Chapter 3 - DIFFERENTIAL EXPRESSION OF HAPTOGLOBIN IN LEPROSY

INTRODUCTION

A protein with hemoglobin binding properties, first described in 1940 by Polonovski and Jayle (see Carter and Worwood, 2007) was later renamed as Haptoglobin (haptein- Greek word for “to bind”). Haptoglobin (Hp) is a positive acute phase α_2_ sialoglycoprotein synthesized primarily in liver and secreted in blood. Haptoglobin concentrations increase in inflammation, infection, tissue injury and even cancerous conditions, whereas the levels decrease in hemolytic conditions. The primary function of haptoglobin is to bind to hemoglobin (Hb) released from RBC’s following hemolysis, transport it to liver and thus prevent iron loss and damage to kidney (Langlois and Delanghe, 1996). Major portion of the Hp-Hb complex is cleared from circulation by liver hepatocytes (90%) and monocyte/macrophages also clear a small fraction (10%). CD163 present on monocytes and macrophages acts as a receptor for Hp-Hb complex (Kristiansen et al, 2001). Haptoglobin shows genetic polymorphism in humans resulting in three major phenotypes Hp 1-1, 2-1, and 2-2 (see later for further details). These phenotypes exhibit structural and functional differences and are consequently associated with resistance/susceptibility to various diseases (Carter and Worwood, 2007). Haptoglobin, is present at concentrations of about 0.3-2.08 mg/ml of blood, and hence is clinically an important protein.

Structure of Haptoglobin

Haptoglobin is composed of two subunits, α and β linked via disulfide bonds. The α and β polypeptide chains associate as α_2_β_2_ to give the tetrameric structure for haptoglobin (Wejman et al, 1984a). Haptoglobin is a glycoprotein by virtue of the N-linked oligosaccharides at four Asn-X-Ser/Thr sequons (Asn_23, Asn_46, Asn_50, Asn_80) in the β chain, and carbohydrates make up 19.4% of its mass (Kurosky et al, 1980). β chain of haptoglobin binds irreversibly to each αβ dimer of hemoglobin (Valette et al, 1981, Lustbader et al, 1983, Wejman et al, 1984b). These studies suggested that α chain is not involved in interacting with hemoglobin at all. However, Melamed-Frank M et al (2001), using recombinant Hp α proteins demonstrated Hb binding site in α chain also.
In humans, haptoglobin is coded by three alleles- $Hp^{1F}$, $Hp^{1S}$ and $Hp^2$ (Maeda et al, 1984). β chain (approx 40 kDa) coded by all three alleles is identical unlike the α chains. $Hp^{1F}$ and $Hp^{1S}$ alleles code for α chain polypeptides α1F (approx 9 kDa) and α1S (approx 9 kDa) respectively, which are of equal length, but differ in two amino acid residues. The amino acids aspartic acid and lysine at position 52 and 53 in α1F chain are replaced by asparagine and glutamic acid in α1S chain. This variation results in two electrophoretically distinguishable, α1F (Fast) and α1S (slow) forms. $Hp^2$ allele codes for α chain polypeptide α2 (approx 16 kDa) and is a partial gene duplication product arising from non-homologous crossing over between $Hp^{1F}$ and $Hp^{1S}$ alleles (Maeda et al, 1984, Fig. 3.1).

These 3 alleles give rise to six Haptoglobin phenotypes -1F-1F, 1S-1S, 1F-1S, 2-1F, 2-1S and 2-2. These phenotypes have been recently identified by analyzing haptoglobin α chain tryptic peptides by MALDI-TOF MS and MALDI-quadrupole ion trap-TOF-MS (Mikkat et al, 2004). However, conventional haptoglobin phenotyping methods such as starch gel electrophoresis of Hb supplemented serum followed by peroxidase staining and SDS-PAGE followed by immunoblotting (Langlois and Delanghe, 1996) can mainly discriminate 3 major phenotypes - Hp2-2 (containing only α2-β chains), Hp 2-1 (containing both α2-β and α1-β chains) and Hp 1-1 (containing only α1-β chains) (Fig. 3.2.A). Haptoglobin structures exists in form of dimers, linear polymers and multimeric/ cyclic polymers (Fig. 3.2.B) in Hp 1-1, 2-1 and 2-2 phenotype respectively (Wejman et al, 1984a).

