INTRODUCTION
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Work directed towards the understanding of the regulatory mechanism of cytokines on lymphocytes, has revealed an unexpectedly complex and interlocking cytokine network. Interaction of cytokines and cells provide a critical regulatory influence on the ongoing immune response \textit{in vivo} where a variety of cell type may be stimulated to produce different cytokines, some inhibitory, some stimulatory at varying times and in varying amounts. For these reasons, cytokines have been found associated with several disease processes and complications.

Tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) mediates diverse pathophysiological events (similar to those of hemolytic transfusion reactions) such as fever, intravascular coagulation and organ failure (Davenport \textit{et al}., 1991). There are several reports, which points to the fact that RBC hemolysis is accompanied by TNF-\(\alpha\) production. In the \textit{in vitro} model of whole blood transfusion reaction, it has been demonstrated that hemolysis of incompatible red cells was accompanied by TNF-\(\alpha\) production induced by TNF-\(\alpha\) gene in the buffy coat of leukocytes (Davenport \textit{et al}., 1991). The occurrence of TNF-\(\alpha\) and TNF-\(\alpha\) soluble receptors in erythrocyte concentrates has been reported employing ELISA technique (Kristiansson \textit{et al}., 1996a,b). Earlier, we have observed altered level of TNF-\(\alpha\) (unpublished observation) in partially hemolyzed mouse plasma and immunoblot analysis of plasma/serum revealed additional band other than 17 kDa peptide that cross-react with antibodies to mouse TNF-\(\alpha\) (Paul \textit{et al}., 1993, Paul and Saxena, 1997). The pathophysiological concern over the hemolytic transfusion of whole blood components (Kristiansson \textit{et al}., 1996a,b; Fong \textit{et al}., 1990) and transfusion induced immunomodulation (Klein, 1996) drove us to study the pattern of cross reactivity
between the major protein of erythrocyte lysate and antibodies to TNF-α. Hemoglobin comprises more than 90% of the total protein present in the erythrocyte lysate. Our objective is to investigate whether human hemoglobin can be recognized by antibodies to human TNF-α and shares bioactivities previously ascribed to TNF-α. This study will help in understanding non-bacteremic febrile transfusion reaction, which can occur after transfusion of stored (not fresh) blood. Alternatively, it will explain the source of pyrogenic cytokines, especially TNF-α, during the transfusion of stored blood.

OBJECTIVES

The present study was undertaken with the following objectives:

➢ Preparation of purified hemoglobin from fresh human erythrocytes
➢ Recognition/cross-reactivity of hemoglobin by antibodies to human TNF-α
➢ Determination of affinity constant
➢ Bio-activity studies with human hemoglobin
➢ Mapping of TNF-α like epitope on human hemoglobin
REVIEW OF LITERATURE
2. REVIEW OF LITERATURE

Biologically, TNF-α and hemoglobin are two different molecules performing diverse functions. The former molecule is a cytokine of pleotropic activity (Le et al., 1987; Wingfield et al., 1987; Tracy et al., 1987), while the latter is responsible for cell respiration, energy formation, organ perfusion and oxidative biotransformation (Lu et al., 1983; Chertkov et al., 1991; Lavrovsky et al., 1994; Chang et al., 1999; Alayash et al., 1999). Both, the TNF-α and hemoglobin differ in their molecular weights, structures, amino acid sequences and bioactivities.

Hemoglobin resides in the red blood cell cytosol. There are several reports appearing nowadays, revealing that red blood cell lysis is accompanied by TNF-α production. Interleukin-1β, interleukin-6 and TNF-α or the chemokines like interleukin-8 and monocyte chemoattractant protein-1; and interleukin-1 receptor antagonist may mediate many of the sequels of hemolytic transfusion reactions. Experimental models of both the acute and delayed hemolytic transfusion reactions demonstrate the production of these molecules (Davenport et al., 1996). Earlier, Davenport et al., (1991), demonstrated in the in vitro model of whole blood transfusion reaction, the lysis of incompatible red cell is accompanied by TNF-α production induced by TNF-α gene in the buffy coat of leukocytes. The rise in TNF-α in the early two hours is related to the rise in plasma hemoglobin. Heddle et al., (1994), had demonstrated the rise in IL-1 and IL-6 during febrile non-hemolytic transfusion reaction but the release of TNF-α was not shown. Increase in TNF-α level and the presence of IL-1 and IL-6 in the plasma of stored platelet concentrates have been reported (Muyllle et al., 1993). Stack et al., (1995) reported the
accumulation of IL-8 and IL-1 in the supernatants of RBC suspended in additive solution despite cold-storage conditions. They described IL-8 levels of >1000 pg/ml in some RBC units. They observed RBC with mean leukocyte contamination of 4760±3780/μL. WBC filtration early in storage prevented the accumulation of IL-8 and IL-1. They did not detect IL-6 in any unit and did not measure TNF-α levels in erythrocyte concentrates. Kristiansson et al., (1996) also reported the presence of TNF-α as well as IL-1 and IL-6 in erythrocyte concentrates. The concentrations of TNF-α and its soluble receptor were analyzed using enzyme immunoassay kit. The detectable concentrations for TNF-α, sTNF-R1 and sTNF-R11 were 3, 50 and 100pg/mL, respectively. The coefficients of variation, inter-assays and intra-assays were less than 10%. Consistently, high level of TNF-α in α- and β- thalassaemic patients was reported (Butthep et al., 2002), where the release of globin chains in peripheral blood, accompanied by senescence of immature red blood cell and increased phagocytosis was observed (Rachmilewitz et al., 1980; Schroit et al., 1991; Borenstein et al., 1993; De Jong et al., 1997).

The TNF-α has been shown to inhibit erythropoiesis in vitro (Rusten et al., 1995) accompanied by alteration in the red blood cell kinetics (Moldawer et al., 1989). The increased level of TNF-α was observed when monocytes with Plasmodium falciparum schizont stage parasitised erythrocytes ruptured (Pichyangkul et al., 1994). The authors proposed that the pigment isolated from ruptured schizont specifically induce the release of large quantity of TNF-α and IL-1 from monocytes. In the test tube models of intravascular hemolysis and IgG mediated red cell incompatibility, the release of IL-6, IL-8 and TNF-α have been demonstrated (Davenport et al., 1990; Hoffman et al., 1991; Davenport et al., 1993).
Earlier, Stack et al., (1994), suggested that cytokines could be derived from three possible sources in the setting of blood transfusion: 1) synthesis by recipient WBC in response to transfusion; 2) synthesis by donor WBC after transfusion to the recipient; and 3) synthesis by donor WBC in vitro during storage prior to transfusion (Dzik et al., 1992). Rakic (1999) had shown that TNF-α, IL-1, IL-6 and IL-8 play a major role in the pathophysiology arising due to acute hemolytic transfusion reaction. The factors affecting the outcome of hemolytic transfusion reaction includes shock, inflammation and disseminated intravascular coagulation. In a case report, the 67 years old patient (Blood group O, D-negative) with a negative pre-transfusion antibody screen was given a massive transfusion because of arterial bleeding. Ten days later the patient developed delayed hemolytic transfusion reaction and mediators like TNF-α, IL-6, IL-8, IL-10, neopterin, elastase, C-reactive protein and fibrinogen were found elevated (von Zabern et al., 1998). Increased release of IL-6 and TNF-α in women with syndrome of hemolysis has been reported by others (Haeger et al., 1996). Weisbach et al., (1999) showed generation of cytokines in whole blood leukocyte depleted and temporarily warm blood cell concentrates. The level of TNF-α and IL-1β rose during storage of whole blood and red blood cells. However, pre-storage filtration of RBC prevented the accumulation of TNF-α and IL-1β.

There are several studies indicating that hemoglobin share some of the bioactivities previously ascribed to TNF-α. Donghul et al., (1997, 1999) had shown in their study that hemoglobin infusion augments the TNF-α response to bacterial endotoxin (LPS) in mice. Administration of recombinant TNF-α has been shown to induce septic shock or increase mortality in mice (Tracy et al., 1986). Griffith et al., (1995), demonstrated that
hemoglobin mediates sepsis in the mouse model. Van Hinsbergh (2001), demonstrated that the endothelial cells are equipped with a number of mechanisms that prevent thrombus formation in the circulatory system. It harbors factor that interrupts the coagulation cascade, such as antithrombin III, the protein C receptor thrombomodulin, and tissue factor pathway inhibitor. It prevents platelet activation by the production of nitric oxide and prostacyclin, exonucleotides and surface heparan sulphate. Furthermore, it can trigger and control fibrinolysis by the synthesis and release of tissue-type plasminogen activator and its inhibitor PAI-I. The general properties of the endothelium are subject to adaptation by environmental factors, such as inflammatory mediators and shear forces. IL-1 and TNF-α reduces the antithrombotic properties of the endothelium.

In cultured endothelial cells, Matsumura et al., (1989) and Saito et al., (1989), showed the synthesis and release of the potent vasoconstrictor peptide, endothelin-1 (ET-1). However, in the culture of endothelial cells, thrombin (Schini et al., 1989) and transforming growth factor-β1 (Kurihara et al., 1989) have been shown to stimulate the release of ET-1 and the expression of ET-1 m-RNA. Ohkita et al., (2002), showed the release of ET-1 following TNF-α treatment in cultured vascular endothelial cell. Release of ET-1 following TNF-α treatment in the epithelial cells of normal and injured renal tubules and its influence on renal interstitial fibroblast was also reported (Zhi, 1999).

