Discussion
There have been continuous efforts in the development of more efficient immunodiagnostic system, effective vaccine and immunotherapy for the prevention and therapy of HIV/AIDS and AIDS related complexes. But, unfortunately even after about two decades of the discovery of HIV, we neither have effective vaccine nor have good immunotherapeutic agent. Although a few diagnostic systems exist developed but a continuous effort is required to further evolve more and more efficient and precise diagnostic systems. In diagnostics, a number of different formats have been evolved with different kinds of antigens, i.e. natural, recombinant and synthetic, through the four generations of development (Kerchoven, et al., 1991, Constantine et al., 1992, Contantine, 1993, Zaaijer et al., 1992, Stetler et al., 1997, Desai et al.). Synthetic antigens have been found more specific than recombinant antigens which in turn is more specific than the natural antigens. The synthetic antigenic epitopes, though being more specific and economical, have the limitation of less immunoreactivity on the solid surface due to lesser accessibility to the corresponding antibodies, steric hindrance in antigen-antibody interactions and shielding of the epitope by blocking agent. Moreover the synthetic p24 antigenic epitopes (both M and O), linear in conformation, which was found less immunoreactive when compared with the native antigenic epitope i.e. recombinant p24, whereas, other synthetic epitopes were found immunoreactive to their corresponding antibodies. This study showed that:

i. For optimum immunoreactivity a synthetic epitopes should bear the conformation equivalent to the native epitopes. Hence, specific conformation of antigenic epitopes is required for immunoreactivity. The small change in the conformation affects the reactivity. Getting conformation of synthetic epitopes equivalent to the native epitope, if
the native conformation is not linear, is not possible by chemical synthesis. To cope with the problem of p24 epitope conformation, one has to go for recombinant p24. Although, recombinant p24 has the native epitope conformation and hence higher immunoreactivity, but has been limited due to the non-specific reactivity. In order to have highly specific epitope only synthetic p24 epitope was advisable as the testing of people for HIV is highly sensitive arena.

ii. In order to enhance the immunoreactivity of synthetic p24 epitopes, this study was targeted to make the synthetic epitope more freely exposed to its antibodies by conjugating it with a protein molecule i.e. BSA. The conjugated epitope was freely accessible to react with its antibodies on the solid surface. The comparative immunoreactivity of the conjugated, free synthetic epitopes and recombinant p24 was studied which showed that the reactivity of conjugated epitope was considerably higher than free epitope and was found equivalent to that of the recombinant p24. The Western blot non-reactive clinical specimens, which were found reactive with recombinant p24, found nonreactive. This finding showed that by conjugating the p24 epitopes with BSA, it takes the conformation equivalent to native p24 conformation.

iii. The extent of the immunoreactivity of synthetic p24 epitopes was further enhanced by
employing following two approaches.

a. reduction in steric hindrance and

b. reduction in shielding effect of blocking agent

The above mentioned two approaches were implemented by conjugating the epitopes with BSA molecule through a decadeptide spacer. The comparative immunoreactivity of the epitopes conjugated through decapeptide spacer to BSA, conjugated to BSA, free synthetic epitopes and recombinant p24 was studied which showed that the immunoreactivity of epitope-spacer-BSA was considerably improved on the immunoreactivity of epitope-BSA. This finding showed that by conjugating the p24 epitopes with BSA through spacer, epitopes are more exposed to their corresponding antibodies with lesser steric hindrance. In addition to the epitopes which are more freely available to react with their antibodies due to the spacer arm, the shielding effect of blocking agent was also reduced.

iv. Moreover, the delta (δ) value of the assay was also improved considerably using epitope-BSA conjugate which was further improved using spacer in the conjugate. The δ values provide statistical estimates of the test sensitivity and specificity and permit differentiation between ELISAs of similar sensitivity and specificity and help to see the comparison of the efficacy of ELISAs to separate the negative and positive antibody
serum populations from the cut-off value. It also reflects the ability of an ELISA test to produce consistently high sample/cut-off ratios; sample’s optical density (OD) lies far above or below the cut-off value for HIV antibody positive and negative sera, respectively. The higher the positive and negative delta values the greater the probability that the test will correctly identify antibody positive or negative sera.

