

8-31-2009

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DEVELOPMENT OF A POTENTIAL VACCINE FOR ASTHMA
USING IgE AND VIRUS LIKE PARTICLES

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ABSTRACT

Highly dense, repetitive antigens such as virus particles induce strong immune responses. Correspondingly, Virus Like Particles (VLPs), which consist of the viral structural proteins, can be used as molecular scaffolds to increase the antigenicity of normally poorly immunogenic antigens. This ability to elicit strong antibody responses is not limited to foreign antigens, but also to self-antigens, which are normally subject to B-cell tolerance. The intention of this proposal is to develop a system for rapid identification of asthma vaccine candidates based on potent immunogenicity of antigens (IgE) displayed in dense repetitive arrays on virus-like particles. The technology is based on virus-like particles (VLPs) of the RNA bacteriophage MS2. MS2 VLPs can be viewed as a modular self-assembly system in which a highly immunogenic protein scaffold can be decorated with diverse target sequences using recombinant techniques. MS2 VLPs can be modified to present diverse target sequences on their surfaces. These recombinant VLPs induce strong antibody responses against the target sequence. I propose to generate MS2-based immunogens targeting domains involved in IgE receptor binding. A vaccine that induces antibody responses against IgE may be beneficial in treating asthma and allergies.

BACKGROUND AND RATIONALE

Once a microorganism or toxin that causes an illness has been identified, scientists pursue a number of approaches to develop a vaccine. All approaches to vaccine development focus on the immune system and the body's natural defenses against foreign invaders. Basic research focuses on the biological mechanisms disease-causing microbes use to cause damage. Such research also takes into account physical characteristics of an organism, which might be used as a vaccine component, or as a drug to prevent or interrupt the disease process². Two major approaches to active immunization have been employed: the use of live (generally attenuated)

infectious agents or the use of inactivated, (detoxified), agents or their extracts. For many diseases (including influenza, poliomyelitis, typhoid, and measles) both approaches have been employed. Live, attenuated vaccines are believed to induce an immunologic response more similar to that resulting from natural infection than do killed vaccines. Inactivated or killed vaccines can consist of inactivated whole organisms (e.g., Japanese Encephalitis), detoxified exotoxin (e.g., diphtheria and tetanus toxoids), soluble capsular material either alone (e.g., pneumococcal polysaccharide) or covalently linked to carrier proteins (e.g., *Haemophilus influenzae* type b conjugate vaccines), or purified extracts of some component or components of the organism (e.g., hepatitis B, subunit influenza, acellular pertussis vaccines)¹.

Most approved vaccines rely on the induction of humoral immune responses. A vaccination mimics a primary immune response that is effectively cleared by a host. Antigen from vaccine that binds to a B-cell is internalized and degraded then displayed on MHC II on the B-cell surface. The first signal required for B-cell activation is delivered through its antigen receptor. For thymus dependant antigens, a helper T-cell that recognizes degraded fragments of the antigen as peptides bound to MHC II molecules on the B-cell surface delivers the second signal. The interaction between CD40 ligand on the T-cell and CD40 on the B-cell is an essential part of the second signal³. The specific interaction of an antigen-binding B-cell with a helper T-cell leads to the expression of the B-cell stimulatory ligand CD40 on the T-cell surface and to the secretion of the B-cell stimulatory cytokines IL-4, IL-5 and IL-6, which promote proliferation, and differentiation of the B-cell into antibody secreting plasma cells³. A portion of these activated B-cells will become memory cells and provide a rapid response with antibodies if the antigen is encountered again. As secondary and tertiary immunization occurs, the memory B-

cells help to promote affinity maturation and an increase in more protective IgG antibodies compared to other antibody isotypes³.