Similarly, hemoglobin has been shown to increase the production of ET-1 in endothelial cell culture (Cocks et al., 1991). Although hemoglobin is a potent vasoconstrictor of cerebral arteries, it has been postulated that extravascular hemoglobin may be at least one of the contributing factor involved in cerebral vasospasm following subarachnoid hemorrhage. Exposure to TNF-α or inflammatory mediator can rapidly lead to pro-
coagulant response activated by bacterial endotoxin and decrease of the endothelial anti-
coagulant membrane components. Hemoglobin has been demonstrated to enhance the
production of tissue factor in LPS treated endothelial cells (Roth et al. 1994). It also
possesses procoagulant activity with dose dependent rise in hemoglobin concentration
(Smith et al., 1992).

Hemoglobin and certain proinflammatory cytokines (TNF-α, IL-1, IL-6, IL-8) play a
role in metabolic response during trauma and infection (Kristiansson et al., 1996a,b).
Bunn et al., (1969), demonstrated the release of hemoglobin into the circulation resulting
in the dissociation of 64 kDa hemoglobin tetramer present in the RBC into two (α-β)
dimers with molecular weight of 32kDa which easily pass through the glomerulli (half
life 10-30 min.) and is cleared by mononuclear system (Jones et al., 1995). The heme
oxygenase and hemopexine also play a role in the degradation of hemoglobin by
reticuloendothelial system (Waener et al., 1997). In circulation, the dissociation of
hemoglobin from the 64kDa tetramer to 32kDa dimer is dependent on the concentration
of free hemoglobin; more the hemoglobin tetramer, less is the formation of 32kDa dimer.
The reverse is also true. The kidney subsequently clears these.

Hemoglobin, has a multiple immunoregulatory effect, including the induction of cytokine
production such as TNF-α, IL-1, IL-8 in the mononuclear cell culture (Novogrodksky et
al. 1989; Simoni et al., 1994; Mc Faul et al., 1994; Hess, 1995) and also constitute a
major danger to vascular integrity (Balla et al., 1991; Balla et al., 1991 Balla et al.,
1993). The TNF-α at high concentration initiates potential lethal microvascular
coagulation (Beutler et al., 1986) and is a crucial mediator in septic shock and cerebral
malaria as demonstrated for hemoglobin (Kwiatkowski et al., 1989; 1990).
Patients undergoing major surgery and those critically ill, some time require massive transfusion of whole blood, demonstrated several changes that suggest modulation of immune response. Blood transfusion may be associated with negative effect on cancer recurrence and survival (Burrows et al., 1982), increased susceptibility to post operative infection (Blumberg et al., 1988; Murphy et al., 1991), activation of latent viruses (Ward et al., 1989), amelioration of recurrent abortion (Taylor et al., 1981) and suppression of immuno-inflammatory response. Most of these reactions are the result of ABO blood type incompatibilities (Davenport et al., 1991)

TNF-α is a 17 kDa polypeptide (Beutler et al., 1986) and has been shown to play a significant role in mediation of septic shock. Biological activity attributed to TNF-α includes fever, shock, capillary leakage, leukocyte chemotaxis, activation of leukocyte and endothelial cell, and activation of intravascular coagulation cascade. These symptoms are also observed during hemolytic transfusion reaction (Pineda et al., 1978) and have been also demonstrated with the release of cell free hemoglobin (Velky et al., 1987) Simoni et al., (1998), evaluated the effect of unmodified hemoglobin on human endothelial cells. They found it to be a potent scavenger of nitric oxide, induces significant depletion of reduced glutathione, acceleration of lipid peroxidation, greater influx of Ca^2+, great activator of nuclear-κβ and a stimulator of intracellular adhesion molecule-1 expression. The formation of 8-iso prostaglandin F_2α increased in comparison to control endothelial cells. Abassi et al., (1997), showed the significant induction of both constitutive endothelium and induces nitric oxide synthase in aortic tissue after 120 to 200min in test animal. Cassidy et al., (2000), demonstrated that the hemoglobin on incubation with poly-morphonuclear neutrophils induces cell death mediated by release
of nitric oxide. Simoni et al., (1994), demonstrated the reaction of human endothelial cells to TNF-α and bovine hemoglobin solution. Direct stimulation of endothelial cell by hemoglobin for the production of IL-1 was found limited. However, GM-CSF expression in endothelial cells was increased with treatment of hemoglobin. In contrast, intermediate levels of GM-CSF expression in endothelial cells was observed in coculture with TNF-α (400pg/ml). The induction of proinflammatory cytokine (IL-1, IL-6, IL-8, TNF-α) has been demonstrated on incubating hemoglobin with whole blood leukocyte but they did not measure the cytotoxicity (Mc. Faul et al., 2000). However, Kim et al., (1999), demonstrated receptor mediated endocytosis of hemoglobin and induce cytotoxicity in polymorphonuclear neutrophil. Mc Faul et al., (1994), showed stimulation of mononuclear leukocytes by hemoglobin to release IL-8 and TNF-α. They demonstrated the PMN/endothelial cell adherence activity by using antibodies to TNF-α and found higher adherence activity for hemoglobin treated mononuclear cell supernatant in comparison to human serum albumin treatment.

The release of hemoglobin has major role in oxidative damage. It may be due to rise in TNF-α in the case of sepsis (Donghui et al., 1999). Both, the TNF-α and hemoglobin mediate oxidative stress by releasing reactive oxygen species (Larrick et al., 1990; Simoni et al., 1990; Robert Motterlini et al., 1995), which interfere in the mitochondrial respiration. The role of nitric oxide in these processes has been implicated. The role of hemoglobin in inducing apoptosis (Oghihara et al., 1999) or cell death or oxidative stress is thought to be due to release of reactive oxygen species by monocytes (Simoni et al., 1989), endothelial cell (Gorbunov et al., 1997) via the release of nitric oxide and ADP-ribosylation. The role of NF-κβ has also been implicated. Hemoglobin has been shown to
induce oxidation of low density lipoprotein, which is a major cause for initiation and propagation of atherosclerosis (Ziouzenkova et al., 1999; Jeney et al. 2002). Recently, hemoglobin has been shown to induce oxidative damage by two apparent pathways. One is via release of superoxide radical or hydrogen peroxide and others is caspase mediated. However, inhibitors of superoxide radical or hydrogen peroxide and caspase did not extend additional protection in rat cerebral cortical neurons (Wang et al., 2002). Coincidentally, TNF-α also releases reactive oxygen species and mediated oxidative damage.

It is well known that TNF-α is cytostatic/cytotoxic for many tumor cells in vitro (Sugerman et al., 1985), but has been also shown to stimulate the growth of normal fibroblast (Vilcek et al., 1986). Stimulation of growth was demonstrated at TNF-α concentration of $10^{-12}$ to $10^{-13}$ M. However, at an equal or lower concentration of TNF-α in the presence of mitomycin induce cytotoxicity in highly sensitive transformed cell line. The growth stimulatory activity was seen with TNF-α in WI-38 cell line of diploid human embryo lung fibroblast and FS-4 cell line of human fore skin fibroblast indicating that TNF-α in general is a fibroblast growth factor. Monocyte/macrophage-derived fibroblast growth factor activity is also due to the presence of TNF-α (De Lustro et al., 1980; Glenn et al., 1981; Wharton, et al., 1982; Wahl et al., 1985). Likewise the hemoglobin or globin without the heme group has been shown to possess stimulatory activity on some of the human fibroblast cell lines such as TIG3-20, TIG7-20, TIG-103 and WI-38 at a concentration of $1.5\mu$M for 16 hours. They demonstrated slow induction of urokinase-type plasminogen activator after 12 hours of incubation (Yoshida, et al., 2001).
Many cell activation pathways initiate the metabolism of arachidonate. Phospholipase A2 plays a central role in this pathway by generating arachidonic acid from plasma membrane bound phospholipids. Inhibitors of the arachidonic acid pathway reduce TNF-α mediated cytotoxicity. Suffys et al., (1987), demonstrated that the dexamethasone, a potent corticosteroid, inhibits the release of arachidonic acid and TNF-α mediated cytotoxicity. Apparently TNF-α also induces a PLA2-activating protein in endothelial cells (Clark et al., 1988). Takenaka et al., (1993), demonstrated the oxyhemoglobin-induced cytotoxicity and arachidonic acid release in cultured bovine endothelial cell line and showed reduction in cell cytotoxicity by using 4- bromophenacyl bromide, a phospholipase A2 inhibitor. In contrast, treatment with indomethacin (cyclooxygenase inhibitor) did not show protection against oxyhemoglobin induced cytotoxicity. Dexamethasone was also shown to inhibit the TNF-α mediated cytotoxicity in U937 cell line (Neale et al., 1988).