Hp α polypeptides are homologous to the fifth crinkle region of plasminogen and β polypeptide shows homology to the serine proteases family (Kurosky et al, 1980). As serine protease family members are synthesized as one chainzymogens and subsequently cleaved into constituent polypeptide chains, the homology data gave indications that haptoglobin is synthesized as a single chain. (Kurosky et al, 1980). Moreover, amino acid sequences in the α-β junction of haptoglobin show strong homology with tissue type plasminogen activator which shows similar processing (Yang et al, 1983). The hypothesis that biosynthesis of haptoglobin occurs as a single precursor chain was supported by mRNA studies in rats (Hanley et al, 1983) and rabbits (Haugen et al, 1981; Chow et al, 1983; Chow et al, 1984). Characterisation of human haptoglobin cDNAs (van der Straten et al; 1983; Raugei et al, 1983; Yang et al, 1983) further showed that DNA’s specifying haptoglobin α and β polypeptides are linked and contiguous in sequence. These studies also suggested that Hp αβ polypeptide chain is broken
by limited proteolysis at arginine α2-143 or α1-84 of the haptoglobin precursor (Fig. 3.3). This –COOH terminal arginine in α chain is then removed by a circulating carboxypeptidase.

**Haptoglobin Phenotyping**

Haptoglobin phenotypes show functional differences (Table 3.1) and thus have clinical implications in various diseases and metabolic disorders. Thus the methods used in clinical settings to phenotype haptoglobin should be fast and sensitive enough to distinguish the polymorphic forms distinctly. Conventional methods were based on electrophoretic separation of Hb-supplemented serum in starch gels and subsequent peroxidase staining (Langlois and Delanghe, 1996). Recently, a new chemiluminiscent based method after PAGE for developed for Hp phenotyping (Huang et al, 2004). This offers a sensitive way of detecting Haptoglobin phenotypes in human sera, even without the addition of hemoglobin, with a detection limit of 1.21 ng. Tubbs et al, (2005) have developed a high throughput MS based phenotyping method. This method, which also detects the post translational modifications, is based on the affinity capture post extraction reduction of Hp α chains. MS analysis in a linear mode gives unique mass spectra of α chain for each of the three phenotypes. Sandwich ELISA using single chain antibodies from phage library also differentiates the major 3 haptoglobin phenotypes (Levy and Levy, 2004). A PCR based method has been developed for differentiating haptoglobin genotypes (Koch et al, 2002).

Most of the above listed methods fail to separate and quantify the different isoforms of α and β chains, arising due to amino acid variation and glycosylation respectively. A combined 2D-PAGE separation with HPLC analysis was developed to characterize the N-linked oligosaccharides of the major and minor isoforms of haptoglobin β chain (He et al, 2006). Haptoglobin α chain isoforms show a complex pattern of spots on 2DE in the theoretical M.wt / pl range of 9-16 KDa / 4.99-6.10. MALDI-TOF MS and MALDI Quadrupole ion trap-TOF-MS of haptoglobin α chain tryptic peptides revealed the cause of migration differences of haptoglobin α chains on 2D gels by assigning the post translational modifications to each isoform (Mikkat et al, 2004). This also helped in assigning the six phenotypes Hp 1F-1F, 1F-1S, 1S-1S, 2-1F, 2-1S and 2-2 on the basis of the Hp-spot pattern of α chains obtained on 2D gels.
Functions of Haptoglobin

The primary function of haptoglobin is to bind to hemoglobin released from RBCs following hemolysis. Free Hb in circulation can cause renal damage by passing through the glomerular filter. Hp-Hb complex formation can prevent this damage by getting transported to liver. This binding is also responsible for some of the important functions of haptoglobin, including it’s antioxidant activity, bacteriostatic effect and anti-inflammatory activity (reviewed in Langlois and Delanghe, 1996). Hp-Hb binding also ensures heme-iron recovery and thus haptoglobin is an important modulator of iron homeostasis (Fagoonee et al, 2005).

