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Review

The Density Code for the Development of a Vaccine?

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ABSTRACT

The development of prophylactic vaccines remains largely empirical in nature and rarely have general rules been applied in the strategic decision and the formulation of a viral vaccine. Currently, there are a total of 15 virus agents from 12 unique virus families with vaccines licensed by the U.S. Food and Drug Administration. Extensive structural information on these viral particles and potential mechanisms of protection are available for the majority of these virus pathogens and their respective vaccines. Here I review the quantitative features of these viral surface antigens in relation to the molecular mechanisms of B-cell activation and point out a potential correlation between the density of immunogenic proteins displayed on the surface of the vaccine antigen carrier and the success of a vaccine. These features help us understand the humoral immunity induced by viral vaccines on a quantitative ground and re-emphasize the importance of antigen density on the activation of the immune system. Although the detailed mechanisms behind this phenomenon remain to be explored, it implies that both the size of antigen carriers and the density of immunogenic proteins displayed on these carriers are important parameters that may need to be optimized for the formulation of a vaccine.

The Impact of Vaccination on Human Health

The recent outbreak of zika virus in Latin America and the Caribbean[1] is growing rapidly. Up to 1.3 million suspected cases have been reported in Brazil by December 2015. By March 2016, 33 countries and territories in the Americas have reported confirmed cases of zika virus infection.[2] Less than 2 years ago, the devastating Ebola virus outbreak occurred in West Africa.[3] With more than 11,000 deaths reported, this Ebola outbreak has become the most extensive Ebola outbreak in human history.[3]

Besides the necessary social and administrative measures that must take place in the events of these infectious disease outbreaks, effective vaccination against these infectious diseases,[4,5] if feasible, might offer one of the long-term solutions to these public health threats. Indeed, vaccination has made a major impact on global population health and has controlled more than a dozen of human diseases. In looking through the human history, perhaps few scientific discoveries can rival vaccination in its efficacy and magnitude in the elimination of disease burden and suffering. In this context, antibiotic does not even compete with vaccination, because only safe water, which is considered to be a basic human right by the World Health Organization,[6] performs better.[7]

Based on the statistics from the Centers for Disease Control and Prevention in the United States, vaccination has eliminated several diseases from the Americas. Notably, the percent reduction in the annual morbidity before and after routine vaccination is 97% or more for smallpox, diphtheria, measles, mumps, polio, rubella, congenital rubella syndrome, tetanus, and *Haemophilus influenza*.[8] More than 88% reduction in annual morbidity is also achieved for pertussis (i.e., 100-day cough), hepatitis A, and varicella.[8] Among these different diseases, smallpox, measles, mumps, polio, rubella, hepatitis A, and varicella are all caused by viral infections.

A Short List of Licensed Viral Vaccines

A survey of vaccines currently licensed for immunization and distribution in the United States identified over 90 pharmaceutical products (http://www.fda.gov/BiologicsBloodVaccines/Vaccines/). Among these, 15 virus agents from a total of 12 unique virus families have been targeted. These virus agents are listed in Table 1.[9-22]

As seen from Table 1, these virus agents include viruses that use different forms of nucleic acids as their genetic materials. Of pharmaceutical scientists' interests, 9 virus agents of 15 have live

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0022-3549/

Table 1
Virus Agents With Licensed Vaccines for Immunization and Distribution in the United States

Virus Agents	Virus Family	Virus Genus	Virus Diameter (nm)	Genetic Material of the Virus	Form of Vaccine	Suggested Mechanism of Action
Adenovirus type 4 and 7	Adenoviridae	Mastadenovirus	92[9]	dsDNA	Enteric-coated live virus	Ab
Hepatitis A virus	Picornaviridae	Hepatovirus	27-32[10]	ssRNA, positive sense	Suspension of inactivated virus	Ab suspected
Hepatitis B virus	Hepadnavirus	Orthohepadnavirus	42[11]	Partially dsDNA	Suspension of subunits	Ab
Human papillomavirus type 6, 11, 16, and 18	Papillomaviridae	Alpha-papillomavirus	60[12]	dsDNA	Suspension of viral-like particles	Ab suspected
Influenza type A and B	Orthomyxoviridae	Influenza viruses A, B	120[13]	ssRNA, negative sense	Inactivated or live attenuated virus	Ab suspected
Japanese encephalitis virus	Flaviviridae	Flavivirus	40-50[14]	ssRNA, positive sense	Suspension of inactivated virus	Ab
Measles virus	Paramyxoviridae	Morbillivirus	50-510[15]	ssRNA, negative sense	Live attenuated virus	Ab
Mumps virus	Paramyxoviridae	Rubulavirus	100-600[16]	ssRNA, negative sense	Live attenuated virus	Ab
Poliovirus	Picornaviridae	Enterovirus	31[17]	ssRNA, positive sense	Suspension of inactivated virus	Ab
Rabies virus	Rhabdoviridae	Lyssavirus	100-430 nm long, 45-100 nm diameter[18]	ssRNA, negative sense	Suspension of inactivated virus	Ab
Rotavirus	Reoviridae	Rotavirus	100[19]	dsRNA	Live attenuated virus	Not clear
Rubella virus	Togaviridae	Rubivirus	55-90[20]	ssRNA, positive sense	Live attenuated virus	Ab
Variola virus	Poxviridae	Orthopoxvirus	360 × 270 × 250[21]	dsDNA	Live vaccinia virus	Ab and cellular immunity suspected
Yellow fever virus	Flaviviridae	Flavivirus	50[22]	ssRNA, positive sense	Live attenuated virus	Ab
Varicella-zoster virus	Herpesviridae	Varicellovirus	Not well characterized	dsDNA	Live attenuated virus	Ab and cellular immunity suspected

dsDNA, double-stranded DNA; ssRNA, single-stranded RNA; dsRNA, double-stranded RNA; Ab, antibody.

attenuated vaccines available, 5 have suspensions of inactivated viruses as vaccines, 1 vaccine formulation is made of suspensions of viral-like particles (human papillomavirus type 6, 11, 16 and 18), and 1 vaccine formulation is made of suspensions of subunits (hepatitis B virus).

