pAb preparations from human serum have a long history of effectiveness. There are two main methods of artificial passive immunization against influenza for humans, namely convalescent plasma or intravenous immunoglobulin preparations made from pooled human plasma (Hui *et al.*, 2013). If suitable donors are available, passive immunotherapy with convalescent plasma or with hyperimmune globulin prepared from convalescent plasma is a good option to treat severe influenza. However, when such donors are not available, intravenous immunoglobulin preparations which are manufactured in consecutive series reflecting immune status of a population remain an interesting alternative (Kubota-Koketsu *et al.*, 2012). Efficiency of convalescent plasma was described by meta-analysis provided by Luke and co-workers (Luke *et al.*, 2006), who reported that during 1918 H1N1 pandemic there was an overall reduction in mortality from 37% to 16% after such a treatment. Case study for H5N1 influenza treatment with convalescent plasma was also reported (Zhou *et al.*, 2007). Recently, two clinical trials with convalescent plasma and hyperimmune IV immunoglobulin (H-IVIG) treatment have been described (see the Clinical trials chapter).

If a donor is an animal, usually some kind of immunization protocol is used (Wang *et al.*, 2008; Wu *et al.*, 2014; Zebedee & Lamb, 1988). In case of a human donor, antibodies against influenza were obtained from vaccinated subjects (Dreyfus *et al.*, 2012; Ekiert *et al.*, 2011; Throsby *et al.*, 2008), convalescents (Hu *et al.*, 2012; Kashyap *et al.*, 2008; Sun *et al.*, 2009) and from defined populations, for example people born in 1915 or earlier (Yu *et al.*, 2008), described also as a 1918 pandemic survivors (Mancini *et al.*, 2011) or designated as "non-immune", which means that no special effort has been done to increase or measure the influenza-specific Abs titer of donors (Lim *et al.*, 2008; Thathaisong *et al.*, 2008). Additionally, some ready to use (usually "non-immune") libraries, like an Ab phage display library (Sui *et al.*, 2009) or intrabodies library (Mukhtar *et al.*, 2009) were screened for Ab fragments neutralizing influenza viruses. Material for Ab screening can be collected either from a single individual (Beerli *et al.*, 2009; Krause *et al.*, 2011; Liu *et al.*, 2004) or it can be pooled from a few or even tens of donors (Herbreteau *et al.*, 2014; Simmons *et al.*, 2007; Sui *et al.*, 2004; Thathaisong *et al.*, 2008; Yoshida *et al.*, 2009).

Polyclonal antibodies

pAbs purification techniques depend mostly on the used source. While many mammalian Abs are bound by protein A, G and L, main Abs of chicken eggs, IgY, do not, and have to be purified in other ways.

Before the development of molecular technologies, serum transfusions from immunized animals or from convalescents were used for treatment of severe diseases. However, one should remember that such serum could contain potentially harmful pathogens and factors causing anti-serum response of the recipient immune system. Challenge experiments in mice confirmed the protective effects of hyperimmune anti-influenza serum from ferrets (Fang *et al.*, 2012), sheep (Simmons *et al.*, 2007), chicken (Shahzad *et al.*, 2008) and guinea pig (Howard *et al.*, 2011).

Polyclonal immunoglobulins and their fragments from different organism have been used in passive immunization experiments. Two recent examples of such pAb-based approaches include highly purified equine specific F(ab')$_2$ against H5N1, also named Fabenflu (Herbreteau *et al.*, 2014) and ovine polyclonal IgG purified with protein G from lactating ewes (Rinaldi *et al.*, 2014), both very effective in mice protection against influenza virus. It is also worth to mention the successful examples of using influenza-specific IgY from chicken egg yolks in mice challenge experiments; this inexpensive and simple technique of pAb production and purification has many advantages (Xu *et al.*, 2011). At least two independent groups were able to demonstrate in mice models high cross-reactive potential of anti-H5N1 and anti-H1N1 IgY extracted from eggs of hens immunized with the inactivated H5N1 (Wallach *et al.*, 2011; Yang *et al.*, 2014).

Monoclonal antibodies

Several selection techniques resulting in successful isolation of broadly neutralizing anti-influenza antibodies are presented below.