Free Hb can promote accumulation of hydroxyl radicals by Fenton reaction, which subsequently causes cell damage. Thus Hp acts as an anti-oxidant by binding to Hb and protecting against free radical mediated damage (Reviewed in Zvi and Levy, 2006). Iron in the free Hb is a source of iron for the pathogenic microbes. During hemolysis, binding of Hp to Hb results in the removal of iron pool and thus Hp acts as a natural bacteriostat (Eaton et al, 1982). Hp-Hb binding leads to sequestration of heme compounds that catalyze oxidation of arachidonic acid for prostaglandin synthesis. Thus by inhibiting prostaglandin synthesis, Hp acts as an anti-inflammatory agent (Langlois and Delanghe, 1996).

In addition to the functions attributed to Hp by virtue of it’s binding to Hb, Hp also functions independently. In its role as an acute phase protein, haptoglobin interacts with leucocytes through various receptors - CD11b/CD18 on monocytes, macrophages, granulocytes, NK cells and a small subpopulation of CD8+ T cells (El Ghmati et al, 1996), CD22 on B cells (Hanasaki et al, 1995) and to mast cells via a receptor different from CD11b/CD18 and CD22 (El-Ghmati et al, 2002). Hp modulates the immune system by acting as a potent anti-inflammatory agent (Table 3.2) and forms a link between innate and adaptive immune responses (Arredouani and Ceuppens, 2002).

During our analysis of serum proteome of leprosy patients, differential expression of Haptoglobin isoforms and the Hp phenotypes was found when compared to healthy controls. We used high resolution 2D gels to separate and quantify haptoglobin isoforms. These results and the clinical implications are described in this chapter.
RESULTS

3.1 Profile of haptoglobin isoforms by 2DE

Haptoglobin α chain was initially identified by comparing 2D protein profile with SWISS-2DPAGE plasma map (Golaz et al, 1993) and the molecular weight and pI of the protein spots on 2D gels were compared with that of the theoretical values given earlier (Mikkat et al, 2004; Table 3.3; Fig. 3.4). The haptoglobin phenotype for each serum sample was then assigned on the basis of the pattern of haptoglobin α chain observed (Fig. 3.4).

In the Coomassie stained gels, (Fig. 3.5), 3 spots of α2 chains (Experimental mass/ pI -21Kda / 5.5-6.25 respectively) and 3 spots corresponding to α1 chains (Experimental mass/ pI-15.4 Kda / 4.95-5.8 respectively) were observed as reported earlier (Mikkat et al, 2004). The identity of these spots was also confirmed by MALDI analysis (as given in Chapter 2). In the Hp 2-2 phenotype (see Fig. 3.5; b, c and e), only spots corresponding to α2 chains (spot 2L, 2M, 2R) were observed in all samples, whereas in Hp 2-1 phenotype (see Fig. 3.5; a and f), in addition to α2 spots, α1 protein spots (spot 1L, 1M, 1R) were detected. However, in one unique case, (Fig. 3.5.c) five spots of α2 chain were observed. In this sample, apart from the usual three spots (spot 2L, 2M, 2R), another spot (2L’) was observed at the acidic extreme and spot number 2M was a doublet (see spot 2M’ at the right edge of spot 2M Fig. 3.5.c). Fig. 3.6 represents magnified view of the spot 2M’. The above rare Hp2-2 phenotype has been reported earlier in Caucasian population (Mikkat et al, 2004, Koy et al, 2003). Hp 0-0 phenotype was uniquely observed in some leprosy cases, which is characterized by complete absence of haptoglobin spots (Fig. 3.5.d). The absence of low amount of low amount of haptoglobin isoforms was confirmed by staining the gels with ultrasensitive ammoniacal silver (detection limit, 1-10 fmoles) and MALDI compatible silver staining.