Findings of this study would not only contribute to the development of an immunoassay for the detection of antibodies to HIV-1 with better sensitivity, specificity and higher $\delta$ -values but also can be employed in the development of immunodiagnostics for other diseases.

The findings of p24 epitopes conjugates led to the approach of conjugating the other synthetic epitopes, though they are highly immunoreactive due to their conformation equivalent to the native conformation i.e. the native epitopes bear linear conformation, with each other to reduce steric hindrance and getting the epitopes more exposed to their antibodies. Following sets of bivalent epitopes were studies and compared with their univalent epitopes.

- gp41(M)-gp120(M)
- gp41 (M)-p24 (M)
- gp41(M) - gp 36
- p24(M) - gp 36
- p24(M) - gp120(M)
- gp36 - gp120(M)
- gp41(O)-gp120(O)
- gp41(O)-p24(O)

This study showed that the bivalent were having better immunoreactivity than univalent epitopes.

The potential for using antibodies to enhance the capacity of the immune system to combat tumors and infectious disease has stimulated imagination for the last 25 years (Weiner, L.M. 1999). A number of antibodies show considerable efficacy as immunotherapeutics. In order to improve the therapeutic utility of antibodies different modifications have been tried. One of the concerns, however, was the generation of bi-specific antibody (BsAb) (Fanger, M.W. 1995 and Van de Winkel, J.G.J. et al. 1997). BsAb comprises two specificities, and can redirect effector cells towards therapeutic targets. These molecules may limit complement activation, which is responsible for side effects in many therapeutic settings, and this profoundly enhance target selectivity. Although, T cells have received most initial interest as effector cells for BsAb, recent insights into the (patho) physiology of immune responses have shifted attention towards phagocytes and antigen-presenting cells (APCs) as effector populations. New concepts for the use of BsAb in autoimmune and inflammatory disorders are also being developed. These studies have been focussed on the development of bispecific antibodies specific to p24, a structural core protein, and protease of HIV-1 which would effectively reduce the HIV-1 infectivity by
neutralizing HIV-1 protease and p24 which appears first in the process of protease digestion of the viral polypeptide to construct the virus.

BsAb is a hybrid protein that was produced by chemical followed by biological methods. In this, a natural bispecific antibody was studied. Since viruses are so small they must make maximum use of the minimal genetic information that they have. HIV does this by making a long polypeptide chains that contains many proteins. These protein precursors, gag and pol must be cleaved by protease at 9 specific points in order to produce functional proteins. The gag precursor will eventually give rise to structural proteins and pol precursor will give rise to enzymes such as reverse transcriptase, integrase, and protease. Thus, an HIV specific protease is necessary for the HIV to make functional virus. The HIV protease is not found in mammalian cells. The HIV protease is unique in that it can cleave between a phenylalanine and tyrosine or proline. This is a very important fact because no human enzyme can cleave between either tyrosine or phenylalanine and proline. The HIV Protease is an enzyme with two symmetrical subunits. The active site is located where the two subunits meet. HIV proteases are aspartic acid proteases and thus, aspartate 25 plays a key role in binding the substrate. The objective of the study was to block the ability of the protease by its antibody to cut the viral polypeptide into the viral particles and reduction of infectivity of HIV Rezacova P (2002) and Franke L (1992). However, This antibody may not lead to 100% reduction in infectivity. Thus, the use of bispecific antibody therapy including specificity to p24, a gag protein of HIV which appear first during the assembling of HIV particles as evident by its seroconversion, was targeted.
In this study, p24 was conjugated to HIV-1 protease and injected into the rabbits to develop its antibodies. It was found that p24-Protease conjugate induces immune response to both p24 as well as protease of HIV-1 when immunized with their conjugate. The ELISA titre for p24, protease and p24-Protease conjugate were 200000 (1), 80000 (2), and >200000 (3), respectively.

