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Heme-regulated eukaryotic initiation factor 2α kinase—a molecular indicator of haemolytic anemia*

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Abstract. The heme-regulated enkaryotic initiation factor-2*α* (eIF-2*α*) kinase, also called the heme-regulated inhibitor (HRI), is a key regulator of protein synthesis in mammalian reticulocyte. HRI is almost undetectable in blood samples of normal rabbits and it increases by 12–15-fold in the reticulocytes of anemic rabbits. In order to determine if such an increase in the quantity of HRI is gradual during anemia, and if it could be an indicator of anemia, we have carried out a detailed analysis on the expression of HRI and its eIF-2*α* kinase activity in rabbit reticulocyte lysates during various stages of acetylphenylhydrazine (APH)-induced anemia. In a 9-day schedule of induction of anemia, using an anti-HRI monoclonal antibody, HRI was detectable immediately after completion of fourth injection (day 5) and it increased gradually during the entire period reaching its maximum (24-fold) on day 9. Furthermore, when rabbits recovered from anemia due to individual response to the drug, quantity of HRI decreased significantly. Northern blot analysis results were similar to those of the Western blot. The other parameters that are generally used to monitor anemia in human patients, namely, reticulocyte count, haematocrit level and haemoglobin content although changed at the onset of anemia, did not change significantly during its progression. These results thus indicate that HRI could be a more appropriate and sensitive indicator of drug-induced anemia.

Keywords. Heme-regulated inhibitor; eIF-2α kinase expression; drug-induced anemia; molecular indicator.

1. Introduction

In mammalian reticulocytes and their lysates, inhibition of protein synthesis during heme-deficiency occurs due to activation of the heme-regulated eukaryotic initiation factor-2*α* (eIF-2*α*) kinase also called the heme-regulated inhibitor (HRI) (Maxwell *et al* 1971; Ochoa 1983; London *et al* 1987; Chen and London 1995; Pal *et al* 1996). HRI is a cyclic AMP-independent protein kinase. Upon activation it undergoes autophosphorylation and subsequently phosphorylates the 38 kDa *α* subunit of eIF-2 at ser-51 residue (Levin *et al* 1976; Kramer *et al* 1976; Ranu and London 1976; Farrell *et al* 1977). Phosphorylated eIF-2 (eIF-2*α* P) binds to guanine nucleotide exchange factor (GEF) also known as eIF-2B or reversing factor (RF), and forms a stable complex which sequesters RF. As a consequence, due to unavailability of RF, which is required for the exchange of GTP for GDP for recycling of eIF-2, initiation of protein synthesis ceases (Amesz *et al* 1979; Matts *et al* 1983, 1986; Ochoa 1983; Panniers and Henshaw 1983; Matts and London 1984; Thomas *et al* 1984).

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HRI has been purified almost to homogeneity from rabbit reticulocyte lysates and has been extensively characterized. HRI is a dimer of 92 kDa polypeptide (Trachsel *et al* 1978) and it sediments as 6·6 S component in glycerol gradient (Levin *et al* 1980). Both purified and rabbit reticulocyte HRI *in situ* can be activated by a number of conditions and reagents, viz., heme-depletion or deficiency, heat shock, high partial pressure of O_2, sulphydryl reagents (N-ethylmaleimide and iodosobenzoate), oxidized glutathione, heavy metal ions and denatured polypeptides (Duncan and Hershey 1984; De Benedetti and Baglioni 1986; Hurst *et al* 1987; Matts *et al* 1993; reviewed in London *et al* 1987; Chen and London 1995). However, the mechanism of HRI activation except during heme-deficiency has not been understood well. Hemin has been shown to bind to purified HRI and promote intersubunit disulphide bond formation, which has been proposed as a mechanism for negative regulation of HRI (Yang *et al* 1992).

