T LYMPHOCYTES AND IRON OVERLOAD: NOVEL CORRELATIONS OF POSSIBLE SIGNIFICANCE TO THE BIOLOGY OF THE IMMUNOLOGICAL SYSTEM

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This paper is written in the context of our changing perception of the immunological system as a system with possible biological roles exceeding the prevailing view of a system concerned principally with the defense against external pathogens. The view discussed here relates the immunological system inextricably to the metabolism of iron, the circulation of the blood and the resolution of the evolutionary paradox created by oxygen and iron.

Indirect evidence for this inextricable relationship between the two systems can be derived from the discrepancy between the theoretical quasi-impossibility of the existence of an iron deficiency state in the adult and the reality of the WHO numbers of people in the world with iron deficiency anemia.

With the mounting evidence that TNF, IL-1, and T lymphocyte cytokines affect hemopoieisis and iron metabolism it is possible that the reported discrepancy is a reflection of that inextricable interdependence between the two systems in the face of infection. Further direct evidence for a relationship between T cell subset numbers and iron metabolism is presented from the results of a study of T cell populations in patients with hereditary hemochromatosis. The recent finding of a correlation between low CD8+ lymphocyte numbers, liver damage associated with HCV positivity and severity of iron overload in B-thalassemia major patients (unpublished data of RW Grady, P. Giardina, M. Hilgartner) concludes this review.

Key words: T lymphocytes – iron – hemochromatosis – extracellular matrix

A growing number of publications in the immunological literature indicates that our perception of the immunological system is changing (Fougereau, 1991). The system can no longer be seen as a system committed predominantly to protection from the invasion of external pathogens or the continuous surveillance of threats within (Thomas, 1959).

Accumulating evidence indicates that, contrary to earlier dogma (Burnet, 1959) one sizeable component of the fluid phase of the immunological system is constituted by antibodies directed against components of self (Fougereau, 1991).

For those of us who, for many years, have

had an interest in the continuous non-random circulation of lymphocytes between blood-and lymph, as the physiological manifestation of a system capable of discriminating between tissue domains and vascular endothelia within self, maintaining surprisingly stable proportions of its recirculating subpopulations (de Sousa, 1973, 1981; de Sousa et al., 1991), this changing view from "the circulating antibody lobby" is greatly welcome. By meeting over the same ground, the old cellular and humoral lobbies of the system as a "physiological system", may join efforts to come to a better understanding of the system itself.

It is nice that the 1st European – Oswald Cruz joint meeting should also bring for the first time Portuguese immunologists representing these two lobbies together. For my part, I am greatly thankful to André Capron for having contributed to this kind of reverse movement of the Discovery of Portugal by Brasil.

This is my first visit to this country; I shall try to honour the occasion by presenting some of the work that we have been doing in my own lab in Oporto in the last 5 years and the hope that research in parasitic diseases may come to encompass young immunologists working in Portugal.

As a visiting card, however, I shall start by refering to my fairly isolated standing twenty years ago, at a time when many of you were in nursery school and external antigens were thought as the great motivating reason of the development of the immunological system and of the recirculation of lymphocytes itself. I only bring it up because it may help you to undestand why I fear not standing again fairly isolated in my current views of the importance of the inextricable interdependence of the two great mammalian cell circulations: the circulation of lymphocytes and the circulation of the red blood cells.

SELECTIVE LYMPHOCYTE POSITIONING (ECO-TAXIS) AS EVIDENCE OF RECOGNITION OF SELF WITHIN SELF

I have had the opportunity of reviewing this topic recently (de Sousa et al., 1991). Here, I shall simply remind you that one of the mechanisms postulated in 1971 to explain the clearcut differences between the peripheral positioning of lymphomyeloid cell populations, involved differences in what was then called "cell adherence" (de Sousa, 1971).

