Vaccination against disease causing pathogens is the most cost effective and widely practiced public health intervention. Majority of the vaccines in use today are prepared from either live attenuated or killed organisms. Although successful in eradicating or drastically decreasing the incidence and morbidity associated with a large number of infectious diseases such as smallpox, polio and diphtheria, the current procedures for vaccine preparation suffer from crucial drawbacks. These include the inability to prepare large amount of material, safety of vaccines and personnel involved in vaccine preparation, possible reactogenicity of the vaccine preparation due to its inadequate attenuation or inactivation and the fear of their reversion to virulent form. Apart from these considerations, the mass distribution and immunization of several vaccines are often limited by their high production cost. These concerns have prompted the testing of various approaches, majority of which focus on the development of subunit vaccines. Antigens considered for subunit vaccine development are usually purified from a range of expression systems which include bacterial, yeast, baculovirus and mammalian expression systems.

The idea that recombinant antigen expressing yeast cells could themselves serve as antigen delivery vectors and induce immune response to the recombinant protein has been less explored. The fact that *Saccharomyces cerevisiae* has a 'generally regarded as safe status' (GRAS), is not normally recognized as a pathogen and can be easily cultured, makes it a suitable organism for the development of cost effective and safe subunit vaccines. Use of whole recombinant yeast as an antigen delivery vector avoids the tedious preparation of purified protein antigen and the use of adjuvant for effective immune response generation as *Saccharomyces cerevisiae* itself serves as an adjuvant [1,2]. However, despite these advantages, so far only two groups have reported the use of whole recombinant *Saccharomyces cerevisiae* yeast to generate immune responses. The first report by Schreuder *et al.* [3] showed weak antibody responses to Hepatitis B virus surface antigen (HBsAg) in mice immunized with heat killed recombinant *Saccharomyces cerevisiae* yeast expressing HBsAg on the surface. In the second report [4], T cell responses to OVA and HIV-1 antigens were demonstrated in mice immunized with whole live recombinant yeast expressing either OVA or HIV-gp160 protein.

In this study, we have re-investigated the potential of whole recombinant *Saccharomyces cerevisiae* to generate antibody responses to heterologous antigens. As in the previous report [3], surface antigen expression was adopted to ensure that the antigen can be directly recognized by the B cells. We initially explored the feasibility of this whole

recombinant-yeast immunization strategy to generate antibody responses to a heterologous protein using GFP as a model antigen. GFP was selected as a model antigen since its expression in the yeast cell can be easily monitored due to its intrinsic fluorescence as well as the availability of GFP as a bacterially expressed soluble protein that could be used for screening anti-GFP antibody responses by ELISA. GFP gene was subcloned in the MCS of the yeast surface expression vector pYD1 and pYD1-GFP transformed yeast cells were analysed for surface GFP expression using polyclonal anti-EGFP antibodies by flow cytometry and immunofluorescence microscopy. Having established yeast cell surface GFP expression, BALB/c mice were immunized with live recombinant yeast expressing GFP on its surface (GFP-yeast) and the sera collected after booster doses were screened for anti-GFP antibody response. Mice immunized with GFP-yeast, generated good antibody responses to GFP after a single prime and boost regimen. Antibody response was found to increase upon further booster doses of GFP expressing yeast.

We extended our study by using this immunization strategy to generate immunity to JEV which is a leading cause of viral encephalitis in Asia. Protective immunity to JEV has been well studied and has been documented to be antibody-mediated that is predominantly dependent on antibodies generated to the E protein and NS1 protein to a lesser extent [5]. Between these two proteins, E protein is a major inducer of neutralizing antibodies and can alone confer protection against JEV infection. Thus, the applicability of our immunization strategy was tested on the E protein of JEV

The full-length E gene of JEV was cloned by RT-PCR from JEV RNA isolated from JEV infected Porcine Stable (PS) cells into cloning the vector pBluescript II KS+. The cloned E gene was verified by sequencing and further subcloned into bacterial expression vector, pRSET-A for purification and antibody generation while subcloning into the yeast surface expression vector pYD1 was done to generate recombinant yeast expressing E protein on its surface. Yeast cells were transformed with pYD1 plasmid containing the E gene and the expression of E protein on yeast cell surface was analysed. Surface expression of E protein on the yeast cell could be detected by polyclonal anti-E antisera using flow cytometry and immunofluorescence microscopy. However, several other anti-JEV antibodies (polyclonal anti-JEV i.p. fluid, anti-JEV Mabs 203, 204, 301 and protected mouse sera obtained from JEV challenged survivors) failed to recognize full-length E protein expressed on the yeast cell surface.

BALB/c mice were parenterally immunized with E protein expressing yeast (E-yeast) at various doses and the antisera collected at different intervals were screened for anti-E antibodies by ELISA with purified E protein coated plates. Good antibody response was observed to E protein in E-yeast immunized mice. However, three booster doses were required to generate an optimum antibody response.

