Immunodrugs: Therapeutic VLP-Based Vaccines for Chronic Diseases

Gary T. Jennings and Martin F. Bachmann

Cytos Biotechnology AG, Zurich-Schlieren 8952, Switzerland; email: gary.jennings@cytos.com, martin.bachmann@cytos.com

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safety, vaccine toxicology, self-antigen, autoantibodies

Abstract

Worldwide, the prevalence of noncommunicable chronic diseases is increasing. The use of vaccines to induce autoantibodies that neutralize disease-related proteins offers a means to effectively and affordably treat such diseases. Twenty vaccines designed to induce therapeutic autoantibodies were clinically tested in the past 12 years. Immunodrugs are therapeutic vaccines comprising virus-like particles (VLPs) covalently conjugated with self-antigens that induce neutralizing autoantibody responses. Four such VLP-based vaccines have been clinically tested and one has achieved proof of principle: a reduction of blood pressure in hypertensive patients. To facilitate preliminary clinical testing, novel nonclinical study programs have been developed. Safety study designs have considered the underlying B and T cell immunology and have examined potential toxicities of vaccine components and primary and secondary pharmacodynamic action of the vaccines.

INTRODUCTION

Immunodrugs are a diverse group of therapeutic, virus-like particle (VLP) vaccines for treating chronic diseases (1). These VLP vaccines can be designed in multiple ways to induce therapeutic humoral, cell-mediated, or immune-modulating responses. Immunodrugs differ from conventional prophylactic vaccines in that the effector responses they induce often target self-molecules associated with maladies such as inflammatory autoimmune diseases, CNS disorders, allergy, and cancer. The term Immunodrug was coined to differentiate these vaccines from conventional vaccines and to reflect their immunotherapeutic nature and use. Immunodrugs have been the subject of a decade-long research and development program that has seen nine projects enter the clinic. To date, three Immunodrugs have achieved clinical proof of concept (2–4).

VLPs are nanoparticle icosahedrons with diameters of 25 to 100 nm (**Figure 1**). They are comprised of multiple copies of one or more viral structural proteins that, upon expression, spontaneously assemble into particles that lack genetic information with replicative capacity (5). VLPs have been acquired from many sources, including animal and plant viruses, bacteriophages, and yeast Ty retrotransposons. Immunodrugs principally utilize VLPs derived from RNA bacteriophages. VLPs can be recombinantly expressed in a variety of hosts, including bacteria, yeast, plant, insect, and mammalian cells, and manufactured economically in large scale following current Good Manufacturing Practice (cGMP.) In addition to being virus-like in structure, VLPs are potent immunogens, often antigenically indistinguishable from the virus from which they were derived (6). The superb immunogenicity of VLPs arises from the fact that they incorporate key immunological features of viruses such as repetitive surfaces, a potent stimulator of B cell

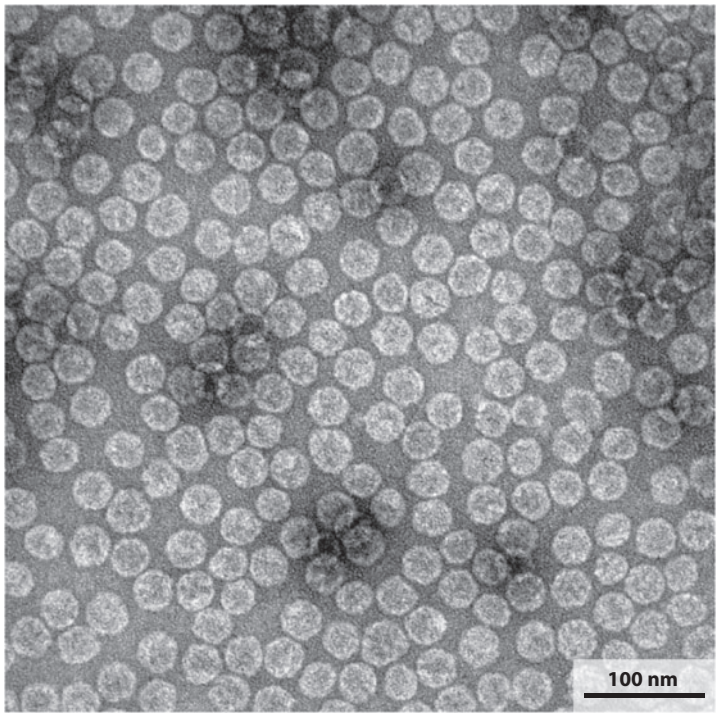

Figure 1

Virus-like particles (VLPs). Electron micrograph of purified recombinantly expressed VLPs derived from the RNA phage Qβ (308,000 X magnification). VLP icosahedrons are approximately 30 nm in diameter.

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responses (7); particulate structures; and sometimes induction of innate immunity through activation of pathogen-associated molecular pattern–recognition receptors (8). The potency of antibody responses induced by VLPs is highlighted by the recent successful development and marketing of the vaccines Gardisil® and Cervavix™ to prevent human papilloma virus (HPV) infection (6).

VLPs can also be used to generate antibody responses toward antigens not derived from the VLPs parental virus. This is achieved by linking a foreign antigen to the surface of a VLP. In this way, the underlying viral fingerprint of the VLP is imparted to the attached epitope, thereby rendering it as potent a B cell immunogen as the VLP (6). The most commercially efficient way to achieve this is by chemical conjugation. This method has been used to couple a diverse range of antigens, including proteins, polypeptides, capsular polysaccharides, and small organic molecules. A novel use of this principle is to attach disease-associated self-molecules to VLPs and to use the resultant conjugate vaccines to overcome B cell tolerance and induce neutralizing autoantibodies (6, 7). This technological innovation has greatly broadened the scope of use for VLP vaccines and is the foundation that underlies Immunodrugs.

The concept of using active vaccination to induce autoantibodies for therapeutic benefit is relatively old. Humans were immunized with crude preparations of renin, with the aim of decreasing blood pressure, in the 1950s (9). Indeed, the past decade has seen more than 14 different self-molecules targeted by autoantibody-inducing vaccines (**Table 1**). The potent B cell responses induced by Immunodrugs in the absence of powerful adjuvants means this technology is able to efficiently induce autoantibodies while providing safety benefits (discussed below). Preclinical proof

Table 1 Clinically tested B cell vaccines targeting self-antigens

Target-antigen[a]	Vaccine	Indication(s)	Organization
$A\beta_{1-40/42}$	AN1792	Alzheimer's disease	Wyeth/Elan (19)
	ACC-001		Wyeth/Elan
	CAD106		Novartis/Cytos (20)
	V950		Merck and Co.
ATII/I	AngQb	Hypertension	Cytos (3)
	PMD3117		Protherics (21)
CETP	CETi	Hyperlipidemia	Avant (22)
hCG	HSD-hCG	Fertility management	Indian Government (23)
EGF	SAI-EGF	NSC-lung cancer	Micomet/CancerVax (24)
FSH	oFSH	Fertility management	Indian Government (25)
Gastrin	G17DT	Pancreatic cancer	Aphton (Gilliam) (26)
Ghrelin	GhrQb	Obesity	Cytos
GnRH	GnRH-DT	Fertility management	Academic group (27)
	D17DT	Prostate cancer	Aphton (28)
Her2	Her2AutoVac	Breast cancer	Pharmexa
IgE	RP 01	Allergic asthma	Resistentia
IL-1β[b]	IL1bQb	rheumatoid arthritis	Cytos
INFα	i-IFN-alpha	HIV/AIDs	University of Milan (29)
Mucin	Stimuvax/STn-KLH	Cancer	Oncothyreon/Merck KGaD (30)
TNFα	TNFQb	Psoriasis	Cytos
	TNFAutoVac	Cachexia	Pharmexa (31)

[a]ATII/I, angiotensin I/II; CETP, cholesterol ester transfer protein; hCG, human chorionic gonadotropin; EGF, epidermal growth factor; FSH, follicle stimulating hormone; GnRH, gonadotropin releasing hormone; IFNα, interferon α; TNFα, tumor necrosis factor alpha.
[b]Trial is scheduled to commence in 2009.