Later, in collaboration with Adam Curtis, we had the opportunity of doing what I believe were the first experiments exploring the role of adhesive interactions in lymphocyte positioning. The results of these experiments demonstrated that cells of one population are capable of diminishing the adhesiveness of the unlike population (Curtis & de Sousa, 1975). We observed also that the frequency of what is today known as "homotypic interactions" in normal mouse lymph node cell populations was significantly higher than that of unlike cells. Administration of anti-lymphocyte antiserum, by altering the balance of T and B lymphocytes, resulted in an increase in the frequency of unlike T/B cell pairs and B/B cell pairs (de Sousa & Heston, 1976).

If single cell/cell interactions may be of importance in the regulation of "fine" cell positioning and B cell activation, cell positioning in tissue compartments or in whole organs may

be dictated by additional interactions of the circulating cells with extracellular matrix components (Kupiec-Weglinsky & de Sousa, 1990). The fact that some of these components may themselves contribute to T lymphocyte activation (Nojima et al., 1990) signifies, that it will become increasingly difficult to dissociate lymphocyte migration from lymphocyte positioning and lymphocyte activation. But even if we begin to unravel a role for the ECM in positioning and activation and come to accept that the balance of the major lymphoid cell populations in the context of the ECM per se is sufficient to determine interactions relevant to T and B cell activation, cytokine and immunoglobulin production, we are still left with the question of a possible fundamental biological role of the system as a system, not just of the interactions within itself but also "dialoguing" with other systems.

I have proposed that one of the most important components of this dialogue, has to do with the resolution of the oxygen paradox imposed by the evolution of the circulation of the red blood cell (de Sousa, 1989).

EVOLUTIONARY STEPS OF THE TRANSPORT AND DELIVERIES OF OXYGEN BY HEMOGLOBIN

The evolutionary steps that placed oxygen at the heart of aerobic life and selected hemoglobin for the transport and the delivery of oxygen to the tissues, created a major biological paradox. As pointed out by Grishman and McCord, "although oxygen is essential for aerobic life, too much oxygen or inappropriate oxygen metabolism is highly toxic" (Grishman & McCord, 1986).

Analysis of the mechanism of toxicity of O_2 has shown that although some of the damaging effects are mediated by O_2^-, most involve the production of other species in the presence of metal complexes. Of these, the hydroxyl radical is perhaps the most damaging. Its formation occurs from the interaction of O_2^- and H_2O_2 according to the iron catalyzed Haber Weiss reaction:

$$O_2^-. + Fe^{3+} \qquad O_2 + Fe^{2+}$$

$$H_2O_2 + Fe^{2+} \qquad HO. + OH^- + Fe^{3+}$$
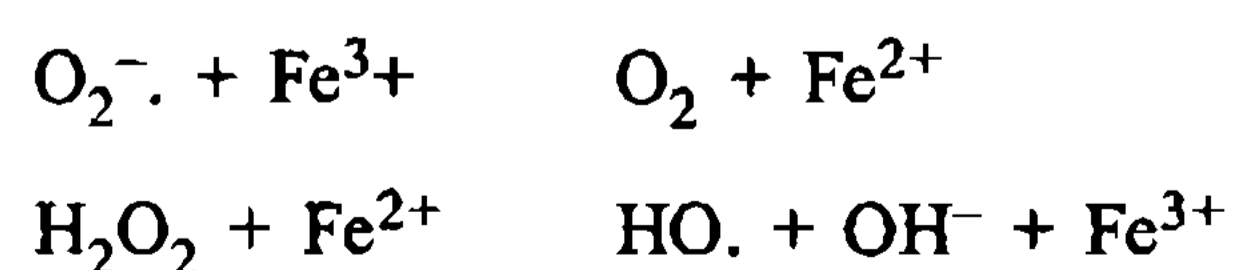
Iron is also involved *in vivo* in accelerating lipid peroxidation by stimulating the decomposition of hydrogen peroxide (for review, see Gutteridge, 1989).

The evolution of oxygen transport – As a result of the poor solubility of O_2 in water, if our arteries contained a simple aqueous solution, one liter of such a fluid would contain only approximately 3 ml of O_2. To meet the demands of energy exacted by the muscles involved in a strenuous run, a heart would have to pump over 1000 liters of that solution per minute, for O_2 to be adequately distributed to the tissues (Weibel, 1984).