E protein of JEV has been well documented in its ability to generate neutralizing antibodies and to protect from JEV infection [5]. Since we observed a good antibody response to full length E protein that was expressed on the yeast surface, it was also examined whether this antibody response would neutralize JEV and protect E-yeast immunized mice from lethal JEV challenge. However, we did not observe neutralizing antibodies in sera collected from E-yeast immunized mice. Further, upon lethal challenge with JEV all the E-yeast immunized mice died within 8-10 days indicating a lack of protection.

Lack of neutralizing antibody generation and protection could be due to incorrect conformation of the E protein on the yeast cell surface or other factors both of which were not investigated further. Instead, several other shorter sub-fragments of E protein known to generate neutralizing antibodies and protection in mice were selected for expression on the yeast cell surface and subsequent immunization in mice. A total of 4 regions were selected within the envelope protein which has 500 amino acids. These included aa1-398, aa238-398, aa373-399 and aa373-500. Yeast were transformed with constructs containing the nucleotide sequence of the selected regions and their expression on yeast cell surface was analysed using polyclonal anti-E antisera, polyclonal anti-JEV i.p. fluid, anti-JEV Mabs 203, 204, 301 and protected mouse sera obtained from JEV challenged survivors. For reasons which are unclear to us, the polyclonal anti-E sera generated to bacterially expressed E protein, failed to recognize the E sub-fragments expressed on the yeast cell surface. However, the surface expression of all E sub-fragments except aa1-398 could be detected by polyclonal anti-JEV i.p.fluid. Among other antisera used to monitor surface expression of E protein sub-fragments, only anti-JEV Mab 203 and protected mouse sera obtained from JEV challenged survivors could bind the aa238-398 E sub-fragment whereas these and other antisera failed to bind the other E protein sub-fragments.

Since the surface expression analysis using various antibodies could not establish the surface expression of aa1-398 E protein sub-fragment, the antibody response to this fragment was not studied further. Yeast cells expressing the other E protein sub-fragments were

immunized into mice and sera collected after booster doses were screened for the presence of anti-E antibody response. As before, we observed good anti-E antibody response in mice immunized with these E protein sub-fragments. However, sera collected from mice immunized with yeast expressing the various E protein sub-fragments failed to neutralize JEV and these mice were not protected from lethal JEV challenge.

The lack of generation of neutralizing antibodies and absence of protection observed above could be either due to the inability of the neutralizing epitope to be recognized by B cells or the inability of the E sub-fragments expressed on the yeast cell surface to attain the right conformation to generate neutralizing antibodies. It is also possible, that the neutralizing epitopes are lost due to partial proteolysis of the E protein sub-fragments by yeast secretory pathway proteases during their transit to the cell surface. We have not investigated these possibilities in detail but have carried out an indirect analysis by neutralization assays to check for the presence or absence of neutralizing epitopes in the aa238-398 and the aa373-399 E protein sub-fragments. This analysis suggests that these fragments may not have been correctly folded to generate neutralizing antibodies and hence no protection was observed in mice immunized with yeast expressing these E sub-fragments.

Our study demonstrates the efficacy of whole live yeast expressing heterologous antigens on its cell surface in generating a good antibody response to the surface expressed foreign protein. However, to generate immunity to JEV using this immunization method, modifications at the level of protein and in the yeast surface expression strategy may be necessary to achieve neutralizing antibody production. Since we have observed good antibody responses to several antigens that we have tried, this immunization approach could be extended to other therapeutically relevant antigens.

References

1. Rios-Hernandez, M., Dos-Santos, N.J., Silvia, C., Bello-Garciga, J.L., and Pedroso, M. (1994). Immunopharmacological studies of beta-1,3-glucan. *Arch Med Res.* 25(2): 179-180

2. Shibata, Y., Metzger, W.J., and Myrvik, Q.N. (1997). Chitin particle-induced cell-mediated immunity is inhibited by soluble mannan: mannose receptor-mediated phagocytosis initiates IL-12 production. *J Immunol.* 159(5): 2462-2467.

3. Schreuder, M.P., Deen, C., Boersma, W.J., Pouwels, P.H., and Klis, F.M. (1996). Yeast expressing hepatitis B virus surface antigen determinants on its surface: implications for a possible oral vaccine. *Vaccine.* 14(5): 383-388.

4. Stubbs, A.C., Martin, K.S., Coeshott, C., Skaates, S.V., Kuritzkes, D.R., Bellgrau, D., Franzusoff, A., Duke, R.C., and Wilson, C.C. (2001). Whole recombinant yeast vaccine activates dendritic cells and elicits protective cell-mediated immunity. *Nat Med.* 7(5): 625-629

5. Kurane, I. (2002). Immune responses to Japanese encephalitis virus. *Curr Top Microbiol Immunol* 267:91-103