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Modern vaccinology — From empiricism to a science

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Vaccination has undoubtedly remained the most effective means of combating infectious diseases. Today the death toll from such ailments is down to just 1–2% in industrialized countries. In developing countries however the mortality from infectious diseases is still needlessly high and vaccination offers the only real way of controlling and ultimately preventing this condition. Indeed the most expensive health care policy in relation to many infections is to do nothing to prevent it. To take an example, the cost of treating just one child with tuberculosis is the same as the cost of vaccinating 7000 children against this disease. To vaccinate every child in the world against diphtheria, whooping cough, tetanus, poliomyelitis, measles and tuberculosis costs as little as US $5–15 per child. In spite of the widely acknowledged cost effectiveness of a mass vaccination program, the fact remains that the basic price of many vaccines is still out of reach of many developing countries and there is a pressing need to develop even cheaper versions.

Origin and development

THE foundation for immunoprophylaxis was laid in ancient China where it was observed that small pox sufferers who recovered were protected against future attacks of the disease. As a result people were encouraged to develop a mild form of infection by inhaling a powder made from grinding the dried crusts of pustules from small pox patients. As an active science however vaccinology had its advent with the landmark demonstration in late-eighteenth century by Edward Jenner that inoculation with the cowpox virus could protect humans against small pox. This was conceptually extended by Louis Pasteur almost a century later who instead of using live virulent material to confer immunity developed attenuated strains of the rabies virus as an anti-rabies vaccine. The use of attenuated non-pathogenic strains as vaccines has achieved spectacular success and accounts for many of the vaccines commercially available today e.g. oral polio vaccine, BCG, measles and influenza. An alternative to live vaccines has been to use inactivated vaccines which consist of suspensions of killed microorganisms. Examples of these include pertusis, tetanus, and the injectible polio vaccine. While these two strategies have proved enormously successful in controlling and perhaps even eliminating (as in the case of small pox) many infectious diseases there are other pathogens for which such a straightforward approach is unlikely to bear fruit. This is particularly true of those microrganisms which have learnt to evade the immune defense mechanism of the host.

One way of looking at an infection is as an interactive competition for dominance between the pathogen and host. On the one hand pathogens in general have an evolutionary advantage due to their high multiplication rates in host which may result in progeny that are even more adept at eluding immune surveillance. At the other end of the spectrum however is the immune system of the host which, though evolutionarily static over the course of an infection, is nevertheless extremely adaptive and versatile with an enormous repertoire capable of recognizing diverse protein sequences[1]. This potential is accentuated by the fact that the repertoire is dynamic in nature and the existing repertoire can be further amplified by appropriate stimulation. It is this expanse of the repertoire coupled with its adaptability that allows our bodies to eventually prevail over most infections encountered in everyday life.

In spite of the formidable immune defense mechanism presented by the host some pathogens have nevertheless acquired the ability to confound it. Two notable examples of this are the malaria parasite *Plasmodium falciparum* (Pf) and the etiologic agent of AIDS the human immunodeficiency virus type-1 (HIV-1) Polymorphism within the antigenic determinants of Pf serves as an effective evasive mechanism More recently the possibility of separating parasite chromosomes on

gels has revealed that not only the genes but also the chromosomes of malaria parasites are variable in size[2]. This reflects the high genetic flexibility of this organism, allowing for successful immune escape. Further the parasite also presents immunodominant decoy sequences such as the repeat regions of the circumsporozoite protein. Such sequences elicit high levels of antibodies during infection which are however incapable of neutralizing the parasite. In effect such decoy sequences serve to distract the immune system from recognizing those determinants which are critical for initiating infection. The combination of antigenic polymorphism and decoy antigens, perhaps along with other factors, is so effective that in most cases there appears to be no sustained, long-term immunity to malaria[2]. At present the relative importance of humoral versus cellular immunity in protection is also not clear. While earlier studies emphasized on antibody-mediated protection[3–5] more recent results suggest that the role of cytotoxic T cells is crucial[6,7]. Perhaps a reasonable middle-ground would be to suggest that an effective malaria vaccine must recruit both the humoral and cellular arms of acquired immunity.