Hybridoma

Hybridoma is the oldest techniques enabling to obtain a relatively stable cell line producing desirable mAb. During the standard hybridoma procedure Ab-producing mice splenocytes are fused with immortal myeloma cells, then the resulting aneuploid cell line supernatant is screened against a given antigen, which is followed by hybridoma cell cloning on agar or by limiting dilutions and re-screening (Lerner, 1981). This technique has proven to be useful for producing Abs against M2 (Fu *et al.*, 2009; Liu *et al.*, 2004; Zebedee & Lamb, 1988), HA1 (Du *et al.*, 2009; Oh *et al.*, 2010) and full-length HA of subtypes H1, H2, H3 and H13 (Yoshida *et al.*, 2009). More recently, anti-HA neutralizing Abs were isolated with a modified hybridoma technique. One type of those modifications uses the Epstein-Barr virus immortalized (EBVi) human B cells (Clementi *et al.*, 2011; De Marco *et al.*, 2012; Krause *et al.*, 2010); second EBVi PBMC and human B cells fused to myeloma (Krause *et al.*, 2011). Those techniques allowed for selection of 2D1 and 2B12 mAbs with neutralizing activity against both, 1918 and 2009 pandemic strains (Krause *et al.*, 2010), mAb 5J8 recognizing a conserved non-linear epitope (residues 133, 137 and 222) on the globular head of H1N1 HA (Krause *et al.*, 2011) as well as a broad specificity mAb such as PN-SIA49 that recognizes conserved epitopes located on the stem region of HA (De Marco *et al.*, 2012) and PN-SIA-28 (Clementi *et al.*, 2011) that is able to neutralize influenza viruses A of groups 1 and 2.

Other methods related to supernatant screening

A technique similar to hybridoma but using non immortalized B-cell lines was used to obtain broadly neutralizing HA-specific Abs (Corti *et al.*, 2011). Supernatants of single plasma cell cultures in a medium supplemented with interleukin 6 (IL-6) were screened with multiple parallel binding assays and cDNA of the selected Abs were rescued by single-cell reverse transcription polymerase chain reaction (RT-PCR). Single cell RT-PCR was also used by other teams to obtain cDNA sequence of Abs broadly neutralizing H1N1 viruses, however in these cases the cDNA libraries were created and screen-

ing of Abs produced by transfected 293T cells (Whittle *et al.*, 2011) or 293A (Wrammert *et al.*, 2011) was performed.

Phage display

Phage display has been used for years and it remains the gold standard for recombinant Abs production (Hairul Bahara *et al.*, 2013). This technique consists of construction of a library expressing peptides or proteins as fusions with phage capsid protein which permits to find phages with proteins interacting with a given DNA, peptides or proteins. The cDNA fragments encod-

Table 2. Monoclonal and polyclonal antibody preparations in clinical trials according to Cinicaltrials.gov database [http://clinicaltrials. gov]).

Responsible party	Material tested	Trial ID	Purpose	Phase /Status*	Results (if known)
Monoclonal antibody preparations					
Crucell Holland BV	CR8020	NCT01938352	To compare prophylactic efficacy against H3N2, safety, tolerability, pharmacokinetics, and potential immunogenicity of CR8020 vs. placebo.	2/C	Completed March 2014.
	CR8020	NCT01756950	To compare the safety, tolerability, pharmacokinetics and immunogenicity of single escalating doses of CR8020 vs. placebo in healthy subjects.	1/C	Antibody admitted to Phase 2.
	CR6261	NCT01406418	To compare the safety, tolerability, pharmacokinetics and immunogenicity of single escalating doses of CR6261 vs. placebo in healthy subjects.	1/C	Antibody initially admitted to Phase 2.
	CR8020 and CR6261	NCT01992276	To evaluate the rate of decline in quantitative viral load measured in hospitalized patients with influenza A infection.	2/W	Withdrawn prior to enrollment due to preliminary efficacy results from an influenza challenge trial.
Theraclone Sciences, Inc.	TCN-032	NCT01719874	To compare safety and efficacy of TCN-032 vs. placebo given to healthy adult volunteers that have been inoculated with the influenza A virus.	2/RP	Estimated study completion date was March 2013, but recruitment is still ongoing.
	TCN-032	NCT01390025	To compare the safety profile of a single intravenous administration of TCN-032 vs. placebo in healthy volunteers.	1/C	Antibody admitted to Phase 2.
Visterra, Inc.	VIS410	NCT02045472	To assess the safety, tolerability, immunogenicity and pharmacokinetics of single escalating doses of VIS410 in healthy volunteers.	1/NB	
Functional Genetics Inc.	FGI-101-1A6	NCT01299142	To determine the safety and tolerability of the anti-TSG101 human monoclonal antibody (FGI-101-1A6) administered to healthy volunteers.	1/U	
Polyclonal antibody preparations					
The University of Hong Kong	Plasma treatment	NCT01306773	Patients with severe 2009 pdm H1N1 infection treated with antivirals were offered treatment with plasma with a neutralizing antibody titer of ≥ 1:160, harvested from patients recovering from the 2009 pdm H1N1 infection. Clinical outcome was compared with that of patients who declined plasma treatment after matching patients by age, sex, and disease severity scores.	NP/C	Mortality and temporal levels of IL-6, IL-10, and TNF-α were lower in the plasma treatment group.
	H-IVIG Treatment	NCT01617317	Patients with severe 2009 pdm H1N1 infection on standard antiviral treatment, requiring intensive care and ventilatory support were randomized to receive H-IVIG (hyperimmune intravenous immunoglobulin) fractionated from patients who recovered from the 2009 pdm H1N1 or normal IV immunoglobulin manufactured before 2009 as control.	NP/C	H-IVIG treatment was the only factor that independently reduced mortality. This treatment was associated with significantly lower post-treatment viral load. The initial serum cytokine level was significantly higher in the H-IVIG group but fell to a similar level 3 days after treatment.

