Panning of libraries constructed from immunised non-human primates (NHP) has not been widely used, even though this has proven to be a successful approach for the isolation of human-like antibody fragments with affinities in the nanomolar to the picomolar range. As recently demonstrated, after initial isolation of antibodies with such high affinities, germline humanization may be applied to these Fabs or scFvs to increase the similarity of their framework regions with those encoded by human germline genes. ‘Germlinized’ antibody fragments may be converted to full size IgGs; indications are given that these IgGs could be better tolerated in clinical use than human antibodies. The use of the combination of NHP immune libraries and germline humanization thus may compete with use of libraries of human origin, whether naïve or immune, and with synthetic libraries. In this report, the various approaches will be compared, and advantages of the two-step NHP-based method, as well as corresponding intellectual property aspects, will be discussed.

Introduction

The therapeutic efficacy of antibodies generally depends on their ability to bind certain epitopes with high affinities, although there are exceptions such as virus neutralization, which is considered to be more dependant on virus “coating” than on precise epitope targeting.1 As a general trend, targeting many epitopes with high affinity is the best way to isolate efficient antibodies for therapy; this efficiency however has to be matched with good tolerance in order for the candidate to have practical medical utility. Tolerance of recombinant antibodies is generally regarded as dependent on the similarity with their human counterparts, especially in the framework regions,2,3 so that an immune response is not induced in the patient. Tolerance of antibodies also depends on their lack of cross-reactivity with non-targeted human proteins, a quality that is very similar to the absence of autoreactivity for naturally expressed antibodies. This latter determinant of antibody tolerance is generally less emphasized compared to the former, but it is of considerable importance because the existence of such cross-reactivity, which should be detected by preclinical and clinical studies or later by pharmacovigilance, would practically forbid any clinical use.

Methods for isolation of new antibodies frequently includes use of naïve and synthetic libraries, which constitute the core asset of several companies.4-7 These libraries are constantly improved, especially in size and diversity, so as to target more antigens with improved affinities. They fill a need for isolation of “fully human” antibodies, as opposed to chimerized or humanized versions, although the exact significance of this term and the improved tolerance it suggests remain in question. Because of their convenience, these libraries might become the dominant method for isolation of recombinant antibodies intended for therapeutic use. Apart from naïve or synthetic libraries, human immune libraries, which may be constructed from the lymphocytes of vaccinees, volunteers in vaccine trials or convalescents, are less frequently utilized.

Here, the pros and cons of producing antibodies by isolation from non-human primate (NHP) immune libraries followed by germline humanization will be compared to use of naïve, synthetic and human immune libraries. For each approach, we will consider various qualities of the antibodies, including affinity, capacity to target various epitopes, tolerance by the human immune system and lack of cross-reactivity with non-targeted human proteins. An overview of legal aspects suggests that the NHP approach is accessible to many scientists, although variations among countries were observed.

Antibody Efficacy

Efficient therapeutic antibodies generally bind selected epitopes with high affinities. In order to reach those affinities, naïve libraries mostly recapitulate the IgM diversity, part of the innate immunity. Construction of synthetic libraries increases that diversity by
adding randomly engineered complementarity determining regions (CDRs), mainly CDR3. Use of the immune library approach results in the harvesting of antibodies that underwent an in vivo antigen-driven affinity maturation process, involving the somatic hypermutation mechanism that brings diversity to the variable domain, followed by a selection step.\(^8\),\(^9\) This affinity maturation process is reiterated after each encounter with the immunogen, and is a crucial characteristic of adaptative immunity, which is known to provide much more efficient protection than innate immunity. In the case of NHP immune libraries, four immunogen injections are generally given with Freund's adjuvant to induce immune responses ranging from 1:10\(^5\) to 5:10\(^5\), as evaluated by post-immune serum titres. Such responses allow antibody fragments with nanomolar to picomolar affinities to be harvested,\(^10\)\(^-\)\(^13\) and a sensogram corresponding to an scFv with a picomolar affinity (reviewed in refs. 14 and 15) is shown on Figure 1. Affinities of antibodies originating from NHP immune libraries are thus 10 to 1,000 better than those typically obtained from high-quality naïve antibodies originating from NHP immune libraries are thus 10 to 1,000 better than those typically obtained from high-quality naïve or synthetic libraries,\(^16\),\(^17\) and compare favorably with the affinity of antibody fragments isolated from human immune libraries.\(^18\)