Recently, HRI has also been purified from Ehrlich ascites cells (Olmsted *et al* 1993) and mouse erythroleukemia (MEL) cells (Mellor *et al* 1993). However, they appear to be different from rabbit reticulocyte HRI. Similarly, although heme-dependent eIF-2α kinase activity has been detected in a number of other tissues and cell types, namely, rat liver (Delaunay *et al* 1977), wheat germ lysate (Ranu 1980), HeLa cells (De Benedetti and Baglioni 1986; Duncan and Hershey 1984), human reticulocyte lysate (Petryshyn *et al* 1984), human K562 (Pal *et al* 1991), *Plasmodium* (Surolia and Padmanaban 1991) and *Drosophila* cells (Duncan *et al* 1995), no cross reacting HRI to anti-rabbit HRI antibodies has been detected in them (Pal *et al* 1991; Chen and London 1995).

HRI cDNAs from rabbit reticulocyte (Chen *et al* 1991) and rat brain have been cloned and they show 82% homology to each other with greatest variation concentrated in the central region of 135 amino acids located between kinase subdomains IV and V (Mellor *et al* 1994). Using rabbit reticulocyte HRI cDNA, expression of HRI was found to be erythroid-specific (Crosby *et al* 1994). However, Mellor *et al* (1994) using rat brain HRI cDNA, demonstrated the expression of HRI in a number of non-erythroid tissues as well, albeit to a much lesser extent (10 times). In all those tissues the size of the HRI transcript was the same as that of the reticulocyte (3·1 kb).

It has been shown that the quantity of HRI protein increases by 12–15-fold in the reticulocyte lysates of anemic rabbits as compared to that of normal, while the mRNA increases by two-fold only (Crosby *et al* 1994). In the light of this report we addressed the following questions (i) is the quantitative increase in HRI during anemia gradual in erythroid cells, and (ii) how early during anemia can the increase be detected. In this report, we present data which suggests that HRI can be used as a molecular indicator for detection of anemia of various degrees in rabbits.

2. Materials and methods

2.1 *Materials*

New Zealand white rabbits were obtained from Institute of Veterinary Biological Products (IVBP), Pune. All the fine chemicals used in the present study were purchased from Sigma and Gibco BRL. Goat anti-mouse IgG-peroxidase and goat anti-mouse IgG-alkaline phosphatase were purchased from Gibco BRL. Anti-rabbit HRI monoclonal antibody (mAb F) was prepared by the senior author in the laboratory of Dr I M London (MIT, USA) (Pal *et al* 1991). Rabbit HRI cDNA was

cloned in the laboratory of Dr I M London and was provided by Dr J-J Chen (Chen *et al* 1991). Anti-hsp 70 antibody (N27F3–4) was kindly provided by Dr William J Welch (University of California, San Francisco, USA). eIF-2 was kindly provided by Dr KVA Ramaiah (University of Hyderabad, Hyderabad). Chemiluminescence detection kit for Western blot analysis was purchased from Boehringer Mannheim. Random priming DNA labelling kit was obtained from New England Biolabs (USA). Nitrocellulose and nylon membranes were purchased from Schleicher and Schuell and Pall Biodyne (USA), respectively. X-ray films were purchased from Nieue films, Japan. [α–^{32}P] ATP and [γ–^{32}P] ATP were purchased from Board of Radiation and Isotope Technology (BRIT), India.

2.2 *Induction of anemia and preparation of reticulocyte lysate*

Rabbits were kept in the animal house for a period of one week for acclimatization prior to their use for experiments. Blood samples (control) were collected through a small cut made in the marginal ear vein of the rabbit. Rabbits were injected with 1·25% acetylphenylhydrazine (APH) for four consecutive days. Experimental bleedings were performed on the 5th, 7th and 9th day from the first injection (table 1). On the 9th day animal was sacrificed and blood was collected in a heparinized tube and filtered through cheese cloth. A small aliquot was kept separate for routine analyses for monitoring anemia as mentioned below. An hour before sacrifice rabbits were injected with 1000 units of heparin through the marginal ear vein. Blood was centrifuged at 600 *g* at 4°C for 10 min. Cell pellet was washed and reticulocyte lysate was prepared as described by Jackson and Hunt (1983).

2.3 *Determination of reticulocyte count, haematocrit level or packed cell volume and haemoglobin content*

Blood was collected in a heparinized tube and mixed with equal volume of Brilliant cresyl blue (1%). The mixture was incubated at room temperature for 30 min and smeared on clean slides. Slides were air dried and observed under light microscope. Reticulocyte count was expressed as % of RBCs.

