CHAPTER – IV
4.1. Introduction

Generally the diet will contain mixed proteins from different sources. Invariably a mixed protein diet is considered the best because a balance in the content and proportion of aminoacid could be struck. When a protein concentrate at a high proportion is used, it may have unwarranted effects. Since the present study involved the use of protein isolates to understand experimentally the effect on induced HCC and MI, the level of Immunoglobulin E (IgE) expression was studied as a measure of allergic reaction. The assessment was done to identify whether a protein from a particular source, when included at high concentration, had elevations in the titers of IgE. In addition differential white blood cell count was also carried out.

4.2. Materials and Methods

The weaned mice, just introduced to solid food were taken for the present study. As already explained they were divided into groups and provided with protein isolates from different dietary sources. Group I mice were used as the control, fed with 20% protein diet (the preparation used here is given in Table 5.1). Animals from groups II to VI were pretreated with five different protein isolates and they were sacrificed and IgE levels in serum and different WBCs cells in blood were analyzed.

The IgE level in serum was measured using ELISA kit and the different white blood cells in blood were analyzed using Sysmax cell counter (Germany).

4.2.1. Immunoglobulin E

Serum Immunoglobulin E was assayed using Kit BioSource, (USA), according to manufacturer’s instructions.

**Principle**

The IgE present in serum sample reacts with the anti-IgE antibodies which have been adsorbed to the surface of polystyrene microtitre wells. After the removal
of unbound serum proteins by washing, anti-IgE antibodies conjugated with horseradish peroxidase are added. These enzyme-labeled antibodies form complexes with the previously bound serum IgE. Following another washing step, the enzyme bound to the immunosorbent is assayed through the addition of a chromogenic substrate, 3, 3', 5, 5'-tetramethylbenzidine (TMB). The quantity of bound enzyme varied directly with the concentration of IgE in the sample tested; thus, the absorbance, at 450 nm is a measure of the concentrations of IgE in the test sample.

**Reagents**

1. Diluent Concentrate: Phosphate buffered saline solution containing bovine serum albumin, 0.25% Tween, and 0.25% Proclin 300 as a preservative.
2. Wash Solution Concentrate: Phosphate buffered saline solution containing 1% Tween.
3. Enzyme - Antibody Conjugate: Affinity purified anti-Mouse IgE antibody conjugated with horseradish peroxidase in a stabilizing buffer.
4. Chromogen-substrate solution: 3, 3', 5, 5'-tetramethylbenzidine and hydrogen peroxide in citric acid buffer at pH 3.3
5. Stop solution: 0.3 M sulfuric acid.
6. Anti-Mouse IgE Elisa Micro Plate: Affinity purified anti-Mouse IgE coated onto the wells.
7. Mouse IgE Calibrator

**Procedure**

100 µL of the diluent was added to all assigned wells. Later 100 µL of standards 400 ng/ml, 200 ng/ml, 100 ng/ml, 50 ng/ml, 25 ng/ml, 12.5 ng/ml, and 6.25 ng/ml were pipetted out into the respective assigned wells. 100 µL of diluted serum samples from various animals was pipetted out into the assigned wells. Microtitre plate was incubated at 22°C for 30 minutes. The contents of the wells were aspirated following incubation. All the wells containing standards and samples are completely filled and appropriately diluted with wash solution and then aspirated. Washing was repeated for around four times. 100 µL of diluted enzyme-antibody conjugate was added to each well and then incubated in the dark at 22°C for exactly 30 minutes. The
contents of the wells were aspirated following incubation. Washing was repeated for about four times. Finally 100 \( \mu \text{L} \) of TMB substrate solution was added to each of the wells and incubated in the dark at room temperature for 10 minutes. After 10 minutes, 100 \( \mu \text{L} \) of stop solution was added to each well and the absorbance was correspondingly determined by using a 450 nm filter.

In our analysis of results, the data of IgE in serum and white blood cells are analyzed separately and discussed in general.