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of concept has been demonstrated with numerous disease models such as rheumatoid arthritis, osteoporosis, experimental autoimmune encephalitis, myocarditis, hypertension, Alzheimer's disease, obesity and atherosclerosis (10–18; M.F. Bachmann & A.C. Tissot, unpublished data). Four Immunodrugs targeting therapeutically relevant polypetides have been trialed in humans and researchers will soon commence clinical testing on a fifth. These vaccines are AngQb, TNFQb, CAD106, GhrQb, and IL1bQb; they target angiotensin II (ATII), tumor necrosis factor α (TNFα), β-amyloid, ghrelin, and interleukin 1β (IL-1β), respectively.

Clinical proof of concept has been achieved with AngQb. Three immunizations with 300 μg of AngQb reduced blood pressure in patients with mild to moderate hypertension during the daytime and especially in the early morning (3). Importantly, this was associated with no serious adverse events (SAEs); most adverse events were consistent with local or systemic responses similar to those seen with other vaccines. The potential for therapeutic vaccination as an economic and effective treatment option for chronic diseases that can satisfy unmet medical needs is broadly acknowledged (1). This encouraging result has provided evidence that this potential may be realized.

All facets of Immunodrug development, including nonclinical studies, cGMP manufacture, and clinical testing, have built on the accrued knowledge and experience from preceding programs. The foundation of safety to support the clinical development and eventual marketing of these novel medicines is a continuum of development that has seen three distinct phases (**Figure 2**).

First, safety and immunogenicity of the platform technology in combination with foreign antigens were established. The Immunodrug DerQb comprised a 21–amino acid peptide, derived from a cysteine protease, Der p1, from *Dermatophagoides pteronissinus* conjugated to VLPs. The antigen was essentially inert; antipeptide antibodies do not recognize full-length Der p1. Hence, nonclinical safety studies were modeled on programs typically required for prophylactic vaccines.

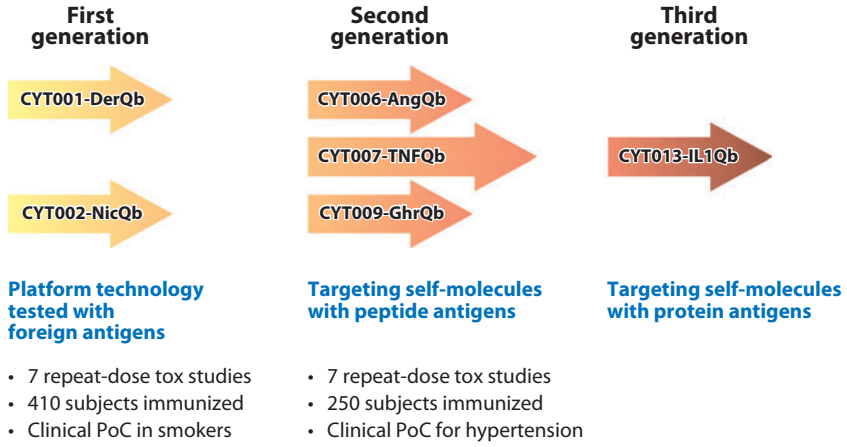

Figure 2

Generational approach to Immunodrug development. The path to the clinic for Immunodrugs targeting self-molecules has followed a sequential approach. The first phase tested safety and efficacy of the platform technology with foreign antigens. The antigens of DerQb and NicQb were a 22–amino acid peptide and nicotine derivative, respectively. From these programs, the magnitude and half-life of antibody responses to the virus-like particle (VLP) and conjugated antigen were determined. Effects of dose, regimen, and Alum adjuvant were also established. The second development phase tested the concept of targeting self-peptides (ATII, ghrelin) or protein (TNFα) using peptide antigens. Safety, tolerability, immunogenicity, and reversibility of the immune response were determined. Third-generation products now beginning clinical testing utilize protein antigens.

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Nonclincal and clinical testing demonstrated DerQb was safe and well tolerated. An assessment of immune response showed all subjects produced anti-Der p1 peptide IgG after only one immunization in the absence of added adjuvant, thus providing proof of concept for the technology (32). Testing of NicQb was similar in concept; the antigen (a nicotine derivative) was foreign but capable of inducing therapeutically beneficial antibodies. Phase I and II studies explored the effects of dose, regimen, and exogenous adjuvant, and demonstrated efficacy in smokers (2, 33).

AngQb, TNFQb, and GhrQb are second-generation vaccines comprising small-peptide antigens that target small peptides or protein. Nonclinical and clinical studies examined potential toxic effects of these Immunodrugs and further extended knowledge of safety and efficacy. The groundwork was thus laid for the more challenging development of Immunodrugs comprising protein self-antigens such as IL1bQb. Each successive phase has required more complex and considered nonclinical safety and toxicity studies to provide the best support for first-in-man studies.

Owing to their similarity to conventional vaccines, many of the established principles of nonclinical testing of vaccines can be applied to Immunodrugs. However, safety aspects unique to Immunodrugs have meant novel approaches are required. This review discusses the safety and toxicological principles concerning the development of Immunodrugs designed to induce autoantibody responses. General principles of toxicology are discussed, followed by in-depth discussion of the more complex safety issues. Examples of nonclinical safety studies to support entry of therapeutic vaccines such as Immunodrugs into early-phase clinical testing are described. Importantly, the principles discussed herein are also of relevance to other types of therapeutic vaccines.

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GENERAL CONSIDERATIONS FOR SAFETY STUDIES

Regulatory Aspects

To date, there are no dedicated regulatory guidelines for therapeutic vaccines. Regulatory guidance for Immunodrugs has been drawn principally from the following documents: "Preclinical Safety Evaluation of Biotechnology Derived Pharmaceuticals" (CPMP/ICH/302/95), "Preclinical Pharmacological and Toxicological Testing of Vaccines" (CPMP/SWP/465/95), and WHO guidelines on "Nonclinical Evaluation of Vaccines. Although a difficult task, it is necessary to address the lack of specific guidelines because of the large number of therapeutic vaccines being developed for clinical trials; for B cell vaccines alone, more than 20 trials were conducted in the past decade (**Table 1**). There is also a need to harmonize advice provided by individual national agencies, and the regulators who assess these challenging new drugs would benefit from guidance. Biotechnology and pharmaceutical companies, too, would profit from guidelines providing a framework for the most efficient investment of resources into appropriate studies. Guidelines would also enable independent consultants to provide consistent advice and approaches, and the healthcare community would be assured that best practices were used for safety testing.

Toxicology

Nonclinical safety studies for Immunodrugs inducing B cell responses against self-antigens should include a combination of good laboartory practice (GLP) toxicity and non-GLP safety studies. The design of safety studies must consider the underlying immunological principles and mechanisms of the intended immune therapy, the biology of the target molecule and its role in disease, and the intended clinical regimen. Planning nonclinical safety studies requires input from immunologists, toxicologists, disease and therapeutic area experts, clinicians, and regulatory authorities.

Toxicity studies should be designed to maximize the chances of measuring toxic effects from the Immunodrug or from immune-mediated pathology resulting from primary or secondary pharmacological action of the vaccine (discussed in detail below). Pivotal GLP toxicity studies typically include an assessment of toxic effects following repeated dose administration and local tolerance. Studies should simulate the intended clinical dose, dosage, and schedule. Frequency and timing of administration and the intended clinical formulation and route of injection should be used. The maximum human dose and/or the dose that induces the maximal antibody response should be tested. In a relative sense, this represents a high multiple of the intended human dose and provides a confident evaluation of safety with respect to toxic effects due to the Immunodrug components (discussed below). To ascertain potential toxic effects from secondary pharmacological actions of autoantibodies, necropsy and histology are often performed at peak antibody responses. Dose-free observation periods are also included to measure recovery of any toxic effects.

The species chosen for toxicity studies must produce the expected immune response and be responsive to the intended pharmacodynamic action of the induced antibodies. Toxicity studies performed with Immunodrugs have used mouse, rat, rabbit, and primates as test species. The ATII sequence in the previously mentioned AngQb is identical to rat ATII. This makes the rat a suitable species for repeat-dose toxicity testing. Indeed, preclinical efficacy studies demonstrating AngQb-induced antihypertensive ATII antibodies were performed in rats (16). As a general principle, species-specific vaccines must be used. In the event the sequence of the human antigen differs from that of the test species, it is then necessary to produce a homologous vaccine for toxicity testing. Human IL-1β is 77% and 96% identical to murine and primate IL-1β, respectively. Hence, in addition to the human vaccine, species-specific versions of the vaccine were produced for toxicity testing in mice and rhesus monkeys. By testing homologous vaccines, the chances of revealing toxic effects arising from reactogenicity of the antigen, antibody-dependent cellular cytotoxicity, immune complex disease, unexpected cross-reactivity to endogenous molecules, and enhancement of target-antigen activity will be maximized (discussed below).