National Institute of Allergy and Infectious Diseases (NI-AID)	Pharmaco-kinetics of H-IVIG	NCT02008578	To determine the pharmacokinetic profile of Flu-IVIG (a type of H-IVIG) and assess whether antibody levels observed following Flu-IVIG transfusion are similar to those predicted in individuals with influenza A or B.	1/O
	H-IVIG preparation study	NCT00383071	To determine how best to use a vaccine for generating high levels of H-IVIG in people, which can be then collected and used to develop a possible treatment for avian influenza.	2/O
	Plasma collection/ preparations	NCT01136057	To collect blood samples from people who have been exposed to the influenza virus and/or who have received a seasonal influenza vaccine. The blood plasma will be processed into fresh frozen plasma and used in a future clinical trial to treat people hospitalized with influenza.	NP/O
	Plasma collection	NCT00984451	To collect plasma samples from people who have high levels of antibodies against the influenza A virus because they either have been previously infected with the virus or have been vaccinated against the infection.	NP/O
	Pharmaco-kinetics of H-IVIG	NCT02037282	To evaluate safety, tolerability and the pharmacokinetics of an anti-influenza H-IVG.	1/RP
	Plasma treatment	NCT01052480	To compare the safety, efficacy, and pharmacokinetics of anti-influenza plasma vs. standard care in hospitalized participants with H1N1 or H3N2 infection at risk of severe disease.	2/RP
Shanghai Public Health Clinical Center	Blood fix treatment	NCT01055990	To treat critical H1N1 patients with blood fix and further observe the effectiveness and safety.	2/U

C, completed; W, withdrawn; RP, recruiting participants; NB, not yet begun; U – status unknown; NP, without designated Phase; O, ongoing

ing such peptides or proteins are inserted as a part of phage's genetic material, and their nucleotide sequence can be easily determined. Usually, this procedure is used to clone scFv (Schirrmann *et al.*, 2011). Phage display succeeded in selection of many broadly neutralizing human anti-HA mAbs (Dreyfus *et al.*, 2012; Ekiert *et al.*, 2012; Kashyap *et al.*, 2008; Sui *et al.*, 2009; Throsby *et al.*, 2008). Also, influenza-neutralizing chicken Fab (Pitaksajjakul *et al.*, 2010) and camel VHH (Tillib *et al.*, 2013) have been obtained by this technique. The phage display library can be used not only for isolation of Abs but also for epitope's mapping (Khurana *et al.*, 2009; Wu *et al.*, 2014). A technique called an *in vivo* phage display is known since 1996 (Babickova *et al.*, 2013).