Table 1. Schedule of induction of anemia in rabbits and its determination by measuring various parameters.

Day	−7	1	2	3	4	5	6	7	8	9
Injection		+	+	+	+					
Bleeding	+					+		+		+
Reticulocyte count (%)	1·60 (±0·10)					20·96 (±0·47)		40·75 (±7·95)		42·10 (±6·00)
Haematocrit level {PCV (1/1)}	0·44 (±0·00)					0·24 (±0·03)		0·24 (±0·03)		0·28 (±0·01)
Haemoglobin content (g%)	10·10 (±0·23)					6·25 (±0·25)		5·16 (±0·62)		6·66 (±0·47)
HRI*	01·0					16·0		21·7		23·8

*These values were obtained by scanning the intensity of HRI bands in Western blot (figure 2a).

Heparinized capillaries were filled with blood to approximately 3/4th the length, and sealed at one end with sealant (Critoseal) and were centrifuged at 10,000 *g* at room temperature for 10min. Haematocrit level or packed cell volume (PCV) was determined using a standard leading device and these values are expressed as length/length (1/1).

Haemoglobin (Hb) level in the blood samples was estimated by using a standard hemometer (Top, India).

2.4 *SDS-PAGE analysis*

Reticulocyte lysate samples were analysed on a SI) SDS-polyacrylamide gel (10 %). along with low molecular weight markers (Laemmlli 1970). (Gel was run at a constant current of 25 mA at room temperature Proteins were stained by Coomassie brilliant blue-R 250. Protein profile was analysed on UVP gel documentation system (Ultra-violet products Ltd., UK) and photographed.

2.5 *Western blotting*

Following SDS-PAGE. proteins were electrophoretically transferred to a nitrocellulose paper (0·45 μM: Schleicher and Schuell. USA) according to Fowbin *et al*. (1979), Western blots were then processed for immunoreaction involving chemiluminescence detection as per manufacturer's (Boehringer Mannheim) protocol. In brief, blots were saturated with 1% blocking reagent (overnight. 4C) and incubated with primary antibody in 0·5% blocking reagent overnight at 4C and then with peroxidase conjugated secondary antibody in 0·5% blocking reagent for 4 h at room temperature. Following each antibody incubation, blots were washed twice (15 min each) with 0·1% Tween 20 in Tris buffered saline (pH 7·5). Blots were developed in dark using the substrate (luminol) and intensity of the signals detected in X-ray film was compared by densitometric scanning and UVP gel documentation system.

2.6 *RNA extraction and Northern blot analysis*

Total RNA was extracted from 1·0 ml of blood samples collected from rabbits at different days during induction of anemia by guanidinium thiocyanate method as described by Chomovynski and Sacchi (1987). Samples containing equal amount of total RNA (50 μg) were electrophoresed on a formaldehyde agarose (1%) denatureing gel. Following electrophoresis, RNA was transferred onto nylon membrane (Pall Biodyne, USA). The membrane containing RNA was hybridzed to (α-^{32}P) ATP-labelled HRI cDNA probe (Chen *et al* 1991). HRI cDNA was labelled by random priming reaction using New England Biolabs random priming kit as per the manufacturer's protocol. The membrane was prehybridized in a buffer containing 5X SSPF, 50 mM NaH_2 PO_4, IX Denhardt's solution. 0·1% SDS, 100 μg/ ml of salmon sperm DNA and 50% formamide for 6h at 42° C and hybridized in the same solution containing radiolabelled HRI cDNA (specific activity = $2{\cdot}1 \times 10^8$ cpm/μg) for 16h at 42C. The blot was washed with 2X SSC, 0·1% SDS, thrice (15 min each), at room temperature and subsequently in 0·1 X SSC. 0·1% SDS twice (15 min each) at 65C. The membrane was dried, exposed to X-ray film and autoradiographed.

2.7 *In vitro eIF-2α kinase assay*

Protein kinase assay mixture (25 µl) contained 20mM Tris-HCI (pH 7·6, 40- 60mM KCl, 2 mM $Mg(OAc)_2$ and 5·8 µCi [γ–^{32}P] ATP (specific activity = 3000 Ci/mmol). Reticulocyte lysate was added to the reaction mixture and incubated for 20 min at 30° C. The details of other additions are mentioned in the figure legends. Reaction was stopped by adding NaF-EDTA, and proteins were precipitated at pH 5 by adding acetic acid (Pal *et el* 1991). Protein pellet obtained was treated with SDS-sample buffer (l.aemmli 1970). Proteins were separated by SDS-PAGE and stained. Gels were dried and autoradiographed with Nieue films (Japan).