Because the more similar a vaccine is to the disease-causing form of the agent, the better the vaccine can mimic the immune responses to the agent during its natural infection,[23] and it is perhaps not a surprise that more than half of the licensed viral vaccines are live attenuated viruses. The live attenuated vaccine is able to replicate in the vaccinated subject, which generates immune responses very similar to that of the natural infection by the wild-type virus, but with much milder adverse reactions. On account of these features, live attenuated vaccine is usually administered in a single dose, which yields long-lasting protective immunity. However, replication of the vaccine may also accumulate mutations that on rare occasions revert the original attenuated virus back to a virulent strain. This scenario has been documented for the live attenuated Sabin type 3 oral polio vaccine,[24] which underscores the cautions necessary for the development of a live attenuated virus. For viruses that are known to integrate into human chromosomes such as human immunodeficiency virus type 1 (HIV-1), a live attenuated vaccine may not even be an option given the potential risks associated with the viral gene integration. The other forms of vaccines may not be as effective as live attenuated vaccines. These include inactivated viruses, subunits, or viral-like particles. Some of these vaccines may require multiple doses of administration before long-lasting immunity can be achieved, and hepatitis B virus vaccine is just one such example. However, they are generally considered to be safer because these vaccines cannot cause disease from infection.

The mechanisms of protection by these licensed vaccines are almost always complex in nature. In addition, to achieve durable protection, certain vaccines will have to require specific administration schedules. As seen from Table 1, the mechanisms of protection are not even certain or clear for several of these licensed vaccines. However, it appears that the protective immune responses to inactivated vaccines, viral-like particles, or subunit vaccines are often correlated with antibodies. This makes sense given the fact that there are no mechanisms to replicate these viral vaccines in the cytosol, unlike the live attenuated vaccines, and thus rarely can these antigens be presented through the canonical major histocompatibility complex class I pathway for recognition by virus-specific cytotoxic T lymphocytes. As a consequence, the key to the success of these types of vaccines will be the optimal activation of viral-specific B cells for eventual production of memory B cells. And here is the question: how can one formulate these viral vaccines to best activate B cells for production of potent protective antibodies?

High-Density Display of Surface Antigens on Virus Particles

In this review, I would like to point out a potential correlation, the further test of which may help the formulation or consideration of viral vaccines, especially when inactivated vaccines, viral-like particles, or subunit vaccines are being considered for elicitation of humoral immunity. Central to this correlative study is the density of the viral immunogenic proteins displayed on the surface of the vaccine antigen carrier, a biophysical parameter that has not been quantitatively compared across different viral agents previously, yet the variation of which may produce different B-cell responses. In order to identify common features that may have the potential for application in the formulation of a vaccine, I will focus on viral vaccines currently licensed in the United States and examine the correlation between this biophysical parameter and the efficacy of the viral vaccine. For the convenience of this review, the vaccine antigen carrier is defined as the biochemical or biophysical entity that is directly recognized by the immune cells and elicits immune responses when it is administered *in vivo*, to distinguish from DNA vaccines, which need be expressed in the host cell and it is the expression product that is recognized by the immune system for induction of desired immune responses. Based on this definition, all the licensed vaccines listed in Table 1 are vaccine antigen carriers by themselves. In realizing the complex immunological mechanisms that can give rise to a successful vaccine, this review makes no attempt to conclude that the density of antigens is the sole factor

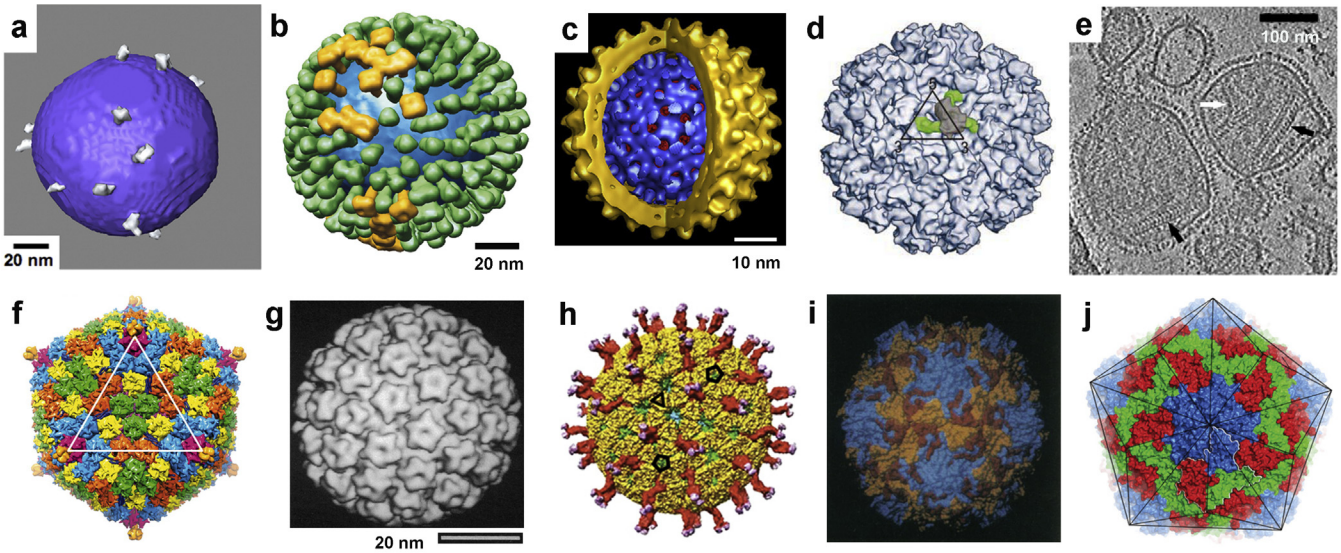


Figure 1. Comparison of protein densities on virion surface. (a) HIV-1. (b) Influenza virus. (c) Hepatitis B virus. (d) Yellow fever virus. (e) Measles virus. (f) Human adenovirus. (g) Human papillomavirus. (h) Rotavirus. (i) Poliovirus. (j) Hepatitis A virus. Models for a-e, g, and h are from cryoelectron microscopy, and models for f, i, and j are from crystal structures. Whereas a-e and j are enveloped viruses, f-i are considered as nonenveloped viruses. All these viruses have licensed vaccines available except HIV-1.