3.2 Haptoglobin Phenotypes

In all the serum samples analysed, three phenotypes Hp 2-2, 2-1, 0-0 were distinctly observed (Table 3.4). In controls, the incidence of Haptoglobin phenotypes 2-2 and 2-1 is 77.7% and 22.2% respectively. In leprosy patients, the overall incidence of Hp2-2, 2-1, 0-0 is 70.3%, 14.8% and 14.8% respectively. In all the 36 serum samples (including controls and leprosy patients) analysed, Hp 1-1 phenotype was not detected. The incidence of haptoglobin phenotypes in controls and each group of the leprosy spectrum is given in Table 3.4. Hp 1-1 phenotype was not found either in healthy controls or any patients in the leprosy spectrum.
control samples, only Hp 2-2 and Hp 2-1 were observed whereas in leprosy serum samples, apart from these two phenotypes, Hp 0-0 was also detected. Patients carrying Hp 0-0 phenotype were recruited for a follow up study.

### 3.3 Acquired Anhaptoglobinemia

Among all the samples examined, three ENL patients (ENL15, ENL14 and ENL149; Table M.1 in Materials and Methods) and one RR patient (RR2) showed Hp0-0 phenotype. Rare form of primary anhaptoglobinemia, due to the deletion of the haptoglobin gene was reported from Asian population (Koda et al, 2000). However, secondary anhaptoglobinemia is more prevalent under pathological conditions (Nandi et al, 1970; Koda et al, 2000; Carter and Worwood, 2007).

In order to identify the nature of Hp 0-0 in our samples, we examined the follow up serum samples of all the Hp 0-0 patients. Serum of patient RR1 showed reversion of Hp0-0 to Hp 2-1 (Fig. 3.7.1 and 3.7.2) phenotype. As shown in Fig. 3.7.1.A, during treatment, absence of haptoglobin α1 and α2 chains (Box 2) is accompanied by the absence of haptoglobin precursor chain (Box 1) as well. The identity of the missing Haptoglobin chains in the serum profile in Hp 0-0 cases was further confirmed by the presence of transthyretin (T) in the expected haptoglobin α chain region (Fig. 3.7.1A). Around the 45 kDa region, where the haptoglobin precursor chains proteins are present, Apolipoprotein-AIV protein (A) could also be detected. This data also confirms that the absence of haptoglobin precursor spots was not due to experimental conditions. In addition, the spots corresponding to haptoglobin isoforms could not be detected even after ammoniacal silver staining. In the follow up study, the serum sample of the above Hp 0-0 phenotype patient collected after the completion of the drug treatment (Fig. 3.7.1B) showed the presence of haptoglobin α2 and α1(Box 4 and 5) proteins as well as the haptoglobin precursor protein (Box 3). Similar results (Fig. 3.8) were observed for another patient (ENL14), but this patient showed reversion to Hp 2-2 from Hp 0-0 phenotype.