Standard parameters measured in repeat-dose studies include viability, clinical observations, injection site observations, body weights, food consumption, opthalmoscopy, hematology, clinical chemistry, urinalysis, necropsy organ weights, and extensive organ and tissue histology. Pharmacodynamic assessment principally involves an assessment of antigen-specific IgG antibodies by ELISA. Consideration should be given to modifying basic study designs and measuring target-related parameters to enhance the assessment of potential vaccine toxicities (discussed below). For example, during repeat-dose toxicity studies with IL1bQb, biomarkers of IL-1β activity (IL-6 and cortisol) were measured after each immunization. This allowed assessment of reactogenicity owing to the IL-1β component of the vaccine. Additionally, immunized animals were given an intravenous dose of IL-1β. This experiment assessed both the pharmacodynamic action of the vaccine and the potential for anti-IL-1β antibodies to enhance IL-1β activity. Safety studies performed in disease models can also be of value for studying the potential pathological consequences of immunization. Induction of antibodies in a disease setting where the target molecule is overexpressed may amplify toxic effects due to antigen-antibody interactions. A caveat is that interpretation of results from studies performed in disease models may be complicated by lack of background data and uncertain pathology.

Five categories of potential toxicity have been described for vaccines (37). These provide a framework for nonclinical testing of Immunodrugs and include intrinsic toxicity of the components of the vaccine, toxicity due to the pharmacodynamic action of the vaccine (i.e., due to antibodies and T cells), activation of preexisting disorders, toxicity of contaminants and impurities, and adverse reactions due to interactions among the various components of the vaccine. The last two

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categories are issues of quality control and combination vaccines respectively and thus will not be addressed. Nonspecific activation of autoimmune disorders or preexisting disease by immunization is a contentious issue. There is little evidence to support the phenomenon, but it is difficult to exclude and difficult to test for because of a lack of validated models (37). The subject is treated cautiously by regulatory authorities. This approach is exemplified by recent FDA decisions to place clinical trials of Heplisav™ and Pollinex® Quattro on hold following single SAEs in phase III clinical trials even though both SAEs were believed to be unrelated to the vaccine. The most important potential toxicities for Immunodrugs are related to the components of the vaccine and its pharmacodynamic action and are discussed in detail below.

TOXICITY OF COMPONENTS

To assess the potential toxicity of an Immunodrug's constituent elements, an analysis of their molecular nature is required. As shown in **Figure 3**, the principal components are the proteinaceous Qβ VLP capsid, *Escherichia coli* RNA incidentally packaged into the VLP during assembly, the heterobifunctional chemical cross-linker that attaches the target-antigen to the VLP, and the antigen. Often, the adjuvant aluminum hydroxide is included in the final vaccine formulation. Toxicity testing performed with high relative doses in appropriate species provides a best means for assessing component-related toxicities.

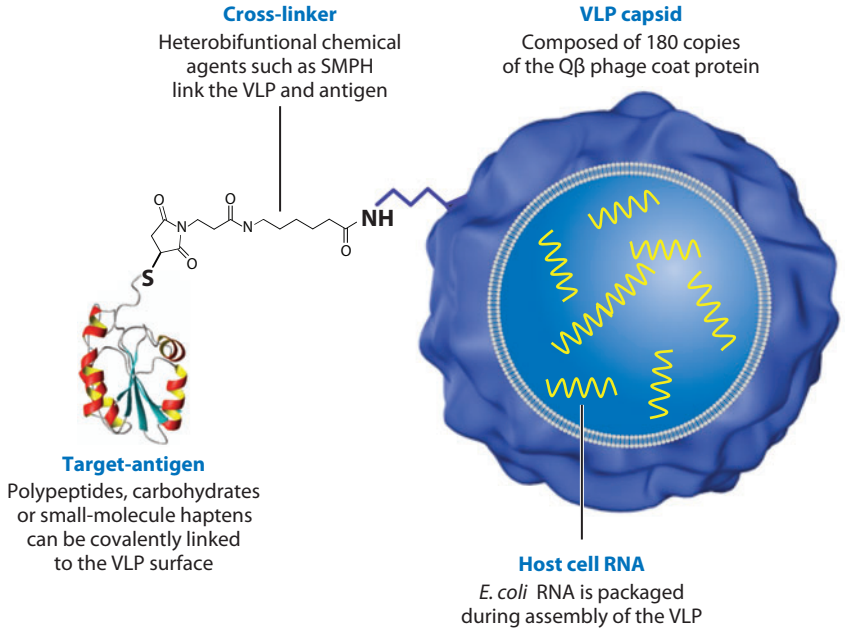

Figure 3

The principal components of a virus-like protein (VLP)-conjugate vaccine. A schematic representation of the elements comprising an immunodrug. They include the VLP capsid, incidentally packaged *Escherichia coli* host RNA, heterobifunctional chemical cross-linker, and target-antigen containing an engineered cysteine to facilitate conjugation (typically, for small peptides, hundreds of antigens are attached to each VLP). Diagram is not to scale.

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The Virus-Like Particle Capsid

All Immunodrugs tested in humans to date have utilized VLPs derived from the bacteriophage Qβ. Although the ability of Qβ to act as a carrier capable of augmenting the immunogenicity of a protective B cell epitope was only recently established (38), the phage was discovered more than 40 years ago. Qβ is an *Allolevirus* that solely infects *E. coli*. Both the Qβ phage and VLP have been extensively studied. The gene encoding the 14-kDa coat protein can be expressed in *E. coli*, resulting in assembly into VLPs with a mass of 2.54 MDa that are morphologically and immunologically indistinguishable from the parental phage (39). The crystal structure of the Qβ phage has been solved, and details of coat protein interactions, assembly, and RNA binding have been determined (40). Qβ VLPs are 30-nm icosahedrons made of 180 coat protein monomers. The basic structural unit is a noncovalently stabilized dimer. Each coat protein has two cysteines that disulphide bridge with other coat proteins to form pentamers and hexamers. Assembly into stable icosahedrons occurs within the cytosol of the production strain. Qβ VLPs are produced under cGMP and classified as a key intermediate of Immunodrug production. In addition to having a structural role, the coat protein binds viral RNA, which represses translation of the replicase gene and initiates phage assembly (41). The coat protein has no known enzymatic or toxic properties and, from a toxicological perspective, is essentially inert.

E. coli RNA

During VLP assembly, nucleic acids of the host, predominately RNA, are packaged. Approximately 25% of the mass of a VLP consists of internalized *E. coli* RNA. The RNA has a role in particle formation and immune response and is defined as an integral component.

Single-stranded RNA (ssRNA) is the natural ligand for Toll-like receptors 7 and 8 (TLR7/8) (42). Engagement of these receptors upregulates costimulatory molecules and cytokines, resulting in induction of effective immunity. Naked, single-stranded RNA is highly unstable in vivo owing to degradative enzymes and conditions. Because RNA is packaged into the Immunodrug, it is stabilized and available for interaction with TLR7/8. Owing to its particulate nature, the VLP is targeted to antigen-presenting cells (APCs) such as dendritic cells and B cells where it activates the endosomal TLR7/8 and promotes antigen-specific IgG2a responses (43). Indeed, B cell responses are enhanced by RNA-containing VLPs when compared with VLPs devoid of TLR agonists (43). Experimental evidence in mice indicates that VLPs containing *E. coli* RNA induce relatively weak T cell responses compared with those containing deoxycytidyl-deoxyguanosine oligonucleotides (CpGs) and are T helper (Th) cell dependent, an important safety consideration (44; M.F. Bachmann, unpublished data).

The relatively recent discovery of the immunostimulatory properties of RNA means there is little clinical experience concerning their use as adjuvants per se. However, there is broad experience with topically or orally administered ssRNA analogues such as Imiquimod, Resiquimod, and Loxoribine, which are TLR7/8 agonists. These compounds were developed as general immunomodulators for use in antiviral [HPV and herpes simplex virus (HSV)] and cancer therapies (45). Preclinical and clinical pharmacodynamic studies with orally or topically administered Imiquimod have shown production of several cytokines, in particular INFα, from a number of different cell types. Toxicology programs indicate a high degree of safety with no target organ toxicity other than that attributed to exaggerated pharmacological activity of INFα (46). Hence, toxic effects due to the RNA contained within the Immunodrug are expected to be of little significance. The likelihood of toxicity is further minimized given the low dose and dosage administered and the episodic and relatively infrequent administration. Packaging within the VLP and targeted

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delivery to APCs is expected to reduce systemic distribution of the ligand and lessen systemic activation and toxicity. These assumptions have been born out by numerous repeat-dose toxicity studies in which high relative doses were tested, and from clinical testing.