that should be considered for vaccine development. Among a myriad of factors that contribute to protection, including dose and vaccination schedule, the attempt is made to first describe the density of surface antigens. To examine the potential correlation between the efficacy of a viral vaccine and the density of immunogenic proteins displayed on the surface of the vaccine antigen carrier, we derive the protein density based on the known structural features of a set of viruses and then provide some potential rationale that is based upon current understanding of the early events in B-cell activation.[25]

Figure 1 shows the high-resolution structural models or images for 10 different viruses. Licensed vaccines are available in the United States for 9 of them (Figs. 1b-1j). The various features or protrusions on the surface of individual virions are viral proteins essential for infection and also important immunogens for virus neutralization by the host immune system. Figure 1a shows a model of HIV-1 on the basis of cryoelectron tomography of individual HIV-1 virions derived from chronically infected T-cell lines.[26] The white protrusions on the surface of the virion show the presumptive viral envelope glycoprotein (Env) gp120/gp41 trimers, the number of which varies from 4 to 35 with an average of 14. Although it remains to be determined in the future what is the gp120/gp41 density on primary HIV-1 virions, this low density of Env on HIV-1 is consistent with recent single-molecule measurements for HIV-1 virions derived from transfected 293T cells,[27] where the average number of gp120 molecules per virion varied from 0 to 9 depending on transfection conditions. In contrast to HIV-1, the majority of viruses that have licensed vaccines carry significantly higher densities of viral-encoded proteins on particle surface. This is illustrated using the high-resolution structural models or images available for 9 of the viral agents listed in Table 1, as shown in Figures 1b-1j. Figure 1b shows a model of influenza virus type A particle (H3N2) based on cryoelectron tomography,[13] in which the viral glycoprotein hemagglutinin is shown in green and the neuraminidase is shown in gold. A spherical influenza virion with an average diameter of 120 nm will display ~375 such protein "spikes" on its surface. Among these spikes, hemagglutinin is 6- to 7-fold more abundant than neuraminidase. Along this line, it is interesting to note that antihemagglutinin antibody is the major antibody that mediates influenza neutralization in the lower respiratory tract, although both antihemagglutinin and antineuraminidase antibodies offer protection in humans.[28] This dense distribution of glycoproteins on individual influenza virions is also confirmed by a later report on influenza type A virus using Zernike phase-contrast electron microscopy, which reported ~450 glycoprotein spikes on the surface of virions with average diameters of 120 nm.[29] Figure 1c shows a model of a native hepatitis B virion based on cryoelectron microscopy.[30] The yellow projections extending from the virion surface show the surface glycoproteins, also known as HBsAg. For each single particle, there are 120 such projections that are spaced ~6 nm apart. It is exactly this surface glycoprotein, produced from baker's yeast, that has been formulated into a licensed vaccine.[31,32] Although the licensed hepatitis B vaccine is regarded as a subunit vaccine, it is worth mentioning that the purified surface glycoproteins from yeast actually self-assemble into homogenous particles about 20 nm in diameter, which are clearly visible under an electron microscope.[31-33] Each particle can thus present multiple copies of the surface glycoprotein molecules. Figure 1d shows a model of an immature yellow fever virus particle based on cryoelectron microscopy.[22] For each single particle, there are 60 icosahedrally organized envelope glycoprotein trimers on the particle surface. One such asymmetric unit is outlined in black, and the envelope glycoprotein is colored in green. Based on structural studies on dengue virus, another virus within the same flavivirus genus,[34] extensive conformational changes will occur to envelope glycoproteins upon virion maturation, which result in a display of 90 envelope glycoprotein dimers on the surface of a single mature virion. The neutralization antibody response to these envelope glycoproteins has been shown to be the most important in the protective effects induced by the live attenuated yellow fever vaccines.[35] Figure 1e shows a cryoelectron tomography image of measles virus.[15] The tubular structures of nucleocapsid inside the virions are denoted by arrows. Compared to all the other viruses shown in Figure 1, measles virus appears special in that it is highly pleomorphic (Table 1), as seen from the different sizes of virus particles in this image. The dense striated features on the surface of these virions represent glycoproteins that are important for binding and entry[36] of the virus into the host cell. These proteins are also the sole targets of neutralizing antibodies (nAbs) induced by

measles virus vaccines.[37] Figure 1f shows a model of a human adenovirus based on the high-resolution crystal structures of an adenovirus capsid.[38] One of the 20 facets on this icosahedral virus is outlined in white. Each facet contains 12 hexon trimers and a penton at each vortex (pink), and there are a total of 240 hexon trimers on a single particle. Indeed, the hexon protein contains most of the epitopes recognized by nAbs against human adenovirus.[39,40] Figure 1g shows a model of a human papillomavirus type 1 virion based on a cryoelectron microscopy study.[12] This particle has a diameter of 60 nm. The outer shell of the virus particle is made of 72 capsomers, and each capsomer is a pentamer of the major capsid protein L1. The human papillomavirus vaccine is made of viral-like particles that are self-assembled from this L1 protein, which induce high titers of neutralizing antibody and offer protection.[41,42] Figure 1h shows the structure of a rotavirus particle determined by cryoelectron microscopy and single-particle analysis.[43] In each particle, there are a total of 60 "spikes" (red stem with purple top) protruding from the surface of the virion, which is made of viral protein VP4. In addition, the extended shell (yellow) below these spikes is made of viral VP7 protein. Although the mechanism of protection by the live rotavirus vaccine is not yet clear, both VP4 and VP7 induce nAbs and these antibodies are believed to contribute to protection.[44,45] Figure 1i shows a model of a poliovirus based on the high-resolution crystal structures of the Mahoney strain of type 1 poliovirus.[17] The viral proteins VP1, VP2, and VP3 are colored in blue, yellow, and red, respectively. A single poliovirus particle is approximately 31 nm in diameter, and each particle has 60 copies each of the VP1, VP2, and VP3 proteins. Based on the crystal structures, the neutralization epitopes have been mapped to all of these 3 proteins. Figure 1j shows the overall structure of the hepatitis A virus obtained from high-resolution X-ray crystallography,[46] where the black lines depict the facets of this icosahedral virus. The viral-encoded proteins VP1, VP2, and VP3 are colored in blue, green, and red, respectively. nAbs, likely those against VP1 and VP3, are correlated with the protection offered by the inactivated hepatitis A virus vaccine.[47,48]