HIV-1 is another example of a pathogen whose adaptability has superseded the defense of the host. Here again antigenic polymorphism plays a critical role. The first recognition of an invading organism by host immunocompetent cells is through the envelope proteins on pathogen. Polymorphism at the level of critical determinants on the envelope proteins serves as an effective and highly successful elusive strategy[8]. Immune recognition is almost always sequence-specific in that responses generated against one sequence do not necessarily crossreact with its variant. The 'secret-of-success' so to speak of HIV-1 survivability lies in the error-prone copying of its genome[9]. Being a retrovirus reverse transcription of its RNA genome is an essential first step both for its replication and disease pathogenesis. Reverse transcriptase enzymes are notorious for their low fidelity in copying which is further compounded by the lack of proof-reading ability. This results in a significant number of errors incorporated for every round of genome duplication (about one mismatch for every 2000 nucleotides). The consequence of this is that viral replication results in a diversity of mutant progeny at least some of which have an increased survival advantage because the original immune response generated against the parent infecting strain is incapable of recognizing them. Indeed there are some who believe that the number of variants produced in the process of several rounds of viral multiplication may eventually overwhelm the available repertoire of immunocompetent cells.

From the two examples described above it should become obvious why the currently employed approaches of using either attenuated or killed organisms are unlikely to be productive in such cases. Immunization with a given strain (either live or killed) will, in all probability, not provide a protective cover against its variants. On the other hand producing a vaccine formulation that includes a cocktail of various strains of a pathogen is also not easily achieved. Indeed developing vaccines against malaria and HIV-1 represent the two most formidable challenges for vaccinologists today.

The paradigm shift

Concomitant with the increased awareness of the complexity of some infectious agents has been a greater understanding of the nature of immune responses and their role in preventing or limiting infection. Though large gaps still remain this knowledge has helped to refine our approaches towards vaccine development. From this standpoint one of the most important findings has been that only a small proportion of the antibody response against an infectious microorganism is actually effective at neutralizing it[10]. Thus though a pathogen may present an array of protein antigens only that component of the immune response directed against a handful of these will have neutralizing ability. While the precise mechanism of neutralization may vary with pathogen such proteins have often been found to play a critical role in the establishment of infection, e.g. binding to a receptor on the target cell thereby facilitating entry. What this has meant to vaccinologists is that immunization with the whole microorganism (either live or killed) may be unnecessary. Rather immunization with such protective antigens (i.e. antigens capable of eliciting a protective immune response) should be sufficient to confer protective immunity. In addition to providing for a more flexible approach another advantage is that individual proteins can easily be produced in bulk by recombinant DNA methodologies to generate sufficient material for mass vaccinations. The most successful example of this is the hepatitis B vaccine where the major envelope protein of the hepatitis B virus (HBV) produced in yeast has proved extremely effective at conferring long-term immunity against hepatitis B[11]. This vaccine is now commercially available. A caveat however is that this approach can only be employed when the protective antigen (or antigens) are known – which unfortunately is not true in many instances. Identification of such protective antigens remains an active area of investigation.

While in theory the entire sequence of a protein is antigenic (i.e. able to provide potential antibody binding sites) in practice however antibody responses are restricted towards a fraction of it. The explanation for this lies in the fact that all proteins fold into specific three-dimensional structures where some proportion of its sequence is buried (usually hydrophobic stretches) and some, usually represented by hydrophilic sequences, exposed to the environment. As a rule the antibody

producing B cells interact with antigens in the native state which predicts that recognition and subsequent antibody production is restricted to the accessible domains on the protein. Such sequences to which the anti-protein antibodies are specific are called epitopes. This situation is also true for invading microorganisms where the host antibody response is polyclonal in nature containing specificities for diverse epitopes displayed by the various antigenic constituents of the pathogen.

We have said earlier that the protective immune response represents a fraction of the total immune response being specific for only a few of the pathogen proteins. Another way of looking at this is that protective immune response is specific for a fraction of the total epitope repertoire presented by the pathogen. This seemingly slight change in perspective – from thinking in terms of proteins to epitopes – has galvanized research in vaccinology. An important outcome of this shift has been the realization that not all epitopes on a protective antigen elicit protective immunity. Antibodies specific for only some of the epitopes displayed by such a protein will have the ability to neutralize infection. Such epitopes are called neutralization epitopes[10] and often represent domains on proteins which are functionally important. For example the hepatitis B virus attacks humans by infecting hepatocytes. Studies have shown that a 26 amino acid sequence in the large envelope protein of this virus plays a critical role in establishment of infection by acting as a ligand for a specific receptor on the hepatocyte cell surface[12]. As might then be expected antibodies to this sequence have been shown to be virus-neutralizing[13]

Reducing the problem to epitopes rather than whole proteins has allowed for the exploration of more ingenious strategies which though still in the laboratory stages hold promise for the future[14]. Some of the more prominent approaches will be considered here. It must be cautioned however that for a successful application of any of these approaches one must know the precise neutralization epitope(s) for a given system – which in many instances have remained elusive.