Thus, a number of oxygen carriers evolved, of which hemoglobin became the one selected for oxygen transport in mammalian life. If human hemoglobin itself circulated in soluble form, its half life would have been approximately 40 min; the excess weight imposed by the circulation of such a viscous fluid to carry O_2 throughout the body of a normal adult man, would have been equivalent to a 20 l volume (Lehmann & Huntsman, 1961). The erythrocyte appears thus as one critical step in the evolution of mammalian life. Its disc shape in man gives the erythocyte a surface of 163 μm^2, a surface 70% larger than that of a sphere of equal volume, one better adapted to a greater gas exchange. In an analysis of the morphology of red blood cells separated in a density gradient, we have now evidence that older red blood cells have a spherical shape, in marked contrast whith the disc shape of the younger cells (Moura, 1990). Erythrocytes have a red color which is due to their high hemoglobin concentration. Hemoglobin occupies about a quater of the volume of an erythrocyte internal space; the presence of highly soluble hemoglobin at high concentration increases blood O_2 capacity. As a result, in one liter of human blood we find about 150g of hemoglobin (15g/100ml) which can bind 8.7 nmol of O_2, thus increasing the O_2 by about 30 times that of a single water solution (Weibel, 1984).

The large occupation of the mammalian red blood cell by hemoglobin coincides with the absence of other intracellular structures, namely the nucleus, ribosomes, organelles, etc. A mature mammalian red blood cell has lost its capacity to synthesize proteins, consequently its repair ability and probably the capacity to maintain its disc shape, becoming a cell with a limited lifespan, that, in man, lasts about 120-140 days. This signifies that 0.7% of all erythrocytes i.e. 175 x 10^9 cells must be replaced by new ones every day, at the end of what has been estimated as a life time journey of 700 miles (Lehmann & Huntsman, 1961).

In retrospect, if 175 x 10^9 erythrocytes were lost daily, two major threats followed: a) a dependence on environmental supplies of iron sufficient to maintain the production of an equal number of new red blood cells; b) the potential tissue toxicity associated with the "unattended" hemolysis of such large volumes of blood (175 x 10^9 red blood cells correspond approximately to 35cc of blood).

It becomes thus "logical" to watch evolution move with the development of a tight iron recycling system rendering erythropoiesis practically independent of external iron supply. Because the major threat posed by the breakdown of the effete red blood cells could be resolved by their clearance, it is not surprising that clearance and recycling of the iron associated with the hemoglobin, became the job of cells capable of scavenging other cells, namely, the macrophages.

This solution ensured that oxygen delivery and iron utilization for hemoglobin synthesis would not be left to the mercy of variations in the environmental supply of iron or to depend on its limited biovailability. An illustration of the exquisite individual stability of red blood cell numbes maintained in circulation in the face of serial phelebotomies in an idiopathic hemochromatosis patient is shown in Fig. 1. In a strict anatomical sense, iron recycling, which normally takes place in the spleen, stands at the frontier between the oxygen transport and the immunological systems. In a wider biological sense, this frontier, could represent itself a major motivation for the development of an immune system in which the capacity of macrophages to discriminate between old and young blood cells in order to recycle iron only from the old ones, could be the first critical expression of recognition of self.