Cryoelectron tomography studies of purified rubella virus were also published recently.[20] The envelope glycoproteins of the virus were shown to form extended rows of density that were separated 9 nm apart on virion surface. By the time this article is written, there are no published high-resolution structural models available to derive the density of surface antigens for Japanese encephalitis virus, mumps virus, rabies virus, smallpox virus, or varicella-zoster virus. However, structural models of similar viruses or viruses within the same family or genus are available. For example, Japanese encephalitis virus and dengue virus belong to the same flavivirus genus in the flavivirus family. The organization of their envelope glycoproteins might be similar. Indeed, Luca et al.[49] have solved the crystal structure for the ectodomain of the Japanese encephalitis virus envelope protein, which shows high similarity to that of the dengue virus. As mentioned above, mature dengue virus has 90 envelope glycoprotein dimers on a single virion surface.[34] Cryoelectron tomography studies of rabies virus revealed discontinuous spike-like objects on the surface of virions that are compatible with viral glycoproteins.[50] Although it is difficult to derive glycoprotein densities from these images, studies of the vesicular stomatitis virus (VSV), which belongs to the same family of rhabdoviridae as rabies virus, might offer some hints. Electron microscopy studies of VSV revealed a layer of density outside the viral membrane[51] that is clearly made of proteins, which can be removed upon trypsin digestion.[52] Ensemble estimations based on RNA and proteins present in viral particles yielded approximately 500 glycoproteins per VSV particle.[53] High-resolution structural models of varicella-zoster virus also lag behind. However, high-resolution structural models are available for the herpes simplex virus, which is another human virus within the same herpesviridae family as varicella-zoster virus. Based on cryoelectron tomography, herpes simplex virus particles are pleomorphic. Their diameters range from 170 to 200 nm, and each virion carries 600 to 750 glycoprotein molecules on the virion surface.[54]

Molecular Mechanisms That Relate Density of Surface Antigens to Antibody Neutralization

From the analysis above, it is clear that a majority of the viruses that have licensed vaccines display high densities of viral proteins on the surface of individual virions. Most of these proteins are the targets of nAbs induced upon vaccination, which offer protection to the vaccinated subjects. At the molecular level, there are 2 potential mechanisms that can relate the density of surface antigens to nAbs. First, high-density display of viral antigens facilitates bivalent binding by the immunoglobulin G (IgG) antibody, which is important for the potency of the antibody to neutralize viral infectivity. Second, high-density display of viral antigens facilitates B-cell activation, which is the prerequisite for antibody secretion and memory B-cell formation. I will elucidate these 2 aspects, especially the second aspect, in detail below.

High Density of Surface Antigens Facilitates Bivalent Binding by IgG

Among different classes of antibodies in the body, IgG is the major class of antibodies that binds various pathogens. An IgG molecule has 2 identical "arms" of Fab, and antigens bind to the tip of each Fab. The distance that can be reached or bridged by the 2 antigen-binding sites in each of the IgG is typically 15 nm.[55] As a result, low-density display of viral antigens on the virion surface, if the distance between antigenic molecules is greater than 15 nm, will impede the bivalent binding of IgG. This indeed occurs on HIV-1 virions as demonstrated recently by Galimidi et al.[55,56] Failure to bind antigens bivalently will increase the off rate of antibodies from the bound target and leads to low efficiency in neutralizing virus infectivity. In contrast, as shown in Figure 1b for influenza virus, the average center-to-center spacing between the densely packed glycoproteins on virion surface is about 11 nm,[13] which is short enough to promote bivalent binding of IgG. Experimental data on neutralization of influenza viruses support this conclusion.[57] Similarly, based on the published protein data bank structures available for human adenovirus (protein data bank entry 1VSZ and 3IYN), the center-to-center distances between 2 neighboring hexon trimers that this author calculated range from 89 to 98 Å, which is again sufficient to promote bivalent binding of IgG, and consistent with the fact that the hexon protein is the major neutralization target in human adenovirus.[39,40] In summary, low-density display of viral antigens can actually be used by the virus to evade host neutralizing antibody responses.

High Density of Surface Antigens Facilitates B-Cell Activation

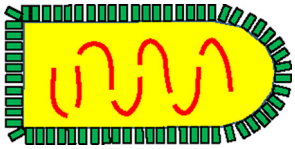
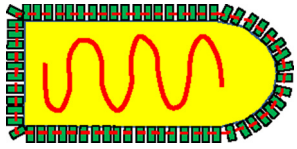
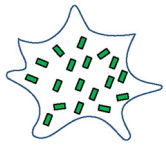
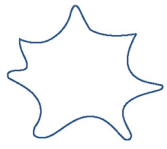
The second mechanism that might operate in those licensed vaccines is that the high-density display of surface antigens on a vaccine antigen carrier facilitates B-cell activation. The sensitivity of the immune system to the density of protein antigens has long been appreciated. Back in 1993, Bachmann et al.[58] demonstrated that the biophysical organization of protein antigens has a direct influence on B-cell responsiveness and antibody production. In this work, they used the envelope glycoprotein of VSV as a model immunogen (VSV-G) and presented this antigen in different biophysical forms for immunization in mice followed by testing the IgM and/or IgG responses from the animal. As mentioned above, individual VSV particles have a high density of envelope glycoproteins on their surface.