Recombinant live vector vaccines

This represents an attractive and exciting new approach where the gene coding for the antigenic determinant of interest (e g. neutralization epitope) is used to transform another live microorganism which is non-pathogenic in the host. Multiplication of such a transformed vector in the host results in a continuous expression of the inserted gene whose product in turn serves as a sustained stimulus of the immune response. An obvious advantage of such a vaccine is that a single dose is sufficient to elicit long-term immunity. Also, delivery of an antigen via live vectors results in priming of both the humoral and cellular arms of immunity including cytotoxic T cells – the importance of which in anti-viral immunity has only recently been realized. Depending on the vector one uses it may also be possible to develop vaccines that can be administered orally; a preferred route as it is more cost-effective, allows for easier distribution and ensures a higher compliance rate.

Both viral and bacterial systems have been explored as vehicles for antigenic delivery. Among the viral vectors the most popular is the vaccinia virus[15] which was successfully used for the eradication of small pox. Indeed its widespread use in humans has more than adequately demonstrated its safety. The vaccinia virus belongs to the pox family of DNA viruses which replicates within the cytoplasm rather than the nucleus of infected cells. It has a broad host range making it suitable for both human and veterinary use. Construction of expression vectors is usually achieved[15] by homologous DNA recombination with a recombination efficiency of about 0.1%. It has been shown that heterologous proteins expressed in vaccinia are processed and transported in accord with the primary structure of the protein and the inherent capabilities of the host cell. In addition they are capable of undergoing the entire gamut of post-translational modifications such as glycosylation, phosphorylation, myristylation etc and consequently retain their biological activity. Recent experiments have demonstrated that experimental animals inoculated with recombinant vaccinia viruses that express genes from a variety of DNA and RNA virus families are usually partially or completely protected against disease by subsequent infection.

Among the possible bacterial vectors that can be used, the human tuberculosis vaccine, Mycobacterium bovis bacillus Calmette-Guerrin (BCG) is the most promising[16]. Immune responses to BCG have been extensively studied and with nearly two billion immunizations it has a proven safety record. It can be given at birth and engender longlived immune responses with a single dose. It is also the most heat stable of live vectors – a particularly important consideration for developing countries. Transformation of BCG is routinely achieved with autonomous replicating plasmid vectors which are usually *E. coli* – mycobacteria shuttle vectors that include the *E. coli* plasmid replicon, an antibiotic resistance marker and an expression cassette containing a mycobacterial (usually heat shock protein) promoter, multiple cloning sites and a transcriptional terminator[17]. Several antigens have been expressed in BCG and, in animal models, the induction of antibodies, T helper cell and cytotoxic T cell activities demonstrated[16,18].

Attenuated non-pathogenic strains of salmonella are also being actively investigated as vectors[19]. This is a particularly attractive system for administration by the oral route since attenuated strains have been shown to retain the ability to invade across the gut epithelium and persist in the gut-associated lymphoid tissue (GALT), Peyers patches and spleen, thereby allowing for the

stimulation of both mucosal and systemic immunity. A variety of antigens of viral, bacterial and parasitic origin have been expressed in Salmonella. In animal models the induction of both mucosal (secretory IgA) and systemic immunity was demonstrated. The systemic immune responses included both antibodies and cytotoxic T cells

Synthetic peptides

Many years ago it was shown that synthetic peptides derived from antigenic proteins of a variety of pathogens can elicit a cross-reactive humoral response. More recently peptide-mediated stimulation of cross-reactive T helper and cytotoxic T cell responses have also been demonstrated. Cumulatively these results offer the distinct possibility of eventually being able to develop synthetic peptide-based vaccines[20]. Synthetic peptides are generally cheap and easy to prepare and purify. Also, since they represent chemically defined entities the fear of contaminating toxic substances (as with recombinant DNA-derived proteins) does not exist. Peptides are also expected to be chemically and thermally more stable than proteins. All these potential advantages have generated a lot of interest in this area. Although much effort has been spent three major obstacles have severely impeded progress[21]. The first relates to a phenomenon known as genetic restriction. Simply translated it means that immunization of an outbred population with a protein results in a differential immune response among the recipients and more specifically recognition of different T helper cell determinants on the protein. As a consequence a synthetic peptide which represents a fraction of the total sequence of protein (and therefore a fraction of the total T helper cell epitope repertoire) would be unable to induce the same number of responders as would the parent protein. However the recent identification of promiscuous T helper cell epitopes i e epitopes that are universally recognized offers a solution to the problem[22].