Antibodies produced from a humoral immune response contribute to immunity in three ways. First, antibodies provide “neutralization” of pathogens. They may prevent bacterial or viral adherence and therefore entry into a cell. Next, antibodies coat a pathogen to enhance phagocytosis. This is known as “opsonization.” Opsonization occurs when antibodies coating the pathogen are recognized by Fc receptors on phagocytic cells that bind to the antibody C-regions. Lastly, the bound antibodies on a pathogen surface can trigger activation of the compliment system. Compliment activation results in the binding of compliment proteins to the pathogen surface, which in turn recruit phagocytic cells for increased opsonization or directly lyse and inactivate some microorganisms—particularly bacteria³.

Virus-like particles (VLPs) are highly immunogenic and mimic viral capsid antigens but contain no viral nucleic acid⁴. They are the basis for the newly FDA approved bivalent (HPV-16, -18) and quadrivalent (HPV-6, -11, -16, -18) human papilloma virus vaccine and Hepatitis B virus vaccines. VLPs can also be modified to induce immune responses against heterologous targets. Recombinant VLPs have the potential to provide strong immunity to many antigenic epitopes⁵.

VLPs have also been used as a scaffold to present heterologous antigens to the immune system. This has been done in two ways; First, by chemical conjugation of peptides to the surface of the VLP⁵; and, second, by inserting target peptides into the viral coat protein by recombination⁵. The Chackerian lab has used several VLP systems to do this. The purpose of the proposal is to use the MS2 phage as a vaccine platform for IgE involved in asthma.

MS2 is one of the single-stranded RNA bacteriophages that infect *Escherichia coli*⁶. A few properties about MS2 VLP are important to mention: They are easily purified; an antigen can be either cloned in to the RNA of the MS2 VLP or physically fused to the phage coat; they are highly immunogenic⁵, and they have only one protein which allows their synthesis by coupled transcription/translation in vitro^{7,8,9}. This single protein functions to encapsidate and package the phage RNA. These properties of the MS2 VLP allow the recombinant bacteriophage to be easily mass replicated.

Furthermore, these VLPs are so efficient in providing strong immune responses that they can be used to illicit autoimmunity when conjugated with target antigens, including self-peptides¹⁰. It has been demonstrated that a self-protein-derived peptide can induce autoantibodies against the native proteins when it is presented within a highly ordered context as part of the regularly assembled viral capsomeres¹¹. Antibodies were elicited only when the self peptide was presented in an organized fashion on whole virions and not when the peptide is disorganized and displayed on soluble envelope protein suggesting that antigen rearrangement is crucial in developing B-cell activation to self-peptide¹².

The intention of this proposal is to develop asthma vaccine candidates based on potent immunogenicity of antigens (IgE) displayed in dense repetitive arrays on virus-like particles.

IgE ASSOCIATED WITH ASTHMA AS A MODEL

Asthma is a chronic inflammatory disorder of the airways that causes recurrent episodes of wheezing, breathlessness, chest tightness, and cough—particularly at night and/or the early morning¹³. Patients with asthma experience disabling attacks of severe dyspnea, coughing, and wheezing triggered by sudden episodes of bronchospasm. These attacks can be triggered by cold

and exercise or by exposure to an allergen to which the patient has been previously sensitized—but no trigger can be identified¹³.

Three major etiologic factors have been described. They include atopy (predisposition to type I hypersensitivity), acute and chronic airway inflammation, and bronchial hyperresponsiveness¹³. Type-2 helper cells and histamine secreting mast cells are the prominent cells of inflammation in the bronchioles, but the relationship between cytokines and inflammatory cells that precede airway hyperreactivity is not fully understood¹³. Airway remodeling, which includes hypertrophy of bronchial smooth muscles and deposition of subepithelial collagen, also occurs in asthmatics and contributes the symptoms of the disease¹³. Inhaled allergens (antigens) illicit a T_H2-dominated response favoring IgE production and eosinophil priming. On re-exposure to the antigen, an immediate reaction is by Ag cross-linking of IgE bound to IgE receptors on mast cells in the airway mucosa. This triggers bronchospasm, increased vascular permeability, and mucus production. The mast cell activation promotes release of other cytokines that promote the influx of other leukocytes¹³.