Chemical Cross-Linker

A heterobifunctional chemical cross-linker is used to covalently conjugate the antigen to the VLP. The reagent used is succinimidyl-6-[(β-maleimidopropionamido) hexanoate] (SMPH). SMPH is a highly reactive compound containing two distinct functional groups that couple to different targets, one on the VLP and the other on the antigen. The conjugation process is performed sequentially. SMPH is first reacted with solvent-exposed amines of the VLP via its amine-reactive NHS-ester, forming a covalent amide bond and releasing *N*-hydroxysuccinimide. Unreacted SMPH is principally hydrolyzed to SMPH free-acid. Reaction by-products are subsequently removed by tangential flow diafiltration. Next, the derivatized VLP is reacted with antigen under conditions permitting conjugation of SMPH's maleimide group to a sulfhydryl group of the antigen. Thus, an irreversible thioether linkage is formed between the cross-linker and cysteine residue of the antigen (**Figure 2**). Unreacted maleimide groups are hydrolyzed to nonreactive maleimic acid and diafiltration is used to remove uncoupled antigen. SMPH is of negligible toxic potential because of the highly labile nature of SMPH's functional groups in aqueous solution, the relatively low concentration used in manufacture (1–2 mM), and the more than millionfold depletion factor resulting from diafiltration.

Target-Antigen

Antigen toxicity has been of issue since vaccination inception. The use of attenuated or killed whole-organisms and chemically or genetically detoxified protein subunits has provided a means whereby potentially noxious agents are rendered relatively harmless. For Immunodrugs, the potential for toxicity due to the target-antigen exists because Immunodrugs may include self-molecules with potent biological activities. The conjugated antigen may retain its activity and residual unlinked antigen may also exert a biological effect. The latter can be avoided by manufacturing processes that remove free antigen or reduce it to levels of no effect. If this cannot be achieved, then potential toxic effects must be considered and addressed experimentally if necessary. An assessment of antigen toxicity should take into account the dose of antigen, route of injection, its potential for systemic distribution, and its degradation/metabolism. Subcutaneous injection and binding to adjuvants limit systemic distribution.

For some Immundrugs, such as DerQb, TNFQb, and GhrQb, the coupled antigens are small peptides that lack the activity of the parental polypeptide from which they were derived. Hence, the issue is irrelevant. In contrast to the antismoking vaccine NicQb, the antigen nicotine is a pharmacologically active, acutely toxic compound. Ingestion of 40–60 mg is assumed to be lethal for humans. However, for NicQb, the potential for nicotine-induced toxicity is negligible. Each NicQb VLP has approximately 570 conjugated nicotine molecules (33). This is less than 1.5 μg of nicotine per 100 μg dose of NicQb, a pharmacologically and toxicologically irrelevant dose equivalent to 0.2% of the nicotine content of one cigarette.

Protein antigens have greater potential for toxic effect. For example, cytokines are potent biological agents, active at doses <10 ng kg^{-1}. The amount of conjugated antigen administered in an Immunodrug is typically in the microgram range. This situation has parallels with subunit vaccines such as diphtheria toxin (DT), which is lethal to humans at 0.1 μg kg^{-1}. To render DT vaccine safe for use, its ADP-ribosylase activity is reduced by formaldehyde treatment, artfully

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detoxifying the protein but preserving neutralizing epitopes. For the anti-IL-1β vaccine (IL1bQb), the detoxification of IL-1β is less critical. The maximum tolerated dose of IL-1β in humans is approximately 300 ng kg^{-1} (47). On the basis of the IL-1β content of IL1bQb, a nominal 100 μg dose would deliver <20 μg of IL-1β, which does not exceed the reported maximum dose for a 70 kg individual. Nevertheless, such a dose is potentially capable of inducing fever, myalgia, arthralgia, and hypotension. To minimize this risk, we produced a genetically detoxified version of IL-1β.

Molecular studies of human IL-1β identified numerous amino acids that influence its biological activity. Most loss-of-activity mutations reduce receptor binding by altering tertiary structure, an unsuitable trait for an antigen that should induce neutralizing antibodies. Therefore, we screened more than 20 different IL-1β mutants for their ability to bind IL-1 receptor, trigger IL-6 release, and induce neutralizing antibodies. From the matrix of results we identified a mutant IL-1β antigen that has the desired traits of low bioactivity and authentic structure and antigenicity (**Figure 4**).

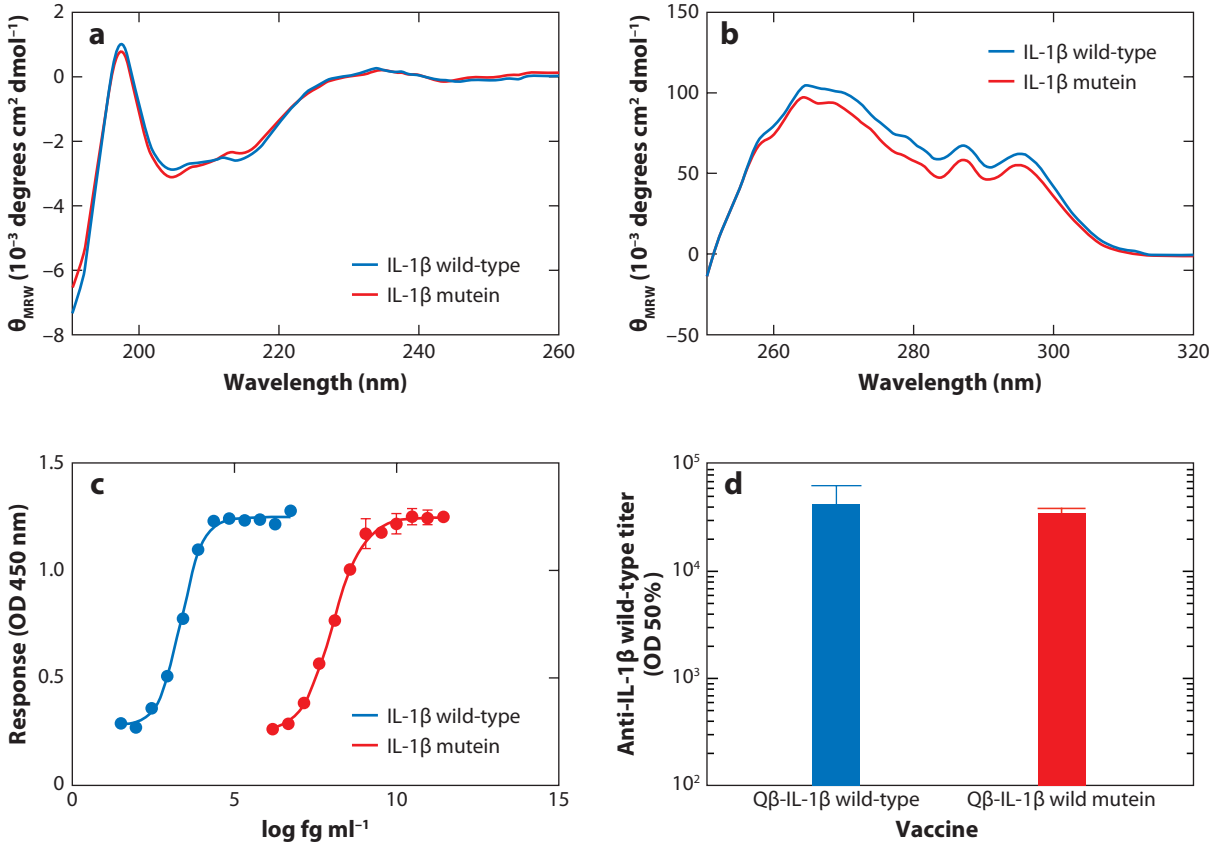

Figure 4

Comparison of IL-1β wild-type and IL-1β mutant antigens. The structure of wild-type and mutant IL-1β were compared by (*a*) far-UV CD spectroscopy and (*b*) near-UV CD spectroscopy. Almost identical spectra indicate that secondary and tertiary structural properties are conserved. Bioactivity (*c*) of wild-type and mutant IL-1β was compared by measuring IL-1β-induced release of IL-6 from HeLa cells. Bioactivity of the mutant was reduced more than 10,000-fold. Wild-type and mutant IL-1βs elicit antibodies that similarly recognize wild-type IL-1β (*d*). Groups of mice ($n = 4$) were immunized with either wild-type or mutant IL1bQb vaccine. IgG antibody titers against wild-type IL-1β were determined by ELISA. Titers are expressed as those serum dilutions that lead to half maximal OD450 nm in ELISA.