The second obstacle often encountered arises from the fact that epitopes expressed by a protein antigen frequently result from conformationally restrained stretches of amino acid sequence and can be either contiguous or discontiguous. The flexibility inherent in a short amino acid sequence permits synthetic peptides to occupy multiple conformational states of which only a fraction may be reminiscent of the 'native' state of cognate sequence in the parent protein. As a result only a small proportion of antibodies elicited by such a peptide would crossreact effectively with the parent protein from which the peptide was derived. Finally the poor immunogenicity (viz ability to elicit an immune response) of peptides vis-a-vis proteins also presents a problem For a vaccine it is imperative that it must invoke a vigorous response in order to provide long-term immunity.

Over the past few years our group has attempted to address these problems systematically for which we chose the surface antigen of hepatitis B virus as the working system. As discussed earlier the hepatitis B surface antigen (HBsAg) has been shown to afford protective immunity against hepatitis B and constitutes the presently available vaccine[11]. Protection is antibody-mediated and interestingly the putative neutralization epitopes on the protein are dependent on conformation which in turn is induced by disulphide bonds In early experiments we were able to generate a cysteine-rich synthetic peptide from HBsAg that spontaneously oligomerized to regenerate a conformational, disulphide-dependent epitope of HBsAg which, in the native state, is also a homo-oligomer[23,24]. In subsequent studies we showed that this epitope was common to all strains of HBV and was immunodominant in the context of the human immune system. We next demonstrated that antibodies induced by the peptide were conformation-specific, of the desired epitope-specificity, that they recognize the native protein as well as they do the peptide and that they can immunoprecipitate HBV viral particles[23]. Thus at least for this system we were successfully able to overcome the problem of conformational epitopes and its attendant complications. At this point however we were faced with the second impediment, namely, the poor immunogenicity of synthetic peptides. A solution revealed itself in parallel studies where we noticed that a small proportion of this oligomeric peptide tends to form non-covalent, macromolecular aggregates in solution. These macro-aggregates could be visualized by transmission electron microscopy and appeared to encode additional epitopes dependent on the quarternary structure of the native protein oligomer[25]. On the premise that increased aggregation would result in enhanced immunogenicity we prepared a lipidated derivative of the peptide to foster intermolecular association via micellar interactions. This strategy proved extremely successful in that we could achieve near quantitative aggregation[26]. These aggregates were highly immunogenic in the primate model giving comparable levels of epitope-specific antibodies with native HBsAg (vaccine) at comparable doses[26]. Finally we were able to show that, in the context of the human immune system, this peptide presents a multiplicity of HBsAg-relevant T helper cell epitopes resulting in a widespread T cell response[27].

From the standpoint of eventual viability of peptide vaccines our results suggest that the obstacles described above can be surmounted. We have called this approach the 'self-assembling' peptide approach as it centres around the generation of a cysteine-rich peptide capable of spontaneous self-assembly to regenerate an epitope mimetic. In recent efforts to extend these observations we have demonstrated the feasibility of designing such

self-assembling peptides by generating a chimaeric peptide that expresses a conformational epitope presented by the envelope proteins of HIV-1[21].

Polyvalent immunogens

The dialectics of scientific reductionism has brought us to the point where we now think of vaccines in terms of epitopes rather than whole microorganisms. However some complexity still remains principally because for many pathogens protective immunity is comprised of specificities for more than one epitope. Even in instances where a single epitope is sufficient for protection the problem may not be simple. For example in HIV-1 the principal neutralization determinant (PND) has been localized to a ten to fifteen amino acid sequence within the V3 loop of the envelope protein gp 120. However this epitope represents a hypervariable region of the protein and antibodies against one PND sequence may not crossreact with a variant PND[8]. Thus in both the above instances the obvious solution is to use a formulation of a cocktail of relevant epitopes.

Broadly speaking there are two ways in which such cocktails can be formulated. One is to simply use a physical mixture of appropriate epitopes and the other is to use constructs in which the individual epitopes are covalently linked together in a suitable fashion of these two generally considered strategies. The latter approach, at least prima facie, appears more promising principally because in such constructs all of the component T helper epitopes may be expected to provide T cell help for simultaneous antibody production against all of the component B cell epitopes consequently overriding the limitation of genetic restriction.