Specifically, they prepared (1) ultraviolet (UV)-inactivated VSV particles (VSV_{UV}, represented by the viral particle with a damaged genome in Table 2), which retain high densities of VSV-G (green) on virion surfaces and can still infect cells, whereas VSV-G can be displayed on an infected cell surface without producing viral progenies; (2) VSV particles treated with formaldehyde (VSV_{fmd}, represented by the viral particle with a dashed circle around VSV-G in Table 2), which retain high densities of VSV-G on virion surfaces but cannot infect host cells; (3) mice peritoneal macrophages that were incubated with either of the 2 treated virus particles and then washed. These macrophages can display VSV-G on the cell surface upon infection by VSV_{UV}, but display nothing upon incubation with VSV_{fmd}. Strikingly, upon injection of these different forms of VSV-G antigens into the spleens of ICR mice, the animal mounted high-titer IgM responses toward either VSV_{UV} or VSV_{fmd}, but not to VSV-G displayed on an infected cell surface. These results are summarized schematically in Table 2. To examine this in more detail, they generated a transgenic mice expressing VSV-G. In this case, the VSV-G will be a self-antigen to the animal. Indeed, intravenous immunization of the transgenic mice with soluble VSV-G purified from a baculovirus expression system did not induce IgM or IgG production in the animal. However, upon immunization with infectious VSV particles, the animal mounted strong IgM and IgG responses that were comparable in magnitudes to that of control animals. These results indicate that the biophysical forms of the antigens used for immunization of animals matter. Although they were the same protein antigens in nature, these proteins were presented in different forms and yielded different immune responses. For VSV-G antigen in particular, the antigens presented in a high-density format on a viral particle is the most potent in inducing neutralizing antibody responses in mice. These results also indicate that there exist mechanisms in mice that can recognize the forms of the immunogens presented and that the immune system can sense the "density" of the immunogen and responds differently. The density of viral proteins on the surface of individual virions may be a key signal for the immune system to mount antibody responses.

Similarly, Chackerian et al.[59] demonstrated that autoreactive IgG can be induced by immunization of mice with bovine papillomavirus viral-like particles that carry peptides from mouse self-antigens. Furthermore, they have developed this technique through chemical modification of papillomavirus viral-like particles by biotin-containing N-hydroxysuccinimide ester. Upon making a fusion protein between streptavidin and a mouse self-antigen, the self-antigen can then be noncovalently conjugated to viral-like particles through this fusion protein. This approach may be generally applicable for attachment of self-antigens. As it turns out, high titers of autoreactive IgG can be induced by immunization of mice with these conjugate viral-like particles in the absence of any adjuvants.[60] This approach has therapeutic potential, as they showed that the induction of autoantibodies against a mouse self-antigen, tumor necrosis factor alpha (TNF-α), through this vaccination approach inhibited the development of type II collagen-induced arthritis in mice. A systematic study using these conjugated particles in mice further showed that the density of self-antigens on these particles was critical for efficient induction of autoreactive IgG.[61]

There were still differences between the 2 systems above that were carefully studied. First, in terms of IgM responses from animals upon immunization of foreign antigens, Bachmann et al.[58] observed significant differences in ICR mice between VSV-G displayed by viral particles and VSV-G displayed by infected cells (Table 2), whereas Chackerian et al.[59] observed similar IgM responses against TNF-α in C57Bl/6 mice between TNF-α conjugated to viral-like particles and TNF-α fused with streptavidin in a soluble format. Second, in VSV-G transgenic mice, the soluble VSV-G purified from a baculovirus expression system, which was a self-antigen to the transgenic animal, did not induce either IgM or IgG responses. However, Chackerian et al.[59] reported that the soluble TNF-α fused with streptavidin can induce both IgM and IgG responses against TNF-α in C57Bl/6 mice. The detailed mechanisms behind these differences are not yet clear. However, the consensus that emerges from these studies is that the biophysical forms of protein antigens displayed by the vaccine antigen carrier matter a lot and that the antibody responses mounted by the immune system are highly sensitive to the density of protein antigens. Protein antigens displayed at a high density can induce high titers of IgM and IgG regardless of the foreignness of the antigen to the immune system and in the absence of any adjuvants. Intriguingly, this high-density display of protein antigens is also suspected to be the cause of autoantibodies for recombinant human protein therapeutics,[62] because the undesired aggregation of these protein molecules may display epitopes at high density to B cells. Although it can be argued that both VSV and bovine papillomavirus viral-like particles may contain substances other than densely displayed proteins, which may act as "special" adjuvants to help break the self-tolerance in both cases, the rise of autoantibodies as a result of protein therapeutics argues against this notion. It further supports that the protein density may be a stand-alone signal to break the self-tolerance. In this regard, it is worth noting an early study by Dintzis et al.,[63] in which they prepared linear polymers of acrylamide that bear varied density and spacing of hapten groups along the polymer chain; these polymers were then used to immunize mice for testing IgM responses from the animals thereafter. The results from their study support a threshold model of IgM responses to these nonprotein polymers, that is, only with sufficient density and total number of hapten molecules within a single polymer chain can an IgM response be mounted. Although the immunogens used in this study were T-cell-independent antigens, which are different from protein-based antigens that can induce T-cell-dependent antibody responses, these studies overall paint a consistent picture for immune system activation, that is, the density of immunogenic molecules presented to the immune

Table 2
Responses of ICR Mice to Immunization of VSV-G Displayed in Various Forms

Form of VSV-G Antigen	VSV_{UV}	VSV_{fmd}	Macrophage + VSV_{UV}	Macrophage + VSV_{fmd}
Cartoon representation of the form of the VSV-G antigen				
IgM titer from the immunized mice	+++++	+++++	+	0

system matters. Given the importance of antibody responses demonstrated for the majority of the licensed viral vaccines, how to best activate B cells is a topic that formulation scientists have to face for intended delivery of immunogens for the purpose of vaccination, because this may directly relate to the efficacy of the vaccine formulation to be developed.

The above studies demonstrate that the density of immunogens can be recognized by the immune system. However, far less is understood regarding how this information of antigen density is relayed from viral particles or particulate antigens to the immune system. One thing for certain is that the B cells that are specific to the antigen must be activated in order to produce IgM and/or IgG, either through T-cell-independent or T-cell-dependent mechanisms. From a historical perspective, this process of B-cell activation starts with antigen binding to specific B-cell receptors, which leads to formation of receptor micro-clusters, or so-called "crosslinking" of receptors. The formation of receptor micro-clusters sets off a chain reaction to downstream cellular events, including the initiation of calcium signaling and the induction of gene expression. Recently using imaging approaches, the spatiotemporal dynamics in B-cell recognition of foreign antigens has been extensively studied (for a review on this topic see Harwood and Batista[25]). Using transgenic B cells that carry B-cell receptors specific for hen egg lysozyme as a model system, Fleire et al.[64] demonstrated that the process of antigen recognition by B cells involves a series of well-orchestrated steps. Using membrane-bound antigens that presumably mimic the antigens acquired on the surface of antigen-presenting cells such as follicular dendritic cells or macrophages, they showed that B cells first spread over the surface that bear the specific antigen molecules and then contract and collect bound antigens into a central aggregate. This contact surface between a B cell and the antigen-bearing membrane is dynamic but as large as 25 μm^2. Based on fluorescence intensity from live cell imaging, they also estimated that 16,000 to 20,000 molecules of antigen can be accumulated by a single B cell in this event. During this process of antigen accumulation, the response of B cells was sensitive to both the density of antigen on the membrane and also the affinity of the B-cell receptors toward the specific antigen. Indeed, B cells were not responsive to hen egg lysozyme that was displayed at a density of 15 molecules per μm^2. Therefore, this process may well display a threshold behavior regarding B-cell activation. In addition to the surface density of antigen, it is likely that a threshold number of B-cell receptors need to engage with the antigen molecules in order for B-cell activation to occur, as they indicated that the quantity of antigens accumulated through this spreading and contracting process may directly determine the degree of B-cell activation.

