DISCUSSION
Most vaccines require multiple administration to achieve effective immunity. This multiple injection schedule of vaccines leads to many dropouts among subjects to be immunised causing failure of protection from vaccine preventable diseases. This has lead to the emphasis on the development of a suitable controlled release system for vaccine delivery. In this study, attention has been focussed on the formulation and evaluation of a biodegradable delivery system for two vaccines - DT and a birth control vaccine (HSD-DT) inducing antibodies against hCG.

Commercially available biodegradable polymers were first fully characterised using GPC, DSC and TGA for various parameters such as glass transition temperature, weight average molecular weight, number average molecular weight and polydispersity index. The need for these parameters in a study of this nature are indispensable. The glass transition temperature provides an indicator as to when the nature of polymer changes from glassy to rubbery stage. The molecular weight and polydispersity influence the degradation profile of the polymer and consequently the end release profiles of the vaccines from the microspheres. The same is also required to tailor a pre-programmed delivery system for vaccines and also to control antigen release pattern.

**Optimisation of formulation method**

The vaccines used in the study were characterised by HPLC for their purity and their estimation methods standardised. The microencapsulation of the vaccines was carried out using the modified solvent evaporation method (Singh et al. 1991a) with polylactide (L and DL) and polylactide-co-glycolide (50:50, 65:35, 75:25 and 85:15) polymers. The vaccine loading efficiency of the microspheres for the two vaccines under study varied from 72% to 98%. The percentage of vaccine to polymer ranged from 1.5 % to 2.0% (w/w) for HSD-DT and DT.
In view of the variability observed in microsphere size and structure, parameters like concentration of polymer, stirring speed, volume of external phase and nature of stabiliser were studied to derive optimum formulation conditions. The findings suggested that a 10% polymer concentration using gelatin at 2.5% w/w as a stabiliser and 500 ml of external phase containing 1% PVA gave reproducible results with respect to microsphere size and loading efficiency.

The size of microspheres did not influence the cumulative vaccine release confirming that these microspheres were of monolithic type and therefore total vaccine release was based on the bulk erosion of the polymer and was not surface dependant. Also the molecular weights of DT and HSD-DT are sufficiently high to prevent significant molecular diffusion in the matrix and most of the release is based on erosion of the polymers. Scanning electron microscopy studies of the microspheres also confirmed the nature of polymer erosion. The formation of the hydrophillic channels in the matrix network of the microsphere sets in within three to four weeks of exposure to hydrolysis, which results in slow leaching out of the vaccine. This information is important to study matrix breakup period and internal structural properties. The lymphocyte proliferation test did not indicate significant findings simply because in three to five days very little of the vaccine is expected to release. Hence significant uptake by the lymphocytes is not seen in the MTT assay.

The polymer molecular weight and monomer ratio had a significant influence on the cumulative vaccine release profiles. Furthermore by choosing a polymer of increasing lactide concentration in a co-polymer one could retard the rate of release (Hutchinson et al. 1985). The delay in antigen release observed from PDLLGA (65:35 and 85:15) and PLLA microspheres were significantly different. While PDLLA microspheres released the total antigen in 9 weeks, PDLLGA (85:15) released it in 13 weeks. It was thus possible to programme the release profiles of antigen under study for a desired duration.
Another phenomenon which is observed from the release profiles is the bi, tri and quadra-phasic erosion pattern of individual polymers. This information therefore is of interest to simulate the conventional three injection schedule of the vaccine more closely. This is also validated subsequently by the fact that a better immune response was observed with a combination formulation comprising of two polymers of varying molecular weights than individual polymers.

The effect of pH on the kinetics of antigen release from the microspheres was investigated. The slowest release of antigen was observed to occur at or near neutral pH. Any change in pH significantly altered the release pattern. The polymers constituting the microspheres are known to degrade by random hydrolytic cleavage of the ester bond (Pitt et al. 1981; Sanders et al. 1985). Any increase in $H^+$ or $OH^-$ ions will have a positive influence on the rate of hydrolysis.

The vaccine loaded microspheres were also subjected to in vitro release kinetics study over extended periods of time. The resultant cumulative percent release profiles indicated a first order release kinetics indicating the nature of vaccine release from these microspheres. These findings are in concurrence with those reported by Bathurst et al. (1992) for a polypeptide analogue of Pfs 25. The cumulative release observed is due to three main factors either acting alone or in combination (Langer 1981; Tice & Lewis, 1983; Hutchinson & Furr, 1985; Sanders et al. 1985). The initial release varying from 2-10% is constituted by the "burst effect" or "rapid release" from surface entrapped protein. This amount is dependant on the nature of antigen, the polymer type and its concentration. The second or middle phase is constituted by the slow diffusion and slow erosion within the matrix. Here too parameters like diffusivity of the vaccine within the polymer matrix, type of polymer, nature of antigen etc. seem to influence the observed profile. The final or last phase of the curve is constituted by the
total combination effect of widening of channels within the matrix, loosening of the polymer network and break-down of the matrix to small particles. As the whole phenomenon is based on too many parameters, precise prediction of the cumulative vaccine release at any given time based on the erosion constant of the polymer is difficult to achieve.