The possibility of targeting certain epitopes is also of importance when searching for specific antibody activities, such as neutralization or signalling, and to avoid any enhancing effect, which occurs when incompletely neutralizing antibodies increase the deleterious effect of pathogens by targeting them to Fc-bearing cells acting as host cells.\(^19\) In the case of a naïve library screened against botulinum toxin A, only a small proportion of the library was found to react with the antigen, which limited the number of epitopes that were bound.\(^20\) This is not surprising because naïve libraries target antigens with a finite number of antibody fragments. In contrast, each antibody in NHP immune libraries is directed against the immunogen of interest. Moreover, in addition to this “focused” character, immunization of NHP might utilize a broad range of immunogens, certainly broader than what is possible in humans. For instance, these immunogens might be recombinant proteins, corresponding to subunits or domains of proteins, in order to best expose desired epitopes as these epitopes would not have been necessarily dominant, or even accessible, if the antigen had been the active protein.\(^12\)

If the immunogen is harmful, inactivated pathogens or toxins might conceivably be used for priming and the first boosts, followed by use of the pathogen itself when immune status has been reached, so that native epitopes will be recognized. Importantly and in contrast to humans, NHP raise a response against human antigens as exemplified by the previous isolation of NHP antibodies directed toward two human surface proteins: B7 and CD23.\(^21\),\(^22\)

Certain aspects of the construction of our libraries explain why they might be regarded as “focused” and how well this character articulates with the use of recombinant antigens. Our libraries are built after a strong immune response has been raised; however, a period of several months without injections is currently allowed to limit the effects of the last boost. After this delay, and utilizing primers specific for the amplification of DNA encoding antibody fragments, no PCR product can be amplified from bone marrow because the animals are healthy and kept in a very clean environment, thus largely isolated from undesired immune stimulation. The last boost raises an immune response against the immunogen, so that PCR products obtained after this boost encode antibody fragments that are very specific for this precise immunogen. Furthermore, NHP allow repetitive bone marrow sampling so that the peak of the response, as evaluated from the number of pairs of primers that allow amplification and from the intensity of amplification, is utilised to build the library. Finally, our strategy aims at obtaining a diverse library of a significant size (around 10\(^8\) clones) that is highly focused on the immunogen such that it should cover many, if not all, of the solvent-accessible regions, especially if the immunogen is a recombinant protein of reasonable size (less than 100 kDa in our examples). We regard these libraries as “hyper-immune,” as are the NHP from which they are constructed. The efficacy of the antibodies isolated from our libraries certainly depends on this “hyper-immune” character.
Abdomen of cross-reactivity with non-targeted human proteins and absence of immunogenicity in humans are the two determinants of antibody tolerance. Antibodies isolated from human naïve libraries (in the strict meaning of the term) derive from IgM; autoreactive IgM have been eliminated by the first “editing” process, which takes place mainly in thymus. Antibodies derived from naïve libraries thus offer the best possible warranty against autoreactivity, except that they are often engineered in vitro for affinity enhancement. As no editing takes place after this in vitro affinity enhancement, there is a risk of autoreactivity for such engineered antibodies. Regarding IgGs, a second, peripheral editing process takes place after in vivo affinity maturation, so that human immune libraries also offer the best possible warranty against autoreactivity and this warranty is not limited by later antibody engineering. The NHP immune libraries offer a slightly inferior level of warranty against cross-reactivity than human immune libraries because the editing processes are done against NHP proteins which generally bear high similarities, but are not completely identical to their human counterparts. These various levels of warranty contrast with the situation of antibodies originating from synthetic libraries, which offer no warranty regarding cross-reactivity with human proteins because no editing has taken place.

The high level of similarity between NHP antibodies and their human counterparts has been presented in various studies, and was summarized as follows: “(immunoglobulin) genes of macaques, for instance, are as closely related to human immunoglobulin genes as human (immunoglobulin) genes are to each other.” In effect, the two NHP antibodies directed against human antigens, mentioned above, have been expressed in the “primatized” form, where variable domains of NHP origin are expressed in fusion with human constant domains and these two primatized antibodies were found to be well tolerated.10,21,22 Beyond those examples and to better appreciate the similarities between NHP and human variable domains, we conceived the “germlinity index” (GI) as the percentage of identity between the framework regions (FRs) of NHP immunoglobulins, and the most similar FRs encoded by human, germline genes. This GI is based on the FR-IMGT delimitations, themselves based on the IMGT unique numbering for V-DOMAIN.28 We have determined (unpublished data) that the GI of variable domains of human IgG is around 95% when it is 90% in the case of NHP IgGs, so that similarities between human and NHP antibodies might not be as complete as anticipated. The GI measurement of NHP antibodies depends on sequence analysis, for instance performed with IMGT/V-QUEST® and IMGT/DomainGapAlign® (available at IMGT®, the international IMunoGeneTics information system®, www.imgt.org), which pinpoint differences existing between FRs of NHP expressed genes and FRs encoded by human germline genes. The latter FRs are encountered by every human, because they are expressed in the variable domains of their IgMs so that these FRs are perfectly tolerated. As a consequence, FRs from human, germline genes are preferable as a basis for humanization.3 In contrast, FRs of IgGs, such as those isolated from human immune libraries, are randomly mutated in the course of affinity maturation and these mutations may in turn be immunogenic. These IgG FRs should thus not be used to obtain the best possible tolerance. Accordingly, germline humanization was conceived as a series of point mutations to turn NHP FRs into human IgM (germline) FRs. While retaining parental affinity, the “germlinized” variant of the parental antibody should, at best, reach a GI of 100% (to obtain FRs identical to FRs entirely encoded by human germline genes, such as FRs found in IgM), and more realistically reach at least a GI of 95% (to obtain FRs that would be 95% identical to FRs encoded by germinal genes, such as FRs found in IgGs). In an example of germline humanization,11 we started from a GI of 92% to reach a GI of 97.8%, while retaining parental affinity and neutralization properties. Quite remarkably, the GI that was reached was superior to the GI of a fully human antibody (83K7C), which had similar affinity and neutralization properties.18 The “germlinized” version of our antibody retained four macaque residues that were showed to be so scattered at the surface of the variable domain that they could not form a B cell epitope, which suggests that the antibody would not be immunogenic in humans. In line with this reasoning, it appears that germlinized antibodies should be the goal, in order to reach the best conceivable tolerance, rather than “fully human” antibodies.32 Finally, the use of NHP immune libraries combined with germline humanization presents significant advantages when compared with the three other approaches, at least until the germline humanization process is also applied on the antibodies obtained with these latter methods.