Based on these findings, one can imagine the following scenario for B-cell recognition of viral particles: the higher density for the antigens on the surface of viral particles, the easier to activate specific B cells. If the total number of antigen molecules available on a single virion particle is below the threshold number of B-cell receptors required for activation, binding of multiple virion particles needs to occur; as a result, both particle size and the density of antigens need to be considered for the efficiency of B-cell activation, as schematically shown in Figure 2. For small virus particles with high surface density of antigenic proteins, they are likely to be very effective in this activation process (Fig. 2a). In contrast, large viral particles with low surface density of proteins will be much less effective in triggering B-cell activation (Fig. 2b).

In formulating various scenarios above regarding B-cell responses to viral antigens, there are 3 important issues that we have not discussed. First, we have not considered explicitly how B cells acquire these particulate antigens *in vivo*, which is directly relevant to the mechanism of antigen accumulation by B cells. Given the extensive literature on the mechanisms of antigen acquisition by B cells, a variety of different routes of antigen acquisition are suspected. For example, for virus particles that have entered systemic circulation, they can be bound by the rare antigen-specific B cells before their entry to the lymph nodes provided that the affinity with B-cell receptors is strong enough for virus attachment to B cells. Interactions with toll-like receptors on B cells may also help in this case. Because many viral particles are less than 200 nm in diameter, they can freely drain into the lymph nodes.[65] Following entry of these particles into the lymph node, the viral particles can be presented to antigen-specific B cells through various cell-mediated mechanisms, including subcapsular sinus macrophages,[66,67] and dendritic cells.[68,69] These cell-mediated mechanisms may help B cells to accumulate viral antigens on the B-cell surface, the detailed mechanisms of which remain to be studied in the future. Second, in considering the efficiency of B-cell activation, we have not explicitly discussed the fate of B cell after its activation. Whether the B cell will proceed to T-independent activation and production of IgM antibodies, or proceeds to T-dependent activation, class switching and production of IgG has not been specified or investigated in response to the density of antigens. The conventional definition for type II T-cell-independent antigens often uses the phrases "highly repetitive structures" that can induce "extensive crosslinking" of B-cell receptors. In light of the recent work into the early events of B-cell activation, especially the 2-stage process of activation, that is, spreading followed by contraction, this "extensive" crosslinking used for type II T-cell-independent antigens has become less precise and clearly needs to be quantitatively defined. As a matter of fact, the magnitude of antigen molecules involved in the study by Fleire et al.[64] is huge: 16,000 to 20,000

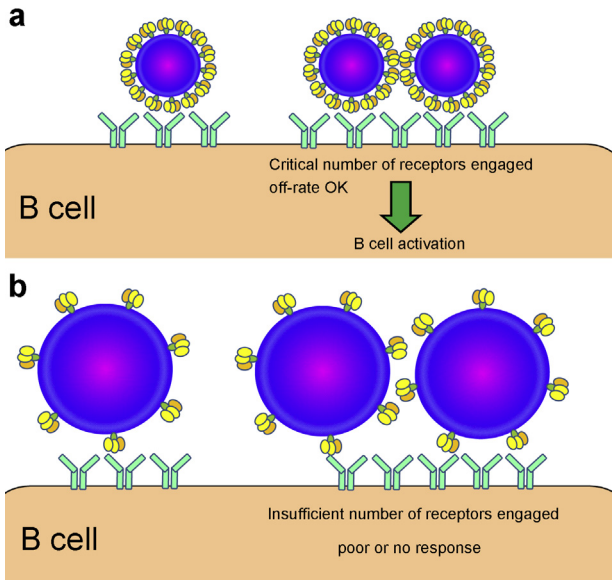


Figure 2. Possible scenarios of recognition of virus particles by B cells specific to antigenic proteins displayed on the surface of individual viral particles. The Y-shaped molecules represent B-cell receptors specific to viral surface antigens. (a) It is potentially very efficient for small virus particles with high-density display of surface antigens to activate B cells, because a small number of viral particles may be sufficient to activate the B cell, provided that the off-rate of the particles is low enough to engage a threshold number of B-cell receptors. (b) For large virus particles with low-density display of surface antigens, the efficiency to activate specific B cells is likely to be low, because to engage the threshold number of B-cell receptors for activation will need more particles to engage the critical number of B-cell receptors required for activation. As implied by these cartoons, the spatial distribution of B-cell receptors on B-cell surface also likely contributes to this recognition process. Although not shown in these cartoons, it is also likely that the viral particles are presented through various cell-mediated mechanisms to B cells instead of just particles themselves. Cartoons are not drawn to molecular scale.

molecules of antigen are involved at the level of single B cells. It will be critical in the future to determine the threshold numbers of B-cell receptors required for both T-cell-dependent and T-cell-independent activation, and quantitatively how these 2 processes differ.

For practical purposes, both types of B-cell activation are desired from the perspective of vaccination, because both types of B-cell activation can generate memory B cells,[70] as demonstrated recently by several laboratories for T-cell-independent antigens.[71-73] The unique advantage of T-cell-independent activation of B cells is the apparent increase in the chemical identity of antigens that can be recognized by the immune system, because these antigens are no longer limited to proteins, but include carbohydrates and other polymeric structures. In fact, T-cell-independent B-cell activation has been used in licensed vaccines in the United States, represented by the 3 polysaccharide vaccines for pneumococcal disease, meningococcal disease, and typhoid fever caused by *Salmonella typhi*, respectively (Table 3).