3. Results

3.1 *Determination of reticulocyte count, haematocrit level* (*PCV*) *and haemoglobin content during anemia*

The degree of anemia at various stages during induction was determined by measuring the parameters that are routinely used, such as reticulocyte count, haematocrit level and haemoglobin content (table 1). These values are representative of several estimations earned out during a number of identical experiments. Although individual variations were there, the general pattern remained the same. Reticulocyte count increased significantly on day 5. but it did not show much increase between day 7 and day 9. whereas the increase in HRI quantity was significant between day 5 and day 9 (table 1 and figure 2a). Haematocrit level went down by two-fold on day 5 as compared to that of control. However it did not show any difference between day 5 and day 7. Furthermore, on day 9 haematocrit level increased although the animal did not show any sign of recovery as indicated by reticulocyte count, haemoglobin content and HRI quantity. Similarly haemoglobin content did not reflect the degree of anemia during day 7 and day 9.

3.2 *SDS-PAGE analysis*

In order to determine the pattern of change in the protein profile during anemia, reticulocyte lysates prepared from blood samples of control (prior to induction of anemia) and anemia rabbits on various days during anemia were analysed by SDS-PAGE using the same amount of protein. Protein concentration of samples was determined by the method of Bradford (1976) using BSA as a standard. Although the SDS PAGE protein profiles of non-anemic and anemic samples (figure 1) were comparable to each other, there were significant quantitative changes of some of the polypeptides as described below. A few polypeptides (indicated with asterix in figure 1) increased in quantity upon induction of anemia, while some others (indicated with arrows) decreased at the begining of induction of anemia, but reappeared with the progression of anemia (figure 1). Protein profiles of the two rabbits (A and B). one of which (B) recovered due to the individual response to the drug, are shown to indicate the differences in their profiles (compare lanes 5 and 9)

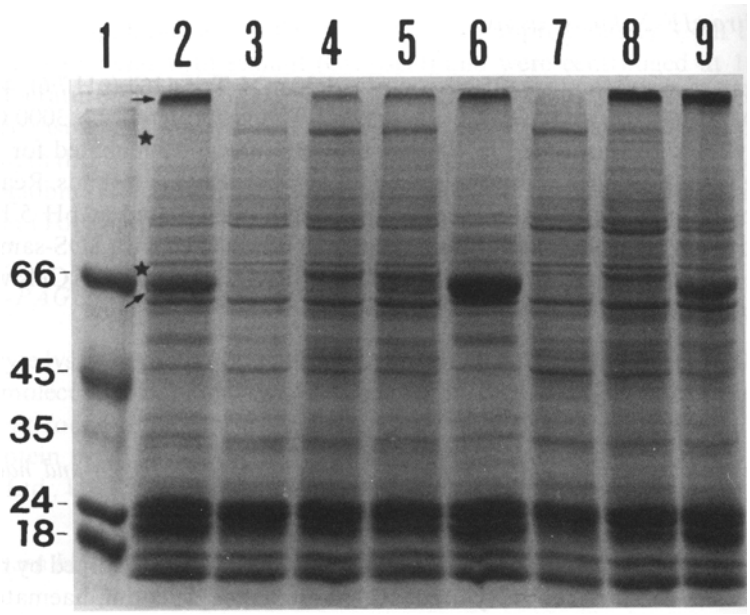

Figure1. SDS-PAGE analysis of reticulocyte lysates from control and anemic rabbits. 650 µg of total soluble proteins were used for electrophoresis Samples were loaded as follows: Control (lanes 2 and 6), day 5 (lanes 3 and 7), day 7 (lanes 4 and 8) and day 9 (Lanes 5 and 9). Asterisk indicates the polypeptides which increased in quantity upon induction of anemia: arrow indicates the polypeptides which disappeared following the last injection. It is to be noted that rabbit B (lanes 6–9)had partially recovered from anemia on day 9 . Lane 1 contained molecular weight marker proteins.