In the case of the other two Hp 0-0 patients (ENL15, ENL 149), serum samples collected before the treatment showed Hp 2-2 phenotype (Fig. 3.9.A and 3.10.A), and serum collected during treatment showed Hp 0-0 phenotype (Fig. 3.9.B and 3.10.B). In case of patient ENL 15, serum profile of the patient after recovery from ENL, showed Hp 2-2 phenotype again (Fig 3.9.C). Albumin and IgG depleted serum of patient ENL 149 was
analysed using 3-5.6 pH range IPG strip (Fig. 3.10), in order to resolve the isoforms clearly. This analysis also confirmed the above results with respect to the absence of haptoglobin precursor and alpha chain spots in the patient during treatment. This clearly shows that haptoglobin expression is affected during drug treatment. The isoform migrating at the extreme basic end (represented by spot 2R in Fig. 3.5) is missing in this narrow range IPG separation covering pH 3-5.6, since the pI of this isoform is 6.25. These results show that the Hp 0-0 phenotypes in our study group are acquired anhaptoglobinemia and the treatment causes the disappearance of haptoglobin protein.

In case of patients ENL 15 and ENL 14, absence of haptoglobin, was accompanied by absence of another protein, hemopexin (see Fig. 3.8 and 3.9), which is similar to haptoglobin in its heme binding function (See discussion). However for patient RR2, the absence of hemopexin during the anhaptoglobinemic condition was not observed (Fig.3.7.2 A and B).

3.4 Differential expression of haptoglobin α isoforms in ENL

In seven out of the eleven (typable haptoglobin phenotype) ENL cases analysed, spot number 2R of the α2 chain was either upregulated or was similar in intensity to spot 2M when compared to control samples. To minimize the error in calculating the intensity difference of spot 2R between the gels, the upregulation of spot 2R was analysed by calculating the ratio of % intensity spot 2M (another isoform not differentially expressed) to 2R (Fig. 3.11A) across all gels. If the spot 2R is upregulated, the ratio of % intensity of spot 2M by 2R will be decreased. As shown in Fig. 3.11B, the ratio of % intensity of spot 2M to 2R in ENL patients decreased $1.104 \pm 0.088$ compared to controls $1.432 \pm 0.262$. This difference was statistically significant ($p=0.0078$). In comparison, there is no significant difference (Fig. 3.12) in the ratio of % intensity of spot 2M to 2L in controls $2.79 \pm 0.514$ and ENL patients $2.51 \pm 1.000$. The spot 2R represents the most basic protein species among the isoforms of Haptoglobin α2 chains. The modification in this isoform is attributed to the presence of C-terminal arginine at the 143rd position (Mikkat et al, 2004). Sequence data (Fig.3.13.b) of the peptide (m/Z value 895. 51 in Fig. 3.13A) which covers this region, was obtained by MS/MS spectra acquisition by Post source decay (PSD). It confirmed the amino acid sequence of this peptide as NPANPVQR (amino acid position 136-143) as shown in Fig. 3.14.
3.5 Comparison of expression of haptoglobin α isoforms in other stages of leprosy spectrum with ENL

Expression of spot 2R of the α2 chain was compared across the leprosy spectrum and healthy controls by 2D gel analysis. The ratio of % intensity of spot 2M to 2R (as represented by histograms in Fig. 3.15) was 1.432 ± 0.262 for controls; 1.316 ± 0.232 for BT; 1.316 ± 0.213 for RR; 1.356 ± 0.072 for BB; 2.08 ± 0.367 for BL; 1.687 ± 0.166 for LL and 1.104 ± 0.088 for ENL cases. Expression in ENL was significantly different compared to all stages of leprosy spectrum except BT.

3.6 Follow up study of ENL patients showing differential expression of isoform 2R

The serum samples of the patients who showed differential expression of isoform 2R of haptoglobin, were followed up post treatment and clinical remission of the reactional condition. These patients, were now diagnosed with LL condition. The upregulation of spot 2R (analysed by calculating the ratio of % intensity spot 2M to 2R separated on 2D gels) in ENL condition decreased significantly (p=0.0016) in LL condition and was comparable to healthy controls (Figure 3.16).