Third, we have not discussed the issue of affinity between antigen and B-cell receptors. For the purpose of vaccination, antibodies with the highest affinity toward the antigen would be desired in the end, which can be produced through the affinity maturation process.[74,75] Previous studies on T-cell-dependent activation of B cells revealed that multivalent presentation of antigens may facilitate activation of B cells even for those antigens with apparently lower affinities toward B-cell receptors.[76] As a result, multiple clonal populations of B cells may be activated toward the same antigens, despite the fact that their B-cell receptors have varying affinities toward the same antigen. Whether this will have a positive or negative influence on the affinity maturation of antibodies remains to be determined.[77]

Implications for Vaccine Formulation

The strong antibody response in mice upon immunization of self-antigens displayed on virus or viral-like particles in the absence of any other adjuvants is very intriguing and may suggest a potential generic strategy to elicit antibody responses to self-antigens. From the perspective of vaccine formulation, there are 2 potential scenarios for interpretation of these results. First, because a strong adjuvant is typically considered to be the key in breaking self-tolerance,[78] one possibility in virus or viral-like particles used in these studies is the presence of other substances in these particles that may inadvertently serve as the adjuvants to help break self-tolerance, such as viral nucleic acids or other factors that get incorporated into these particles as a result of their biogenesis process from eukaryotic cells. To eliminate this concern, particles made of homogenous materials using highly purified immunogenic proteins will be desired to repeat these findings. Alternatively, it is likely that the density of antigens may operate by itself as a signal for B cells to discriminate between self and foreign antigens,[79] and this recognition of antigen density by B cells and subsequent activation of B cells may be truly independent of any adjuvants. The mechanisms of B-cell activation from recent imaging studies[64] suggest that even for foreign antigens, display of antigens in a high-density format should strongly enhance B-cell activation and subsequent responses. These studies altogether have very interesting implications on how B cells may respond to the density of antigens. As schematically illustrated in Figure 3, B cell response to foreign antigens increases with antigen density. When the density of antigens exceeds the threshold of self-tolerance, B cells will start to respond to self-antigens, as indicated by the shaded area in Figure 3. Furthermore, it is likely that the response of B cells to the density of antigens may not be simply monotonic. It has been suggested that very high densities of antigens may lead to unresponsiveness of B cells, or anergy.[80] A recent liposome delivery study indeed suggested that too high a density of antigen display decreased serum IgG responses.[81] As a consequence, it will be very interesting to determine this entire dependence quantitatively, and as part of this effort, to determine whether this quantitative dependence may vary with different B-cell subsets in the body. The quantitative understanding of this dependence will no doubt help the development of vaccines and engineering of B-cell responses.

Based on this model of B-cell response to the density of antigens, one may also come up with a potential explanation for why the spontaneous curing rate of HIV-1 infection in the human population is so low compared to other viral infections. Figure 4 shows the density of key viral surface antigens that one can estimate based on the diameter of individual viral particles (Table 1) and the known number of molecules of viral surface antigen. It is clear that HIV particles have the lowest density among them all. The red dashed line shows the antigen density of 150 molecules/μm^2, the density that is sufficient to observe B-cell activation by Fleire et al.[64] in transgenic B cells. The average density of gp120/gp41 trimers on HIV-1 particles is calculated to be 212 molecules/μm^2 based on the estimation for virions derived from chronically infected T cells[26] and an average diameter of 145 nm.[82] Based on this estimation, HIV-1 virions display sufficient number of envelope glycoproteins for B-cell activation. However, its efficiency may be much lower than other viruses comparatively. The development of nAbs in HIV-1-infected individuals is slow; only after several months of infection can nAbs be detected[83] and the reason for this is unclear.[84] As a matter of fact, only after 2 to 4 years of infection can broadly nAbs be detected in HIV-1-infected individuals.[85] Among other mechanisms that have been well demonstrated in literature, such as high mutation rates, the structural features of HIV envelope glycoproteins such as glycan shields,[86-88] the low density of glycoproteins on the HIV surface that impedes the bivalent binding of IgG,[56] the required accumulation of antigens on a specific B-cell surface may well retard the protective B-cell responses and give rise to an apparent delay in nAb responses.

This model of B-cell response also bears implications on the development of DNA vaccines. In contrast to inactivated virus, viral-like particle, or subunit vaccines, DNA vaccines have to rely on the expression of target protein antigens from the DNA vector for presentation to the immune system. Whether these protein antigens can be presented in a high-density format upon expression

Table 3
Licensed Vaccines That Use T-Cell-independent Mechanism for B-Cell Activation

Polysaccharide Vaccines	Current Product on Market	Dosage and Administration	Duration of Protection
Pneumococcal polysaccharide vaccine	Pneumovax 23	Single 0.5-mL intramuscular or subcutaneous injection	Elevated Ab levels persist at least 5 y in healthy adults
Meningococcal polysaccharide vaccine	Menomune	Single 0.5-mL subcutaneous injection	Reimmunization every 5 y recommended
Salmonella typhi polysaccharide vaccine	Typhim Vi	Single 0.5-mL intramuscular injection	Reimmunization every 2 y recommended under conditions of repeated or continued exposure

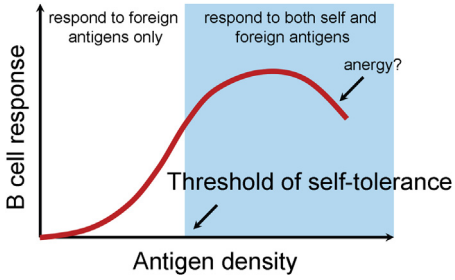


Figure 3. Potential model of B-cell response to antigen density displayed on particulate antigens. In this model, the density of antigens by itself serves as a stand-alone signal for B cells to distinguish between self and foreign antigens. Whether adjuvants are required for this process remains to be determined in the future.

may well influence the B-cell response. Intriguingly, compared to conventional vaccines, DNA vaccines usually induce poor antibody responses.[89] DNA vaccines developed for influenza virus in general have lower immunogenicity than inactivated vaccines.[90] One possibility among others may be the density of the hemagglutinin protein expressed, which may be lower than that in inactivated viruses. To improve the efficacy of DNA vaccines, this issue of antigen density upon expression may have to be investigated for future efforts in the development of DNA vaccines.

