Chapter-VI
SUMMARY

Hepatitis B is an infectious disease caused by Hepatitis B virus (HBV) which infects humans causing inflammation of liver called hepatitis. In India, the estimated prevalence rate was found to be 4% (with approximately 36 million carriers overall) (Tandon, 1996). The highest prevalence rate (5.7%) is reported in Southern India (Kurien et al., 2005). The clinical findings of hepatitis attributed to complications of chronic infection, like cirrhosis and HCC, and only 6% were attributed directly to acute hepatitis B (Goldstein et al., 2005). More than 2 billion people have been infected with the Hepatitis B virus in the world population (WHO, 2009).

The intensive investigations of last two decades on the preparation, experimental and clinical characteristics of relatively new category biologically active substances are called as immunostimulants; they are the products from natural or synthetic origin with different chemical characteristics and mechanism of action (Petrunov, 2004). Immunostimulants are able to activate different elements immune mechanisms of the humans and animals. They reinforce body’s natural resistance in order to successfully cope with various viral and bacterial infections; they stimulate the components of the immune system like phagocytosis, properdin and complement systems, protective secretory IgA antibodies, α- and γ-interferon release, T and B lymphocytes, synthesis of specific antibodies and cytokines and pulmonary surfactant. An immunostimulant is a naturally occurring compound that modulates the immune system by increasing the host’s resistance against diseases caused by pathogens (Peddie et al., 2002). The use of immunostimulants in vaccine formulations has given very good antibody responses in in vivo conditions. Immunostimulants (naturally occurring molecules) can be obtained from
a natural source in large quantities, they can improve the innate defense of the animal providing resistance against pathogens, and under stress.

Muscle tissue in mammals may be divided into three types: skeletal (striated) muscles- used for movement of organs etc, visceral (smooth) muscles - found in the walls of the digestive tract, arteries, veins, uterus, bladder and many glands and cardiac (heart) muscles - a special type of muscle found only in the heart. Skeletal muscles, visceral muscles and cardiac muscles are connected by many intermediate forms. The visceral and cardiac muscles contract independently of voluntary control, while the skeletal muscles are subject to voluntary control. When a bit of fresh skeletal muscle is examined under the microscope, the muscles cells (i.e. muscle fibers) appear as long, spindle shaped bodies which are thickened in the middle and become narrow towards their pointed ends.

In many places in the body but particularly in the skin, striated muscle fibers are arranged parallel to one another in one plane. The muscle fibers are large and multinucleated cells. Most skeletal muscles move bones and cartilages; some also cause movement of soft parts, for example, facial muscles. Skeletal muscle is influenced directly by the central nervous system and is under the control of the will. The muscle fiber (large cell) is multinucleate i.e. it is a syncytium. The fibers are unbranched, each fiber is enclosed in a thin membrane, the sarcolemma, which is a specialized cell membrane. The protoplasm of the fiber, sarcoplasm, contains five longitudinal myofibrils which extend throughout.

Electron micrographs show that each myofibril is composed of two types of short myofilaments which are precisely arranged giving the appearance of transverse banding, the striations. The abdominal muscles are important to provide cardiac and respiratory support when the diaphragm muscles have been damaged by any adverse situations. Muscular dystrophy may lead to decreased function of various muscle group especific
muscles involved in respiration causing breathing difficulties as well as leading to cardiac problems. In humans and in animals, degeneration, necrosis and regeneration of muscle fibers can occur after strenuous overuse of muscles. Reduced tolerance to exercise can result from inherited factors such as a glycogenolytic defect in the muscle and impairment of muscle function because of overuse is known. Split fibers and muscle-fiber necrosis are reported in pathological and physiological conditions. Immunostimulants and/or probiotics (particularly lactic acid bacteria) are generally regarded as safe for their inhibitory and immunomodulatory activities, and are defined as live microorganisms which are beneficial, safe and effective. (FAO/WHO, 2001).