DISCUSSION

Study of Haptoglobin in Leprosy

Haptoglobins are important proteins to be studied in leprosy because of their physiological significance. Few studies have focused on the effect of the genetic variability and the type of leprosy. Studies done previously have analysed the haptoglobin phenotypes (Shenoy et al, 1983; Saoji et al, 1980) or the haptoglobin levels (Sritharan et al, 1981) across the spectrum of leprosy cases. So far no study was undertaken to examine haptoglobins in reactions. No correlation was observed between the leprosy stages and haptoglobin phenotypes (Shenoy et al, 1983; Saoji et al, 1980), but our study using reactional cases show significant changes.

Haptoglobin phenotype has been shown to be an important susceptibility factor for the development of renal complications (Awadallah and Hamad, 2003; Burbea et al, 2004). Renal lesions in leprosy patients manifested as glomerulonephritis, amyloidosis etc. especially at the lepromatous end is a potential cause of death in this disease (da Silva Junior and Daher, 2006). Chronic renal failure, common in ENL condition is attributed to the presence of immune
complexes. Added to that free Hb in circulation (a result of drug or disease induced hemolysis) may pass through the glomerular filter and thus cause renal damage. The haptoglobin phenotype is important in determining the effective clearance of free Hb, which is least in Hp2-2 individuals (Langlois and Delanghe, 1996). Also the type of Haptoglobin determines the resistance/susceptibility to various diseases (Table 3.5), thus making it an important protein to be studied for its role in a spectral disease like leprosy.

**Haptoglobin phenotypes in leprosy**

Among the samples examined in this study, Hp2-2 phenotype is predominant in controls and leprosy patients (Gupta et al, 2007). It has been shown that the Indian population predominantly has a Hp 2-2 phenotype (Langlois and Delanghe, 1996; Shenoy et al, 1983; Saoji et al, 1980). But these Haptoglobin polymorphism studies of Indian population among healthy controls and leprosy samples were done by one dimensional polyacrylamide disc gel electrophoresis (Shenoy et al, 1983; Saoji et al, 1980) or gel filtration technique (Sritharan et al, 1981) which do not resolve the haptoglobin isoforms. The reported Hp2-2 frequency in these studies with respect to controls are similar to our data, but leprosy samples in our study showed lower Hp2-2 frequency. The variance between our data and the earlier reports could be due to the high frequency of Hp 0-0 in our studies. The geographical distribution of the populations examined could also be a likely explanation. Hp 1-1 phenotype was shown to be very rare in Indian populations (Langlois and Delanghe, 1996). The $Hp^1$ frequency varies from about 0.07 in parts in India to over 0.7 in parts of West Africa and South America (Carter and Worwood, 2007). Data in this report clearly shows that Hp1-1 phenotype is absent in both control and leprosy cases examined.

Hp 1-1 protein complex (α-β)$_2$ is ~ 86 KDa in size and these are shown to transmigrate across vascular epithelium freely unlike Hp2-1 and Hp2-2 complexes, which form linear and circular multimers respectively. Population studies in Israel, Belgium and United States have shown that when compared to Hp1-1 phenotype, diabetic patients with Hp2-2 phenotype have five fold increased risk of developing cardiovascular diseases (Melamed-Frank et al, 2001). High rate of death and heart failure were predicted in diabetic patients with Hp2-2 and Hp2-1 phenotype (Suleiman et al, 2005). Hp2-2 phenotype is overrepresented in tuberculosis patients with advanced destruction and dissemination and in autoimmune diseases (Langlois and Delanghe, 1996). In contrast, Hp1-1 phenotype population is shown to be more prone to breast cancer and cervical carcinoma (Langlois and Delanghe, 1996). Lower effectiveness of
multimers in their ability to bind hemoglobin, poor antioxidant capacity and inefficient inhibition of prostaglandin synthesis indicate, perhaps the predisposition of Indian population, which is predominantly Hp2-2 phenotype, to chronic diseases under certain conditions.