The rabbit antiserum which showed the titre of 1: 200000 for p24 and 1: 80000 for protease was passed first through Sepharose – p24 column (A) and depleted the antibodies to p24 as evident by immunoblot. The same anti p24 antibodies depleted serum was again passed through the Sepharose – protease column (B) and depleted the antibodies to protease as evident by immunoblot. Then, the anti p24 and anti protease antibodies depleted serum was again passed through the Sepharose – p24 - protease column (C) and eluted the antibodies to the shared epitope formed during the conjugation of p24 with protease. The finally eluted antibodies were found reactive to p24 as well as protease as shown by the immunoblots.

The immunoblots showed that the IgG reacted with p24, protease and p24-protease conjugate. After depletion of anti p24 antibodies the IgG reacted only with protease and p24-protease conjugate but not with p24 which showed that the anti p24 antibodies are practically undetectable. After depletion of anti p24 and anti protease antibodies the IgG reacted only with p24-protease conjugate but not with p24 and protease which showed that the anti p24 and anti protease antibodies were practically undetectable in the IgG. It also showed that, though IgG did not react with anti p24 and anti protease antibodies but it reacted with p24-protease conjugate.
which confirmed that the IgG had an antibody which was specific only to the shared epitope of p24 and protease in their conjugate.

The studies on the efficacy of the bispecific antibodies to the shared epitopes were carried out by testing the reduction of HIV infectivity. Lathey J (1994), Ho DD (1989), Coombs RW (1991), Holodniy M. (1991), Saag, M.S. (1991), Katzenstein, D.A. (1992), Venet, A. (1992), Coombs, R.W. (1993). This testing was done employing the method of HIV Culture Assay which estimates the number of infectious units of HIV. The MT4 lymphoblastoid cell line infected with HIV-1 III B 100 TC ID 50 in a 24-well microtiter plate for 14 days. Addition of the antibodies (antibody against the share epitopes of p24 and protease) to the culture causes reduction in infectivity. The infectivity level was measured in the supernatants from each culture well by assaying HIV p24 antigen. Infectious Units per mL (IUPM) were calculated based on the pattern of positive culture wells. Following were the finding of the assay.

- The p24 and protease shared antibodies reduce the progression of HIV-1 infection by \( \sim 80\% \).

- The anti p24 antibodies reduce the progression of HIV-1 infection by \( \sim 60\% \).

- The anti protease antibodies reduce the progression of HIV-1 infection by \( \sim 60\% \).

- The antiserum to P24-Protease reduces the progression of HIV-1 infection by \( \sim 70\% \).
• The anti p24 antibodies depleted serum reduces the progression of HIV-1 infection by ~ 70%.

• The anti p24 and anti protease antibodies depleted serum reduces the progression of HIV-1 infection by ~ 70%.

Conclusively the bispecific antibody to HIV-1 protease and p24 was a potential finding which reduced the HIV infectivity ~ 80% in in-vitro culture assay and hence, opens the door for further studies especially in in-vivo conditions.

The development of an effective vaccine is still a question mark to the HIV researchers, I studied the efficacy of the antigenic epitopes in immunogenicity and persistence of the immune response. Since the epitopes were hapten and cannot elicit the immune response, they were targeted to the immune system on the surface of the multilamellar liposomes. Liposome, a micelle is formed spontaneously in aqueous media from an amphiphilic compound, which consists of distinct hydrophilic and lipophilic sections. The lipophilic sections form the micelle core. The epitopes were directly coupled to the surface of bilayer membrane of liposomes. The liposomes were prepared from phosphatidylcholine and phosphatidylethanolamine molecules. The epitopes were incorporated onto the liposome surface by conjugating them with phosphatidylethanolamine and incorporation of the epitopes – PE conjugates in the formulation of liposomes. In addition, the liposome were sterically protected by an amphiphilic derivative of
poly(ethylene glycol) (PEG) or a PEG-like polymer (mPEG20 kD). Ref. : Chemtech, vol 29, No. 11, 27 – 34.