The cells of all living organisms synthesize enzymes and these are rapidly degraded in diseased state and their synthesis may be replenished during disease free state. Microbial infections may denature the enzymes. Enzymes are classified into 6 types according to catabolic reactions - oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. The enzymes present in plasma can be grouped as functional (plasma active) and non functional (exocrine secretions and intracellular enzymes). Functional enzymes perform physiological function in blood which include the pro enzymes of blood coagulation, lipoprotein lipase etc.

Under normal conditions non functional enzymes are found in low concentration in serum but they may reach in high concentration during the destruction of erythrocytes, leucocytes and other cells under pathological conditions. If the cell activity is impaired or damaged, the cell membrane becomes permeable or it ruptures. Among the functional enzymes that can be detected in diseased condition are aminotransferases. They include AST and ALT. AST (SGOT) is found in majority of tissues like liver, heart, muscle, kidney and brain. Phosphatases are characterized by their ability to hydrolyze different organic phosphates such as p-nitrophenyl phosphate, phenyl phosphate and sodium beta
glycerol phosphate. Clinically three types of phosphatases are recognized - alkaline phosphatase (ALP) (of serum, bone, liver, and intestine), acid phosphatase (ACP) (of prostrate, liver and serum) and cell phosphatase (CP).

ALP is present in several different organs, e.g., placenta, liver, intestine, and its metabolism can be inhibited under pathogenic conditions. In mammals, two forms of ALPs, occur one of which is distributed in intestine and the other is widely distributed in a variety of tissues (Van Belle, 1972; Borgers, 1973; Goldstein et al., 1980). Significant activity of ALP has been reported in myeloid metaplasia, infection, and in various stressful conditions (Valentine et al., 1951). Phagocytosis is a feature of the breakdown of dystrophic muscle and the increase of enzyme may be due to enzymes in the macrophages. Acid phosphatase activity was visualized within muscle fibers in injured muscles of rabbits. Lead toxicity caused alteration in the activity of phosphatases and the level of creatinine in heart, liver and skeletal muscles of mice due to cellular destruction and encapsulation. The activity of phosphatases and the level of creatinine in heart, liver and skeletal muscles altered significantly in lead treated mice (Satyalatha and Vardhani, 2000). Free glutamine is maintained at a very high concentration in skeletal muscle and a significant correlation between glutamine concentration and muscle protein synthesis has been reported in malnourished and endotoxaemic rats (Jepson et al., 1988).

From the literature available it is known that viral hepatitis may cause abnormalities in the body’s metabolism and immune response and/or cellular damage in vital organs. Very little information is known with regard to biochemical, qualitative protein profile and histological changes in abdominal muscles due to viral infections. Therefore, the present studies were undertaken to analyze the effect of Gene Vac B vaccine in Immunex DS treated mice with regard to certain biochemical, and protein
quality and histopathological changes in the abdominal muscles. The present work is
designed to determine the following

1) Enzymatic activity of AST, ALT, ALP, ACP, GST, SOD and CAT, SDS PAGE
   analysis of protein and histopathology of abdominal muscles in mice treated with
   a single dose of Immunex DS (150mg/mouse) for one day and vaccinated with a
   single dose of 0.07 ml of Gene Vac B vaccine (on day 7).

2) Enzymatic activity of AST, ALT, ALP, ACP, GST, SOD and CAT, SDS PAGE
   analysis of protein and histopathology of abdominal muscles in mice treated with
   a single dose of Immunex DS (150mg/mouse) for one day and vaccinated with a
   single dose of 0.1 ml of Gene Vac B vaccine (on day 7).

3) Enzymatic activity of AST, ALT, ALP, ACP, GST, SOD and CAT, SDS PAGE
   analysis of protein and histopathology of abdominal muscles in mice treated with
   a single dose of Immunex DS (150mg/mouse) for one day and vaccinated with a
   single dose of 0.2 ml of Gene Vac B vaccine (on day 7).

4) Enzymatic activity of AST, ALT, ALP, ACP, GST, SOD and CAT, SDS PAGE
   analysis of protein and histopathology of abdominal muscles in mice treated with
   a single dose of Immunex DS (150mg/mouse) for one day and vaccinated with a
   single dose of 0.4 ml of Gene Vac B vaccine (on day 7).