Hence, it is necessary to find out, whether, the red cells can produce TNF-α or some other protein present in erythrocyte mimics TNF-α. Human red blood cells are none nucleated and therefore the presence of TNF-α gene is beyond question. Therefore RBCs cannot be induced to secrete TNF-α via induction of TNF-α gene. Monocytes, macrophages, T cells, B cells, NK- cells, Kuffer cells of the liver and glial cells of the central nervous system have been reported to secrete TNF-α after exposure to endotoxin or products of bacteria, viral or parasitic or inflammatory origin (Tracy and Cerami 1992). Because of the pathophysiological concern over the hemolytic transfusion of whole blood components (Fong et al., 1990; Kristiansson et al., 1996(a,b)) and transfusion induced immunomodulation (Klein et al., 1996) we sought to study the
reaction of human hemoglobin (the major component of hemolysate comprising >90% of the total hemolysate) with antibodies to human TNFα. Furthermore, as evident from the review, TNF-α and hemoglobin are interrelated and shares many bioactivities like apoptosis in different cell lines, nitric oxide release etc. It is therefore pertinent to ask the question whether the two molecules, TNF-α and hemoglobin have similarities in term of bioactivities and immunoaffinity. We therefore, sought to study the antibody recognition pattern with their kinetics and bioactivities.
3. MATERIALS AND METHODS

3.1 MATERIALS

All the chemicals/reagents used in this study were of the analytical grade and obtained from Glaxo Laboratories, India; Sisco Research Laboratories, India; E.Merck (India) Ltd., India; Ranbaxy Laboratories Ltd., India and Spectrochem Pvt. Limited, India. Fine chemicals and biochemicals were procured from Sigma Chemical Co.(USA), ELISA assay kits were procured from Genzyme Corporation (USA), and R & D (USA), ficoll hypaque (pharmacia), DEAE cellulose (Whatman, UK), cellulose acetate strip (Oxiod, UK).

3.2 METHODS

3.2.1 Source of blood

Blood was collected from healthy volunteers.

3.2.2 Isolation of erythrocytes

Blood was collected in 1/9 Alsever's solution and centrifuged at 2000rpm for 20min at 25°C. The entire plasma was discarded. Erythrocytes were washed three times with normal saline and centrifuged at 2000rpm for 10 min at 25°C. The erythrocyte packed volume was resuspended in normal saline and leukocytes were removed by 2-3 cycles of
centrifugation at 2000rpm for 20 minutes on ficoll-hypaque cushion. The platelets and
leukocyte contamination were checked by using Neubauer haemocytometer under phase
contrast microscope and bright field microscope respectively. For platelet count, the
haemocytometer with the sample was kept in a humid temperature for 10-15 min. prior to
phase contrast microscopy.

3.2.3 Preparation and purification of hemoglobin
Ficoll- hypaque purified erythrocytes were washed thrice with normal saline and lysed by
giving hypotonic shock with distilled water in a ratio 1:20 for 20min. at room
temperature and centrifuged at 8000rpm for 30 min. The RBC ghost was discarded. The
cytosol rich in hemoglobin (Peri et al., 1999) was collected and applied on DEAE
cellulose column equilibrated with 50mM Tris HCl, pH 8.5 for 12 hours (Huisman and
Dozy et al., 1965). A 0.2 ml of cytosol containing 1.8 mg of hemoglobin was applied on
DEAE cellulose column (15cm x 0.5cm). The first fraction of hemoglobin was eluted
with 50mM Tris HCl (pH- 8.4) and the second fraction with 50mM Tris HCl (pH- 7.5), at
a flow rate of 10mL/hr.

3.2.4 Spectral analysis of hemoglobin fraction
UV spectral analysis of hemoglobin fraction was performed in normal saline at a
concentration of 1 mg/ml and the absorbance spectrum plotted over the range of 350nm to
700nm (Dilorio, 1981) in Cintra 40 double beamed spectrophotometer.
3.2.5 Analysis of hemoglobin fraction by reverse phase HPLC

The purity of hemoglobin was checked by reverse phase HPLC (Waters Pvt. Ltd). Hemoglobin fractions collected at pH- 8.4 and 7.5, and standard hemoglobin (Sigma Co.) were separately performed by reverse phase HPLC column. Reverse phase column (Merck- 50983, Analytical grade, Lichrosphere-100, RP-18, 250 x 4mm) was thoroughly washed with 100% Acetonitrile (filtered thoroughly 0.22μm membrane) at a flow rate of 1ml/min. Finally, hemoglobin fraction were loaded on the column. Injection volume was 10 μL. The different eluted hemoglobin fraction (6 ppm each) were analysed by gradient programme of two solvents comprise of solvent A- 0.1% TFA in Millipore water and solvent B- 0.1% TFA in acetonitrile (filtered by 0.22μm membrane), simultaneously the absorbance was recorded at 220 nm (Jones, 1994).

The gradient programme of two solvents is as follows:

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<th>Time(min.)</th>
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3.2.6 Determination of hemoglobin concentration

The method of Tentori and Salvate (1981) was adopted. Fresh erythrocyte lysate (0.02 mL) was mixed with 5 mL of hemoglobin estimation reagent [Potassium ferricyanide (200 mg) + potassium cyanide 50 mg + potassium dihydrogen phosphate (140 mg) + tritonX-100 (1 mL/litre) dissolved in 1 L; pH 7 to 7.4] and incubated at room temperature in brown bottle for 3 mins. Optical density was taken at 540 nm in a spectrophotometer. The hemoglobin concentration was calculated using formula as below:

\[
\text{Concentration (g/L)} = A_{540} \cdot F \cdot \frac{M}{E_{540}} \cdot L
\]

Where  
F = dilution factor  
M = molecular weight of the Hemoglobin monomer  
L = light path in cm  
\(A_{540}\) = optical density of Hb\(^+\)-CN\(^-\) solution at 540 nm  
\(E_{540}\) = millimolar extinction coefficient of Hb\(^+\)-CN\(^-\) monomer at 540 nm

3.2.7 Total protein estimation of hemoglobin fraction

Total protein was estimated by adopting modified Biuret technique in Autoanalyser (Chemwell, USA). The total Protein assay kit (SPINREACT, Spain Cat. No.1001291) was used in all throughout the experiments.
3.2.8 Determination of molecular weight of hemoglobin fractions by native PAGE and SDS-PAGE

Apparatus

Complete mini slab-gel electrophoresis apparatus (Biotech), electrophoresis constant power supply (Biotech)

Chemicals

Acrylamide, N, N-methylene-bis-acrylamide, tris-(hydroxymethyl) aminomethane (Tris), glycine, sodium dodecyl sulphate (SDS), ammonium persulphate, N', N, N, N-tetramethyl ethylenediamine (TEMED), 2-mercaptoethanol, bromophenol blue, glycerol, glacial acetic acid, hydrochloric acid, coomassie brilliant blue R-250 and methanol.

3.2.8.1 Native poly acrylamide gel electrophoresis

Briefly, the two hemoglobin fractions purified by DEAE cellulose column were characterized by native PAGE (10%) under non-reducing condition by vertical gel electrophoresis and stained with coomassie brilliant blue R-250. For molecular weight determination the protein sample was run under a variety of acrylamide concentration ranging from 4% to 12% (Bollag et al., 1966). The accumulated information from these conditions serves to reduce the effect due to protein charge. Protein mobilities were calculated as the Rf values. A semilogarithmic plot of the Rf relative to the acrylamide concentration provides a line with a slope characteristic for a protein of a specific molecular weight. The proteins of known molecular weight were electrophoresed under
the same condition and the slope generated from the experiments defined a linear relationship with the molecular weight. The molecular weight of the hemoglobin was interpolated from the data with the molecular weight standards. The native protein molecular weight marker kit of Sigma was used (Albumin from bovine serum, Albumin from chicken egg, Carbonic anhydrase) and supplier instructions were followed.

3.2.8.2 Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out following the method of Laemmli (1970), after slight modification, 15% resolving gel and 5% stacking gel concentrations were found suitable for the electrophoresis of hemoglobin fractions.

**Procedure**

Vertical slab gel (11 cm long X 10 cm wide X 1 mm thick) containing 15% resolving gel with a stacking gel (5%) was used. Hemoglobin samples were diluted in sample buffer (containing 0.1 gm SDS, 0.5 mL glycerol, 0.75 mL 2- mercaptoethanol, 5μL of 1% bromophenol blue, 0.625 mL Tris-SDS stock (4x) pH 6.8 in 5 mL distilled water) in 1:2 ratio. Appropriately diluted hemoglobin sample were heated at 100°C in water bath for 5 minutes. The hemoglobin sample (50μg) each were loaded in separate well and the gel was run at 150 V till the bromophenol dye reached upto the end of the gel. After electrophoresis, gels were stained over night in staining solution (40% methanol, 10% acetic acid and 0.05% coomassie brilliant blue R-250) followed by destaining solution (40% methanol and 10% acetic acid). Gels with clear background were photographed.
The relative mobility ($R_f$) of hemoglobin in SDS-PAGE was extrapolated from a calibration curve, prepared by plotting the $R_f$ value of known proteins (bovine albumin, ovalbumin, bovine $\beta$- lactoglobulin, lysozyme) on the abscissa and the corresponding molecular weight in the ordinate of a semi-logarithmic graph (Shapiro et al., 1967).

3.2.9 Characterization of hemoglobin

3.2.9.1 Dot blot analysis

2µl of both of the hemoglobin fractions, total erythrocyte lysate and rhu-TNF-α (100 pg) were dot blotted and air dried at room temperature. Free sites were blocked with 1% BSA and probed with polyclonal goat anti-human TNF-α antibody (R&D system). The second antibody, rabbit anti-goat IgG conjugated with alkaline phophatase (Banglore Genei, India) was used and the substrate reagent kit (Banglore Genei, India) including BCIP & NBT were used for developing the dots.