Yeast two-hybrid display

Yeast Gal4 transcription activator protein is an endogenously expressed yeast protein consisting of the DNA binding domain (BD) and the activation domain (AD). The BD and AD domains can be physically separated and synthesized as chimeric proteins fused with other proteins (X and Y, respectively). If interaction between the partner proteins (X and Y) occurs, it brings the separate AD and BD domains in close proximity, thereby reconstituting the function of Ga14 and driving expression of a downstream reporter gene (Young, 1998). This system was used to obtain Abs targeting the nucleoprotein of influenza virus (Mukhtar *et al.*, 2009). Nucleoprotein gene was inserted into pGBKT7 to create a protein containing the GAL4 DNA-binding domain fused to the NP protein, which was used as a bait. Intrabodies'

cDNA fragments were inserted into pSF50, and then library plasmids were transformed into yeast AH109 cells harboring the bait plasmid. Transformed AH109 cells were grown on a highly selective medium. Single colonies were isolated and transferred onto the QDO medium with X-α-Gal. Blue clones were identified as positive clones indicating interaction between antigen (NP) and the Ab fragments.

Mammalian cell display

Mammalian cell display based on the Sindbis virus expression system was used for selection of Abs recognizing the M2e protein (Beerli *et al.*, 2009). Briefly, in this technique the scFv library from B-cells was prepared in a plasmid allowing for cell surface localization of the recombinant proteins, used for cell transfection. Infected cells were tested by *FACS (Fluorescence Activated Cell Sorting)* analysis for M2-binding to identify virus clones encoding M2-specific scFv antibodies.

Sorting of immortalized memory B-cells stained with allophycocyanin (APC)-labeled HA

Supernatant screening methods usually require testing of large amount of cells; for example 104 000 plasma cells were tested by Corti and co-workers (Corti *et al.*, 2011). A very fast method of Abs screening, based on sorting B cells stained with labeled HA was developed and successfully used to identify anti-HA mAbs (Ekiert *et al.*, 2011). It is worth to mention that mAb CR8020 selected by this method were already tested in Phase 2 clinical trials (see Table 2 and the Clinical Trials chapter).

VERIFICATION OF THE EFFICACY OF ANTI-INFLUENZA ANTIBODIES

Determination of the epitope recognized by mAb gives an important hint for evaluation of its neutralizing activity, especially in the case of Abs against proteins with such well characterized antigenic properties as influenza HA. Nevertheless, even if this epitope is known, the mechanism of neutralization of an antigen might be complex and might be a combination of direct and indirect mechanisms. Because some of them depend on the presence of Fc fragments, differences in virus neutralization by Ab fragments and full-length Abs can be expected (Edwards & Dimmock, 2000). Several *in vitro* and *in vivo* methods can be used for examination of the efficiency of Ab binding and for evaluation of Ab usefulness in virus neutralization.

In vitro methods

Hemagglutination inhibition (HI)

This test depends on natural ability of some viruses (such as influenza virus) to agglutinate red blood cells upon binding to their surface. Incubation of the HA antigen or the entire virus with Abs binding to the receptor binding site of HA will inhibit this process (Brandenburg *et al.*, 2013). Despite theoretical simplicity this test is not fully reliable. It is strongly dependent on subjective assessments, laboratory conditions and other factors, which can introduce some errors (Defang *et al.*, 2012).

Microneutralization (MN)

The theoretical assumption of this test is very similar to the HI test. This highly sensitive and specific test depends on the presence of virus neutralizing Abs in the sample added to the cell culture (e.g. MDCK). The examined Abs or sera are pre-incubated with standardized amounts of the respective virus before its addition to the cells. Subsequently, the ELISA test is used to measure the presence of NP in the infected cells (WHO, 2013).

In vivo methods

This group of methods reflects more accurately immunological reactions of an organism during a viral infection. Challenge with the virus is an unambiguous and the most important test. Each step of such tests should be well justified because of the associated ethical issues and the significant costs. An appropriate animal model has to be chosen depending on the virus strain used and its natural host (Bouvier & Lowen, 2010). Such clinical signs as weight loss, histopathological changes and viral load in tissues (nasopharynges or lung) can be assessed, depending on the animal model. Passive immunization, namely administration of neutralizing Abs combined with challenge with the chosen virus proves most clearly the efficacy and the neutralizing possibilities of the applied Abs. Several animal models are commonly used in immunization study against influenza virus.

Mouse (*Mus musculus*) is commonly used in drug development and challenge experiments. The main advantages are: relatively low cost, high availability, ability of gene manipulations and generally available species-specific reagents. The main disadvantages, from the viewpoint of anti-influenza vaccine efficacy confirmed by challenge, is the lack of typical for humans sialic acid receptors with an α-2,6 linkage to galactose. Without prior adaptation to the mice model, it causes resistance of mice to human influenza viruses.