5) Enzymatic activity of AST, ALT, ALP, ACP, GST, SOD and CAT, SDS PAGE
   analysis of protein and histopathology of abdominal muscles in mice treated with
   a single dose of Immunex DS (150mg/mouse) for one day and vaccinated with a
   single dose of 0.8 ml of Gene Vac B vaccine (on day 7).

6) Enzymatic activity of AST, ALT, ALP, ACP, GST, SOD and CAT, SDS PAGE
   analysis of protein and histopathology of abdominal muscles in mice treated with
a single dose of Immunex DS (150mg/mouse) for one day and vaccinated with a single dose of 1.0 ml of Gene Vac B vaccine (on day 7).

7) Enzymatic activity of AST, ALT, ALP, ACP, GST, SOD and CAT, SDS PAGE analysis of protein and histopathology of abdominal muscles in mice treated with a single dose of Immunex DS (150mg/mouse) for one day and unvaccinated with Gene Vac B vaccine.

8) Enzymatic activity of AST, ALT, ALP, ACP, GST, SOD and CAT, SDS PAGE analysis of protein and histopathology of abdominal muscles in control mice (untreated with IDS + Gene Vac B vaccine).

Male Swiss albino mice (Mus musculus albinus) (6-8 weeks old; Av. Wt. 25-31 gms) used in the present study were purchased from the Mahaveer enterprises of experimental animals, Hyderabad (A.P). They were fed with standard balanced diet and water at ad. libitum. All the experiments were performed according to the guidelines laid down by CPCSEA. Immunex DS (IDS) was given orally on 0 day to mice of group I, and to all the experimental groups (A to F) of mice with a tuberculin syringe fitted with a oral blunt (16 gauze) feeding needle. Control mice (untreated with IDS and HBsAg vaccine) were given distilled water only. HBsAg vaccine was injected into the experimental mice (groups A to F) intramuscularly (using soft pen needles) on day 7 after IDS treatment.

AST, ALT, ALP, ACP, GST, SOD and CAT enzymatic studies, SDS PAGE analysis of protein and histological observations were made from the abdominal muscles of various groups of experimental and control mice.

Estimation of AST and ALT in the abdominal muscles was made by the method of Reitman and Frankel (1957) and estimation of ALP and ACP was made by the method of Bessey et al., (1946). Estimation of GST, SOD and CAT in the abdominal muscles was made by the method of Habig et al., (1974). Misra and Fridovich, (1972) and Sinha,
(1972) respectively. Qualitative determination of protein profile was made by SDS PAGE analysis, and histopathological studies were done by using H and E method.

Two experiments were performed to estimate the activity of AST, ALT, ALP, ACP, GST, SOD and CAT, SDS-PAGE analysis of protein on day 1, 2, 3, 4 and 5 of experiment and histopathological studies (on day 3 and 5 of experiment) in abdominal muscles.

In Experiment I, three groups (ten in each) of mice received IDS orally (@150mg/mouse) on 0 day and Gen Vac B Vaccine @ 0.07 ml/mouse in group A, 0.1ml/mouse in B, 0.2ml/mouse in C, on day 7 of experiment (immunostimulated + vaccinated). Another group (I) of 10 mice was treated with a single dose of Immunex DS @ 150 mg/mouse (0 day) (not vaccinated). Another batch of (group U) 10 mice was kept as untreated and unvaccinated controls for comparison. Two mice from each of the experimental groups A, B and C were sacrificed on day 4, 5, 6, 7 and 8 after vaccination (11\textsuperscript{th}, 12\textsuperscript{th}, 13\textsuperscript{th}, 14\textsuperscript{th} and 15\textsuperscript{th} day of experiment). Similarly, two mice from IDS treated (group I) and controls (group U) were sacrificed on the same designated days.