3.2.9.2 Cellulose acetate electrophoresis

Cellulose electrophoresis was carried out following method of Marengo-Rowe (1965).

Apparatus & Reagent

The apparatus requires a power pack capable of supplying direct current up to 10mA at 200Volts, a tank suitable for electrophoresis on cellulose acetate in the horizontal plane (Biotech), cellulose acetate strip (120x25mm), Tris buffer (pH 8.9) consist of Tris-
(hydroxymethyl)-aminomethane 14.5g/L (0.12M), ethylene-diamine-tetraacetic acid 1.5g/L (0.005M) and boric acid 0.9g/L (0.015M).

**Procedure**

The cellulose acetate strips were immersed in the Tris buffer for five minutes, blotted evenly between two sheets of Whatman #3 filter paper to remove excess moisture and mounted horizontally in the electrophoretic tank. The strips are allowed to equilibrate in the closed unit for 10 min. The loading volume of hemoglobin fraction was 2 µl each placed 0.5 cm away from the cathode side of the mid point of the strip. Reaction between hemoglobin and antibody to human TNF-α was also demonstrated on cellulose acetate strips. Free site were blocked with 1% BSA and finally washed and probed with goat anti-human TNF-α antibody (R&D system, USA; catalogue # AB-210NA). The second antibody was anti-goat IgG conjugated with alkaline phosphatase (Bangalore Genei, India) and the substrate reagents include BCIP and NBT (Bangalore Genei, India.). Peroxidase activity was also demonstrated on cellulose acetate strip by incubating it with substrate reagent (Genzyme Corporation, Cambridge, USA.) of horseradish peroxidase enzyme.

**3.2.10 Measurement of relative antibody affinity**

Hemoglobin (100ng/well) was adsorbed on to 96-well microtiter plate (Immunol, USA) by incubating at 37°C in Tris saline (50mM Tris HCl + 0.1M NaCl, pH 7.5) for 2 hr (Ausvarumgnirum et al., 1988). Relative affinity of antibodies to human TNF-α with human hemoglobin (HbA₀ and HbA₂) was measured by ELISA using thiocyanate elution.
technique (Mac Donald et al., 1988). The plates were rinsed with Tris saline and 100μl goat anti-human TNF-α (1:1000) diluted in Tris saline containing Tween (0.05%) and 1% BSA was dispensed in triplicate on to well coated with hemoglobin or recombinant human TNF-α. The plates were then incubated at 37°C for 2hr. After washing with tris saline Tween, ammonium thiocyanate in 0.1M phosphate buffer, pH 6.0 was added to appropriate wells 100μl/well in triplicate, in concentration ranging from 0 to 5M. The plates were allowed to stand for 15min at room temperature before thrice washing with tris saline, 100μl rabbit anti-goat IgG conjugated with alkaline phosphatase at a dilution of 1:500 was added per well and incubation continued for 2hr. After 3-wash, 100μl substrate solution containing p-nitrophenyl phosphate as substrate and 2-amino-2-methyl-1,3propanediol as buffer (Raichem, San Diego) was added and the optical density of the solution in each well was read with an interference filter at 405nm in ChemWell Auto Analyzer (Awareness Technology, USA). Ascites fluid from the mouse implanted with Ehrlich ascites tumor cell line was used as a negative control. The determination of the affinity index was performed by plotting a graph of log10 (50 % of initial absorbance) vs. molarity of NH₄SCN by third degree polynomial regression analysis as performed by a graph generation programme (MS Excel, Hewlett Packard computer system). Initial absorbance was taken as that obtained with no NH₄SCN present.

**3.2.11 Enzyme activity assays**

Alkaline phosphatase (EC 3.1.3.1) activity of DEAE cellulose column purified hemoglobin fractions were measured spectrophotometrically at a wavelength of 405 nm. The assay kit (Raichem; Hemagen Diagnostics, Inc., San Diego) consists of p-
nitrophenyl phosphate as substrate and 2-amino-2-methyl-1, 3-propanediol as buffer. The formation of the color was followed kinetically in ChemWell Autoanalyser (Awareness Technology, USA). The presence of horse radish peroxidase (EC 1.11.1.7) activity in different fractions of hemoglobin was also monitored at 450 nm in ChemWell Autoanalyser using 1,2 benzene diamine, 2mM (Sigma), hydrogen peroxide (7μL in 10 mL of citrate buffer (2g/L)) substrate reagent for horse radish peroxidase containing hydrogen peroxide and tetramethyl benzidine (Cat. # 2642KK). Lactate dehydrogenase (EC 1.1.1.27) was measured using LDH-P assay kit (Ark Diagnostics, India) at 340 nm.

3.2.12 Bioactivity studies

3.2.12.1 Cell line

Human promonocytic leukemia cell (U937) procured from Prof. Sita Nayak, Dept. of Immunology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, INDIA. was used for studying apoptosis, cytotoxicity and nitric oxide release. Cells were maintained at 37°C in humidified atmosphere containing 5% CO2 in RPMI-1640 media containing 10% FCS, 2mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin, 5x10⁻⁵M 2-mercaptoethanol.

3.2.12.2 Cytotoxicity assay

Cytotoxicity induced by hemoglobin was detected by MTT dye uptake assay (Manna et al., 1998). Briefly, U937 cells (1x10⁴/well) in 100μl media were incubated with either human hemoglobin (4.0, 40, 400 and 4000 nM), TNF-α (0.01, 0.1, 1.0 and 10 nM) or...
human hemoglobin (4000 nM) + anti-hu TNF-α antibody (0.002 and 20μg/well) in triplicate wells for each concentration separately. U937 cells were incubated with serum activated LPS (100μg/ml) for 1hr to see the inhibition of hemoglobin and TNF-α mediated cytotoxicity after 24 hours of incubation. Dexamethasone induce protection of TNF-α induced cytotoxicity in U937 cell line was adopted from the method of Suffys et al., (1987) and Neale et al., (1988). Dexamethasone (100μM) mediated protection of hemoglobin induced cytotoxicity was studied by 2 hrs pretreatment of U937 cells with dexamethasone. Incubation was continued for 24 hours. Thereafter, 25μl of MTT solution (4mg/ml in PBS) was added to each well and incubation continued for 4 hours at 37°C. Extraction buffer (100μL; 20% SDS, 50% Dimethylformamide) was added and incubation continued over night at 37°C. The OD at 590 nm was measured using a 96-well plate ELISA Reader (Auto Analyser, ChemWell, USA) with extraction buffer as a blank.

3.2.12.3 Apoptosis

Apoptosis, was studied by annexin V-affinity assay based on the detection of externalized phosphatidylserine molecule (van Engeland et al., 1998). U937 cells (1x10^7/well) were incubated with either of the test sample: human hemoglobin (40, 400 and 4000 nM), human TNF-α (1ng/mL), human hemoglobin (400 and 4000 nM) + human TNF-α (1ng/mL) or human hemoglobin (4000 nM) + antibodies to TNF-α (1 and 10 μg/well) in triplicate wells in 100μl media for 8hr at 37°C in a humidified atmosphere of 95% and 5% CO2. After incubation, cells were harvested, washed twice with chilled PBS and 50μl of binding buffer (Annexin V-FITC detection kit, BD Pharmpingen) and 5μl
of annexin V was added and incubated in dark. After, 15 min 150μl of binding buffer and 5μl of propidium iodide (PI) was incubated for 15min in dark at room temperature. The stained cells were analysed in FACS scan by flow cytometry (BD-Pharmingen, USA) using a CELLQUEST software. Data were collected from a minimum of 10,000 cells per sample. Stained cell population was defined as viable or undamaged cell (Annexin V'PI'); Cells undergoing early apoptosis (Annexin V'PI'); Necrotic or late apoptotic (Annexin V'PI') and necrotic if (Annexin V' PI').

3.2.12.4 Nitric oxide release

The method of Green et al.,(1982), was adopted for measuring the release of nitric oxide in U937 cell culture supernatant following incubation for different time period (4, 8 and 24 hours) in presence or absence of hemoglobin (40, 400 and 4000 nM) or recombinant TNF-α (1 ng /mL) by the Griess reagent and monitored at an absorbance maxima, 545 nm.