Ferrets (*Mustela putorius*) are the most representative small animals for human influenza disease model. This influenza susceptible animal has few disadvantages, including the relatively large size, high cost of breeding and husbandry requirements. Its main advantage is similarity of influenza virus infection symptoms in ferrets and humans. Ferrets were used as a model for an antiviral drug (olsetamivir, amantadine) and in a virus transmission study.

Guinea Pigs (*Cavia porcellus*), similarly to ferrets, are highly susceptible to human influenza virus. However, in this model a high level of viral titer does not correlate with disease severity (which is always weaker). Moreover, virus infection does not spread throughout the organism and is limited to the respiratory tissues.

Nonhuman Primates. Rhesus macaques (*Macaca mulatta*) are the most genetically similar to a human animals but this model is usually involved only in the most complicated studies, such as tests of antiviral drugs, vaccine efficacy and examination of gene expression during virus infection. More details about animal models used in research related to influenza can be found elsewhere (Bouvier & Lowen, 2010).

Besides conventional immunization methods based on administration of an antigen (active immunization) or a specific Abs (passive immunization) other experimental methods were effective in obtaining protection against influenza viruses. One of these methods, called vectored immunoprophylaxis (VIP), is based on usage of adeno-associated virus (AAV) expressing Abs recognizing the epitope mapped in the HA stem region (Balazs *et al.*, 2013). The authors cloned the cDNA fragments encoding VH and VL of previously characterized neutralizing mAbs (F10 and CR6261) into the AAV-based expression vector containing the Fc of a human IgG1. Next, the efficacy of the VIP method was tested in a mice model. Within 1 week, a single dose ($1{\times}10^{11}$ genome copies) of recombinant AAV resulted in generation of a detectable level of human IgG in the serum. Expected specificity was confirmed in the HI test. What is worth to note, mice that received recombinant AAV bearing the F10 sequence were fully protected against the H1N1 challenge. Unfortunately, the test in ferrets gave much less promising results. The Abs level was 100-fold lower than in mice and no protection was observed in the challenge experiments (Balazs *et al.*, 2013). Another example of a similar approach was reported for mice that were tested in passive immunization with a recombinant adenovirus expressing anti-HA H5-specific single-domain Ab (Tutykhina *et al.*, 2013). Instead of the classic scFv, the camelid single-domain antibody (sdAb) was used. Intranasal administration protected mice that received sdAbs 14 days before the challenge, against challenge with H5N2 virus. Moreover, combination of adenovirus-expressed sdAb and the recombinant sdAb purified from the bacterial expression system indicated a protective property of this approach in both prophylactic and therapeutic applications (Tutykhina *et al.*, 2013). In a similar approach mice were vaccinated with a plasmid encoding full-length Ab isolated from hybridoma cells (Yamazaki *et al.*, 2011). Experimental infection with H1N1 (A/PR/8/34 strain) that was performed 20 days after intramuscular plasmid administration (in 3 muscles) resulted in 100% protection (Yamazaki *et al.*, 2011).

It is worth to mention that some Abs have the virus neutralizing activity only *in vivo*. Neutralization of influenza virus by such Abs requires interaction between the Fc fragment and Ab Fc receptor for IgG suggesting that the mechanism of their neutralizing activity is based on induction of ADCC (DiLillo et al., 2014).

Clinical trials (http://clinicaltrials.gov/)

The ClinicalTrials.gov database [http://clinicaltrials.gov] contains information about publicly and privately supported research studies conducted in the United States and around the world. Search of this database for the study related to artificial passive immunization strategies against influenza returned 20 trials, 3 referring to maternal passive transfer immunity (described in the chapter Maternal antibodies against influenza and maternal cellular transfer of immunity) and the remaining 17 experiments, at various phases of clinical trials or without clear indication of the clinical phase, are shown in Table 2.

Completed trials

The safety studies (Phase 1 of clinical trials) were completed with three mAbs (CR6261, CR8020 and TCN-032). Later, the Phase 2 trial (effectiveness) was finished with CR8020, however trials including both, CR6261 and CR8020, were withdrawn prior to enrollment due to preliminary efficacy results from an influenza challenge trial.