4.3. Results

The appearance and movement of the animals were closely watched for any deviation when they were on the different protein diets. Remarkable deviation in appearance and behavior was observed in whey protein fed animals. These behavioral changes are recorded in Table 4.1.

4.3.1. Immunoglobulin E

IgE levels in the serum of control and experimental animals pretreated with the five protein isolates are shown in Table 4.2. The estimation of IgE is one of the most important indicators of hypersensitivity. It was found that the levels of IgE varied in the groups depending on protein isolate fed. A significant increase in IgE, therefore, was observed in all groups, the highest in the whey treated animals. Among the various groups the least concentration of IgE was recorded in soy protein treated group (105% as compared to control), and it gradually increased in the following order; coconut protein (106%), casein (110%), garlic protein (133%) and whey protein treated (154%). The level of serum IgE in soy protein, coconut protein and casein ranged between 73.16 ng/ml to 77.11 ng/ml, concentration close to the control level. On the contrary garlic protein treated and whey protein treated had values 92.86 ng/ml and 107.16 ng/ml respectively (Figure 4.1).

4.3.2. Total WBC Count

The total WBC count in the blood of control and experimental animals are shown in Table 4.2. A significant \((P<0.05)\) increase in total WBC count was observed in all pretreated animals when compared to the control group. A 158% increase in total WBC count was seen in the whey protein group, which had the highest increase
Table-4.1: Appearance and Behaviour of animals fed control and five different protein isolate diets

<table>
<thead>
<tr>
<th>Behavioural changes</th>
<th>Control</th>
<th>Soy protein</th>
<th>Garlic protein</th>
<th>Coconut protein</th>
<th>Whey protein</th>
<th>Casein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active movement</td>
<td>++++</td>
<td>+++</td>
<td>++++</td>
<td>++++</td>
<td>(_)</td>
<td>++++</td>
</tr>
<tr>
<td>Hair bristling</td>
<td>(_)</td>
<td>(_)</td>
<td>(_)</td>
<td>(_)</td>
<td>+++</td>
<td>(_)</td>
</tr>
<tr>
<td>Restlessness</td>
<td>(_)</td>
<td>(_)</td>
<td>(_)</td>
<td>(_)</td>
<td>++</td>
<td>(_)</td>
</tr>
<tr>
<td>Skin rashes</td>
<td>(_)</td>
<td>(_)</td>
<td>(_)</td>
<td>(_)</td>
<td>++</td>
<td>(_)</td>
</tr>
<tr>
<td>Appetite</td>
<td>+++</td>
<td>++</td>
<td>++++</td>
<td>++++</td>
<td>(_)</td>
<td>++++</td>
</tr>
</tbody>
</table>