Cell culture media supernatant (50μl) was added to each well of a 96well plate. After the addition of 50μl of Griess reagents (1% sulphanilamide and 0.1% naphthylenediamide dihydrochloride in 2% phosphoric acid), the plate was continuously shaken for 10min at room temperature. The quantification of nitrite production was made spectrophotometrically using automated calorimetric procedure. The nitrite concentration in samples was extrapolated from the standard curve of sodium nitrite solution prepared in culture media.
3.2.13 Prediction of antigenic site(s) and molecular modeling

Amino acid sequences of human TNF-α precursor and matured protein were procured from the Swissprot Database (Accession # P01375). Human hemoglobin-α chain was derived from Pir Database (Accession # HAHU) and human hemoglobin-β chain was derived from Swissprot Database (Accession # P02023). Sequence alignment was performed, using CLUSTAL W (1.81) multiple sequence alignment software (Thompson et al., 1994). The hydropathicity plot of matured human TNF-α and human hemoglobin α chain was determined using the method of Kyte and Doolittle (1982). The prediction of antigenic sites of human TNF-α precursor and matured protein and human hemoglobin-α and -β was performed by using EMBOSS: antigenic software (Kolaskar et al., 1990; Parker et al., 1986). Molecular modeling was performed using GRASP software (Nicholls et al., 1991). The structure of TNF-α was derived from PDB file # 1TNF and that of human hemoglobin α-chain was derived from PDB file # 1HHO.
RESULTS
In the present study, the reaction between human hemoglobin and antibodies to human TNF-α was studied using highly purified erythrocytes from fresh human blood. Klutter et al., (1997) observed insignificant level of different cytokines (IL-1β, IL-6, IL-8 and TNF-α) in the supernatant of buffy coat during storage in the first 12 h. We isolated the erythrocytes within 1h from the time of collection of blood. These erythrocytes are practically free from platelet and leukocyte as judged from the microscopic analysis. The platelet and leukocyte counts were <3% and <1% respectively. Depletion of platelets and leucocytes from the erythrocytes was necessary because of the reported effect of buffy coat storage on the generation of inflammatory cytokines and platelet activation (Davenport et al., 1991; Muyllle et al., 1993; Kluter et al., 1997). The purified population of erythrocytes was lysed with brief exposure to hypotonic shock with water and centrifuged. The RBC ghost was discarded to rule out the possible presence of membrane receptor bound TNF-α. The cytosol was used exclusively for hemoglobin isolation. Hemoglobin concentration in the cytosol was 9 mg/mL. Hemoglobin isolated from cytosol by column chromatography on DEAE cellulose yielded two fractions based on pH dependent elution pattern. The first fraction was eluted with 50mM Tris HCl (pH 8.4) and the second one with the same buffer but with pH 7.5. Both fractions were lyophilized and reconstituted to the original column loading volume (0.2mL). Hemoglobin content in the first and second fraction was in the ratio of 1: 40. Earlier the fraction eluted with Tris HCl (pH8.35) has been referred to as HbA₂ and the other with pH 7.5 as HbA₀ (Huisman et al., 1965). In our laboratory, however, the first fraction was
eluted with 50 mM Tris HCl at slightly more alkaline pH (8.4). As this pH is very close to the reported pH 8.35, we presume the first fraction to be HbA₂ in line with Huisman et al., (1965) observation. The second fraction was eluted at the same pH as reported by former group and therefore in line with their nomenclature, we call this fraction as HbAα.

4.1 Analysis of HbAα and HbA₂ by reverse phase HPLC

HbA₀ and HbA₂ were subjected to HPLC analysis by using RP-18 column and fraction were eluted with TFA and acetonitrile developer. The separation of the α- and non-α-globins that are constituents of the hemoglobin tetramer is based on the application of an increasingly hydrophobic environment to the chromatographic column, which is filled with an interacting lipophilic stationary phase. Because of the presence of a constant concentration of TFA in the developers, a pH of 2.0-2.5 is created. Under these conditions, the heme groups are removed from the tetramers, and the different constituent globins dissociate. The order of elution is therefore, primarily based on the hydrophobicity of the individual chains, the faster being less hydrophobic: β, δ, α and γ - chain (Masala and Manca, 1994). Purified human hemoglobin (Sigma) was run as standard. The elution pattern of the standard human hemoglobin shows the appearance of three prominent peaks at 15, 42.008 and 43.003 minutes of retention time (Fig.1A). The first peak represents the most hydrophilic heme group and the second and third represents the β-chain and the α-chain of the human hemoglobin (Schroeder et al., 1986). The δ- and γ -chain occurs in minute quantities in adult hemoglobin and therefore the elution peak for these two chains could not be ascertained. The DEAE-cellulose column purified HbA₀ sample also yielded similar elution profiles with 3 peaks at ~ 15, 42.059 and 43.006
minutes retention time (Fig.1B). HbA₂, however, displayed two peaks at 42.253 and 43.0045 minutes retention time but the peak representing the heme group was absent (Fig. 1C). This could be due to the dissociation of the heme group from the HbA₂ molecule during the DEAE cellulose column fractionation in the alkaline environment. The peak at 43.004 (Fig. 1A), 43.006 (Fig. 1B) and 43.0045 (Fig. 1C) represents the α-chain of human hemoglobin in standard Hb, HbA₀ and HbA₂. The peak at 42.008 (Fig. 1A), and 42.059(Fig. 1B) represents the β-chain of the standard hemoglobin and HbA₀ respectively. The peak at 42.253 (Fig. 1C) of HbA₂ resides between the β-chain and the α-chain of the human hemoglobin and seems to represents the δ-chain. Importantly both the β-chain and the α-chain of the human hemoglobin eluted at retention time very close to the earlier reported retention time. Thus, the typical elution pattern of peptides in reverse phase HPLC confirms the existence of HbA₀ and HbA₂ and is suggestive of the purity of preparation of different hemoglobin species with out any other contaminating peptide(s).

4.2 Characterization of HbA₀ and HbA₂

Both HbA₀ and HbA₂ showed characteristic absorption spectra in the visible range with maxima at 413, 540 and 576 nm (Fig.2). These absorption maxima are similar to the reported values of oxyhemoglobin (Dilorio, 1981). The fractions were subjected to SDS-PAGE under reducing condition. Both the fraction segregated into two major bands, one migrating at 36 kDa and the other at < 14kDa position (Fig.3). Although both the fractions are pH sensitive, the migration pattern of HbA₀ and HbA₂ on SDS-PAGE are of similar nature and both the fractions yielded two bands. 10% native gel electrophoresis
revealed a single band at ~67kDa (Fig. 4). The reported molecular weight of adult hemoglobin is 64 kDa (Alayasht., 1999). It is not clear as to why the migration of HbA₀ and HbA₂ is retarded in the 10% native gel. This may be due to inappropriate gel concentration that we have selected. However, during electrophoresis on cellulose acetate paper, the migration of HbA₂ from cathode was relatively slow in comparison to HbA₀ (Fig. 5; strip 2 & 3). Scanning of cellulose acetate strip revealed that HbA₂ represents only 2.5% of HbA₀ in the erythrocyte lysate of normal human subjects (Fig. 5; strip 1).

4.3 Enzyme activities of HbA₀ and HbA₂

Hemoglobin has been reported earlier to possess intrinsic peroxidase activity. The HbA₀ and HbA₂ purified from DEAE cellulose column, showed substantial peroxidase activity (Fig. 6 & Table-I). Hence peroxidase conjugated detection reagents become unsuitable for immunoreactions studies involving hemoglobin. Incidentally both the hemoglobin species (HbA₀ & HbA₂) lack alkaline phosphatase activity (Table-I). LDH activity was also evaluated and found substantial activity in HbA₀ & HbA₂. Hence the immunoreactivity studies involving HbA₀ and HbA₂ and antibodies to TNF-α was carried out using alkaline phosphatase conjugated detection reagents.

4.4 Immunoreactivity of HbA₀ and HbA₂

RBC cytosol, HbA₀ and HbA₂ probed with polyclonal antibodies to human TNF-α displayed positive reactions in a dot blot assay. However, the dots were not so prominent in comparison to recombinant human TNF-α (Fig. 7). Immunoreactivity of polyclonal antibodies to human TNF-α was also tested on cellulose acetate membrane after
electrophoresis of different hemoglobin fractions. Prominent immunoreactions were observed between polyclonal antibodies to TNF-α and the HbA₀ and HbA₂ (Fig. 8). The relative affinity of polyclonal antibody to TNF-α towards different species of human hemoglobin was also measured by ELISA affinity elution procedure (Fig. 9). The affinity indices were determined from the point of intersection of the elution curve by a line causing 50% reduction in initial absorbance by ammonium thiocyanate, i.e., \( y = 1.699 \log_{10}(50\% \text{ initial absorbance}) \). Both species of hemoglobin demonstrated low affinity indices in comparison to recombinant human (rhu) TNF-α (Table II). Ascitic fluid from Ehrlich’s ascitic tumor bearing mice did not show response above the background level (control blank).

4.5 The effect of hemoglobin on U937 cell cytotoxicity

Incubation of U937 cells with 1ng/mL recombinant human (rhu) TNF-α showed optimum cytotoxicity after 24 h incubation in comparison to control group of U937 cells without rhuTNF-α (Fig. 10). Cytotoxicity displayed by recombinant huTNF-α was found dose dependent in the range of 0 – 10nM. Interestingly hemoglobin also displayed cytotoxicity in U937 cell line but at higher concentration. 4000 nM of hemoglobin showed optimal cytotoxic response in U937 cells. A dose response curve was also observed with varying doses of HbA₀ and HbA₂ (Fig. 11 and 12). In another experiment, U937 cells pretreated with serum activated LPS for 1hr and were subsequently incubated with different concentration of hemoglobin or human TNF-α for 24hrs. Cytotoxicity of U937 cell pretreated with serum activated LPS was inhibited both in the presence of HbA₀ (Fig. 11) and HbA₂ (Fig.12) or rhuTNF-α (Fig.10) separately. However,
RESULTS

dexamethasone (100μM) treatment showed protection from HbA₀ (Fig.13) and HbA₂ (Fig.14) mediated cytotoxicity in U937 cell line. In another experiment, anti-huTNF-α antibody showed complete protection from HbA₀ mediated cell cytotoxicity, whereas addition of 20μg of antibodies to TNF-α with 4 μmole of HbA₀ in U937 cells displayed higher optical density (A₅₉₀) in comparison to normal control well containing only U937 cells. It is not clear to us as to why the OD increased above the control values. Possibly it suggest that 20μg of antibodies to TNF-α with 4 μmole of HbA₀ combination is not appropriate condition for monitoring cell cytotoxicity utilizing ELISA plates. (Table III). Thus, human hemoglobin induced cytotoxicity in U937 cells mimic TNF-α induced cytotoxicity in cell line specific to TNF-α. Furthermore, inhibition of hemoglobin-mediated cytotoxicity in U937 cells by antibodies to TNF-α suggests that hemoglobin acts on the TNF-α receptor on U937 cells.