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Adjuvant

Aluminum hydroxide, namely Alhydrogel™, is frequently included in Immunodrug formulations to boost B cell responses (33). Its inclusion in vaccines is believed to have no impact on safety (3, 33, 48). Aluminum salts were often included in whole-cell vaccines to reduce reactogenicity; thus, in more than 50 years of clinical experience, billions of doses have been administered. Side effects attributed to the use of aluminum in vaccines are limited to local reactions such as swelling, indurations, erythema, and cutaneous nodules (48).

TOXICITY DUE TO PHARMACODYNAMIC ACTIVITY

The potential for toxicity arising from primary or secondary pharmacodynamic activity of an Immunodrug is a complex issue that must be addressed in detail. Safety issues associated with primary pharmacodynamic activity are principally concerned with neutralization of the target molecule. Toxicity arising from secondary pharmacodynamic activity may result from unintended antibody- and T cell–mediated pathology, which is also of concern for conventional vaccines.

Primary Pharmacodynamics

A predictive assessment of toxic effects arising from the intended pharmacological action of an Immunodrug, namely induction of neutralizing antibodies specific to the target molecule, requires knowledge of the outcome of successful target neutralization. This can be provided by nonclinical and clinical studies involving neutralization of the target molecule with small-molecule inhibitors, biologicals (e.g., monoclonal antibodies, receptor antagonists, and decoy receptors), naturally occurring autoantibodies, and in some instances, other therapeutic vaccines. Valuable information can also be garnered from rare human genetic deficiencies or mice rendered genetically deficient for the target molecule. In rare cases, surgical removal of organs may also offer insights into the potential risks of reducing the levels of a particular molecule. The toxic consequences of neutralizing a target molecule have to be assessed in detail on a case by case basis and no general rule can be applied. The following section provides examples to illustrate the concept.

An analysis of the use of small-molecule pharmaceuticals targeting the renin-angiotensin-aldosterone system (RAAS) provided a predictive safety assessment for AngQb. The primary pharmacological effect of neutralizing the action of ATII by anti-ATII antibodies is not expected to be different from that observed for angiotensin-converting enzyme (ACE) inhibitors and angiotensin-receptor blockers (ARBs) (i.e., all three treatments reduce signaling via the ATII receptor 1). The long-term safety of conventional small-molecule pharmaceuticals that inhibit ACE or block ATII receptors has been established in millions of patients. They are generally regarded as safe. Because there are no known safety issues associated with ACE inhibitors and ARBs effecting signaling via alternate receptors (e.g., ATIIR2), it is also expected that the effect of antibody-mediated neutralization of ATII will not be problematic. No current pharmacological intervention completely inhibits RAAS. Similarly, ATII attains equilibrium with anti-ATII antibodies and the law of mass action dictates that complete binding of ATII will not occur. Thus, the effect of long-term blockage of ATII by antibodies is expected to be safe. Preclinical and clinical studies performed to date confirm this analysis.

Similarly, the successful clinical application of monoclonal antibodies, receptor antagonists, and soluble receptors targeting IL-1β or TNFα provides a basis for safety assessment for TNFQb and IL1bQb. For example, extensive clinical experience has been obtained with IL-1α/β-neutralizing biologicals such as the IL-1R1 receptor antagonist Kineret®; an anti-IL-1β monoclonal

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antibody, AZ885; and an IL-1 receptor trap comprising IL-1 receptor and IL-1 receptor accessory protein, Arcalyst. Kineret® is a licensed product that reduces symptoms in patients suffering from diseases characterized by IL-1 overproduction and has been shown to have a therapeutic effect in type 2 diabetes (49, 50). Arcalyst has been reported to be effective in treating inherited auto-inflammatory conditions and AZ885 demonstrated efficacy in patients with Muckle-Wells syndrome and methotrexate-resistant rheumatoid arthritis. Extensive postmarketing surveys of Kineret® and available early clinical data from the other IL-1 antagonists indicate that neutralization of IL-1 activity is generally safe and without systemic toxicities (51).

IL-1 is involved in the host response to infections, especially those involving intracellular pathogens, and IL-1R1- and IL-1α/β-deficient mice have increased susceptibility to infection with *Mycobacterium tuberculosis* and *Listeria monocytogenes*. An analysis of multiple clinical studies performed with Kineret® has shown a slight increase in the incidence of serious infections (1.8% in Kineret®-treated versus 0.7% in placebo group). The type of infection associated with Kineret® treatment does not appear substantially different from what can be observed in a heavily treated rheumatoid arthritis (RA) population (51). Provided that adequate measures are taken, this problem is considered manageable. IL1bQb may actually have safety advantages over Kineret®, which acts as a competitive inhibitor for the binding of both IL-1α and IL-1β to IL-1 receptors on the surface of cells. In contrast, IL1bQb induces antibodies that are highly specific for IL-1β and do not neutralize IL-1α (17). Therefore, immunization with IL1bQb will selectively neutralize IL-1β while maintaining the activity of IL-1α. We have compared mice immunized with either anti-IL-1α or anti-IL-1β vaccines and found that mice immunized against IL-1α are considerably more susceptible to infection with *Listeria* (M. F. Bachmann, T. M. Kündig & G. Spohn, unpublished data). This may provide an additional level of safety in the context of host response to infectious agents.

Reversibility of autoantibody response. A unique characteristic of Immunodrugs that sets them apart from pharmaceuticals and most biologicals, with the exception of a few monoclonal antibodies (52), is the persistence of the pharmacological agent (target-specific autoantibodies) and its potential effect. The relatively long-lived response of active immunization offers potential advantages for the treatment of chronic diseases such as less-frequent administration of medicine, improved patient compliance, and lower costs (1). However, longer-lived therapeutic effect brings with it safety considerations, principally the difficulty in readily reversing the antibody response.

The 4-month half-life of anti-ATII antibodies induced by AngQb (3) is considerably longer than the typical 12-h half-life for ACE inhibitors and ARBs. To turn off the effect of an Immunodrug is less readily achieved. The presence of detectable target-specific antibodies does not necessarily equate to pharmacologic effect. A threshold of antibody concentration is required to achieve an effective neutralization of the target. As discussed above, this is not expected to lead to unforeseen safety issues. Animal models may be used to predict the relationship of titer and response and establish reversibility of the therapy, but ultimately data pooled from clinical studies and pharmacometric analyses will be required. Hence, clinically validated molecules, preferably without irreplaceable function, should be targeted for therapeutic vaccines. In this way, the effects of long-term blockade of the target molecule can be best predicted.

Reversibility of antibody response must be established in animals as part of the routine pharmacological assessment and is done prior to commencing clinical studies. From an immunological perspective, coupling of the antigen to the VLP that contains Th epitope offers a means to prevent irreversible antibody responses (discussed in detail below).

To date, all subjects immunized with Immunodrugs have responded by producing antigen-specific IgG antibodies. The kinetics of the antibody responses have been measured for more than

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12 months. Importantly, for all subjects the antibody response has been reversible (2, 3, 16, 32, 33). The antibody half-life measured for subjects immunized with DerQb or NicQb did not differ markedly from those immunized with self-antigens (2, 3).

Secondary Pharmacodynamics: Antibody-Mediated Toxicity

Antibodies induced by vaccination may result in unintended effects that have the potential to cause toxicity. These include immune complex formation and deposition, altered pharmacokinetics of the target molecule, antibody-dependent cellular cytotoxicity, and cross-reactivity with molecules other than the intended target. Such events represent secondary pharmacological activities and nonclinical safety assessment should be designed to reveal them.