Present (+), Absent (-)
### Table – 4.2: Levels of IgE in serum and Differential white blood cell count in control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum IgE (ng/ml)</th>
<th>Total WBC count (cells /cu.mm)</th>
<th>Lymphocytes %</th>
<th>Monocytes %</th>
<th>Eosinophils %</th>
<th>Neutrophils %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>69.55 ± 1.93</td>
<td>3722.66 ± 134.79</td>
<td>10.66 ± 1.21</td>
<td>5.3 ± 1.03</td>
<td>5.5 ± 1.04</td>
<td>64 ± 1.67</td>
</tr>
<tr>
<td>Soy protein</td>
<td>73.16 ± 2.31&lt;sup&gt;a&lt;/sup&gt; (105 %)</td>
<td>4651.66 ± 146.31&lt;sup&gt;a&lt;/sup&gt; (124%)</td>
<td>12.50 ± 1.87 (117%)</td>
<td>6.6 ± 1.2 (124%)</td>
<td>6.3 ± 1.03 (115%)</td>
<td>67.16 ± 1.47 (104%)</td>
</tr>
<tr>
<td>Garlic protein</td>
<td>92.86 ± 2.78&lt;sup&gt;a&lt;/sup&gt; (133%)</td>
<td>4877.66 ± 83.85&lt;sup&gt;a&lt;/sup&gt; (131%)</td>
<td>16.66 ± 1.21&lt;sup&gt;a&lt;/sup&gt; (156%)</td>
<td>8.5 ± 1.04&lt;sup&gt;a&lt;/sup&gt; (159%)</td>
<td>7.3 ± 0.81 (133%)</td>
<td>67.66 ± 1.86&lt;sup&gt;a&lt;/sup&gt; (105%)</td>
</tr>
<tr>
<td>Coconut protein</td>
<td>74.01 ± 2.19&lt;sup&gt;a&lt;/sup&gt; (106%)</td>
<td>4822.83 ± 30.68&lt;sup&gt;a&lt;/sup&gt; (129%)</td>
<td>15.02 ± 1.26&lt;sup&gt;a&lt;/sup&gt; (140%)</td>
<td>6.5 ± 1.04 (121%)</td>
<td>6.5 ± 0.83 (118%)</td>
<td>64.83 ± 1.47 (101%)</td>
</tr>
<tr>
<td>Whey protein</td>
<td>107.16 ± 3.60&lt;sup&gt;a&lt;/sup&gt; (154%)</td>
<td>5904.50 ± 50.15&lt;sup&gt;a&lt;/sup&gt; (158%)</td>
<td>19.05 ± 1.26&lt;sup&gt;a&lt;/sup&gt; (178%)</td>
<td>8.6 ± 1.03&lt;sup&gt;a&lt;/sup&gt; (162%)</td>
<td>10.66 ± 1.36&lt;sup&gt;a&lt;/sup&gt; (193%)</td>
<td>71.66 ± 1.86&lt;sup&gt;a&lt;/sup&gt; (111%)</td>
</tr>
<tr>
<td>Casein</td>
<td>77.11 ± 3.45&lt;sup&gt;a&lt;/sup&gt; (110%)</td>
<td>4770.16 ± 75.68&lt;sup&gt;a&lt;/sup&gt; (128%)</td>
<td>13.83 ± 1.16&lt;sup&gt;a&lt;/sup&gt; (129%)</td>
<td>6 ± 0.89 (112%)</td>
<td>5.83 ± 0.75 (106%)</td>
<td>65.5 ± 2.25 (102%)</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD for 6 animals. Test of significance: control vs others <sup>a</sup>.

<sup>a</sup> P<0.05 significantly different compared with control animals.

(%) As percent of control group.
Figure 4.1
Levels of IgE in serum and Differential white blood cell count in blood of control and experimental animals

Serum Immunoglobulin E

Total WBC count in the blood

% of Lymphocytes in blood

Percentage of Monocytes

Percentage of Eosinophils

% of Neutrophils

Serum Immunoglobulin E

Total WBC count in the blood

% of Lymphocytes

Percentage of Monocytes

Percentage of Eosinophils

% of Neutrophils
among all the experimental groups. The least count of WBC was recorded in soy group (124%). The rise in WBC total count shows that the protein treatment could have significantly altered the expression of WBCs, which is consistent with the individual WBC cell profile. The most obvious reason for such a rise may be the administration of protein, which could have elicited immune response in the blood of the animals.

4.3.3. Lymphocyte count

The percentage of lymphocytes in the blood of control and experimental animals are shown in Table 4.2. There were significant differences in the lymphocyte count while comparing the control and protein-fed experimental groups. The increase was high in the blood of whey protein (178%) and garlic protein (156%) fed mice, but moderately high in the soy protein (117%), casein (129%) and coconut protein (140%) fed animals. The percentage of lymphocytes was, of course higher in soy protein fed than the control, but not significantly different. The percentage of lymphocytes in soy protein, casein treated and coconut protein treated ranged between 12.5 to 15, values close to the normal. The values in garlic and whey protein were found to be 16.66% and 19% respectively. The presence of higher numbers of lymphocytes in the blood of protein-fed animals indicated that the observed rise in the B and T cells might be owing to increased proliferation of immune cells due to cytokine release due to the antigenic nature of the protein isolates.