Poly(ethylene glycol)-N-succinimide carbonate was prepared by converting methoxy -
Polyethylene glycol (mPEG) into its N-succinimide carbonate derivative. This form of the
polymer reacts readily with amino groups of proteins / peptides. This modified proteins /
peptides / aminophospholipids have PEG chain grafted onto the polypeptide /peptide /
aminophospholipids by means of stable, hydrolysis – resistant urethane (carbamate) linkages.
The carbamate linkages have the following merits which contributes in in-vivo self life and hence
longer exposure to the immune system.

- Higher degree of reaction is achievable in shorter period of time.
- Less effect on the activity of the molecule undergoing modification.
- Those functional groups that do not react with amino groups of the molecule undergo
   fast aqueous hydrolysis producing OHSu, CO₂ AND PEG-OH.

New-generation vaccines that are based on recombinantly made subunit and synthetic-peptide
antigens are usually nonimmunogenic, and the need for immunopotentiation is well recognized.
Although many structurally unrelated agents (immunological adjuvants) are capable of inducing
immune responses to vaccine antigens, most of them are toxic. Surprisingly, for about 70 years
the only immunological adjuvant licensed for use in humans was, until recently, aluminum salts
(alum). However, they are far from ideal: they are not always effective, induce humoral
immunity (HI) but not cell-mediated immunity (CMI), and cannot be lyophilized. The liposomes based vaccines against bacterial and viral infections are under development. Liposomes offer a number of advantages as carrier of vaccines [Gregoriadis G (1990)] in that they are biodegradable and nontoxic, can elicit both HI and CMI [Kersten GFA & Crommelin DJA (1995)], and can be prepared entirely synthetically. Furthermore, they are highly versatile in their structural characteristics, which allow for the precise manipulation of their immunoadjuvant properties. A number of structural variables of liposomes can influence adjuvanticity [Gregoriadis G (1990), Gregoriadis G (1993)]: the lipid to antigen mass ratio, bilayer fluidity, vesicle size, surface charge [Nakanishi T, Kunisawa J, Hayashi A, Tsutsumi Y, Kubo K, Nakagawa S, Fujiwara H, Hamaoka T & Mayumi T (1997)], and the mode of antigen association with the vesicles. Manipulation of these variables usually induces variation in the immune response level of a maximal factor of three [Kersten GFA & Crommelin DJA (1995)].

To boost the response further, different immunostimulants have been tested. Among them are avridine, muramyl-dipeptide (MDP) and MDP-lipid conjugates, nonionic block polymer surfactants, aluminum salts, IL-2, IL-6 and lipid A. In this study, the liposomes were coated with PEG. It is noteworthy that the impact of the incorporation of these immunostimulants into liposomes was not only an increase of their immunological action, but also a reduction of their toxic side effects [Alving CR (1991)]. It is noteworthy that liposomes, in addition to promoting immunity to antigens injected through a variety of parenteral routes, also increase IgA immunity to antigens given orally, probably because of vesicle interaction with the gut lymphoid tissue [Fattal E, Ramaldes GA & Ollivon M (1995)].
Liposomal adjuvanticity appears to depend on several of the structural characteristics of the system which are known to determine its fate in vivo and, thus, the mode of interaction with APC. In this study, liposomes as vaccine-delivery systems appears to be a successful. It has been a landmark on the path of success that the antigenic epitopes carried to the immune system on the surface of liposomes, conjugated with PEG, showed very high immune response and the antibody titre was also found long lasting compared to free epitopes as well as epitope-liposome conjugates.

Enzyme-Linked Immunosorbent Assay (ELISA) is a powerful method in estimating ng/ml to pg/ml ordered materials in the solution, such as serum, urine and culture supernatant. There are different kinds of ELISA employed for different conditions. In this studies, since the antigens / antigenic epitopes / modified antigenic epitopes were developed and very well characterized, indirect ELISA was found most suitable for detecting their corresponding antibodies in the solutions.