3.3 *Expression and activity of HRI in reticulocytes during anemia*

3.3a *Western blot analysis* : Pattern of change of HRI quantity during anemia was determined by Western blotting of proteins from a similar gel as shown in figure 1. As seen in figure 2a. mAb F recognized the expected 92kDa HRI polypeptide in all the anemic samples and there was a faint signal in the control lysate also. Intensity of the signal clearly indicated a gradual increase in amount of HRI during anemia. An identical pattern was also seen with colour detection method using alkaline phosphatase conjugated second antibody, except that HRI signal in day 5 sample was barely detectable (results not shown). Interestingly, when one of the rabbits recovered due to an individual response to the drug, the quantity of HRI went down on day 9 as compared to that on day 7 (figure 2b). while the quantity of another protein, hsp 70 (detected by anti-hsp 70 monoclonal antibody) did not decrease (figure 2c). Differences in the intensity of signals in rabbits A and B were merely due to individual variation at the level of anemic response. Quantitative changes in the level of HRI peptide for rabbit A were estimated by scanning of Western blot signals and the fold increase is indicated in table 1.

3.3b *Northern blot analysis*: We also determined the pattern of expression of HRI at the level of mRNA during anemia. The results presented here (figure 3) were obtained from the blood samples of the rabbit B which recovered from anemia on day 9 (as described above). Northern blot (figure 3) of total RNA extracted from blood

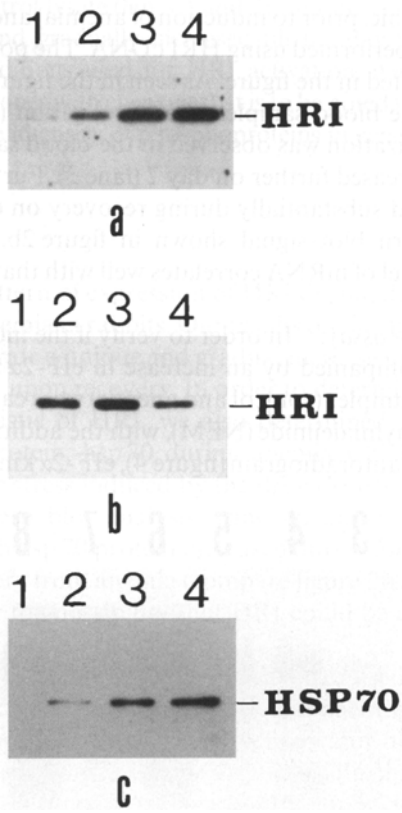

Figure 2. Western blot analysis of reticulocyte lysates during anemia, (**a**) and (**b**) are Western blots with anti-HRI monoclonal antibody, mAb F; in (**b**), the samples of the rabbit B which partially recovered from anemia on day 9 were used. Lanes 1–4 contain samples from control rabbit (lane 1) and anemic rabbit on day 5 (lane 2), day 7 (lane 3) and day 9 (lane 4), respectively. (**c**) Same as (**b**) except that the Western blot was carried out with anti-hsp 70 monoclonal antibody.

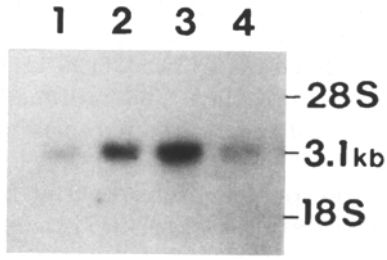

Figure 3. Northern blot analysis of total RNA extracted from blood samples of rabbit during progression of anemia with HRI cDNA. 50 µg of total RNA from control rabbit (lane 1) and anemic rabbit on day 5 (lane 2). day 7 (lane 3) and day 9 (lane 4) were electrophoresed on a denaturing Formaldehyde Agarose gel, transferred to nylon membranes and were hybridized with HRI cDNA probe. In the autoradiogram, HRI mRNA signal (3·1 kb) and the positions of 28 S and 18 S ribosomal RNAs are indicated. The details of conditions of hybridizations and washings are mentioned in the text. It is to be noted that this rabbit (rabbit B) had partially recovered from anemia on day 9.