4.3.4. Monocyte count

The percentage of monocytes in the blood of control and experimental animals are shown in Table 4.2. Upon comparing the percentage of monocytes in the blood of control and the protein pretreated mice, the groups pretreated with the various proteins revealed an increase in the monocytes. The groups pretreated with garlic protein isolate and whey protein showed significant \( P<0.05 \) increase in the number of monocytes. The percentage of monocytes in soy protein, coconut protein and casein treated was of course higher than control animals, but not significantly different. The highest increase was recorded in whey protein group (162%) followed by garlic protein (159%), soy protein (124%) and casein (112%) respectively.
4.3.5. **Eosinophil count**

The total number of eosinophil present in the blood of control and experimental animals has been represented in Table 4.2. A significant ($P<0.05$) increase in the numbers of eosinophil cells was observed in the whey protein pretreated group, when comparing with the control mice. The whey protein fed animals showed almost a 193% increase in the number of eosinophil WBCs when compared to the value seen in the blood of control mice. The rise observed in eosinophil numbers was lower in all other protein fed groups. The lowest increase observed was in the casein fed animals (106%). Soy protein and coconut protein feeding did not elevate eosinophil count to a great extent as that seen in the whey protein fed group.

4.3.6. **Neutrophil count**

Table 4.2 gives an account of the neutrophils in the blood of control and protein-pretreated animals. A significant ($P<0.05$) increase in neutrophil count was observed in the garlic protein and whey protein-fed animals when compared to control mice. The percentage of neutrophil in soy protein, coconut protein and casein was of course higher than control, but not significantly different. Among the various groups the highest percentage was observed in the whey protein animals (111% as compared to control and it gradually decreased in the following order; garlic protein (105%), soy protein (104%), casein treated (102%) and coconut protein treated (101%). The level of neutrophil in coconut protein and casein ranged between 64.83 to 65.5 percentages close to control group. Where as in soy protein and whey protein treated group was 67.16 % and 71.66 % respectively.

4.4. **IgE and differential white blood cells - Discussion**

Food allergy is relatively rare and sometimes when it occurs it may cause violent immune system reaction to food proteins. Food allergy includes syndromes involving several immune mechanisms, each causing a variety of symptoms. The most common is hypersensitivity (type I) mediated by IgE antibodies to food proteins (Wood, 2003; Cordle, 2004).
Protein foods vary in clinical allergy significance. Although all food proteins have the potential to be allergenic for some people, some have been identified as the most frequent human food allergens (milk, eggs, wheat, peanuts, tree nuts and soy) and account for 90% food allergies.

Food antigens generate activated antigen-specific B cells and a special set of helper T cells that direct B cells to differentiate into IgE producing plasma cells. Once secreted, IgE is quickly bound by high affinity IgE receptors mainly on the surface of mast cells. These cells contain large amounts of histamine and other allergic reaction mediators and are the main inducers of symptoms of allergy. The end result is the presence of large numbers of mast cells armed by allergen specific IgE antibodies on the cell surface (Sampson, 1999; Sicherer, 2002; Sicherer et al., 2003).

In the present study a minimal increase in the titers of IgE was observed in soy, coconut and casein groups, and a highly significant elevation in garlic and whey protein treated animals compared to control mice. The level of increase varied with different protein isolates depending upon their antigenic nature to cause hypersensitivity reactions. Even though a rise in IgE values was observed in some groups, their growth and behavior pattern was not disturbed, suggesting that their values were probably within acceptable range. The highest titer of IgE was noted in whey protein group and garlic protein fed animals.