Immune complex deposition. The continual formation of antigen-antibody complexes and their removal by mononuclear phagocytes of the reticuloendothelial system is a normal mechanism of antigen clearance. However, in certain abnormal circumstances persistent immune complexes may be deposited in tissues and organs. This can result in activation of complement and effector cells, which causes inflammation and tissue damage.

The propensity for immune complexes to form, deposit, and cause disease is influenced by antigen valency and concentration, antibody class, concentration and affinity, the concentration and size of the complex, and the complement status of the individual (53). Deposition requires vascular permeabilization and occurs mostly at sites of high pressure and turbulence such as the kidneys. Immune complex disease can result from persistent infection, inhalation of antigenic material, or autoimmune disease. The latter is of consequence for therapeutic vaccination. Immune complex disease is a recognized complication of certain autoimmune diseases in which the continued production of autoantibodies against self-antigens leads to prolonged immune complex formation, deposition, and inflammation with pathological consequences. Because Immunodrugs aim to induce autoantibodies in situations where there is persistent antigen, this issue must be addressed.

The design of a particular Immunodrug and the molecular nature and concentration of its target molecule are key factors to consider when assessing the risk of immune complex disease. Antigen-antibody complexes can form only if the antigen contains at least two simultaneously accessible antibody binding sites. Multi-epitopic antigens induce antibodies that can form complexes. Antibodies specific for a single epitope may also cross-link when the cognate epitope is repeatedly displayed in the context of a multimer. Immune complexes won't form when the target-antigen contains only a single accessible antibody-binding site. Hence, for Immunodrugs that induce antibodies that access only one site within the target molecule, immune complex disease should not occur.

This is the case for AngQb, which comprises amino acids 1–8 of ATII cross-linked to Qβ VLP. The chance of inducing antibodies that recognize two epitopes within ATII is low. Given the number of residues typically involved in antigen-antibody recognition and area of contact (54), it is reasonable to conclude that the small size of ATII virtually precludes binding of two antibodies. Therefore, it is not expected that immune complexes will form following immunization. This expectation has been fulfilled in nonclinical and clinical studies. The kidney is a sentinel for immune complex disease owing to its sensitivity to complex deposition. Hence, toxicity studies must include thorough histological examination of the kidney for signs of glomerulonephritis. This is of particular relevance for AngQb because the levels of ATII in the kidney far exceed those in the blood (55). AngQb has been tested in numerous repeat-dose toxicity studies with in-life phases spanning 10–30 weeks. Histological examination of kidneys and blood vessels showed no signs of

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nephritis, vasculitis, or angitis indicative of immune complex deposition (16; M.F. Bachmann & G.T. Jennings, unpublished data). The same was true for a long-term safety study performed with spontaneously hypertensive rats, a model of disease (16). Data from clinical trials indicate this is the case for humans, too. In phase I, the concentration of activated complement factors (C3a, C5a) and of circulating complexes containing C1, C3, IgM, IgA, or IgG were measured at baseline and 7 and 14 days after immunization. No changes in were observed (16). Similarly, in phase II, the concentration of immune complexes containing C1 and C3, and the level of complement factor C3a in hypertensive patients did not change beyond fluctuations also recorded in the placebo group (3). These findings are supported by clinical studies in hypertensive patients with PMD3117, a vaccine that targets ATI. Concerns about immune complex disease were reported as unfulfilled (21).

Immunodrugs that target larger polypeptide antigens are more likely to induce immune complexes. The antigen conjugated to TNFQb is a 20–amino acid peptide with homology to residues 4–23 of human TNFα. The peptide contains an 11–amino acid core identical to human TNFα. The amino acids VAHVVA within this core form an internal β-sheet involved in intermolecular interactions between the 17.4-kDa TNFα monomers. Antibodies induced against this sequence are thought to disrupt trimerisation of TNFα (15). Additionally, antibodies induced to less well-conserved elements of the antigen may induce antibodies that bind to exposed regions of trimeric TNFα. Hence, there is a possibility TNFα-antibody complexes could form. This is supported by in vitro studies with the anti-TNFα monoclonal antibody Humira®, in which it was demonstrated Humira® binds TNFα and forms stable 598-kDa hexameric antigen-antibody complexes (56). However, in vivo the situation may be different because TNFα is present in the serum at concentrations of 5–30 pg ml^{-1} (57), which would not favor the formation of antigen-antibody complexes. Furthermore, in the event that immune complexes formed, their concentrations would be many orders of magnitude below the normal range of circulating immune complexes found in healthy individuals (58), making the chances of disease remote. This seems to be the case because extensive clinical testing and postmarket surveillance of Humira® have not identified adverse events relating to TNFα-Humira® complexes (59). This also seems to hold true for TNFQb, for which nonclinical and clinical studies have shown no evidence of immune-complex disease. Repeat-dose toxicity studies performed with TNFQb showed no signs of inflammation indicative of immune-complex disease, and a phase I/IIa trial in psoriasis patients showed no changes from baseline in circulating immune complexes (M.F. Bachmann, G.T. Jennings & P. Mueller, unpublished data).

For IL1bQb, the antigen is a multi-epitopic 17-kDa protein. Although there is a chance immune complexes may form, there is no cause to suspect that deposition and inflammation will result. Arguing in favor of this is the fact that like TNFα, the plasma levels of IL-1β are exceedingly low [approximately 50 pg ml^{-1} for healthy individuals and 230 pg ml^{-1} for patients with autoimmune inflammatory disorders (60)]. Under these conditions, the formation of large IL-1β-antibody complexes would not be favored and, in the event that complexes formed, their anticipated concentrations would be low. Evidence that small immune complexes do not cause disease is provided by studies with anti-immunoglobulin E (IgE) monoclonal antibodies, which form trimeric, tetrameric, or hexameric IgE-anti-IgE complexes (61). Although these complexes are relatively small in size, clinical testing has shown that they accumulate in the circulation with concentrations up to 24 μg ml^{-1} without induction of immune complex disease (62). As a general rule, targeting soluble molecules present at low concentrations is not expected to result in toxicity related to immune complex disease. Nevertheless, careful nonclinical assessment is required.

Finally, immune complex disease may also present in a relatively benign Arthus reaction, which occurs when subcutaneously injected vaccine combines with specific antibodies to form immune

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complexes that deposit around the walls of small blood vessels. Activation of complement and polymorphonuclear leukocytes (PMNs) results in inflammation, which manifests as edema and hemorrhage at the injection site. The phenomenon has been noted following administration of prophylactic vaccines such as rabies and diptheria, tetanus, and pertussis (DTaP) vaccines (63, 64). It is not a serious adverse event because it is an interaction of antibody with locally deposited vaccine rather than systemic antigen.

Altered pharmacokinetics. Circulating antibodies specific for bioactive proteins have the potential to alter the pharmacokinetics of the target protein. This has been reported for some anticytokine monoclonal antibodies; instead of binding, neutralizing, and eliminating the cytokine, antibody binding increases the magnitude and duration of the cytokine's effect. Rosenblum et al. (65) reported that an anti-INFα monoclonal antibody increased the plasma half-life of INFα, resulting in prolonged antiviral and antiproliferative activity. Similarly, complexes of IL-3, IL-4, or IL-7 and monoclonal antibody result in prolonged agonistic activity (66). Accumulation of monomeric antibody-IL-6 complexes has been reported in a multiple myeloma patient treated with an anti-IL-6 monoclonal antibody (67). It must be considered that increasing the half-life of a disease-associated protein may worsen disease. To prevent disease enhancement, antibodies should bind a neutralizing epitope and/or multiple epitopes within the target molecule. It has been demonstrated that simultaneous treatment with three epitopically distinct anti-IL-6 monoclonal antibodies induces rapid uptake and elimination of the resultant trimeric IL-6-antibody complex in contrast with treatment with one or two antibodies (68). Immunodrugs comprising large antigens with multiple epitopes typically induce poly-epitopic antibody responses, and thus the likelihood of enhancing the effect of the target protein is minimized. Dedicated safety studies performed in disease models may also generate valuable information. As described for IL1bQb, the inclusion of antigen-challenge experiments in toxicity studies should be considered.

Cross-reactivity. A hallmark of antibodies is their specificity toward their cognate antigen. Despite this, some antibodies bind molecules different from those used for their induction. There is a possibility that for vaccines and monoclonal antibodies such cross-reactivity may have biological consequences. The concern for cross-reactive antibodies is that they may bind cell-surface or extracellular matrix proteins and damage cells and tissues by binding and activating complement and/or effector cells carrying Fcγ receptors (e.g., NK cells, neutrophils, macrophages, and eosinophils). In addition, cross-reactive antibodies could unintentionally neutralize bioactive molecules or cross-link and activate receptors.