samples of control (nonanemic, prior to induction of anemia) and anemic rabbit during progression of anemia was performed using HRI cDNA. The positions of 28 Sand 18S ribosomal RNAs are indicated in the figure. As seen in the figure, HRI mRNA (3·1 kb) was barely detectable in the blood samples of control rabbit (figure 3, lane 1), while a significant level of hybridization was observed in the blood sample of anemic rabbit on day 5 (lane 2) which increased further on day 7 (lane 3). Furthermore, the quantity of HRI transcript decreased substantially during recovery on day 9 (lane 4) which is consistent with the Western blot signal shown in figure 2b. Thus, the pattern of expression of HRI at the level of mRNA correlates well with that at the level of protein.

3.3c *In vitro eIF-2α kinase assay*: In order to verify if the increase in HRI quantity during anemia is also accompanied by an increase in eIF-2α kinase activity, kinase assay of all the four lysate samples (control and anemic) were carried out either as such or upon induction by N-ethylmaleimide (NEM), with the addition of purified eIF-2 as the substrate. As seen in the autoradiogram (figure 4), el F-2α kinase activity was almost

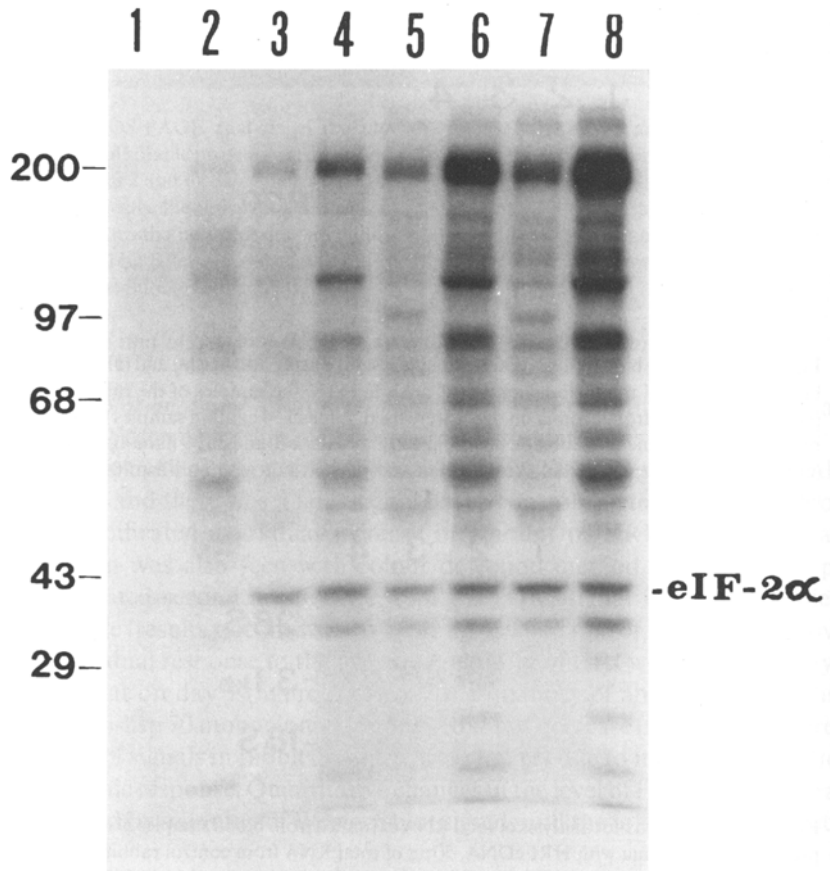

Figure 4. Heme-regulated eIF-2α kinase assay profile (autoradiogram) of reticulocyte lysate during anemia. Samples loaded were from control (lanes 1 and 2) and anemic rabbit on day 5 (lanes 3 and 4), day 7 (lanes 5 and 6) and day 9 (lanes 7 and 8). In each set of two lanes, the left one is reticulocyte lysate without hemin (– hemin) and the right one is the lysate treated with 5 mM NEM (+NEM).

undetectable in the control lysate (lanes 1 and 2), and the activity started increasing upon induction of anemia and gradually increased till the day 9 (lanes 3–8). This was true irrespective of the conditions used for HRI activation, namely, – heme and + NEM (figure 4; compare odd lanes with even ones). It is to be noted that with the same quantity of lysate samples used, the intensity of phosphoproteins in general increased during anemia.