Because IgE plays a central role in the initiation of the allergic response associated with asthma, it is a desirable antigen for vaccine targeting. Currently, omalizumab (Xolair) is being tested for the treatment of asthma. Omalizumab is an IgG monoclonal antibody derived from recombinant DNA that inhibits IgE binding to the high-affinity IgE receptor on Mast cells and Basophils¹⁶. Currently, there are no clinical comparisons of omalizumab with other standard treatments for asthma; therefore, it is difficult to determine its overall place in therapy¹⁶. Omalizumab should be considered as a second-line therapy for patients with moderate to severe persistent allergic asthma¹⁶.

Monoclonal antibodies work by a number of mechanisms such as blocking the function of target molecules, inducing the death of cells that express the target, or by modulating signaling pathways¹⁷. These actions have been exploited in a range of proven and experimental indications. Immune-mediated inflammatory diseases are particularly suitable candidates for this form of therapy. This is because key immune control molecules are secreted or expressed transiently on the surface of cells during the pathogenic process. Blocking these molecules with monoclonal antibodies may have specific effects on the disease¹⁷. While many monoclonal antibody therapies are effective, there are certain drawbacks including¹⁷:

- Expense
- Requirement for parenteral administration
- Adverse effects
- Host immune anti-drug responses limiting ongoing therapy
- Limitations in current concepts of molecular pathogenesis of disease.

The first monoclonal antibodies were mouse derived and anti-mouse antibodies were common although they only occasionally caused adverse effects. Nevertheless, this limited repeated exposures to the drugs. As monoclonal antibodies now resemble human antibodies this problem has been reduced, but not entirely eliminated. On re-exposure to the initial monoclonal antibody an allergic and/or anaphylactic reaction may occur¹⁷.

Monoclonal antibodies have to be administered parenterally. The costs of cannulation and injection site reactions may be considerable¹⁷. New and unexpected serious adverse effects are emerging. There is therefore a need for patients to be informed of these problems when they are considering treatment¹⁷. These problems associated with the administration of monoclonal antibodies could be decreased using a vaccine-base approach.

A mouse monoclonal anti-human IgE antibody (BSW17) capable of recognizing receptor-bound IgE without inducing mediator release from human basophils or mast cells has been described¹⁴. Circular nona- and octapeptides from human IgE associated with induction of asthma have been isolated and are specifically recognized by BSW17¹⁴. They are called ‘mimotopes’ as they mimic at least part of a conformational epitope. These mimotopes do not react with any other anti-human IgE antibody and strongly inhibit the binding of human IgE to BSW17 only¹⁴. The peptide sequence of the epitope associated with the Asthma inducing IgE antibody has been illustrated.

There are several regions of IgE that are thought to be involved in receptor binding to mast cells. The domains are amino acids 343-355, 371-386, and 405-415 of the IgE peptide. The major goal of these experiments is to induce antibodies using amino acid sequences genetically fused to VLPs located within these specific receptor-binding domains that are capable of blocking IgE binding to Mast cells.

HYPOTHESIS

We hypothesize that a recombinant-VLP based immunogen against specific domains of IgE involved in receptor binding can induce receptor blocking of IgE to Mast cells and provide an avenue for the development of a vaccine for asthma.

METHODS AND RESULTS

Display of IgE peptides on MS2 VLPs by genetic fusion.

In order for a site of peptide insertion to be useful for display, it must be accessible on the surface of the VLP and insertions should not interfere with protein folding and assembly. We have used a surface-exposed site on MS2 coat protein, the AB-loop, to present a loop derived

IgE antigen. The AB-loop is a 3-residue β -turn connecting coat protein's A and B β -strands (Figure 1). Peptides inserted here are highly accessible on the surface of the virus particle; and because they are tethered at both ends, are conformationally constrained. Since many epitopes in their native environments are found in surface loops, this is a natural location for peptide display.