In Experiment II, three groups (ten in each) of mice received IDS orally (@150mg/mouse) on 0 day and Gen Vac B Vaccine @ 0.4ml/mouse in group D, 0.8ml/mouse in E, 1.0ml/mouse in F on day 7 of experiment (immunostimulated + vaccinated). Another group (I) of 10 mice was treated with a single dose of Immunex DS @ 150 mg/mouse (0 day) (not vaccinated). Another batch of (group U) 10 mice was kept as untreated and unvaccinated controls for comparison. Two mice from each of the experimental groups D, E and F were sacrificed on day 4, 5, 6, 7 and 8 after vaccination (11\textsuperscript{th}, 12\textsuperscript{th}, 13\textsuperscript{th}, 14\textsuperscript{th} and 15\textsuperscript{th} day of experiment). Similarly, two mice from IDS treated (group I) and controls (group U) were sacrificed on the same designated days.
Mice of all the groups of immunostimulated and vaccinated (groups A to F), immunostimulated (group I) and untreated + uninfected (group U) survived throughout the experimental period (15 days). Mice of groups A to F showed clinical symptoms like skin rashes, hair fall, acute illness, anorexia and disinterest to take food and water. Also, all the experimental groups of mice exhibited hemorrhages and inflammation and severe lethargy and continuous stretching of forelimbs.

**Experiment I:**

**AST activity in groups A to C, I and U:**

IDS treated mice (group I) showed higher level of AST than that of control mice (group U) throughout the experimental period. In group I, the level of AST remained almost same from day 1 (27.97 µmoles of pyruvate formed/min/mg of protein) to 5 (27.97 µmoles of pyruvate formed/min/mg of protein). In mice of groups A (treated with IDS + 0.07 ml of vaccine), B (treated with IDS + 0.1 ml of vaccine) and C (treated with IDS + 0.2 ml of vaccine), increased AST activity was found when compared with controls (group U) from day 1 to 5 of experiment (except on day 1 in group A). In mice of groups A, B and C, there was a gradual increase of AST from day 3 to 5 and decrease on day 1 and 2 in groups A and B and on day 1 in group C when compared with IDS treated mice. The AST level is same on day 3 in groups A (28.90 µmoles of pyruvate formed/min/mg of protein) and B (28.92 µmoles of pyruvate formed/min/mg of protein) and reached zenith (67.56 µmoles of pyruvate formed/min/mg of protein) on day 5 in group C.

**ALT activity in groups A to C, I and U:**

ALT was slightly higher in group I than that of control mice (group U) from day 1 to 5 of experimental period. In group I, ALT remained almost same from day 1 (25.88 µmoles of pyruvate formed/min/mg of protein) to 5 (25.84 µmoles of pyruvate formed/min/mg of protein). Increased ALT activity was found on day 1, 2, 3 and 4 in mice of
group A, on day 1 and 2 in group B and from day 1 to 5 of experiment in group C. There was a decrease of ALT on day 5 in group A and on day 3, 4 and 5 in group B when compared with controls (group U). When compared with IDS treated mice, mice of groups A and B showed a decreased ALT activity from day 1 to 5 and on day 1 in group C. The level of ALT is almost same on day 2 (25.21 μmoles of pyruvate formed/min/mg of protein) and I (25.86 μmoles of pyruvate formed/min/mg of protein) in group C.

**ALP activity in groups A to C, I and U:**

The level of ALP in the IDS treated mice (group I) was higher than that of control mice (group U) from day 1 to 5 of experiment. In group I, ALP remained constant from day 1 (18.64 μmoles of PNP formed/min/mg of protein) to 5 (18.64 μmoles of PNP formed/min/mg of protein). Mice of groups A, B and C exhibited a gradual and marked increase from day 1 to 5 when compared with control (group U). Increased ALP activity was found on day 3, 4 and 5 in mice of group A and from day 2 to 5 in group B and from day 1 to 5 in group C when compared with IDS treated mice.