It was objected to the NHP approach that difficulties were to be expected when assessing the safety of an NHP antibody, candidate for therapeutic use and directed toward a human protein. In effect, the region of a human protein recognised by a NHP antibody should not be shared between human and NHP species, otherwise it would not have been immunogenic in NHPs. In this situation, the candidate antibody will indeed not bind the NHP equivalent of the human protein, thus NHPs—the classical animal model for safety assessment—may not be utilised to study the safety of the antibody. However, this classical dogma of absence of “self” recognition may not be respected when inducing NHP antibodies with strong immunisation protocols, which could cause autoantibodies to be produced. Secondly, it is not compulsory to utilize NHP for safety assessment and a transgenic animal, expressing the human protein, may be utilised. Thirdly, the assessment may utilize a surrogate antibody directed toward the NHP region, functionally equivalent to the human region recognised by the candidate antibody. A murine antibody directed against this NHP region could certainly be raised because, if the region is not shared between NHP and human, it is very likely that it is also not shared between NHP and mice. The surrogate product could thus be a chimeric (murine/NHP) antibody, for instance. Conversely, it could be noted that safety assessment of a NHP antibody directed against a non-human protein (such as of infectious origin) can be performed in a very satisfactory fashion, by utilizing the fully NHP version of the antibody for pre-clinical studies performed in NHPs while the germlinized version would be intended for clinical use.
Legal Aspects Regarding NHP Immune Libraries

The field of recombinant antibodies is full of possibilities, although the scope is more often than not constrained by existing patents or commercial concerns. This is certainly the case regarding access to the best naïve or synthetic libraries, despite the fact that a few naïve libraries are accessible from academic researchers. Regarding the use of NHP immune libraries, the legal situation varies among countries and, due to their general availability, only the situation of macaques (old world monkeys) will be considered here. Japan and the US are on the two extremes of the spectrum: no legal limitation to the medical use of macaque antibodies seems to exist in Japan, and the most restricted situation seems to be encountered in the U.S., due to a patent filed on December 1997 that covers any such use. Europe might be in an intermediate position, with a patent filed on June 1992 that presents macaques as human-like animals with regard to their antibodies, but not regarding any of their other proteins. According to this vision, which is the core of the patent, macaques can be immunized against human proteins and human antibodies are yielded. Only antibodies directed against human antigens are protected by the European patent and, in particular, no rights are due if the antigen is of infectious origin. This patent has allowed us to work freely, to isolate antibodies neutralizing bioweapons. The U.S. and European patents are otherwise comparable, and based on several comparisons of human and NHP antibody sequences. Both patents also claim certain primers for amplification of macaque variable domains, which are different from the primers utilized in our studies. Additional legal advice regarding complex aspects of the patents may be sought, but advice concerning germline humanization should not be required because this strategy was not patented prior to its publication, and indeed never will be.

Conclusion

NHP “hyper-immune” libraries approach, when combined with germline humanization, can be used to isolate therapeutic antibodies with high affinities, directed against various epitopes, which are non-immunogenic in patients and do not cross-react with human proteins. This approach compared favorably with three other types of libraries and legal aspects of the NHP-based approach offer variable possibilities to isolate therapeutic antibodies, depending on the countries where they apply. These possibilities will expand with time and patent expiry. Hopefully, the NHP combined approach will further improve the availability of recombinant antibodies, “Pro Patria et Humanitate.”

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