FIGURE 1

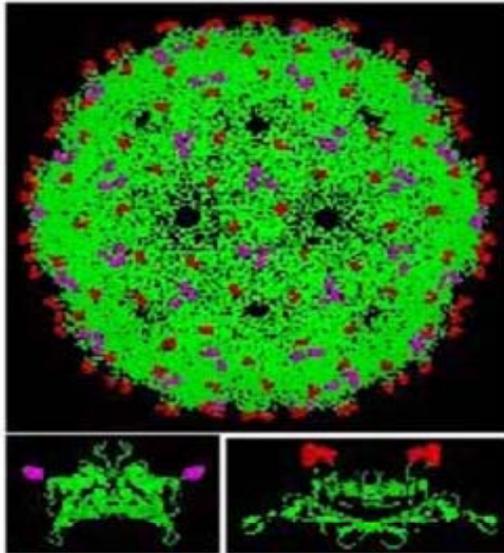


Figure 1. The top panel shows an intact VLP. The lower panels show two views of the coat protein dimer emphasizing to surface structures. Shown in red (at right) the positions of the AB-loops. In purple (at left) the N-termini—shown for reference purposes.

In addition, a variety of sequences can be inserted into this site without interfering with the ability of the coat protein to assemble into VLPs¹⁸.

The IgE peptides described below are naturally found as part of a loop structure (reference), so presentation in the AB-loop may be an effective method for mimicking the structure of this epitope. To generate these recombinant MS2 VLPs, selected sequences derived from IgE were cloned into the MS2 coat protein using standard recombinant techniques. Resulting recombinant coat protein will be over expressed in bacteria

were purified from transformed bacteria by size exclusion chromatography.

We next determined whether each of these newly formed chimeric coat proteins made VLPs, using a gel electrophoresis assay. (Shown in Table 1). Two of the recombinant proteins did not form VLPs, the remaining four (C – F) IgE fused VLPs did.

TABLE 1

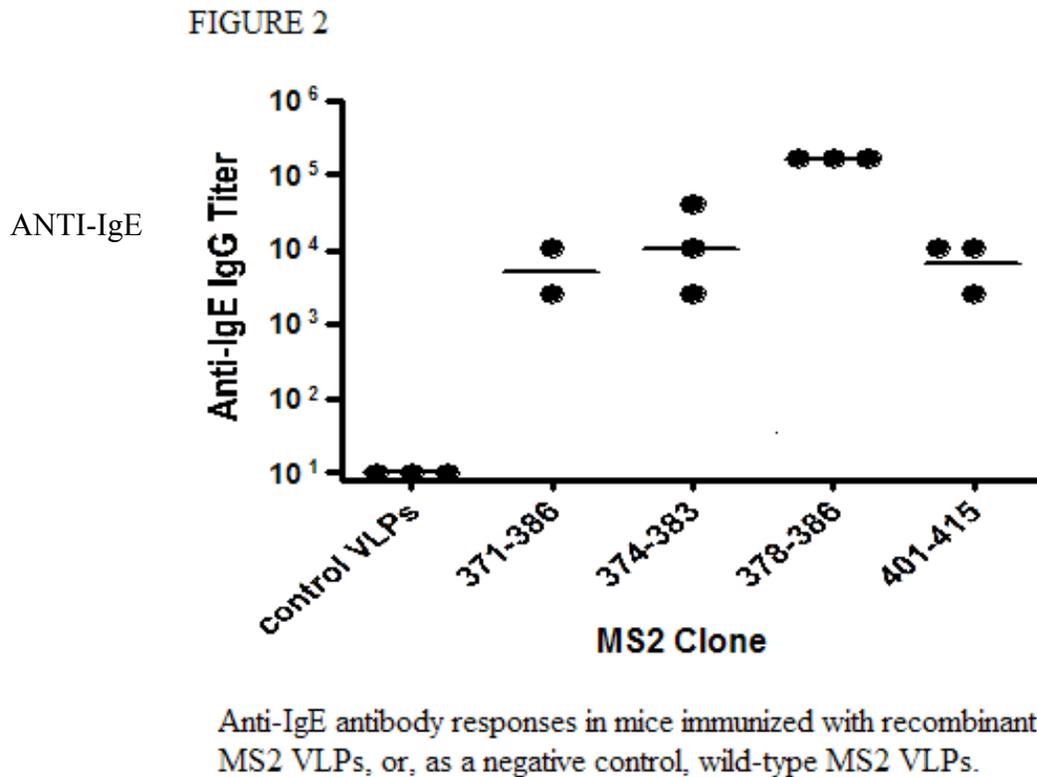
Peptide Sequence	Region of IgE (amino acids)	VLP assembly?
PSPFDLFIKRSPT	343-355	No
NLTWSRASGKG	371-380	No
NLTWSRASGKPVNHST	371-386	Yes
WSRASGKPVN	374-383	Yes
SGKPVNHST	378-386	Yes
VGTRDWIEGET	405-415	Yes