4.6 Apoptosis

HbA₀ induced apoptosis was studied in U937 cells and compared with TNF-α. Different concentration of HbA₀ (4000 to 40 nM) induced early apoptosis (Annexin V⁺ PI⁻) ranging from 19.33% to 12.63% and 6.98% to 1.26% late apoptosis (Annexin V⁺ PI⁺) (Fig.15). 4000 nM hemoglobin showed maximal apoptotic cells. Temporal kinetics of U937 cells undergoing apoptosis was also evaluated in presence of optimal dose of HbA₀ at 0, 4 and 8 hours. Maximum apoptosis (Annexin V⁺ PI⁻ and Annexin V⁺ and PI) was observed at 8 hours (Fig 16). TNF-α also revealed 87.8% late apoptotic cells at a concentration of 1ng/ml in U937 cell line and only 10.46% cells showed early apoptosis (Fig.15E). Incubation of U937 cell line with HbA₀ and TNF-α had synergistic effect on late
apoptosis (Fig. 15F). Antibodies to TNF-α (10μg and 1μg/well) were found to inhibit
HbA0 mediated apoptosis in U937 cells (Fig 15G; Table IV). It indicates that hemoglobin
shares some of the epitope(s) of TNF-α.

4.7 Release of nitric oxide
Nitric oxide was measured in U937 cells following 4, 8 and 24 hours of incubation with
HbA0 in varying concentration (0-4000nM). We observed significant release of nitric
oxide in U937 cell in the presence of HbA0 in comparison to normal control U937 cells
without HbA0 at 4 and 8 hours (Fig. 17). 24 hr culture of U937 with HbA0 reduced the
nitric oxide level drastically. This is possibly because the cells are known to consume the
release nitric oxide during this prolonged incubation period. This situation is not seen in 4
or 8 hr of incubation period. Incubation of U937 cells with 1ng/mL of TNF-α could not
induce the release of nitric oxide in comparison to control at all the time points (not
shown in fig.). Temporal analysis revealed maximum release of nitric oxide by U937
cells following co-culture with hemoglobin A0 at all the doses at 8 hour in comparison to
4 and 24 hours of incubation (Fig.17). We could not detect the TNF-α mediated nitric
oxide release possibly because nitric oxide release is associated with normal healthy
cells. Our result on apoptosis shows that, at 8hour the number of healthy cells was only
1.40% and because of this low percentage of healthy cells there was no significant release
of nitric oxide.

RESULTS
4.3 Comparative homology studies

Comparative homology studies were performed between TNF-α and human hemoglobin -α and -β chain using ClustalW (1.81) multiple sequence alignment software. Sequence homology score between TNF-α and α-chain of human hemoglobin was 11.26% (Fig.18A). Sequence homology score between TNF-α and β-chain of human hemoglobin was 12.2 (Fig.18B). In spite of the low sequence homology between TNF-α and the different chains of human hemoglobin (α and β chain), an interesting sequence VAHV was detected in both TNF-α and α-chain of human hemoglobin (Fig. 19). This sequence resides between 89 – 92 amino acid residues of TNF-α precursor molecule or 13 – 16 amino acid residues in the matured TNF-α. In case of α-chain of human hemoglobin VAHV resides in the 71 – 74 amino acid residues. Although many amino acids like serine, lysine, threonine, alanine resides in the antigenic sites, the occurrence of hydrophobic amino acids like cysteine, leucine and valine (very high antigenic propensity) on the surface of the molecule could be more antigenic (Kolaskar et al., 1990). This unique valine rich sequence unexpectedly lies in the hydrophobic region of both the molecules as seen in the hydropathicity plot of TNF-α and α-chain of human hemoglobin (Fig.20). Therefore we sought to predict the antigenic site present on TNF-α and α-chain of hemoglobin. Prediction of antigenic sites of TNF-α precursor molecule (Figure 21) and α-chain of human hemoglobin (Fig.22) was performed by using EMBOSS: antigenic software based on the findings of Kolaskar et al., (1990) and Parker et al., (1986). 8 sites residing at 27-58, 86-96, 122-179, 65-80, 223-230, 190-202 and 110-117 amino acid residues of TNF-α precursor molecule were predicted. In matured
RESULTS

TNF-\(\alpha\) 5 antigenic sites were detected. This is because of the deletion of the starting 76 amino acid residues during the process of maturation. The antigenic sites predicted in the region 27-58 and 86-96 amino acid residues of TNF-\(\alpha\) precursor or the 10 - 20 amino acid residues of matured TNF-\(\alpha\) had a very high score (1.2). Interestingly, the valine rich sequence VAHV lies in the region 86-96 of TNF-\(\alpha\) precursor molecule or 10 - 20 amino acid residues of matured TNF-\(\alpha\). In case of \(\alpha\)-chain of human hemoglobin 4 antigenic sites were predicted in the region 80-139, 68-75, 43-66 and 14-21 with a score of 1.237, 1.104, 1.090 and 1.042 respectively. The VAHV sequence lies in the 68-75 antigenic site of \(\alpha\)-chain of human hemoglobin. Therefore recognition of hemoglobin by antibodies to TNF-\(\alpha\) can be due to sharing of VAHV sequence in the antigenic sites of the two molecules. The molecular model of both TNF-\(\alpha\) and the \(\alpha\)-chain of hemoglobin derived from X-ray crystallography data were compared to see the location of the antigenic sites containing the homologous sequence in the accessible region. Interestingly, the antigenic site containing VAHV residues lies in the accessible region of both \(\alpha\)-chain of hemoglobin (Fig.23) and TNF-\(\alpha\) molecules (Fig.24). Therefore, it is quite likely for the \(\alpha\)-chain of hemoglobin to share epitope of TNF-\(\alpha\) molecules.
DISCUSSION
5. DISCUSSION

Red cells have limited life span. The catabolic products produced during cell destruction accompany their senescence. Spleen appears to be the major site of sequestration and catabolism of red cells (Grossi and Lydyard, 1992). Elements of reticuloendothelial system, particularly liver and bone marrow, sequester red cells when the spleen is removed (Berlin and Berk, 1975).

The catabolic products formed during red cell destruction are derived from degradation of hemoglobin, iron, globin and bilirubin and to a lesser extent lactic dehydrogenase. Hemoglobin comprises about 90% of the red cell lysate (Riggs, 1981). Abnormal senescence of RBC is seen during hemolytic anemia and malaria. The former disease arises due to a shortened lifespan of red cells leading to their premature destruction and death, and the latter is associated with the rupture of Plasmodium falciparum schizont stage parasitized erythrocytes (Pichyangkul et al., 1994). Sometimes even drugs can induce hemolytic anemia. It is well known that drugs that are harmless to most persons produce hemolytic reaction in a few sensitive persons. This is particularly true of the antimalarial drug, primaquine, and, to a lesser degree, some aniline derivatives such as sulfa-nilamide, phenacetin and acetanilide (Boyd, 1979). Using extremely sensitive and specific techniques, we have found that the human hemoglobin species, HbA₀ and HbA₂, prepared from highly purified human erythrocytes that are practically free of leukocytes and platelets, fractionated/isolated from DEAE cellulose column, react with antibodies to human TNF-α. The purity of both the fractions, HbA₀ and HbA₂, was checked and
confirmed by reverse phase HPLC. Therefore, the recognition of pure HbA0 and HbA2 by antibodies to TNF-α is interesting and it reinforces the earlier findings of Kristiansson et al., (1996), who reported the presence of TNF-α in the erythrocyte concentrates. However, we differ from the findings of Kristiansson and his group by demonstrating that hemoglobin present in the erythrocytes cross reacts with antibodies to TNF-α and for possession of peroxidase activity by the hemoglobin itself.

Further, the occurrence of TNF-α in erythrocyte lysate is not due to the induction of TNF-α in platelets or leucocytes present as contaminants as suggested by Kristiansson et al., (1996). The presence of peroxidase activity in hemoglobin bears a greater significance in techniques like ELISA involving horse radish peroxidase conjugated detecting reagents resulting into false positive reaction.

Hemoglobin encapsulated in liposome (LEH) is an experimental red cell substitute that is being developed as artificial oxygen carrying resuscitative fluid. Encapsulated hemoglobin has been shown to accumulate in organs of the reticuloendothelial system, particularly liver and spleen. Tissue specific accumulation of inflammatory and regulatory cytokines have been measured by ELISA over a period of 24 h after injection of 10% blood volume of mice (Rudolph et al., 1996). Interestingly, increased levels of tissue associated TNF-α, IL-1, IL-4 and IL-10 were observed without significant change in serum level. The authors proposed earlier that LEH elicits local production of inflammatory cytokines in the organs of reticuloendothelial system as a consequence of accumulation in tissue resident phagocytes.

