

**THE RELATIONSHIP BETWEEN RED BLOOD CELL CONCENTRATION AND
INFLAMMATION: STUDIES IN RUNNERS AND RHEUMATOID ARTHRITIS
PATIENTS**

by

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ABSTRACT

This investigation sought to define further the pathophysiology of anemia accompanying two stimuli of an inflammatory response: an exercise-induced inflammatory response to focal disruption of muscle and a disease-linked inflammation of synovial joints in rheumatoid arthritis (RA). Specifically, the contribution of erythrocyte sequestration in the spleen or liver to a variation in intravascular red blood cell (RBC) concentration was studied both in runners (n=6) preparing for and recovering from a marathon competition and in RA patients (n=10) during a disease flare and subsequently throughout four weeks of methotrexate treatment. Plasma concentration of the cytokines tumor necrosis factor-alpha (exercise study), gamma interferon (IFN- γ) (RA study) and interleukin (IL)-1 β , IL-6 (both studies) were serially measured in order to determine their relation to plasma erythropoietin (Epo) concentration, reticulocyte count and erythrocyte content of the spleen and liver, any of which may contribute to a variation in RBC concentration during either type of inflammatory response. Cytokines and Epo were measured using commercially available ELISA kits. All other blood measures were analysed in a professional laboratory using standard techniques. Chromium 51-labeled RBCs (^{51}Cr -RBC) were used to detect variation in the erythrocyte content of an organ. In the exercise study, liver ^{51}Cr -RBC content increased and splenic ^{51}Cr -RBC content tended to increase 24 or 48 hours after the marathon run concomitantly with a decline in RBC concentration. Due to limitations of the method, the contribution of sequestration of red cells to the reduction of RBC concentration following the marathon remains unclear. An increase in mean plasma Epo concentration may have mediated the increase in mean reticulocyte count observed in the runners seven days after the marathon, when RBC concentration had returned to its baseline value. In the RA study, there was no evidence of release of sequestered red cells during methotrexate treatment. An increase in RBC concentration corresponded with an increase in reticulocyte count in three out of four anemic RA subjects, suggesting that erythropoiesis was stimulated as disease activity declined. A

corresponding decrease in plasma Epo concentration suggests that it played no role in the increased reticulocyte count. Further research is required to confirm these findings and to determine the mechanism responsible for the observed increase in reticulocyte count during methotrexate treatment.

Dedication

To the subjects of the study, who made this thesis possible

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Chapter 1. General Introduction

In all higher organisms, oxygen transport from the ambient air to tissue is mediated by binding of oxygen to hemoglobin in the red blood cell. Appropriate tissue oxygenation is determined in part by the concentration of hemoglobin in the blood, which is normally regulated within a narrow range. When a condition arises in which the hemoglobin concentration of the blood falls below a certain level, an individual is said to be anemic. In the developed countries there is a 10% to 20% prevalence rate of anemia (Scrimshaw, 1985). A specific type of anemia is associated with chronic infection, inflammation, malignancy and autoimmune disorders (Means and Krantz, 1992) and is found in about half of all anemic patients admitted to a public hospital (Cartwright and Wintrobe, 1952; Cash and Sears, 1989). The precise pathogenesis of this "anemia of chronic disorders" (ACD) remains unclear, but is most likely multifactorial. Profound metabolic alteration takes place in man during an inflammatory or immune challenge whatever its origin. This investigation sought to define further the pathophysiology of anemia accompanying two stimuli of an inflammatory response: an exercise-induced inflammatory response to focal disruption of muscle and a disease-linked inflammation of synovial joints in rheumatoid arthritis (RA). The following pattern is hypothesized to apply to a condition of continuing inflammation ("inflammation on") then a condition of reduced inflammation ("inflammation off") in both the exercise and RA studies:

INFLAMMATION ON

- ↑ plasma cytokine concentration
- ↓ plasma erythropoietin concentration
- ↓ reticulocyte count
- red blood cells (RBC) sequestered in the spleen
- ↓ intravascular RBC concentration

INFLAMMATION OFF

- ↓ plasma cytokine concentration
- ↑ plasma erythropoietin concentration
- ↑ reticulocyte count
- sequestered RBCs released
- ↑ intravascular RBC concentration

Development of the hypotheses

The inflammatory response

The process of inflammation has as its main objectives neutralization and removal of pathogens and tissue debris and initiation of tissue repair. Phagocytic cells must be summoned to the site of injury and the immune system must be primed (in the case of an invading pathogen). The primary local events in response to injury are histamine release from the mast cell leading to vigorous vasodilation of the local vasculature and increased blood flow to the area (Hurley, 1972) and an increased vascular permeability (Gordon and Koj, 1985). Adhesion molecules expressed on the endothelium mediate the attachment of flowing leukocytes to the blood vessel wall (Bevilacqua 1993). Captured leukocytes then move between endothelial cell junctions into the injured or infected tissue. A local concentration of neutrophils peaks in the first hours, followed by a steady rise in number of monocytes during 48 hours following injury or infection (Gordon and Koh, 1985).