Immunizations.

Because this was a preliminary study to show proof of principle efficacy of this vaccine a relatively small sample size of C57B1/6 mice in groups of three to five were inoculated with the purified the recombinant VLPs or as a control, wild type MS2 VLPs. Incomplete Freund's adjuvant (IFA) in a 1:1 volume: volume ratio was used at two week intervals. Serum samples were collected at each inoculation and 1-2 weeks after the final dose. All animal care was in accordance with University of New Mexico guidelines. Two weeks after the final boost, mice were bled and the resulting mouse sera were tested for the presence of anti-IgE antibodies by ELISA. by ELISA. Immulon II ELISA plates were coated with 200ng of IgE diluted in PBS in a total volume of 50 μ L for 2 hours at 37°C. After blocking wells with PBS-0.5% milk, mouse serum was serially diluted in PBS-0.5% milk and applied to wells for 2.5 hours at room temperature⁵. Reactivity to target antigen was determined using horseradish peroxidase (HRP)-labeled goat anti-mouse IgG at a 1:2000 dilution in blocking buffer. After 1 h, wells were washed and then developed using the substrate ABTS. Upon development, wells were read (OD 405nm) using a microplate reader. OD405 values that were greater than twice background were

considered positive. The end-point dilution titer was calculated as the largest dilution that result in a positive value.

As shown in Figure 2, all four recombinant VLPs induced high titer anti-IgE antibodies.



ANTIBODIES DO NOT BLOCK RECEPTOR BINDING

This is a **pre-clinical proof of principle** study in a mouse model for immunization of IgE specific antibody in asthma using antibody level measurements and determining statistical significance using antibody titers in comparison with wild-type phage immunization. Antibody specificity can be determined using ELISA. Because the goal of these experiments was to induce antibodies that block IgE function, we next tested whether anti-IgE sera could block IgE binding

to the IgE receptor. We tested this by flow cytometry, using RBL-48 cells, which are stably transfected with the human IgE high affinity receptor. We incubated fluorescently-labeled IgE (IgE-FITC) with mouse sera and then applied the mixture to RBL-48 cells for 2 hours at 37 degrees. After washing, cells were analyzed by flow cytometry, to measure the degree of IgE binding.

FIGURE 3

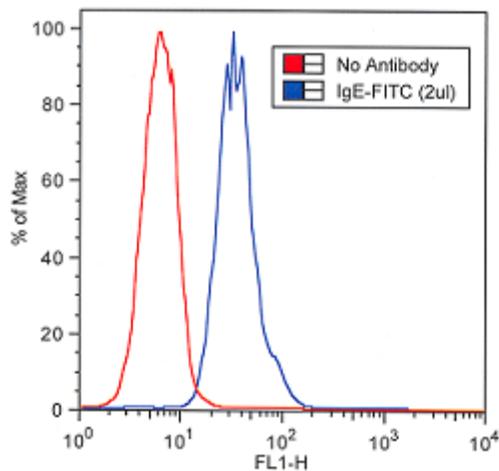
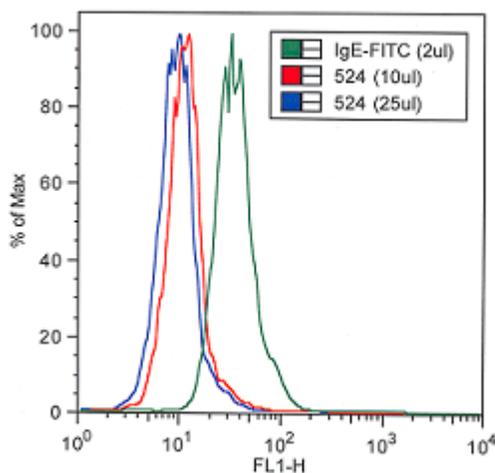