Two other completed clinical trials were connected with treatments of severe pandemic influenza A (2009 pdm H1N1) infections with human polyclonal Ig preparations. In the first one, the outcome of 20 cured patients treated with orally administered oseltamivir (75 mg) along with 500 mL of convalescent plasma containing neutralizing Abs to 2009 pdm H1N1 was compared with an appropriate group treated with oral oseltamivir alone. Mortality, viral load as well as the levels of IL-6, IL-10 and TNF-α were significantly lower in the group that received the double treatment (Hung et al., 2011). Later, the efficacy of the treatment with H-IVIG (0.4 g/kg) has been assessed by the same group (Hung et al., 2013). The treatment correlated with reduced mortality. An interesting discussion regarding further research on H-IVIG can be found in a recent publication (Joob & Wiwanitkit, 2013).

Ongoing studies

All four current studies are carried out by the U.S. National Institute of Allergy and Infectious Diseases (NIAID). One is a pilot study to determine safety of an anti-influenza H-IVIG. The preliminary results of Phase 2 study on development of Ab treatment for Avian Flu (ID No. NCT00383071) are already submitted on-line. The remaining two studies deal with collection of anti-influenza A immune plasma.

Studies still recruiting participants/not yet begun

Three out of four, non-yet started anti-influenza passive immunization clinical studies are currently recruiting participants (ID No. NCT02037282, NCT01719874, NCT01052480). More subjects are envisaged to be involved in the next study on anti-influenza H-IVIG by NIAID in order to assess the pharmacokinetics of such treatment during longer observation period and with higher doses (ID No. NCT02037282).

After successful completion of Phase 1, Theraclone Sciences, Inc prepares Phase 2a, a double-blinded, placebo-controlled study of TCN-032 with subjects challenged with H3N2 influenza A virus (ID No. NCT01719874). The aim of another Phase 2 study is to assess safety, efficacy and pharmacokinetics of an immune plasma preparation for influenza A treatment (ID No. NCT01052480). The authors plan to enroll 150 adults, children and pregnant women hospitalized with influenza A (H1N1, H3N2) at risk of a severe disease.

The mysterious VIS410 mAb (ID No. NCT02045472) developed by Visterra Inc. was submitted as a candidate to Phase 1 clinical trial. The study does not yet recruit participants, however, the company claims that it will start in 2014. Unfortunately, information about this Ab is limited; according to data provided by Visterra Inc. (http://www.visterrainc.com/pipeline/vis410.html, accessed on July 9th, 2014), it binds a unique conformational epitope on the stem region of HA that is resistant to virus mutation and conserved across all influenza subtypes. The VIS410 Ab prevents membrane fusion between the viral and host membranes.

Studies with unknown status

Two studies (ID No. NCT01299142 and NCT01055990) have unknown status that has not been changed for over two years.

MATERNAL ANTIBODIES AGAINST INFLUENZA AND MATERNAL CELLULAR TRANSFER OF IMMUNITY

Every organism which has been either infected or vaccinated with a sufficient dose of an antigen, reacts with a specific response inducing a long-lasting immune memory. During pregnancy and lactation in mammals and during hatching in birds, Abs from mother's circulatory system are transferred to the progeny. In mammals, IgG class antibodies are transferred by placental cells to the fetus, while IgA class antibodies are secreted into milk. In birds, IgY class antibodies are transferred into the egg yolk.

Experiments with animals clearly indicated that maternal vaccination is important for protection of the offspring against influenza. For example, significant maternal protection of young ferrets against influenza virus has been observed (Hwang et al., 2010; Jakeman et al., 1989). In young mice, suckling on vaccinated mouse was apparently more important than thevaccination status of the mother, but both approaches together gave the best results (Zhang et al., 2013). Such effects were observed with both, DNA immunization and live-attenuated vaccines. Another interesting observation was that the presence of maternal Abs interferes with active immunization of mice offspring (Chen et al., 2007). Therefore, it has been proposed by the authors that mothers and their offspring should not be immunized with the same vaccine against influenza. Similar results were obtained in the case of hen immunization against avian influenza (Maas et al., 2011).

Several clinical experiments demonstrated that vaccination against influenza during pregnancy is safe and effective and diminishes a number of episodes of respiratory illness with fever in infants and their hospitalization rate (Benowitz et al., 2010; Brydak & Nitsch-Osuch, 2014; Eick et al., 2011; Zaman et al., 2008). In most completed human trials the detailed breast-feeding-related data were usually not available and therefore these data cannot be correlated with results on vaccine efficiency. Fortunately, this situation has now changed. Recently completed Phase 2 trial was comparing the postpartum immunization of breast-feeding women with two classical

vaccines (ID No. NCT01181323). The first results are already submitted on ClinicalTrials.gov website (http://clinicaltrials.gov) with data on breast milk ELISA IgA and IgG titer to be added. Clinical trials to which participants are recruited (ID No. NCT01496079, no phase and NCT01034254, Phase 3) add the assessments of influenza antibody titer in maternal colostrum and breast milk in women immunized in early or late pregnancy.