In our opinion, the elevated level of TNF-α may be due to the accumulation of hemoglobin in the organs through LEH. The hypothesis that LEH elicits local production
of inflammatory cytokines fail to explain the normal level of serum cytokine (TNF-α) in blood plasma in spite of a large presence of macrophages. Our study reveals for the first time that hemoglobin reacts with antibody to TNF-α and therefore it is quite likely to share epitope(s) with human TNF-α. The VAHV sequence residing in the antigenic site lying between 68-75 amino acid residues of α-chain of human hemoglobin is also present in the antigenic segment of both precursor and matured TNF-α. In the matured TNF-α, the VAHV sequence resides in the 13-16 residues of the N-terminus of TNF-α. This is interesting as antibodies against amino acids 1-15 and 1-31 of the TNF-α has been demonstrated to block its binding to cell surface receptor (Socher et al., 1987). Five synthetic peptides comprising of linear amino acid segments from human TNF-α [hTNF(1-15), hTNF(1-31), hTNF(65-79), hTNF(98-111) and hTNF(124-141)] were prepared and tested in human TNF receptor binding assays and in two biologic assays like cytolysis of tumor cells and suppression of lipoprotein lipase in adipocytes. None of the synthetic peptides were found to act as agonists or antagonists in these assays. However, when all five peptides were conjugated to thyroglobulin, they induced antibody responses to the immunizing peptide and to human TNF. Further when each antiserum was tested for antagonist activity in human TNF binding assays, only antisera raised against hTNF(1-15) and hTNF(1-31) blocked binding. The VAHV residues incidentally happen to reside between 1-31 residues of the matured human TNF-α. Therefore, recognition of hemoglobin by the antibody to TNF-α can be due to the sharing of VAHV sequence in the antigenic sites of the two molecules. Sharing of epitope may lead to functional similarity as suggested by (Gershoni et al., 1997). We studied three bioactivities that were earlier ascribed to TNF-α. These include apoptosis, cytotoxicity
and nitric oxide release in TNF-α specific cell line, U937. Interestingly, we observed that hemoglobin could induce apoptosis and cytotoxicity in TNF-specific cell line but at a higher concentration in comparison to TNF-α mediated apoptosis and cytotoxicity. More interestingly, hemoglobin induced U937 cells to release nitric oxide at all the doses after 4 and 8 hours co-culture with HbA0. After 24 hours of incubation with HbA0 the nitric oxide in the culture medium of U937 culture reduced substantially. Co-culture of U937 cells with TNF-α for 24 hours also displayed reduced nitric oxide in the culture supernatant. Furthermore, serum activated LPS is known to block TNF-α mediated cell cytotoxicity (Manna and Aggarwal., 1999). Incidentally, serum activated LPS was found to inhibit hemoglobin A0 and A2 mediated cell cytotoxicity. Thus, both the species of hemoglobin mimics TNF-α in terms of immuno-reactivity, bioactivity and theoretical prediction of antigenic sites and surface topology.

It is for this reason possibly the presence of TNF-α was observed during transfusion of stored erythrocyte concentrate, hemolytic transfusion reaction and abnormal senescence of RBC. Analysis of hemoglobin on cellulose acetate paper and cross reactivity with antibodies to TNF-α suggest for revaluation of diseases associated with erythrocyte destruction and alteration of TNF-α.

We for the first time demonstrate that antibodies to human TNF-α can recognize human hemoglobin and therefore it becomes pertinent to have a look at the packed erythrocyte volume and erythrocyte count before ascribing the disease to cytokine, especially TNF-α. It is necessary to evaluate the contribution of hemoglobin in altering the TNF-α profile. It is also necessary to note that presence of hemoglobin in test sample like hemolyzed serum or plasma is not suitable for both bioactivity and immunoreactivity tests for
Furthermore, we can now hypothesize that agents that induce hemolysis, may it be a xenobiotics or microorganism, are likely to alter and/or elevate the plasma TNF-α.
FIGURES & LEGENDS
Chromatogram of the purified adult human hemoglobin (Sigma, USA) was used as standard (A), DEAE cellulose column purified adult HbA₀ (B) and adult HbA₂ (C); Numerals against each peak denotes the elution profile peptides in terms of retention time (RT) in minutes.

Fig 1
Absorption Spectra of human hemoglobins. Spectral analysis was performed in normal saline at a concentration ~1mg/mL. Spectra 1, 2 and 3 represents the hemoglobin present in the cytosol fraction of erythrocyte lysate, HbA_0 fraction eluting at pH 7.5, and HbA_2 fraction eluting at pH 8.4, respectively. The spectra showed absorption maxima at 413, 540 and 576nm typical of hemoglobin.
SDS-PAGE analysis to determine the molecular weight and the composition of Human hemoglobins. 10μl of sample containing ~ 50 μg hemoglobin was mixed with 10μl of sample buffer containing 2-mercaptoethanol and SDS was boiled for 5 min, and 15μl was loaded in each well. Lane 1 represents molecular weight marker, Lane 2 represents unfractionated cytosol of erythrocyte lysate, Lane 3 represents HbA₀ and Lane 4 represents HbA₂.

Fig 3.

Native PAGE (10%) of different human hemoglobin fractions. Lane 1, 2 and 3 represents total lysate, HbA₂ and HbA₀ respectively. Lane 4 represents molecular weight marker.
SDS-PAGE analysis to determine the molecular weight and the composition of Human hemoglobins. 10μl of sample containing ~ 50 μg hemoglobin was mixed with 10μl of sample buffer containing 2- mercaptoethanol and SDS was boiled for 5 min, and 15μl was loaded in each well. Lane 1 represent molecular weight marker, Lane 2 represents unfractionated cytosol of erythrocyte lysate, Lane 3 represents HbA₀ and Lane 4 represents HbA₂.

Native PAGE (10%) of different human hemoglobin fractions. Lane 1, 2 and 3 represents total lysate, HbA₂ and HbA₀ respectively. Lane 4 represents molecular weight marker.
Migration pattern of Human hemoglobin on cellulose acetate electrophoresis strips. 2 μL of DEAE cellulose column separated fractions after adjusting to the original column loading volume, was run on cellulose acetate paper. Strip-a represents unfractionated cytosol of human erythrocyte lysate, strip-b and strip-c represents human HbA₂ and HbA₀.

Demonstration of peroxidase activity of human hemoglobin on cellulose acetate electrophoresis strips. Electrophoresis was carried out as in Fig.5 and Horseradish peroxidase activity was observed by adding the specific substrate. Strip-a represents unfractionated cytosol of human erythrocyte lysate and Strip-b and Strip-c represents human HbA₂ and HbA₀ respectively.
Dot blot assay showing immunoreactivity of human hemoglobin with antibodies to human TNF-α. Dot 1 represents recombinant human TNF α; Dot 2 represents unfractionated cytosol of human erythrocyte lysate; Dot 3 and Dot 4 represent human HbA2 and HbA0.