FIGURE 4



Unfortunately, none of the sera blocked binding of IgE to the receptor. This was determined through flow cytometry analysis of transfected RBL-48 cells. Figure 3, left, depicts a control analysis of the IgE-FITC cells against no antibody. The left or back shift of the peak assumes that there is no antibody blocking the IgE-FITC receptors, which is expected considering there are no antibodies in the assay

Figure 4, left, shows the IgE-FITC cells incubated with 10 μ L (red) or 25 μ L (blue) of sera from a mouse (#524) immunized with VLPs displaying IgE(371-386). Although sera did interfere with IgE binding to the cells (the curve is backshifted), this backshift is similar to that observed when IgE was incubated with sera from a negative

control mouse that was immunized with wild-type MS2 VLPs (Fig. 8). Similar results were observed when using sera from mice immunized with VLPs displaying the other IgE sequences.

FIGURE 5

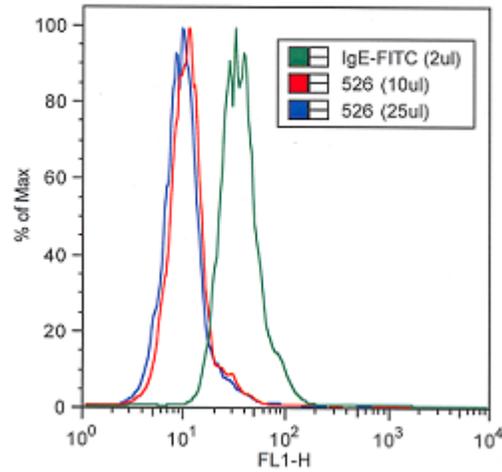


FIGURE 6

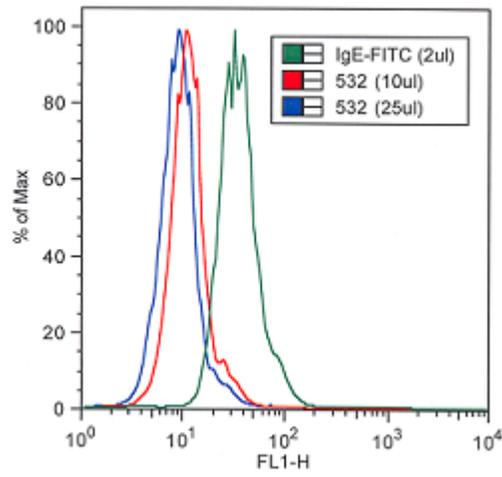


FIGURE 7

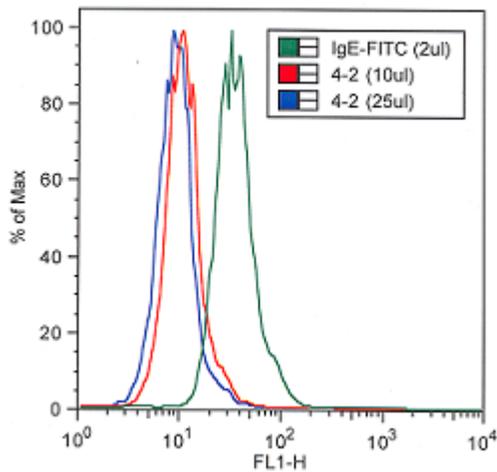
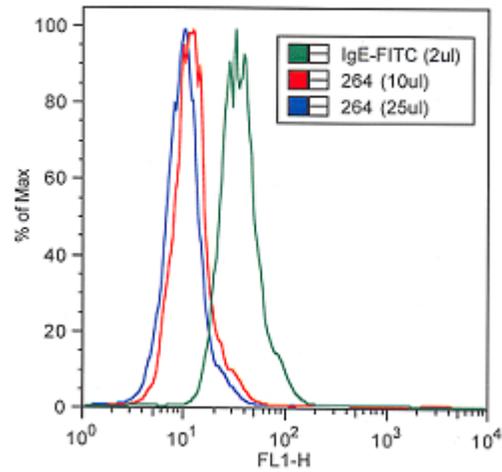