**Acquired Anhaptoglobinemia in Leprosy**

Hp 0-0 phenotype was detected in 14.8% of all the leprosy samples analyzed. The frequency observed is 2.5 fold higher when compared to earlier reports in leprosy (Shenoy et al, 1983; Saoji et al, 1980) Compared to the conventional haptoglobin phenotyping methods used in these studies, 2D analysis and sensitive staining methods used in this study are more accurate, reliable and give information about haptoglobin isoforms which the earlier studies missed completely. Earlier studies attributed the Hp 0-0 phenotype either to the hereditary factor (Shenoy et al, 1983) or to the diseased state leading to the absence of haptoglobin synthesis per se (Saoji et al, 1980).

According to our data, this anhaptoglobinemic condition is acquired, since the follow up study of patients released from drug therapy showed that the patients regained haptoglobin levels (Gupta et al, 2007). In all these anhaptoglobinemic sera, the haptoglobin precursor chains were also not detected. Haptoglobin precursor is a single polypeptide chain synthesized in liver, containing a signal peptide and α and β chains (Mikkat et al, 2004). As proteolytic processing of the precursor leads to the formation of separate haptoglobin α and β chains, the absence of precursor, confirms the absence of expression of this gene in these patients. Even though anhaptoglobinemic condition was reported in other diseases such as in malaria, sickle cell anaemia, diseases associated with liver dysfunctioning (Nandi et al, 1970; Carter and Worwood, 2007) and in leprosy (Shenoy et al, 1983; Saoji et al, 1980), the status of haptoglobin precursor was not studied in these cases.

Absence of haptoglobin could be due to selective proteolytic degradation or loss from the blood circulation following hemolysis. One of the findings in this study, which supports hemolysis as the cause of anhaptoglobinemic condition in leprosy, is the absence of heme binding protein, hemopexin (see A and B in Fig. 3.17) in two of the ENL patients during their anhaptoglobinemic stage (Fig. 3.8.A, 3.9.B). Hemopexin is a non-acute phase plasma glycoprotein, which could also form a defense against hemoglobin mediated oxidative damage during intravascular hemolysis (Delanghe and Langlois, 2001). Hemopexin levels start decreasing in serum only after the Hb-binding capacity of Hp is saturated (Delanghe et al,
1998; Delanghe and Langlois, 2001). The levels of hemopexin were restored alongside haptoglobin in the patients after recovery from ENL condition (See hemopexin and haptoglobin spots in Fig. 3.8 and 3.9). But in contrast, in the Reversal reaction patient (RR2) undergoing anhaptoglobinemia, hemopexin levels remain unchanged (Fig. 3.7.2.A and B; Fig. 3.17.C). This discrepancy could be linked to the differential hemoglobin binding capacity (HBC) of different haptoglobin phenotypes (Table 3.1). Patients 15 (Fig. 3.9.A) and 14 (Fig. 3.8.B) were of Hp2-2 phenotype in contrast to patient RR2, who is Hp 2-1 phenotype (B in Fig. 3.7.1. and 3.7.2) As HBC of Hp 2-2 is low, haptoglobin would be saturated with Hb much sooner in an Hp2-2 individual undergoing hemolysis when compared to Hp 2-1.

Hence hemopexin levels also decreased/depleted (possibly due to its binding to heme) in the Hp2-2 but not the Hp 2-1 individuals during the Hp 0-0 stage. Decreased levels or absence of both Hp and hemopexin in plasma is suggestive of a severe intravascular hemolysis or chronic hemolytic anemia (Delanghe and Langlois, 2001). Falling Hpx concentrations after depletion of Hp indicates continued erythrocyte destruction. These findings suggest the development of anhaptoglobinemic condition in leprosy might be attributed to hemolysis. However, more number Hp 2-2 and Hp 2-1 phenotype patients undergoing anhaptoglobinemia have to be studied to support this data. Although dapsone induced hemolysis leading to hypohaptoglobinemia in leprosy has been shown in a previous study (Sritharan et al, 1981), our study showed anhaptoglobinemic condition not only in MDT (includes dapsone) treated, but also in one rare case of only thalidomide treatment (ENL 14). Thalidomide, an immunomodulatory drug, is used in treating ENL cases as it ameliorates the inflammatory condition rapidly and does not have the side effects of steroids (Haslett et al, 1998). leprosy cases under different drug regimen have to be studied to understand the clinical significance behind the development of this anhaptoglobinemic condition.