Prior approaches to generating such polyvalent immunogens have largely relied on chemical means by either synthesizing linear sequences containing tandemly oriented epitopes or by chemical crosslinking of peptide mixtures. A combination of these two has yielded some success in the efforts toward a vaccine against malaria[28]. In this direction we have also been able to extend our self-assembling peptide approach to co-assembling more than one of such sequences[21].

To explore alternative strategies we recently expressed a designed, chemically synthesized and assembled gene coding for a 100 amino acid polypeptide which included select determinants from the surface antigens of hepatitis B virus[29]. From the standpoint of eventual success of the polyvalent approach an important criterion that has often been overlooked is that each of the incumbent epitopes must elicit comparable levels of immune responses in the host. This is particularly important since prior experience with protein immunogens has already cautioned us that problems may be anticipated. Thus though a given protein expresses a range of epitopes, the antibody profile obtained against it is partisan for a select subset. This phenomenon has been described as intramolecular antigenic competition but its mechanistic basis remains obscure. Indeed we have also encountered this problem with our polypeptide construct in that the predominant antibody response to it is focused on a fifteen amino acid stretch towards its amino terminus. Current efforts are aimed at dissecting the etiology of this immunodominance and preliminary results suggest that it is not a consequence of primary recognition by B cells. Rather immunodominance is established over the course of maturation of primary antibody response. Our hope is that an understanding of the molecular and cellular basis of relative immunodominance will provide pointers on overcoming it thereby paving the way for a rational and viable way of designing and developing such vaccines.

Conclusions

This article was not intended as a comprehensive review of the field of vaccinology as it stands today. It was more of an attempt to trace the thread of intellectual and conceptual growth over the last few years. The story however does not end with having developed a suitable formulation in the laboratory and demonstrating its ability to protect against a given infection. It must now go to the field and meet the generally accepted criteria of good overall performance. An ideal vaccine has been described as one which has the following characteristics:

(a) It must be extremely effective; inducing protection in 90–100% of the recipients.
(b) It should engender lifetime immunity.
(c) It should be heat-stable, not requiring a cold-chain.
(d) It should require only one shot or be compatible with the schedule for other vaccines.
(e) It should be inexpensive and safe.
(f) It should be easy to administer preferably by a non-invasive route.
(g) It should be capable of being given either close to or at birth.

While it may be unrealistic to expect that all vaccines will meet all of the above conditions, any new vaccine must strive to fulfil as many of these requirements as possible in order to be used widely.

To sum up vaccinology today represents a growing, vibrant and interdisciplinary science in its own right; often stimulating because of its call for creativity but also often frustrating because the call comes sans the magician's wand.

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RESEARCH ARTICLE

Surface properties of *Thiobacillus ferrooxidans* and its adhesion to mineral surfaces

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Thiobacillus ferrooxidans **cells grown on sulphur, pyrite and chalcopyrite exhibit higher hydrophobicity than ferrous iron-grown cells. The isoelectric points of sulphur, pyrite and chalcopyrite-grown cells were at a pH higher than that for ferrous iron-grown cells The effect of duration of biotreatment under static and agitation conditions on bacterial adhesion is reported. Further, scanning electron microscopy was carried out to visualize the process of bacterial adhesion to the mineral surfaces.**

THIOBACILLUS *ferrooxidans*, a gram-negative chemoautotrophic acidophile, is the most important microorganism implicated in the bio-oxidation of various sulphide minerals[1]. *T. ferrooxidans* is commercially used in the extraction of copper, uranium, silver and gold from their ores. It can oxidize virtually any sulphide mineral such as that of lead, zinc, nickel, molybdenum and cobalt. Two mechanisms, indirect and direct, are known to be responsible for the biodissolution of sulphidic ores. Indirect mechanism operates by the chemical action of acidic ferric sulphate produced by bacterial metabolism. In this mechanism adhesion of the bacteria to mineral surfaces is not required. Direct mechanism, on the other hand, involves enzymatic attack of the mineral by the bacteria for which intimate contact and hence adhesion is required[2,3]. After the bacteria comes in contact with the mineral the enzymes on the outer membrane carry out the dissolution of the mineral. Previous studies in this laboratory have shown that direct attack plays an important role in the bio-

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