In indirect ELISA, if solutions contains antibodies to the antigenic epitopes, those antibodies bind to the corresponding HIV antigens on the plate. Unbound antibodies are washed out and followed by reaction with anti-human immunoglobulin coupled to an enzyme (e.g. Horse radish Peroxidase). This is the second antibody that binds to human antibodies captured on the plate. Unbound enzyme coupled immunoglobulins are washed out and followed by reaction with the enzyme's substrate and chromogen. The enzyme cleaves the substrate and which further changes the colour of chromogen. The enzymatic reaction is quenched after a optimum time.
period by denaturing the enzyme. The colour of final product is measured colorimetrically and interpreted.

When considering the binding capacity of adsorbent plastic surfaces for biomacro-molecules, it is essential to distinguish between the total amount of molecules that can be bound to the surface and the amount that can be bound and still remain biologically active. Both quantities are very much dependent on the nature of the molecules and the character of the surface. The adsorption of molecules to a polystyrene surface is due to inter-molecular attraction forces (van der Waals forces), to be distinguished from true chemical bonds, i.e. covalent bonds (through electron share) and ionic bonds (through stoichiometric charges of opposite signs). There are four main types of possible bonds between macromolecules.

a) "True" chemical bonds are represented by a covalent disulphide bond
(b) Ionic bond between a carboxyl ion and an amino ion
(c) Van der Waals mediated bonds are represented by a hydrogen bond between two dipoles and an alternating polarity bond between hydrocarbon residues protruding from the macromolecules' backbones (d), where the encircled area indicates a water-deprived zone.

There are two different types of adsorbent polystyrene surfaces are available from Nunc

- POLYSORP surface
- MAXISORP surface
PolySorp predominantly presents hydrophobic groups, MaxiSorp has in addition many hydrophilic groups, which results in a fine patchwork of hydrophobic and hydrophilic binding sites. In this study MaxiSorp surface was used for facilitation of the adsorption of hydrophilic synthetic epitopes, because not only can this surface compete with the water molecules for binding the macromolecules by hydrogen bonds, but the molecules can also be captured from a much longer distance by the long-range hydrogen bond forces for establishment of both hydrogen bonds and eventually hydrophobic bonds. However, with MaxiSorp peptide epitopes binding events are more likely to occur, which means that adequate incubation conditions are more easy to establish.

As mentioned above, van der Waals mediated bonds are relatively weak, wherefore they may be insufficient for stable bonding when they are few in number, i.e. when the molecules are small. For binding of small molecules, strong chemical bonds are needed. Ionic bonds would not do, because they normally dissociate in aqueous solution, leaving covalent bonds as the only possibility for direct, stable binding of small molecules e.g. peptides. However, this difficulty may be overcome by using small molecules linked to (indifferent) carrier macromolecules as supported by this study.

Three bivalent epitopes from the immunodominant domains of envelop region of HIV were selected based on their immunoreactivity. These bivalent epitopes not only covers the detection of HIV of subtype ‘M’ but also ‘O’. Since antibodies to these epitopes prevail till the infection persists, there was no need to include the epitopes from other domains. Since, antibodies to p24
appear first after seroconversion, the epitopes of p24 from both the subtypes ('M' as well as 'O') were also selected to reduce the window period of diagnosis.

Antigen coating efficiency is dependent on immobilization pH (Coating buffer), ionic strength, immobilization time (incubation period), concentration of epitopes, temperature of immobilization.

Bivalent epitopes showed maximum adsorption on solid phase at pH 9.5 whereas epitopes coupled to BSA showed maximum adsorption at pH 7.2. Since there was no much difference in the adsorption of bivalent epitopes between pH 7.2 and 9.5 but there was considerable difference for epitopes coupled to BSA. Hence, the common pH 7.2 was selected for the immobilization of cocktail of epitopes.

All the epitopes showed maximum adsorption on solid phase between 50 mM to 100 mM but reactivity falls down at 500 mM. The saturation of the adsorption of all the epitopes on solid phase took place within 12 hours.