For the past decade, a variety of different nanomaterials have been developed for potential delivery of various immunogens.[91,92] These nanomaterials provide excellent scaffolds on which antigens of interest can be attached and optimized.[81] Based on the model of B-cell response to antigen density discussed here, a potential approach that may be complementary to these nanomaterials can be envisioned as follows for elicitation of antibody responses to proteins of interest, regardless of the foreignness of the protein antigen. Liposomes are well-documented drug delivery systems with a safe record of clinical applications.[93,94] Many recombinant proteins can be prepared with hexahistidine tags for purification of the proteins using the well-established nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity technology. Therefore, through incorporation of lipid molecules that carry Ni-NTA as head groups, one can potentially make liposomal particles that display almost any proteins of interest. This antigen delivery system has advantages in 2 aspects. First, the size of liposomes can be well controlled and the use of liposomes by themselves will not elicit an immune response, in contrast to antigen carriers that use

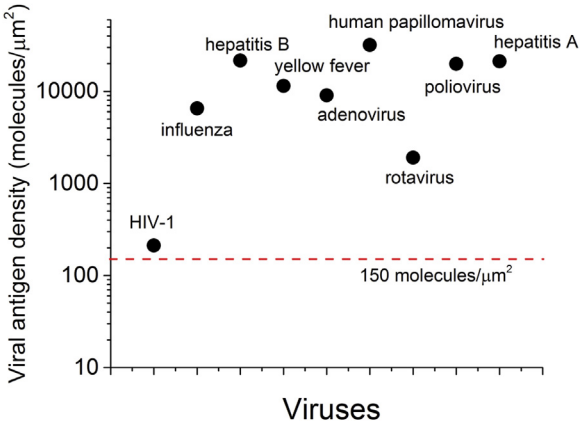


Figure 4. Density of key surface antigens calculated for HIV-1 and 8 virus antigens with licensed vaccines. The density is calculated based on published number of antigenic proteins per virion particle as described in the text.

molecules that are foreign to the human body. Second, the purification of recombinant proteins can ensure the relative homogeneity of the antigen molecules, which is in contrast to certain viral-like particles generated from cell culture systems that may be highly heterogeneous in nature due to the complex biogenesis pathways of these particles involved. The use of this liposome system for antigen delivery has been reported in literature.[95] The key to this design will be the density of proteins to be displayed on the liposomal surface, which likely yields different responses given the model described in Figure 3. A recent example is the conjugation of stabilized soluble trimers of HIV-1 to unilamellar liposomes.[96] A high-density display of these glycoprotein trimers activated B cells much better than the soluble trimers both *ex vivo* and *in vivo*, which provides a promising lead for the development of HIV vaccines. Moreover, the orientation of these protein molecules when attached to liposome surfaces is perhaps critical to ensure the proper engagement of multiple B-cell receptors on a single B-cell surface. How to best align the orientation of these proteins on liposomes remains a subject for future research. In contrast to the above advantages of using liposomes for vaccine antigen delivery, there are drawbacks to this Ni-NTA liposome technology. First, there is heterogeneity in the Ni-NTA coordination complex formed between the hexahistidine tag and the nickel ions. This heterogeneity results in different off rates observed for proteins attached to lipid through this chemistry[97]; therefore, the exact density of proteins on the liposomal surface may not be stable but evolve with time. The second drawback is intrinsic to the liposome structure itself. Because liposomes are formed from self-assembly of lipid molecules, they will disassemble upon significant dilution according to mass action. There are different ways developed in the literature to overcome this issue, for example, interbilayer-crosslinked multilamellar vesicles.[98] Third, nickel ions may have adverse biological effects *in vivo* such as tissue inflammation that is correlated with its distribution.[99] As an alternative to liposomes as the carriers for protein antigens, the licensed subunit vaccines or viral-like particles might be suitable scaffolds for displaying protein antigens at high densities. The use of papillomavirus-like particles for conjugation of mouse self-antigens is an excellent example in this regard.[60] However, the use of streptavidin as the "bridge" for conjugation between protein antigens of interest and the viral-like particle is not optimal apparently because streptavidin protein is foreign to humans, which may defocus the intended immune response to the antigens of interest. Other types of conjugation chemistry that can introduce a well-defined covalent bond between the viral-like particle and the antigen of interest is much desired. The various site-specific chemical conjugation techniques available, such as incorporation of unnatural amino acids[100] followed by chemical conjugation, or the incorporation of the so-called "aldehyde tags"[101] into proteins, will likely contribute to the future development of vaccine antigen carriers with defined protein densities.

Conclusions and Future Perspective

The quantitative examination of the antigen density on viral surface suggests a potential correlation between the antigen density and the efficacy of a viral vaccine. A density at proper level can efficiently activate B cells, which may constitute one of the molecular bases behind this phenomenon. Further exploration of the molecular mechanisms may bear direct implications on vaccine design and formulation. To better activate B cells, a particulate antigen carrier that bears antigens of interest on its surface at an appropriate density appears to be an ideal choice. Both the size of the antigen carrier and the density of immunogenic proteins on the surface of the carrier are important parameters to consider and

potentially optimize for the development of a vaccine. Given the importance of protein density on particulate antigens, it will be desirable in the future to develop nanoparticles with a tailored density of protein antigens on their surface. These materials cannot only be used for the exploration of the immunological mechanisms discussed here but also be useful for vaccine deliveries. Given the fact that T-cell-independent B-cell responses can also elicit memory B cells, it will be critical to determine the number of B-cell receptors that are optimally required for both T-cell-dependent and T-cell-independent B-cell activation, which will be very useful for the future of vaccine design. Recombinant human antibodies have become important drugs on the pharmaceutical market. As an alternative to passively supplying antibodies to the patient, in the future, a possible solution will be to supply antibodies through active immunization using particulate vaccines, which might provide durable antibody responses regardless of the foreignness of the antigenic proteins. This may potentially create an “antibody boost” on demand or serve as a replacement for the passive antibody therapies.

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