In this context, it is of interest to note that "antigen-free" mice contain exclusively in their spleen, as many activated B and T lymphocytes as conventionally bred, healthy animals, in striking contrast with poorly developed lymph nodes or mucosal associated lymphoid tissues (Pereira et al., 1986). Lymphocyte activation in response to autologous red cell recycling could be a significant immunological by-product of a major biological adaptation; "autoreactive" splenic activated lymphocytes have been shown to act as effector cells and to secrete IgM antibodies (Pereira et al., 1986; Hooijkas et al., 1984). Moreover these early
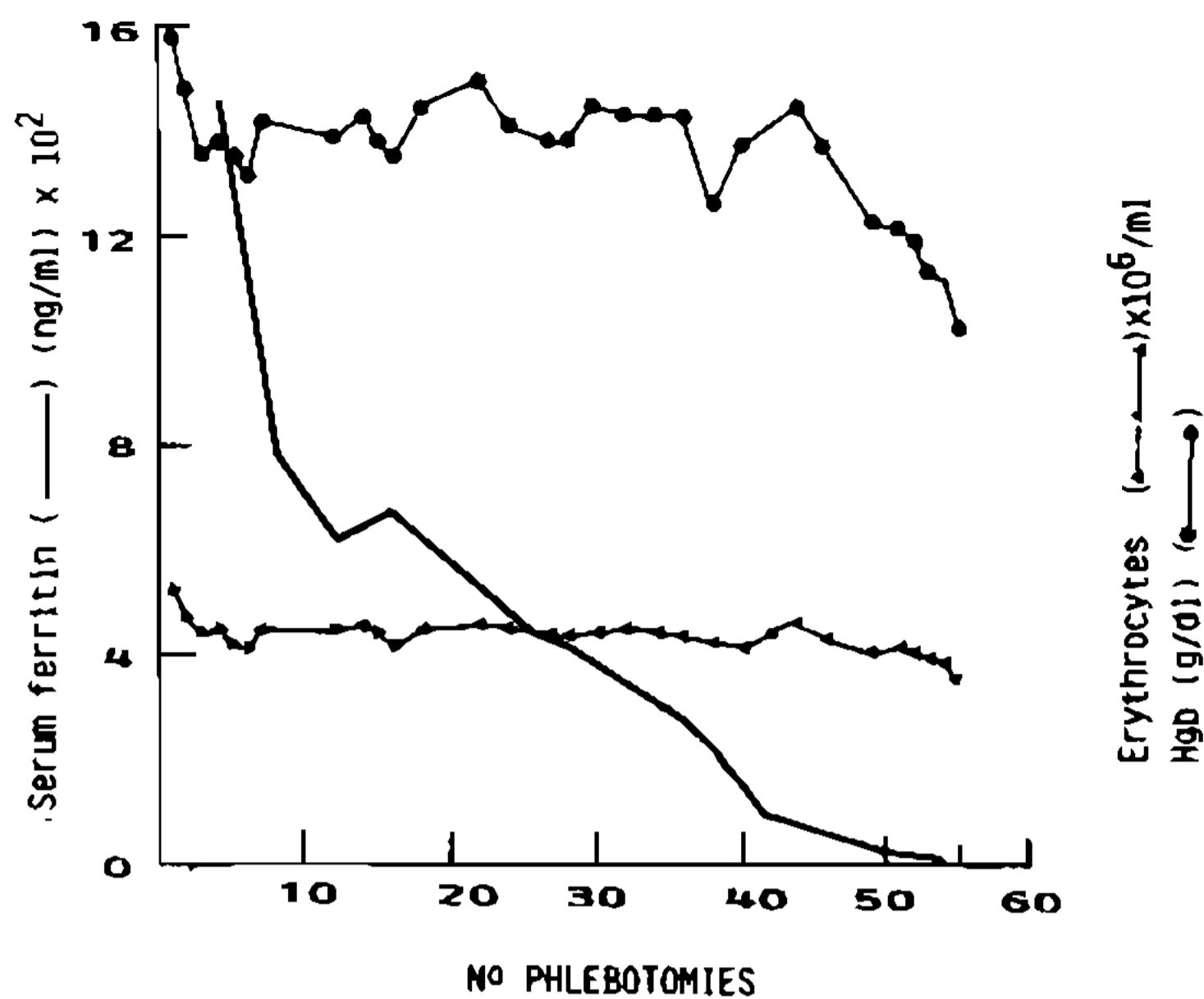
NO PHLEBOTOMIES