**ACP activity in groups A to C, I and U:**

The level of ACP in the IDS treated mice (group I) was higher than that of control mice (group U) throughout the experimental period. In group I, the level of ACP remained almost same from day 1 to 5 (14.73 to 14.70 μmoles of PNP formed/min/mg of protein). A gradual increase from day 1 to 5 was observed in mice of groups A, B and C when compared with control (group U). When compared with IDS treated mice there was a decrease of ACP on day 1 and increase from day 3 to 5 in groups A and B, and an increased ACP level was found from day 2 to 5 in group C.
GST activity in groups A to C, I and U:

The level of GST in the IDS treated mice (group I) was higher than that of control mice (group U) throughout the experimental period. In group I, the level of GST remained almost same from day 1 (57.48 nanomoles of GS-CDNB formed/mg/protein/min) to 5 (57.49 nanomoles of GS-CDNB formed/mg/protein/min). In mice of groups A and C an increased GST level was found from day 2 to 5, and in mice of group B from day 1 to 5 when compared with control. Increased GST level was found on day 4 and 5 in mice of group A, from day 3-5 in group B and from day 2-5 in group C when compared with IDS treated mice (group I),

SOD activity in groups A to C, I and U:

The level of SOD in the IDS treated mice (group I) was higher than that of control mice (group U) throughout the experimental period. In group I, the level of SOD remained almost same from day 1(2.89 units/mg of protein/min) to 5(2.87 units/mg of protein/min). Increased SOD activity was observed in groups A, B and C from day 1-5 when compared with control (group U) (except on day 2 in group A) and IDS treated mice (group I) (on day 4 and 5 in group A, from day 3-5 in group B and from day 2-5 in group C).

CAT activity in groups A to C, I and U:

The level of CAT in the IDS treated mice (group I) was greater than that of control mice (group U) on all the days of experiment. In group I, the level of CAT remained almost same from day 1 (13.88 units/mg of protein) to 5 (13.85 units/mg of protein). CAT activity increased from day 3 to 5 in groups A and C and decreased activity from day 1 to 5 in group B when compared with control (group U). When compared with IDS treated mice (group I), decreased activity was found from day 1 to 2 and an increased
activity on day 4 and 5 in group A and decreased activity from day 1 to 5 in group B, and an increased activity from day 3 to 5 (except on day 1 and 2) in group C.

Experiment II:

**AST activity in groups D to F, I and U:**

The level of AST in the IDS treated mice (group I) was higher than that of control mice (group U) throughout the experimental period. In group I, the level of AST remained almost same from day 1 (27.97 μmoles of pyruvate formed/min/mg of protein) to 5 (27.97 μmoles of pyruvate formed/min/mg of protein). Group D (treated with IDS + 0.4 ml of vaccine), E (treated with IDS + 0.8 ml of vaccine) and F (treated with IDS + 1.0 ml of vaccine) showed an increased activity of AST from day 1 to 5 of experiment (except from day 3-5 in group F) when compared with control (group U) and IDS treated (group I) mice. Increased AST level was observed from day 3 to 5 and constant value on day 1 and 2 in group D and an increase from day 2 to 5 in group E and on day 1 and 2 in group F (except from day 3 to 5).

**ALT activity in groups D to F, I and U:**

The level of ALT in the IDS treated mice (group I) was higher than that of control mice (group U) throughout the experimental period. In group I, the level of ALT remained almost same from day 1 (25.88 μmoles of pyruvate formed/min/mg of protein) to 5 (25.84 μmoles of pyruvate formed/min/mg of protein). Gradual increase of ALT level from day 1 to 5 of experiment was found in groups D, E and F when compared with control (group U) mice. Group D showed an increased ALT activity from day 2 to 5 and a constant value on day 1, from day 1 to 5 in groups E (except on day 2 in group E) and F when compared with IDS treated mice and reached zenith (87.63 μmoles of pyruvate formed/min/mg of protein) on day 5 in mice of group F.
**ALP activity in groups D to F, I and U:**

The level of ALP in the IDS treated mice (group I) was higher than that of control mice (group U) throughout the experimental period. In group I, the level of ALP remained almost same from day 1 (18.64 µmoles of PNP formed/min/mg of protein) to 5 (18.64 µmoles of PNP formed/min/mg of protein). When compared with controls group U and with IDS treated mice (group I), mice of groups D, E and F showed a gradual increase of ALP activity from day 1 to 5 and reached zenith (67.29 µmoles of PNP formed/min/mg of protein) on day 5 in group D.