Investigation of immobilization of antigenic epitopes with the variation in the concentration revealed that there is a rise in the concentration of all the epitopes from 10 ng/well to 50 ng/well and then after, there was no effect of higher antigen concentration on immobilization as evident by the experimental data.
Work on the immobilization time at 37°C showed that there was a rise in the adsorption of the epitopes on the surface up till 60 minutes followed by saturation. Although optimum adsorption took place in 60 minutes, it was advisable to prolong the incubation period for another 60 minutes for strengthening the bond between the solid phase and epitopes.

Blocking agents are essentially to be used in ELISA for blocking possible excess solid surface after coating with antigenic epitopes to avoid unspecific immobilization of succeeding reactants. One reason for using a true blocking agent would be to substitute detergent for blocking: if detergent is present during incubation with secondary reactants, it might in some way interfere with the immunologic specificities or cause unspecific immobilization of the reactants (Esser P., 1990); if detergent is present during wash after secondary reactants, possible weak immunologic affinities might be broken by the washing activity of the detergent. Another reason for using a blocking agent would be to stabilize the immobilized epitopes by sterical support (Jitsukawa T. et al., 1989). This is relevant for storage preservation of coated surfaces. A typical blocking agent would be an indifferent macromolecule, large enough to establish a stable attachment to the surface, yet small enough to find its way between epitopes.

Bovine serum albumin (BSA), of MW 67,000 is commonly used as a blocking agent. Also the more heterogeneous casein is often used and may be more effective than BSA (Pratt R.P. & Roser B., 1989. and Vogt R.F. Jr. et al. 1987). In this study Casein digest, hydrolysate was found to be the best blocking agent amongst all the three blocking agents studied. Casein digest, hydrolysate was found better than BSA which was further found better than skimmed milk.
Casein digest, hydrolysate stood to be the best blocking agent amongst all the three studied due to its following features:

1. Due to its heterogeneous nature (different masses of peptides) it blocks the inter epitope space and unimmobilized space on the solid surface more firmly than BSA and skimmed milk.
2. It also has exerted lesser effect on the steric hindrance in antigen–antibody reactions.
3. It also has lesser effect on the shielding of epitopes.

Upto 0.01% of casein digest, hydrolysate, in phosphate buffered saline, 100 mM, pH : 7.2 was found sufficient enough to serve the purpose of blocking.

Assay employing the cocktail of selected modified epitopes was evaluated using the specific antibodies to the epitopes to validate the immobilization of particular antigenic epitope and found that all the epitopes subjected for coating were immobilized optimally. This study was further extended to sero-conversion panels, a set containing only anti p24 antibodies and another containing all most all the HIV antibodies, and W.B. reactive as well as non-reactive clinical samples collected from the local clinics. This assay differentiated the reactive samples from non-reactive samples i.e. W.B. reactive samples were reactive and W.B. non-reactive samples were non-reactive with this assay.

The performance of this assay was compared with that of US FDA approved tests and was found comparable.
Amongst all the reagents in ELISA, anti hlgG-HRP conjugate, solid phase immobilized epitopes and the enzyme substrate-chromogen (TMB-H₂O₂) are very critical, hence an effort was made to stabilize them. In the conjugate, HRP is very prone to peroxidation which was protected by a reducing agent (TMB). Protein part of the conjugate was protected from deterioration by protein stabilizers (casein digest, hydrolysate), preservatives (thiol containing compounds), surfactant (tween -20) and antibacterial compounds. Stability studies of the conjugate showed that it was found stable at 4°C for the study period of 24 months and at accelerated temperatures, 25°C retained 77% activity in 7 weeks and 37°C retained 50% activity in one month.

Since the synthetic epitopes have linear conformation, there was no effect of temperature up to 37°C on the stability of solid phase immobilized epitopes observed up to a study period of 12 months. The enzyme substrate-chromogen (TMB-H₂O₂) reagent was also stabilized by protecting the hydrolysis of H₂O₂ and studied up to 12 months, > 90% activity was observed at the end of the study.

Conclusively, this study on the efficacy of antigenic epitopes of HIV-1 and HIV-2 was very informative for the development of better diagnostic system and led to a step on the development of neutralizing antibodies to HIV-1 and opened a door to move towards a reliable vaccine development.