Fig. 1: changes in serum ferritin levels throughout a therapeutic course of 55 serial phlebotomies in a HH patient aged 45 years at the time of diagnosis and start of therapy. Note the stability of erythrocyte numbers.
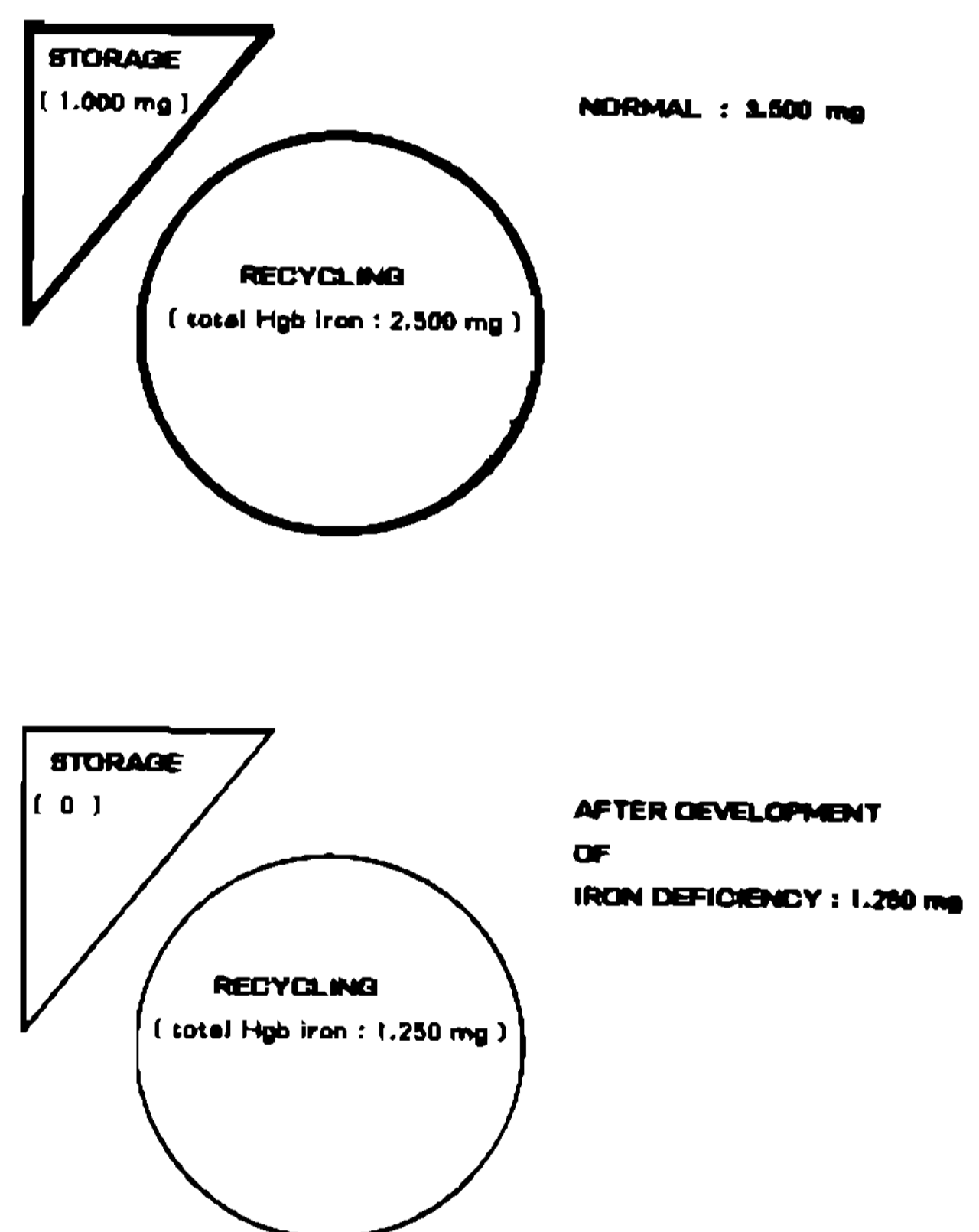
storage and thus reducing external requirements throughout life to less than 1 mg per day (Burman, 1974). Harris & Kellermayer (1974) have estimated that "the development of iron lack-anemia in the adult male and post-menopausal female because of lack of ingestion of iron is therefore for practical purposes just about impossible. For iron deficiency to occur iron loss must be increased. Their estimations, shown in Fig. 2, stand in manifest conflict with the reported prevalence of iron deficiency anemia in 500-600 million people in the world (Cook & Lynch, 1986).