**Differential expression of α2 isoform in leprosy**

One of the isoforms of the haptoglobin α2 chain (spot 2R in Fig 3.5) showed increased expression across the ENL samples analysed (Gupta et al, 2007). The uniqueness of this haptoglobin α2 isoform is that it has an arginine containing C-terminus unlike the other two isoforms (represented by spot 2M and 2R in Fig 3.5). This arginine residue is the single amino acid separating the α and β chains of Haptoglobin and is the site of proteolysis leading to the conversion of the precursor to separate α and β chains (Mikkat et al, 2004). In this study, the MS/MS data of one of the peaks in the PMF of spot 2R also confirms the presence of C-
terminal arginine. Canine blood analysis from an animal undergoing acute inflammation reaction has shown that 30% of the haptoglobin α chains have C-terminal arginine residue (Mikkat et al, 2004). ENL condition in leprosy also represents a severe inflammatory condition with increased systemic concentrations of TNF-α. Hepatic synthesis of haptoglobin is known to be induced by TNF-α (Langlois and Delanghe, 1996). Thus in ENL condition, increased in haptoglobin synthesis would result in presence of more of newly synthesized haptoglobin α chains with the C-terminal arginine residue. Deamidation of asparagine residue, a post translational modification of haptoglobin, which results in Hp α isoforms with negatively charged chains (spot 2L in Fig. 3.5), has been proposed to affect the molecular clock of proteins, such as, protein turnover, development and aging in peptide model studies (Mikkat et al, 2004). Thus relative amount of haptoglobin α chain isoforms in a physiological or diseased state, would give information about haptoglobin turnover and perhaps other events.

**Significance of differential expression of Haptoglobin in ENL and its comparison to other stages in leprosy spectrum**

Hp serves as an intrinsic negative regulator of acute inflammatory responses. Initiation of inflammation increases Hp levels, following which Hp exhibits its anti-inflammatory activity, which helps in restoring the normal homeostasis (Hanasaki et al, 1995; Arredouani et al, 2003; Oh et al, 1990; reviewed in Langlois and Delanghe, 1996). Though the variations in haptoglobin levels and it’s potential as a biomarker has been described in various diseases (Table 3.6), the specific, most basic alpha isoform increase shown in this study in a diseased condition has not been reported before. Increase in serum haptoglobin alpha levels has been shown in Ovarian cancer (Ye et al, 2003) but here total alpha levels were measured by ELISA and western blot. In our study, by 2D analysis we specifically show the differential expression amongst haptoglobin alpha isoforms. The isoform was significantly (p<0.05) upregulated in ENL compared to other stages of leprosy spectrum too (except the BT cases). This has to be confirmed with larger number of samples across leprosy spectrum using a high throughput validation technique. Our data also shows that, post treatment for ENL, when the patients are clinically diagnosed as LL cases, the isoform levels are comparable to that of healthy controls. The data also clearly shows that compared to non-reactional LL and BL and reactional RR condition, the Hp α2 2M isoform is specifically upregulated in ENL condition.
Our study showing the demonstrable variation that is statistically significant (p=0.0078), with respect to α2 haptoglobin isoforms (spot 2R) levels could be studied further for it’s use as a putative diagnostic marker in ENL cases. These acute phase changes could either be used as independent biomarkers before the clinical signs develop or could be used to confirm the diagnostic criteria. In a reactional condition like ENL, validating clinical biomarkers responding to drug therapy will help in better disease management and treatment.