4. Discussion

We report here the pattern of expression of HRI during drug (acetylphenylhydrazine) induced haemolytic anemia in rabbits. Our results on the quantification of HRI protein in particular, demonstrate a unique and gradual increase in HRI during anemia and its concomittant decrease upon recovery. In order to determine the specifity and uniqueness of this characteristic of HRI, we also determined the pattern of quantitative changes of another protein. hsp70 during anemia which is known to overexpress during haematopoietic stress induced by the drug (Matts *et al* 1992; Gross *et al* 1994; Pal *et al* 1996). Western blot analysis using an anti-hsp 70 monoclonal antibody indicated that although hsp 70 protein increased during anemia, unlike HRI, it did not decrease during recovery from anemia (compare figure 2b and 2c). These results taken together, thus indicate the possibility that HRI could be used as a specific molecular indicator of anemia.

While comparing with other parameters that are used for clinical investigations, namely, reticulocyte count, haematocrit level and haemoglobin content, it is evident that HRI is a more appropriate and sensitive indicator of anemia, in determining its degree of intensity in particular (see table 1). Our results thus, for the first time indicate the possibility of detecting anemia using anti-HRI monoclonal antibody as a sensitive probe. Thus, we suggest that an anti-human HRI monoclonal antibody based Western blot kit may be very useful, along with other existing clinical investigations, for a better analysis and assessment of the status of anemia in human patients.

Protein profiles of reticulocyte lysates prepared from the blood samples of rabbits during progression of anemia, indicate a number of significant quantitative changes: some of the proteins (indicated by asterisk in figure l)show substantial increase in the quantity upon induction of anemia. A large number of heat shock and stress related proteins have been shown to be synthesized under adverse conditions of survival (reviewed in Pal *et al* 1996). Drug induced anemia being a haematopoietic stress to the rabbit as discussed above, may lead to the synthesis of such proteins. However, further characterization of these proteins will be necessary to establish their relationship with heat shock/stress proteins.

It is well established that during anemia, inhibition of globin synthesis is due to the activation of the heme-regulated eIF-2α kinase (reviewed in Chen and London 1995). Therefore, we carried out *in vitro* eIF-2α kinase activity of the reticulocyte lysate samples under two conditions, namely, absence of heme and presence of NEM which is also known to induce el F-2α kinase activity even in the presence of hemin (reviewed in Pal *et al* 1996). Results presented in figure 4 which indicate a gradual increase in the heme-dependent eIF-2α kinase activity during anemia, therefore, further confirm the validity of our schedule of induction of anemia.

A simultaneous increase in the quantity of HRI and its kinase activity in the blood samples during anemia is not well understood. Based on the earlier observations that

HRI is abundantly present in the reticulocytes and not in the mature erythrocytes (reviewed in London *et al* 1987), we speculate the following. During anemia when peripheral blood gets enriched with reticulocytes, there is an automatic increase in the quantity of HRI. However since there is about 8-fold difference between the quantities of HRI mRNA and protein (Crosby *et al* 1994), there may be a preferentially high rate of translation of HRI mRNA. However, further research is necessary to determine the mechanism of translational regulation of HRI mRNA. With regard to the high level of heme-dependent eIF-2*α* kinase activity, heme-deficiency alone perhaps cannot account for. It has been reported earlier that hsp 90 interacts with HRI and results an increase in its eIF-2*α* kinase activity (Matts *et al* 1992; Pal 1994, reviewed in Pal *et al* 1996). Since hsp90 is overexpressed during anemia, we speculate that more of hsp 90 could be mobilized to form complex with HRI and thereby results in increased kinase activity of HRI. Thus, both these two mechanisms may be operating in the reticulocytes *in situ* to cope up with the adverse condition of anemia.

In conclusion, we propose here that HRI could be a molecular indicator for detection of drug induced anemia in rabbits. Further experiments are in progress to establish the universality of HRI as an indicator for various other types of anemia either associated with a number of pathological disorders, such as thalassemia and haemophilia, or caused by other factors, such as heavy metal poisoning.

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