The assembly fabricated during the course of the study to simulate the 'depot' conditions of the formulation, surprisingly did not show any significant difference from the results obtained from the commonly used shaking water bath method indicating that one could utilise the shaking water bath maintained at 37°C for in vitro release studies and prediction of release kinetics, without significant differences from in vivo depot conditions. The findings highlight the classical behavior of the polymer to degrade in a homogeneous manner and amount of dispersion medium does not alter its degradation profile.

Immunogenicity Studies

Animal models chosen for immunogenicity studies were Balb/C or Wistar rats. As the response varies from individual to individual, statistically significant number of animals were used in each study to draw statistically valid conclusions. Confirmation of good response on microsphere formulation in rodents was obtained in bonnet monkeys. Standardised quality control assays were employed for antibody titration carried out at various time points. Data was subjected to statistical analysis to determine levels of significance.

The anti-DT response in Balb/C mice by a single injection of DT loaded in PDLLA microspheres suggests that the system can be used to deliver three doses of the vaccine at a "single contact point" to elicit a response similar to the three injection conventional schedule (Singh et al, 1992). Both the duration and the type of response in the two cases were found to be comparable. Bathurst et
al. (1992) have recently reported similar enhancement in antibody response with microspheres containing a 25 kD surface protein of *P. falciparum*.

The kinetics of anti-DT response obtained with microspheres prepared from polymers of varying molecular weights appears to be based on the degradation profile of the polymers taken up for the study. The high molecular weight polymer PDLLGA (65:35) gave a delayed peak titer whereas low molecular weight polymers like PDLLA gave earlier peak titers.

Employing HSD-DT as an antigen, a single injection of microspheres prepared from PLLA and PDLLGA induced in wistar rats essentially a similar anti-hCG response which was comparable to that elicited by the conjugate given on alum at monthly interval. As expected the maximum anti-hCG titers were attained at different time points; with PLLA at day 110 and with PDLLGA at day 150. Attempts were therefore made to see the effect of 50:50 mixed formulation of these polymers on the antibody response. The composite formulation evoked an apparently better response than the individual formulations. Similar improvement in immunogenicity has been reported for other antigens also. Stass et al. (1991) have observed that a mixed formulation of microspheres containing Staphylococcal enterotoxin B induced a better response. This can mainly be attributed to a cumulative triphasic release profile from this formulation, which gives a prominent release from the low molecular weight polymer initially for early priming of the immune system, followed by a booster response by the delayed release of the antigen from the high molecular weight polymer (Tice & Gilley, 1986; Wise et al. 1987).

Mixed microsphere formulation proved to be equally effective in bonnet monkeys. Peak anti-hCG titers were produced of the order of 1000-1400 ng/ml hCG binding capacity. The kinetics of response was comparable to that obtainable with alum formulation indicating that this biodegradable microsphere delivery system can substitute the three injection conventional
schedule for HSD-DT vaccine. Antibodies generated were of bioneutralising type suggesting that there was no apparent loss in the integrity of the antigen during the process of microencapsulation.

The above studies have clearly demonstrated that the slow release of the antigen from microspheres over a prolonged period of time does not cause tolerance or immunosuppression in immunised animals. These findings are contrary to general belief that a continuous release of an antigen over an extended period of time might generate low dose tolerance or immunosuppression; a slow and continuous release of an antigen could lead to neutralisation of the antigen at source by the circulating antibodies and consequently a depletion of the antigen reservoir would occur.

Employing microspheres, induction of a potent immune response has also been observed against other antigens such as, Staphylococcal enterotoxin B (Eldridge et al. 1989), ovalbumin (O'Hagan et al. 1989), CFA/I (Edelman et al. 1993; Reid et al. 1993), HIV antigen (Kreuter et al. 1991) and a polypeptide analogue of surface protein of P. falciparum (Bathurst et al. 1992). The probable mechanism of antigen presentation through microspheres (Eldridge et al. 1989) is internalisation of the less than 10 um particles by macrophages and subsequent antigen release intracellularly. As the larger particles (greater than 10 um) also breakdown to smaller fragments by slow erosion, which can be internalised by the macrophages and the antigen processed intracellularly.

In this study no significant difference was observed in the antibody response obtained in rats following immunisation with HSD-DT encapsulated microspheres of the size range of less than 10 um and greater than 10 um suggesting that the mechanism of antigen release, uptake and presentation through the microspheres is in a manner that is not influenced by the size of the microspheres. Some investigators (Eldridge et al. 1991; Gilley et al. 1992) have, however obtained reduced immune response with microspheres more than 10
um in size. The difference in our results and those of other workers may reside in the fact that these observations have been made by employing different antigen, different dose levels and different microsphere compositions. Antibody titers have also been estimated at different time points.

The stability studies are a vital aspect of the findings as the encapsulated vaccine showed no loss in its immunogenicity and activity till an observation period of nine months. Microencapsulation of the vaccines by the biodegradable polymers seems to impart a protective coating to the entrapped antigen thereby increasing its thermal stability.

The study presented above demonstrates the feasibility of the biodegradable microsphere delivery system to engender a response comparable to the conventional three injection schedule on alum. Indications that the single injection of these microspheres can substitute for the presently administered three injections at monthly interval for DT and HSD-DT are available.