The protein diet used in the present study was formulated in such a way that it contained 20% protein (Table 5.1). Since the exclusive specific protein diets were administered for the first time to the experimental animals a minimal rise in IgE levels and differential white blood cell count was observed. In animal groups fed soy protein and coconut protein no visible external allergic symptoms were seen in these groups except a small elevation in IgE levels.

In the present study a significant rise in the value of differential WBC count was observed in garlic protein fed animals. Allergic reaction to garlic protein has been reported in human studies. Previous studies by others (Shao et al., 2004)) have shown that purified proteins from garlic elicited IgE mediated hypersensitive responses in patients with garlic allergy. Recent study (Ghazanfari et al., 2002) has shown that garlic contains two major proteins of molecular weight 14 and 40 kDa respectively. Of these 14 kDa was reported to significantly enhance delayed type hypersensitivity
response, whereas a protein fraction from garlic R10a was shown to be a good immunomodulator for trial studies on human diseases.

Soy protein fed mice did not show much elevations of differential WBC count. However, a significant rise in IgE was observed. An increase in soy protein specific IgE in normal female adult population was reported by Goodwin (1982); and this was associated with lower levels of adverse effects.

In the present study a significant rise in the values of serum IgE and total WBC, lymphocytes, and neutrophils, monocytes, eosinophils was observed in whey protein fed animals compared to control animals and an increase over and above the values of soy and casein fed animals (Table 4.2). Similar findings were reported by Jason et al. (2001) who in addition had reported a significant (P<0.05) higher levels of CD4+ and CD8+, in whey protein fed animals compared to casein and soy protein fed mice.

The so called milk allergy is an adverse reaction to the proteins present in milk. Several of the biologically active peptides released by enzymatic hydrolysis of milk proteins are known to affect cells of the immune system (Coste and Tome, 1991; Kayser and Meisel, 1996). Our observations make us suggest that it may partly be due to the whey proteins.

Whey proteins α-lactalbumin and β-lactoglobulin are powerful inducers of allergy in infancy (Carolina et al., 2007) and is the most abundant of the whey proteins. They are also a potential modulator of lymphocyte responses (Guimont et al., 1997).

Since β-lactoglobulin is one of the components of whey, the allergic reactions to whey proteins may reasoned out as follows. Immunoglobulin E antibodies against all major milk proteins are commonly found in the blood sera of allergenic subjects, whereas β-lactoglobulin is considered the dominant milk allergen present only in cow milk but not in human milk. When milk is ingested β-lactoglobulin resists enzymatic digestion by pepsin in the stomach and passes undigested intact through the gut epithelial cells into the blood circulation. The immune system recognizes this milk protein as a foreign molecule, causing white blood cells to produce allergy antibodies (IgE) (Monaci et al., 2006). The antigenicity of the protein to induce an allergic reaction depends on its aminoacid sequence and spatial conformation. Trypsin
digestion of β-lactoglobulin has shown many allergic epitopes spread over its surface (Rosendal et al., 2000).

In the present study, coconut protein fed animals had an elevated total WBC count. A significant increase in IgE level was observed in this group. Coconut intake was reported (Kidon et al., 2009) to elicit IgE response in two Asian patients. In contrary coconut intake in children did not cause allergy (Rangsithienchai et al., 2009).

As the present investigation is only a preliminary type and as there are only limited studies involving the use of proteins causing allergic reactions, further insight are necessary to prove the actual mechanism of action of various proteins in elevating WBCs and related allergic reactions.
PART-II

THE EFFECT OF THE CANDIDATE PROTEINS IN DISEASE CONTROL
The Part-II, ‘THE EFFECT OF THE CANDIDATE PROTEINS IN DISEASE CONTROL’ deals with two experiments.

Experiment –IV:

*The preventive role of selected protein isolates on induced hepatocellular carcinoma in mice.*

This experiment is presented in chapter –V

Experiment –V:

*The protective role of selected protein isolates on induced myocardial infarction in mice.*

This experiment is presented in chapter –VI

Each experiment has its introduction, review of literature, materials and methods, results and discussion.