IgM antibodies seem to play a major role in the initiation of idiotype networks (Coutinho, 1988). Considering that in an adult man, splenic macrophages recycle 3-4 million old red blood cells per second (de Sousa, 1989), the power of such a biological stimulus to maintain a pool of so-called "autoreactive" lymphocytes should not be underestimated. It generally is.

A tight iron recycling system having evolved primarily to secure the stability and the autonomy of erythopoiesis and of oxygen delivery, may serve equally well the demands of other steady state functions dependent on iron. These include the oxidative production of cellular energy in the form of ATP, dependent on the electron transport enzymes, the cytochromes a, b and c, other non-haem compounds involved in oxidative metabolism including NADH-dehydrogenase, the reduction of endogenously generated hydrogen peroxide by catalase and peroxidase. Oxygen storage for utilization during muscle contraction is assured by myoglobin, the red pigment of the muscle. Iron is also necessary for the synthesis of DNA for the reduction of the ribonucleotides to deoxiribonucleotides by ribonucleotide reductase (Graslund et al., 1982; Chitambar et al., 1988).

To secure the autonomy and the stability of the supply of iron for all these vital functions, evolution created in addition a back-up system, placing sufficient amounts of iron in

Fig. 2: diagrammatic representation of the two major sources responsible for an almost complete impossibility for an adult man to develop a negative iron balance, according to Harris and Kellermeyer: storage iron, which in the normal is 1,000 mg and total hemoglobin iron 2,500 mg (recycled through macrophages at the rate of 3-4 x 10⁶ red blood cell per second). After development of iron deficiency, considering storage iron 0, the total haemoglobin iron is still 1,250 mg.

Assuming that no iron is absorbed and 1 mg is excreted per day, it would take 2,250 days (6.3 years) of negative iron balance for iron deficiency anemia to develop. According to the WHO, 500 to 600 million people in the world are believed to have iron deficiency anemia.

EVIDENCE FOR THE CONTROL FROM INSTANCES OF "MISCONTROL"

Participation of products of the activation of the immunological system in the pathogenesis of iron deficiency anemia – Studies of the effect of well-characterized products released by cells of the immunological system on iron kinetics and erythropoiesis *in vivo* (Marchal & Milon, 1986; Moldawer et al., 1989) and on bone marrow cell differentiation *in vitro* (Zoumbos et al., 1984) have, for some time demonstrated that activated macrophages as well as activated lymphocytes are likely candidates to provoke iron deficiency and anemia. Recognizing that the immunological system has a major biological role in controlling the hemopoietic system via the control of the iron kinetics leads us to consider that the widespread occurrence of iron deficiency anemia in the world may not be simply a problem of nutritional iron dificiency to be resolved by iron intake, but perhaps the result of a much more complex series of interactions involving the immunological system. These interactions could involve infection, inflammation, response of the immunological system directed to the infection with "misdirected" effects on the hemopoietic system. The complex nature of these interactions gains further support from the reported instances of widespread and lethal infections associated with the administration of iron in populations vulnerable to infection, particulary in areas with endemic malaria (Oppenheimer, 1989). These occurrences have been linked to the known utilization of iron by microorganisms (Weinberg, 1978); if, as we shall see later, one of the responses of the immunological system to iron overload appears to be a differential expansion of the two major T cell populations the uncontrolled infections could result from the imbalance of T cell populations and not simply from a direct nutritional cause effect relationship between iron and the micro-organism (Weinberg, 1978).