**ACP activity in groups D to F, I and U:**

The level of ACP in the IDS treated mice (group I) was higher than that of control mice (group U) throughout the experimental period. In group I, the level of ACP remained almost same from day 1 to 5 (14.73 to 14.70 µmoles of PNP formed/min/mg of protein). Mice of groups D, E and F showed an increase from day 1 to 5 when compared with control (group U) and IDS treated mice. ACP levels reached a peak value on day 5 in mice of group E.

**GST activity in groups D to F, I and U:**

The level of GST in the IDS treated mice (group I) was higher than that of control mice (group U) throughout the experimental period. In group I, the level of GST remained almost same from day 1 (57.48 nanomoles of GS-CDNB formed/mg/protein/min) to 5 (57.49 nanomoles of GS-CDNB formed/mg/protein/min). An increased GST activity was found from day 1 to 5 in groups E and F on day 1 and 2 in group D when compared with control. Decreased GST activity was observed from day 1 to 5 in group D,
and increased GST activity from day 3 to 5 in group E and from day 1 to 5 in group F and a same value on day 2 in groups E and F when compared with IDS treated mice (group I).

**SOD activity in groups D to F, I and U:**

The level of SOD in the IDS treated mice (group I) was higher than that of control mice (group U) throughout the days of experimental period. In group I, the level of SOD remained almost same from day 1 (2.89 units/mg of protein/min) to 5 (2.87 units/mg of protein/min). An increased SOD level was found in groups D, E and F from day 1 to 5 compared with control (group U) and with IDS treated mice (group I) and reached zenith on day 5 (27.60 units/mg of protein/min) of group F.

**CAT activity in groups D to F, I and U:**

The level of CAT in the IDS treated mice (group I) was greater than that of control mice (group U) on all the days of experiment. In group I, the level of CAT remained almost same from day 1 (13.88 units/mg of protein) to 5 (13.85 units/mg of protein). A decreased CAT activity was found on day 1 and 2 in group D, from day 1 to 5 (except on day 5 of group F) in groups E and F when compared with control (group U) and IDS treated mice.

Statistical analysis showed a significant increase of AST in groups A to E and significant increase of ALT in groups C, D and F when compared with controls. The increase of AST was found to be non significant in groups A, B, D, E, F and the increase of ALT was found to be significant in groups A, B, C, D, F when compared with IDS treated mice. No significant difference was found in the level of AST and a significant difference was found in the level of ALT in all the experimental groups when compared among themselves. Increase of ALP and ACP was found to be significant in all the experimental groups when compared with controls and IDS treated mice. However, no
significant difference was found in the level of ALP and ACP in groups A to F when compared among themselves. GST showed non significant difference in all the groups A to F when compared with controls, IDS treated mice and among themselves. SOD showed significant increase in groups A to F when compared with controls and IDS treated mice; comparison among the experimental groups showed non significant difference. The level of CAT was found to be decreased and increased significantly in groups A, C, D and B, E, F in comparison with controls. When compared with IDS treated mice there was a significant decrease in groups A, C, D and no significant difference was found when comparison was made among themselves.

SDS PAGE analysis on qualitative protein profile in abdominal muscles revealed the presence of some common, additional and dominant polypeptides in various groups of test mice; synthesis of stress proteins is also found. Histopathological observations of abdominal muscles of normal, IDS treated and IDS + vaccine treated animals revealed interesting findings: muscle fibers were healthy in controls, and in IDS treated mice. Marked changes were observed in the histology of abdominal muscles of different groups of test mice. They showed muscle destruction, necrosis, degeneration, cloudy swelling, loss of striation and cellular infiltration. Though the histological changes in muscles were not depended on HBsAg vaccine dose, mice received heavy doses of vaccine (groups D to F) showed comparatively much abnormal/pathological changes.

These changes like increase or decrease of transaminases, phosphatases, GST, SOD and CAT, and, polypeptide changes (qualitative protein profile) and histological observations clearly suggest that IDS may act as a better adjuvant to stimulate the immune response and boost up the synthesis of molecules even at the level of abdominal muscles.