The use of in silico, in vitro, or in vivo methods to assess whether antibodies generated against a particular epitope will cross-react, resulting in toxic effects, is contentious. There are reports that certain microbial infections induce antibodies with cross-reactivity to host cell proteins. For example, the M protein from group A streptococcus is structurally and immunologically similar to host proteins myosin and laminin, and anti–M protein antibodies that cross-react with them are postulated to be involved in postinfection immune sequelae (69). However, there are no proven examples of pathogens inducing disease in humans owing to cross-reactive B responses, nor are there proven cases of vaccines inducing pathological cross-reactive antibodies. Finally, the presence of cross-reactive autoantibodies is not indicative of autoimmune disease (70). Nevertheless, despite the lack of a convincing precedent, an assessment of safety must pay heed to this matter.

Computer-based homology searches using the primary amino acid sequence of the target-antigen can be performed. However, such analyses invariably yield numerous irrelevant identities with contiguous sequences from unrelated proteins. The relevance of a significant hit requires analysis of protein structure and cellular location. In the event that conserved linear epitopes are

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identified on the surface of secreted or outer-membrane proteins, further investigation may be warranted. Currently, there are no satisfactory in silico approaches for predicting conformational epitopes, principally owing to limitations of data sets and predictive programs (71, 72). Because it is estimated that >90% of B cell epitopes are discontinuous (73, 74), in silico prediction of cross-reactive epitopes is of little value.

Toxicity assessment of monoclonal antibodies often examines potential cross-reactivity by immunohistochemistry utilizing panels of human tissue derived from multiple donors. Such studies are not part of the routine battery of toxicity tests required for vaccines. Individual cross-reactive clones will be heavily diluted in a polyclonal response and the use of sera renders interpretation of results difficult.

Complicating in vivo toxicity assessment of cross-reactivity is a lack of suitable models to assess risk of disease arising from autoantibodies (70). Hence, emphasis is placed on the species chosen for toxicity studies. Owing to the high sequence similarity between human and nonhuman primates such as rhesus macaques (75), toxicity studies performed in primates provides a best-practice approach.

When the target molecule is a member of a family of related proteins, undesired cross-reactivity with homologous family members can be readily determined. For example, nonclinical assessment of IL1bQb examined the ability of antibodies generated against IL-1β to bind IL-1ra or IL-1α, proteins with approximately 30% identity to IL-1β. In a worst-case scenario, unintentional neutralization of IL-1ra could exacerbate autoimmune inflammatory disease (76). However, it was demonstrated by ELISA and in vivo methods that IL-1β-specific antibodies induced by IL1bQb do not cross-react with IL-1ra or IL-1α (17).

Secondary Pharmacodynamics: T Cell–Mediated Toxicity

The ability of autoreactive CD4 and CD8 T cells to contribute to pathology is well documented in human diseases such as multiple sclerosis and type 1 diabetes. Moreover, there are numerous T cell–mediated disease models in which autoreactive T cells are induced by immunizing with T cell epitopes and strong adjuvants. There has long been an association of infection and molecular mimicry with T cell–mediated autoimmune disease. Some examples are *Streptococci* and rheumatic fever, B3 coxsackieviruses and myocarditis, and *Borrelia burgdorfii* and arthritis. Animal models have provided direct evidence that infection can invoke a particular autoimmune disease. However, apart from the expected pathology arising from T cell effector functions in infected organs, and the possible exception of Lyme's disease, there is no verified example of disease due to cross-reactive T cell responses in humans (77). Attempts have been made to link vaccination with induction of autoimmune disease (e.g., hepatitis B vaccination and multiple sclerosis) but no cause-effect relationship has ever been established (78).

In contrast, the possibility to elicit unintended autoreactive T cells by therapeutic vaccination aimed at inducing humoral responses to self-antigens has been realized. The single case among the >20 clinically tested therapeutic self-specific B cell vaccines was the first Alzheimer's disease (AD) vaccine, AN1792. AN1792 was designed to induce antibodies to $A\beta_{1-40/42}$ and comprised fibrillar $A\beta_{1-42}$ peptide in combination with the adjuvant QS21. AN1792 was safely tested in phase I, but an ensuing phase IIa trial involving AD patients with mild to moderate disease reported 18 cases of meningoencephalitis among the 298 treated subjects (79). Consequently, the development of AN1792 ceased. The inflammatory response was attributed to Aβ-specific T cells rather than antibodies (19, 79). Indeed, T cell epitopes are contained within the middle and carboxy-terminal regions of $A\beta_{1-42}$. Involvement of T cells was supported by autopsy from two affected patients. Subsequent testing of animal models also provided evidence for induction of T cell responses

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(80, 81). Second-generation vaccines designed to minimize the risk of induction of autoreactive T cells have now entered clinical testing.

In addition to inducing autoreactive T cells, vaccination against self-antigens has the potential to overcome Th cell tolerance, which may have the undesired effect of making B cell responses to self-antigens irreversible. Hence, an understanding of the immunology that governs the induction of autoimmune T cells and incorporation of these principles into vaccine design is the best way to minimize risk or avoid this secondary pharmacodynamic effect.

The immune system has several mechanisms to prevent autoimmune responses. The main mechanism is tolerance, whereby self-specific T and B cells are deleted or rendered unresponsive during ontogeny or in the periphery. Additionally, regulatory T cells contribute to self-tolerance by preventing exaggerated immune responses. Tolerance mechanisms exist for both T and B cells but are stricter for T cells. T cells are tolerant of soluble self-proteins. In contrast, B cells are not tolerant but are simply unresponsive because of Th cell tolerance for the particular antigen. True B cell tolerance is observed only for highly expressed membrane proteins and other antigens (e.g., DNA) that cross-link B cell receptors, resulting in deletion of specific B cells. Membrane-bound proteins of low abundance induce B cell anergy (7).

In general, B cells do not respond with long-lasting IgG titers to antigens in the absence of specific Th. This is the case for carbohydrates, which do not contain Th cell epitopes and for self-antigens, which fail to induce specific Th due to tolerance. B cells respond, however, to such antigens if recognizable Th cell epitopes are provided. This is the underlying principle of all conjugate vaccines, which display carbohydrates or self-antigens and provide multiple Th cell epitopes via the conjugate protein. By linking a B cell epitope to a foreign T cell epitope, Th cell tolerance for self-antigens is not broken but circumvented (1). This principle has important safety consequences for therapeutic B cell responses as illustrated by AN1792. In this case, the antigen $A\beta_{1-42}$ was not linked to a carrier containing a foreign Th cell epitope. Hence, to induce antibodies, it was necessary to break Th cell tolerance rather than circumvent it (79). This was achieved through the use of the strong adjuvant QS21, which probably resulted in the activation of normally unresponsive Aβ-specific T cells and disease in 18 patients.

Self-specific B cell responses are transient and reversible because endogenous self-antigen does not continuously stimulate antibody production. This is because memory B cells do not respond to antigenic stimulation in the absence of T cell help. Immunization with conjugate vaccine containing Th epitopes is required for boosting B cells responses. However, in the course of vaccination, should Th cell tolerance toward a self-antigen be overcome, then an irreversible antibody response could ensue. Hence, nonclinical studies should measure target-specific antibodies following administration of target-antigen to immunized animals. In this way, boosting of antigen-specific antibody responses by endogenous self-antigen can be formally excluded.

T cells respond to peptide epitopes presented on major histocompatibility complex (MHC) class I or class II molecules. The minimal length of a peptide that can bind to MHC class I [recognized by cytotoxic T lymphocytes (CTLs)] is 8 amino acids, and MHC class II molecules (recognized by Th cells) is 10–12 amino acids. Peptides <8 amino acids are unable to induce T cell responses. This principle has underpinned the design of second-generation therapeutic AD vaccines, which utilize small Aβ peptide fragments conjugated to either VLP (CAD106) or DT (AC-001) (12, 20, 81).

For the Immunodrug AngQb, the risk of inducing autoreactive T cells is also negated by the fact that the target-antigen is only 8 amino acids. Preclinical studies and clinical trials have shown no evidence of autoreactive T cell pathology. However, the use of small epitopes is limited to the few cases where the target-antigen is a small peptide. For protein targets, small peptides often fail to induce neutralizing antibodies and therefore full-length proteins or domains must be used.