FIGURE 8



[Fig. 5; IgE(374-383) Fig. 6 IgE(378-386), Fig. 7 IgE(401-415)]. Thus, all of the sera, including negative control sera had the same ability to block IgE binding, suggesting that the inhibition of IgE binding was not specific.

DISCUSSION

Despite the inability of the IgE antibodies to block the IgE receptor, there were some positive aspects to this research. For example, Table 1 illustrated the fact that producing recombinant VLPs that display known IgE epitopes is a possibility. Several regions of the IgE peptide are thought to be involved in receptor binding. Four of the six regions of the known amino acid sequences of these regions were successfully cloned into recombinant VLPs that correctly assembled. These VLPs were subsequently inoculated into mice and ultimately produced an anti-IgE IgG antibody response (Figure 2).

Although the recombinant VLPs produced an anti-IgE response, the antibodies were not specifically targeted to block binding to the IgE receptor. In some respects these data were not unexpected. Omalizumab (Xolair) binds to a complex conformational IgE epitope. IgE has a complex secondary structure so it is likely that targeting linear epitopes will not be sufficient for neutralizing IgE.

The Chackerian and Peabody lab has developed a phage display system that might be more appropriate to identify vaccines targeting IgE and should allow for the identification of mimics of more complex epitopes. This phage display system involves use of an a library of VLPs that display random peptides on the surface of the VLP¹⁸.

Another important component of the phage display system is the genotype-phenotype linkage where the MS2 VLPs will encapsidate its own RNA that codes for the selected peptide into the VLP. By encoding a diverse display of random peptides (epitopes), one can affinity-select ligands of specific antibodies from libraries of random-sequence peptide or antigen fragments¹⁸. Reverse transcription and amplification by PCR of RNAs extracted from affinity selected VLPs will allow the recovery of the selected sequences, which can then be subjected to

additional cycles of synthesis, assembly and selection¹⁸. These MS2 VLPs should provide the means both to identify epitopes and to present them directly to the immune system at high density on a single structural platform¹⁸.

Given the initial success of this phage display system, the next steps in our project would be to screen it using a monoclonal antibody against IgE. Omalizumab (Xolair) is a humanized monoclonal antibody that targets IgE and has been used to treat allergic asthma patients. Multiple clinical trials have shown that Xolair is safe and effective in treating these patients. Unfortunately, a disadvantage of Xolair is that it needs to be administered intravenously, and frequent (every 2 weeks) infusions are required. This makes Xolair extremely expensive and impacts the quality of life of the patient. This may be why Xolair is used almost exclusively as a treatment for allergic asthma, which is a chronic condition. Xolair is not given to those who suffer from the more moderate and temporary symptoms of seasonal allergies.

Targeting IgE has several advantages. First, vaccines elicit long-lasting responses, alleviating the need for costly and frequent injections. One can envision a yearly vaccine boost, given prior to the start of the allergy season. Also, monoclonal antibodies can sometimes induce immune responses that limit the effectiveness of the drug. While this can be mitigated somewhat but not completely upon humanization of the antibody, it is still an issue in a certain percentage of patients. An inactivating anti-antibody response would not be an issue with an IgE vaccine. We believe that these advantages--cost, frequency of administration, and effectiveness—would give an IgE vaccine a significant role in asthma treatment

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