T lymphocyte sets in iron overload: a novel correlation – We were led to consider the question of the complexity of the interactions between iron load and cells of the immune system, with the results of a study of T lymphocyte sets in idiopathic hemochromatosis. Idiopathic hemochromatosis is an MHC I linked genetic disease of iron overload. For the past six years, Drs Graça Porto and Berta Martins da Silva in Oporto, have been HLA typing and characterizing clinically patients and their families and the corresponding control populations for iron metabolism parameters (Porto et al., 1988, 1989, 1991).

In addition, the patients have been followed throughout their serial phlebotomies therapy protocols. This has given us some insight into the extraordinary stability of all lymphoid cell populations in the blood. The details of the results of this study are published elsewhere (Reimão et al., 1991). Briefly throughout the serial phlebotomy treatment, each individual manifested a great stability of its CD4: CD8 ratios. This stability was apparent whether the results were looked at as absolute numbers of cells or percentages; in addition, the study permitted to demonstrate the existence of a statistically significant positive correlation between the relative proportions of CD4+:CD8+ T lymphocytes, the severity of iron overload and length of the response to therapy by repeated phlebotomy; the higher the CD4:CD8 rations seen in the patients, the higher the number of phlebotomies required for correction of the excessive iron balance.

After completion of therapy, i.e., starting from a corrected iron balance, in patients with unusually high (> 2.9) CD4:CD8 ratios, the re-entry of iron in the serum transferrin pool is occurring at a faster rate, reaching abnormal ($\geq$ 60%) transferrin saturation values more rapidly than in patients with normal or close to one CD4:CD8 ratios.

The finding of a novel correlation between a higher rate of iron reentry into the transferrin pool and higher CD4/CD8 ratios, was found to be related both to the low numbers of CD8 cells and to the high numbers of CD4 cells. This led us to see if in thalassemia major, in a situation of transfusional iron overload, we could use T cell proportions as probable indicators of increased iron absorption in individual patients.

In the transfusional iron overload of β-thalassemia major, the analysis of the absolute numbers of cells and of CD4/CD8 ratios is complicated by the effect of the iron overload on the percentages of circulating CD4+ cells and by the effect of splenectomy on the absolute numbers of cells in the blood (for review see de Sousa, 1989).

It was thus decided to subdivide a group of 30 HIV negative β-thalassemia major patients according to their percentages of CD8+ cells

(whether lower than the minimal control value or within control values). We subdivided further the patients in HCV+ and HCV– and could then observe the separate correlations of low or normal CD8+ cells with iron overload and liver damage measured by liver enzymes.

The data are presently being finalized for publication (Grady et al., in preparation). The results show that HCV+ patients have significantly higher liver enzymes when their percentages of CD8+ cells are low than HCV+ patients with low percentages of CD8+ cells. Liver enzyme levels were similar to those in HCV+ with normal CD8+ percentages. In HCV negative patients, liver enzymes increase proportionally to serum ferritin levels. This correlation is lost in the HCV+ group.

These results represent our first opportunity of finding ourselves at the crossroads of two immunologies: a "classical" immunology where CD8+ cells are expected to protect from tissue damage related to a viral infection (Zinkernagel & Doherty, 1974) and the immunology that we have committed ourselves to explore, in which iron status is inextricably related to immunological function (de Sousa, 1978, 1987, 1983, 1989) the expansion of the two major T cell subpopulations (de Sousa et al., 1991) and perhaps even to the establishment of the peripheral T cell repertoire in man (J. M. Cabeda, M. J. Saraiva & M. de Sousa, in preparation). The latter will have to wait for confirmation.

CLOSING REMARK

As a closing remark, I would like to say that for someone who has been walking alone on an immunological side track for the last 13 years (de Sousa, 1978) this sense of hitting the main road again brings an undescribable sense of peace.

The kind of peace experienced by those who return home, after many years of travel abroad.

It is only appropriate that the Institute Oswald Cruz should provide this sense of home to a Portuguese speaking immunologist. For, at a distance, the Institute has been a home to all of us in the Portuguese speaking world.

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