It has been known for a long time that not only Abs but also immune cells can be transferred via placenta and milk. Maternal leukocytes were discovered for the first time in the cord blood over 40 years ago (Desai & Creger, 1963). Recent data indicating that transplacental transfer of cells from mother to child (known as maternal microchimerism) takes place in 42% of normal pregnancies (Eikmans et al., 2014; Jeanty et al., 2014), provide a new perspective concerning the significance of transfer of immune system cells via placenta. It can be a potentially additional source of immunity against influenza or other infectious diseases gained by unimmunized and not-yet-infected child. Also in milk, besides the secretory IgA antibodies and previously known factors preventing bacterial infection such as lysozyme or lactoferrin, presence of leukocytes and stem cells has been confirmed (Hassiotou et al., 2012). Moreover, human milk contains numerous proteins, many of them known to possess immune properties (Roncada et al., 2013). Therefore, although passive immunity definition excludes cell transfer, complete research on immunity acquired from mothers should also take this phenomenon into consideration. Apparently, passive immunization works mainly as maternal immunity transferred after vaccination or infection during or before pregnancy/breast feeding. Reasonable vaccine programs for pregnant women can benefit by lowering hospitalization rates and fatal cases in infants less than 6 month old to whom vaccination against influenza is not recommended.

CONCLUSIONS

Not always the preventive active immunization is sufficient to effectively protect against influenza virus infection. Highly immunogenic epitopes of surface-exposed antigens of influenza virus are under strong evolutionary pressure to escape Ab-mediated neutralization. Therefore, cross-reactive broadly neutralizing Abs are relatively rare because they are usually directed against epitopes with lower immunogenic potential. This phenomenon increases the probability of mismatch between vaccine strains, which are selected six month before influenza season, and dominant/most dangerous infection in the vaccinated population. Therefore, administration of purified broadly-neutralizing antibodies is sometimes the only way to succeed in virus clearance, despite high cost and time-consuming production of such antibodies. For the purposes of passive immunization Abs can be applied topically, by systemic injection or as a component of multipurpose vaccines. A short lasting protection provided by artificial passive therapies makes their massive usage unreasonable in prevention of infectious diseases. The costs and logistics of such treatments set this technique as a second line of defense against influenza virus, after antiviral drugs, and limit it to the very special cases and groups of high risk patients. Still, antibodies with broad-neutralizing activity might save lives during influenza pandemics.

Most of research on passive immunization against influenza focus on monoclonal full-length antibodies. They have longer half-life and can induce CDC and ADCC response. In the authors' opinion they are the most promising candidates for the artificial passive treatment. Nevertheless, simple and quick purification of polyclonal antibodies from convalescent sera remains the most frequent way to save lives in cases of severe infections with influenza virus non-responding to standard antiviral treatment.

Further research is needed to confirm a possibility of virus repression by intracellulary expressed or internalized recombinant antibody fragments targeting internal viral proteins. The HI and MN tests are the main in vitro, and animal challenge is the main in vivo, way of confirming passive immunization efficacy. Unfortunately, many antibodies are screened only against a narrow range of strains and administration protocols which complicates (if not makes impossible) their comparison. 'Gold standard' techniques like hybridoma and phage display are the most frequently used for selection of specific mAbs, however new technological approaches like sorting of immortalized memory B-cells stained with a labeled target, have already resulted in isolation of the most promising mAbs. Multiplicity of Abs and the sources of genes encoding Abs as well as diversity of selection methods increase a chance to achieve success.

Naturally acquired passive immunity — both in a form of Abs transferred through the placenta or milk and by maternal cellular transfer, is also investigated with growing interests. Those mechanisms are very important for prevention of influenza infection in children under 6 months old for whom vaccination is not recommended, as well as for vaccination programs with high failure risk due to covering the vaccine antigen by maternal Abs.

Both artificial (with mAbs and pAbs) and natural passive immunizations are subjects of ongoing clinical trials. Their results might be the milestones for passive immunization approaches against influenza in the future.

Finally, it is necessary to mention that various anti-influenza Abs can be useful in other applications, for example in immunodiagnostics.

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