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Hence, to minimize the probability of inducing self-specific T cells, a number of other factors should be considered.

Adjuvants such as QS21 that provide strong stimulus to the innate immune system and induce self-specific T cells should be avoided. Vaccination should be spaced by at least two weeks. Indeed, daily injection of antigen may lead to much stronger T cell responses than biweekly injection (82). In addition, proteins expressed in secluded sites such as the brain or membrane-bound proteins not expressed in the thymus or secondary lymphoid organs should not be used as antigens because such proteins usually fail to induce tolerance.

However, because induction of self-specific T cells cannot be formally ruled out for protein antigens, nonclinical testing is necessary. It is generally thought that Th cells rather than cytotoxic T cells are responsible for autoimmune diseases (83). Therefore, our T cell safety experiments focus primarily on Th cells. It is essential to demonstrate that the regimen and adjuvants intended for human use do not induce self-specific T cells. To achieve this, the target-antigen derived from the test species must be used because potential T cell tolerance is usually species specific.

There is a paucity of animal models for evaluating pathology arising from unintended T cell responses to self-antigens. The difficulty of finding and establishing reliable and predictive models is exemplified by studies performed with AN1792. Immunization of transgenic mice (Tg) that overexpress a mutated human amyloid precursor protein under the control of platelet-derived growth factor promoter (PDAPP) with AN1792 revealed no evidence of T cell infiltration or T cell–mediated pathology. Similarly, age/strain studies using DBA/2, SW, C57Bl/6, and SJL7j mice, guinea pigs, and primates showed no evidence of pathology. By chance, efficacy studies performed with PDAPP Tg mice revealed conditions under which T cell infiltration and inflammation occurred in the brain (81). Twelve-month-old PDAPP Tg mice treated repeatedly with AN1792 plus MPL-SE and GM-CSF developed chronic meningitis, vascular degeneration, inflammation of the meninges and cerebrum, and perivascular leukocytes and lymphocytes after 4 months (82). The model is difficult, requiring large numbers of aged animals and non-Tg littermates, multiple controls, and a combination of adjuvants. Moreover, there is variable incidence and severity of inflammation from study to study with relatively high background rates in placebo.

Numerous in vitro/ex vivo methods are available to measure T cell responses, including proliferation assays, ELIspot assays, and intracellular cytokine staining. However, it is difficult to measure weak T cell responses, and the absence of a measurable T cell response may not reflect tolerance but rather assay limitations. Hence, inclusion of positive controls is essential and we propose two strategies to establish these: The first approach involves mice deficient for the self-antigen in question. Because no T cell tolerance will be present in knock-out animals, it should be possible to induce T cells by immunization and measure specific T cell responses ex vivo. If no response can be measured, more potent adjuvants and immunization regimens should be tested. In the second approach, neo-T cell epitopes may be introduced into the self-molecule. A measurable T cell response against the neo-epitope may serve as a positive control. We are exploring both approaches for IL1bQb using IL-1β-deficient mice and an IL-1β/T cell p13 epitope fusion derived from the lymphocytic choriomeningitis virus.

CONCLUSION

The treatment of chronic diseases by therapeutic vaccines that induce autoantibodies holds great potential as an affordable and effective therapeutic option for noncommunicable, chronic diseases, which are a major cause of death and disability worldwide (84). These disorders include cardiovascular and respiratory diseases, diabetes, obesity, and cancer, and account for almost half of the

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global disease burden and deaths. The World Health Organization (WHO) estimates that with increasing economic prosperity and life span, noncommunicable, chronic diseases will account for 60% of the global disease quota by 2015 (85).

Despite the long-recognized promise of harnessing the immune system for therapeutic benefit, the development of therapeutic vaccines is only now gaining momentum. The path toward the practical application of therapeutic vaccines has been made possible by fundamental insights gained from immunology, genomics, molecular cellular biology, and vaccinology. Numerous biotechnology and pharmaceutical companies are currently testing therapeutic B cell vaccines for chronic diseases such as AD, hypertension, smoking addiction, and cancer (see **Table 1**). These developments are laying the foundations of efficacy and safety that we hope will produce a new class of therapeutics soon licensed for human use.

Our experience with Immunodrugs serves as a useful paradigm for nonclinical testing, with many of the principles and approaches applicable to other therapeutic vaccines. The basis for understanding the efficacy and safety of therapeutic vaccines is knowledge of the fundamental immunology that governs the B and T cell responses they induce. This knowledge, by way of rational design, has been incorporated into VLP vaccines with the intent of maximizing antibody responses and minimizing safety concerns.

Similarly, this understanding has provided a framework for designing nonclinical safety and toxicity studies that have enabled the transition of these vaccines from bench to clinic. Numerous factors require consideration. An understanding of the biology of the target molecule and the effects of its neutralization is essential. From a safety perspective, not all molecules are tractable by therapeutic vaccination and due care should be taken in selecting suitable targets. The design of safety studies must also consider the components of the vaccine and the toxic effects they may induce. Such assessment will also directly affect vaccine design where it may be required to produce detoxified antigens. Assessment of potential secondary pharmacodynamic activities resulting from unintended antibody and T cell responses is perhaps the most challenging element of Immunodrug safety. Addressing these concerns requires a multiplicity of experimental approaches, animal models, and molecular tools. Finally, prudent clinical development can be achieved by a sequential approach to human testing. For Immunodrugs, a foundation of preliminary pharmacology and safety was first established with vaccines directed against foreign antigens. The knowledge gained from these studies served as a basis for the development and phase II testing of five Immunodrugs targeting self-antigens involved in chronic disease indications. To date, approximately 660 subjects have been immunized with Immunodrugs designed to induce B cell response. The nonclinical safety studies performed in support of these clinical programs have provided useful and reliable predictions of safety for initial human studies. However, long-term safety and efficacy can be established only by continued nonclinical studies, widespread clinical testing, and post-market evaluations.

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SUMMARY POINTS

1. Immunodrugs are therapeutic VLP vaccines designed to induce autoantibodies against disease-related proteins. They are comprised of VLPs derived from the RNA bacteriophage Qβ to which self-antigens are covalently attached. One Immunodrug, AngQb, achieved clinical proof of concept by lowering blood pressure in hypertensive patients.
2. Immunodrugs are representative of a new class of therapeutic agents for which dedicated regulatory guidelines do not exist. More than 20 therapeutic vaccines have been clinically tested in the past 12 years. Novel approaches are required for nonclinical safety and toxicity studies.

3. Clinical development of Immunodrugs has been a generational approach that first tested VLP-based vaccines conjugated with foreign antigens. Second-generation vaccines used small peptides to target disease-related peptides or protein. Approximately 250 people have been immunized with AngQb, TNFQb, or GhrQb. Vaccines comprising full-length protein antigens are now being developed.
4. Nonclinical safety testing is a combination of GLP-toxicity studies and experimental immunological approaches involving animal models and specific molecular tools. Safety studies must assess toxicity due to vaccine components and primary and secondary pharmacodynamic actions of the vaccine.
5. Secondary pharmacodynamic activity involves (*a*) antibody-mediated pathology due to immune complex disease, unintentional cross-reactivity, and antibody-dependent enhancement of target-molecule activity and (*b*) T cell–mediated toxicity.
6. The following are favorable characteristics for target molecules:
   - A validated target: provides predictability of target neutralization.
   - Nonessential: eliminates concerns of extended loss of an irreplaceable function.
   - Not membrane associated: B cell tolerance unlikely, not possible to induce antibody-dependent cell-mediated cytotoxicity (ADCC).
   - If membrane bound, then lowly expressed: no B cell deletion, limits likelihood of ADCC.
   - Low concentration: minimizes risk of immune complex disease.
7. The following conditions limit the likelihood of T cell–mediated toxicity:
   - Antigen linked to a carrier with multiple Th cell epitopes, antibody responses can be generated by circumventing T cell tolerance.
   - Antigens comprise short amino acid sequences or sequences devoid of T cell epitopes.
   - Avoid adjuvants that are strong stimulators of innate immunity.
   - Avoid antigens for which there is no T cell tolerance.
   - Limit antigen persistence and infrequently administer vaccine.

DISCLOSURE STATEMENT

Authors are employees of Cytos Biotechnology AG and are holders of stocks or stock options in the company.

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