Immunoreactivity of polyclonal antibodies to human TNF-α with different human hemoglobin fractions on cellulose acetate strip after electrophoresis. Strip-a represents unfractionated cytosol of human erythrocyte lysate. Strip-b and Strip-c represent human HbA2 and HbA0. The detection reagent was conjugated with alkaline phosphatase.
Fig 9. Effect upon initial absorbance by adding increasing concentration of NH₄SCN in the immunoreaction between human hemoglobin and polyclonal antibodies to human TNF-α (▲) Represents recombinant human TNF-α; (①) HbA₀; and (②) represents HbA₂.
\( R^2 = 0.868 \)
\( y = 2.223x + 3.092x^2 - 28.73x + 99.31 \)
\( R^2 = 0.996 \)
\( y = 0.577x^2 - 3.810x + 11.23x + 100.36 \)
\( y = 0.1 - 0.223x + 5.204x^2 + 69.37x + 8.724x \)
Effect of SA-LPS on TNF-induced cytotoxicity. (●) U937 cells (1 x 10^4 cells/well) pretreated with 100 ng/ml SA-LPS for 1 hour at 37°C in a CO2 incubator with different concentration of TNF for 24 hours. Cell viability was then determined by MTT method as described in Materials and Methods. The results showed the mean (± SEM) of OD in triplicate assay. (⧫) represent U937 cells treated with TNF-α in the absence of SA-LPS.
Effect of SA-LPS on HbA₀ induced cytotoxicity (G) U937 cells (1x 10⁴ cells/well) pretreated with 100ng/ml SA-LPS for 1hour at 37⁰C in a CO₂ incubator with different concentration of HbA₀ for 24 hours. Cell viability was then determined by MTT method as described in Materials and Methods. The results showed the mean (± SEM) of OD in triplicate assay. (▲) represent U937 cells treated with HbA₀ in the absence of SA-LPS.
Effect of SA-LPS on HbA₂ induced cytotoxicity. (○) U937 cells (1 x 10⁴ cells/well) pretreated with 100ng/ml SA-LPS for 1 hour at 37°C in a CO₂ incubator with different concentration of HbA₂ for 24 hours. Cell viability was then determined by MTT method as described in Materials and Methods. The results showed the mean (± SEM) of OD in triplicate assay. (▲) represent U937 cells treated with HbA₂ in the absence of SA-LPS.
Effect of Dexamethasone on varying concentration of HbA₀ induced cell cytotoxicity in U937 cells (■). (▲) represents cytotoxicity in U937 cells induced by HbA₀. Cytotoxicity has been presented as percentage of viable U937 cells.
Effect of Dexamethasone on varying concentration of HbA$_2$ induced cell cytotoxicity in U937 cells. (■) represents cytotoxicity in U937 cells induced by HbA$_2$. Cytotoxicity has been presented as percentage of viable U937 cells.
FACS analysis of PS externalization in U937 cells by using the Annexin V & PI assay. Each panel shows a typical flow of $10^5$ cells/sample for a representative experiment. Early apoptotic cells (Annexin V⁺, PI⁻) are shown in the lower right quadrant (R2), and late apoptotic plasma membrane damaged cell (Annexin V⁺, PI⁺) are shown in the upper right quadrant (R3). A, represents U937 cells without HbA₀. B, C & D represents U937 cells incubated with 4000nM, 400nM & 40nM of HbA₀ respectively. E, represents U937 cells incubated with r-TNF-α (1ng/mL). F, represents the synergistic action with increase in late apoptotic cells on co-culturing of U937 cells with HbA₀ (4000nM) and r-TNF-α (1ng/mL) after 8hr of incubation. G, represents U937 cells incubated with HbA₀ (4000nM) with 10 µg of antibodies to hu TNF-α. The FACS analysis were done after incubation period of 8hr.
Temporal kinetics of late apoptosis (Annexin V\(^+\) PI\(^+\)) and early apoptosis (Annexin V\(^+\) PI\(^-\)). Flow cytometric plot of U937 cells incubated with optimal concentration of HbA\(_0\) (4000nM). A, B and C represents early and late apoptotic cells at 0 hours, 4 hours and 8 hours respectively. Upper right hand quadrant represents Annexin V\(^+\) PI\(^+\) (R3) and lower right hand quadrant represents Annexin V\(^+\) PI\(^-\) (R2).
The release of nitric oxide under varying concentration HbA0 in U937 cells after incubation period of 4hr (□), 8hr (◇) and 24hr (▲).
Amino acid sequence homology between α-chain (A) and β-chain (B) of human hemoglobin with human TNF-α.
Primary structural analysis of \( \alpha \)-chain of human hemoglobin and human TNF-\( \alpha \) precursor. Linear sequence homology between \( \alpha \) chain of human hemoglobin and human TNF-\( \alpha \) revealed unique valine rich segment in the region 89-92 amino acid residues of human TNF-\( \alpha \) precursor.
<table>
<thead>
<tr>
<th>ProTNF-α</th>
<th>Homo</th>
<th>MESTMIRDVELAEHALPKRTGEGGSPRCRELSESLFSLIVAGATELPCLLLHPVGIGPR 66</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MVLS2ADTNVKAAGVGAGGAGEYGGANALEMPFSEPRTTKTFPHF 47</td>
</tr>
<tr>
<td>ProTNF-α</td>
<td>Hae</td>
<td>EEPREDLSLLPSLACAVRSSSRTPSDKPVAVNHVANRFQEGQHQ&gt;NKVRINALLANGVELR 12</td>
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<td></td>
<td></td>
<td>DLSHG3AQVGHGKTVADILNAVVAHVDDAPRILSLALSOLHAEKDNVDPVFWKLL 16</td>
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<tr>
<td>ProTNF-α</td>
<td>Yhe</td>
<td>DQQLVVPSGGLYLLYSQVLPGGCCPSTVQLLTNRTISLAVYQVTWNLSSAIKSPQRE 41</td>
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<td></td>
<td></td>
<td>SHCLVT 23</td>
</tr>
<tr>
<td>ProTNF-α</td>
<td>Rho</td>
<td>TPECA2AKPWYEPITLGVEQLEKGDRLSARINRDPYLDFAE9GQYFGIAL 233</td>
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<tr>
<th></th>
<th>Homo</th>
<th>MESTMIRDVELAEHALPKRTGEGGSPRCRELSESLFSLIVAGATELPCLLLHPVGIGPR 66</th>
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<td>MVLS2ADTNVKAAGVGAGGAGEYGGANALEMPFSEPRTTKTFPHF 47</td>
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<tr>
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<td>EEPREDLSLLPSLACAVRSSSRTPSDKPVAVNHVANRFQEGQHQ&gt;NKVRINALLANGVELR 12</td>
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<td></td>
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<td>DLSHG3AQVGHGKTVADILNAVVAHVDDAPRILSLALSOLHAEKDNVDPVFWKLL 16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DQQLVVPSGGLYLLYSQVLPGGCCPSTVQLLTNRTISLAVYQVTWNLSSAIKSPQRE 41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SHCLVT 23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TPECA2AKPWYEPITLGVEQLEKGDRLSARINRDPYLDFAE9GQYFGIAL 233</td>
</tr>
</tbody>
</table>
Fig 20

Hydropathicity plot of \( \alpha \) chain of human hemoglobin and human TNF-\( \alpha \).
α-chain of Hemoglobin
Fig 21

Predicted antigenic site of human TNF-α.
(1) Score 1.207 length 32 at residues 27 – 58
   Sequence: SRRCLFLSLFSFLIVAGATTLFCLLHE'GVIGP
            ▲  ▲
           27  58

(2) Score 1.207 length 11 at residues 86 – 96
   Sequence: DKPVARVVAMP
            ▲  ▲
           86  96

(3) Score 1.175 length 58 at residues 122 – 179
   Sequence: HQLVVPSEGFLIYQVLQKQGCESTVLLLTMTISRAVITYQTKNLLSAIKSFCQR
            ▲  ▲
           122  179

(4) Score 1.141 length 16 at residues 65 – 80
   Sequence: RDLSLISLAQARSS
            ▲  ▲
           65  80

(5) Score 1.125 length 8 at residues 223 – 230
   Sequence: SQVYFGI
            ▲  ▲
           223  230

(6) Score 1.112 length 13 at residues 190 – 202
   Sequence: WYEPIYLGVFOIL
            ▲  ▲
           190  202

(7) Score 1.094 length 8 at residues 110 – 117
   Sequence: NALLANGY
            ▲  ▲
           110  117

(8) Score 1.063 length 8 at residues 9 – 16
   Sequence: DVELAEEOA
            ▲  ▲
           9  16

*Refers to Maximum Score position
Fig 22.

Predicted antigenic site of α chain of human hemoglobin
Fig 23

Location of VAHV segment on a computer simulated \( \alpha \) chain of human hemoglobin. Carbon is represented in white, nitrogen in blue and oxygen in red of VAHV peptide segment in the accessible region of the \( \alpha \)-chain of human hemoglobin.

Fig 24

Location of VAHV segment on a computer simulated human TNF-\( \alpha \). Carbon is represented in white, nitrogen in blue and oxygen in red of VAHV peptide segment in the accessible region of the matured human TNF-\( \alpha \).
TABLE-I

Inherent enzyme activities (IU/ L) of different fractions of human hemoglobin

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>HbA₀</th>
<th>HbA₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>NIL</td>
<td>NIL</td>
</tr>
<tr>
<td>Horseradish Peroxidase</td>
<td>184.6</td>
<td>179.1</td>
</tr>
<tr>
<td>LDH</td>
<td>116.06</td>
<td>597.01</td>
</tr>
</tbody>
</table>

TABLE-II

Comparative affinity indices of anti human TNF-α antibodies with human Hemoglobin as measured by NH₄SCN elution

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Affinity Index (NH₄SCN molarity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant</td>
<td></td>
</tr>
<tr>
<td>Human TNF-α</td>
<td>&gt; 5</td>
</tr>
<tr>
<td>HbA₀</td>
<td>2.03</td>
</tr>
<tr>
<td>HbA₂</td>
<td>2.40</td>
</tr>
</tbody>
</table>
Effect of varying concentration of antibodies to hu-TNF-α on ciliobrevin mediated cell cytotoxicity in U937 cells after 24 hr of incubation by MTT optical methodology. The results showed the mean ± SEM of 03 in triplicates assay.

<table>
<thead>
<tr>
<th>Antibody concentration (μg)</th>
<th>HbA0 (μM)</th>
<th>MTT uptake (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4</td>
<td>0.96 ± 0.12</td>
</tr>
<tr>
<td>0.01 g</td>
<td>4</td>
<td>0.70 ± 0.01</td>
</tr>
<tr>
<td>0.025 g</td>
<td>4</td>
<td>0.61 ± 0.01</td>
</tr>
<tr>
<td>0.002 g</td>
<td>4</td>
<td>0.44 ± 0.02</td>
</tr>
<tr>
<td>0</td>
<td>4</td>
<td>0.43 ± 0.04</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0.71 ± 0.02</td>
</tr>
</tbody>
</table>

Effect of varying concentration of antibodies to hu-TNF-α on ciliobrevin mediated early and late apoptosis in U937 cells after 3 hours of incubation by flow cytometry.

<table>
<thead>
<tr>
<th>Antibodies to TNF-α (μg)</th>
<th>HbA0 (μM)</th>
<th>Apoptosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Early</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>3.24</td>
</tr>
<tr>
<td>0</td>
<td>4</td>
<td>19.33</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>5.65</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>5.75</td>
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</tbody>
</table>