Local production of cytokines

Direct activation of monocytes after phagocytosis of microbial products or cellular debris leads to local production of the pro-inflammatory cytokines tumor necrosis factor-alpha (TNF- α) and interleukin (IL)-1 (Le and Vilcek, 1987; Helle et al., 1988). IL-6, released by fibroblasts, T-lymphocytes, endothelial cells, monocytes and keratinocytes induces an acute phase response, described below. IL-8, another locally produced cytokine, promotes cell migration to a site of focal disruption or disease (Brennan et al., 1990) and IL-1 and TNF- α induce adhesion molecules (Bevilacqua 1993). In the immune response activated T-lymphocytes produce lymphokines such as IL-2, IL-3, IL-4 and gamma interferon

(IFN- γ) (Tizard, 1984). In addition, activated macrophages and lymphocytes can produce transforming growth factor-beta (TGF- β), involved in wound healing and suppression of pro-inflammatory Th 1 cells (a subset of CD4+ T lymphocytes) (Chen et al., 1994), eicosanoids (prostaglandins and leukotrienes) and free radicals, which have a potent pro-inflammatory action (Das, 1991).

The acute phase response

Locally produced cytokines gain entry into the circulation and mediate a vast number of systemic adjustments, collectively termed the "acute phase response". Many of these adjustments act to limit the harmful effects of an inflammatory response. Adrenal glucocorticoid production increases and by feed-back inhibition reduces production of TNF and IL-1 (Doherty et al., 1992). Natural inhibitors of IL-1 and TNF- α production, such as IL-10, have been detected in the affected joint of an RA patient (Katsikis et al., 1994), apparently in an insufficient amount to control inflammation. Another protective feedback mechanism, activated mainly by IL-6 and glucocorticoid, is an increased hepatic production of the "acute phase proteins", many of which have an anti-inflammatory effect (Table 1.1). Redistribution of trace metals results in an acute hypoferrremia and hypozincemia (Pekarek et al., 1972). Serum ferritin also behaves as an acute phase reactant and its rise accompanies an acute phase response as part of the hypoferrremic response (Baynes et al., 1986). Endocrine changes described in infected and injured subjects include an increased secretion and synthesis of the hormones glucagon, insulin, adrenocorticotrophic hormone (ACTH), cortisol, adrenal catecholamines, growth hormone, thyroid stimulating hormone, thyroxine, aldosterone and vasopressin (Beisel, 1977; Egdahl et al., 1977). Accompanying an increase in the fluid regulating hormones aldosterone and vasopressin is an acute increase in plasma volume and a dilutional pseudoanemia.

Table 1.1 Acute phase proteins.

| Protein | Function | Protein | Function |
|------------------------------|-------------------------------------|-------------------------|-----------|
| <u>Major Changes*</u> | | <u>Negative Changes</u> | |
| C-reactive protein | opsonization | albumin | (?) |
| serum amyloid A protein | alolipoprotein | transferrin | transport |
| <u>Medium Changes+</u> | | | |
| fibrinogen | coagulation | | |
| haptoglobin | bind and remove hemoglobin | | |
| hemopexin | binds heme | | |
| ceruloplasmin | O ₂ scavenger, transport | | |
| serum amyloid P | (?) | | |
| complement C3 | opsonization | | |
| alpha 1-proteinase inhibitor | antiproteinase | | |
| alpha 1-antichymotrysin | antiproteinase | | |
| alpha 1-acid glycoprotein | transport | | |

* concentration usually increases several hundredfold.

+ concentration usually increases 50%-to-fourfold.

If infective agents are destroyed and cellular debris is removed, signs of healing are normally apparent 72 hours later. Should the inflammatory process fail to restore normal structure and function to the injured tissue, the inflammatory response will continue. Cytokines produced by activated leukocytes and other cells at the site of injury or infection are known to alter the functional capacity of the erythron. Mechanisms which describe how the continuous presence of circulating inflammatory cytokines might disturb normal erythropoiesis are described below.

Hemopoietic cytokines and growth factors support erythropoiesis

Hemopoiesis is regulated by a group of soluble and cell-associated molecules known as hemopoietic cytokines. Cytokines important in the support of erythropoiesis are IL-3 (Emerson et al., 1987), IL-4 (deWolf et al., 1991), IL-9 (Birmer et al., 1992), Insulin-like

growth factor-1 (Cotton et al., 1991) and the newly described Stem Cell Factor (Ohneda et al., 1992; Toksoz et al., 1992). The growth factor erythropoietin (Epo) is essential to the proliferation and differentiation of erythrocytic progenitors (Krantz and Jacobson, 1970). In an anemic human with intact kidney function, plasma Epo activity is roughly an inverse exponential function of the blood hemoglobin concentration and the hematocrit (Erslev et al., 1987). Recombinant human (rh)Epo is currently approved for treatment of anemia associated with HIV infection and chronic renal failure and rhEpo trials are ongoing for treatment of anemia in the cancer and RA patient, and in the premature infant (Abels and Rudnick, 1991).

Inflammatory cytokines alter erythropoietin response to hypoxia *in vitro*

The cytokines of inflammation have been implicated in the reduction of the Epo response to hypoxia. *In vitro* studies, using human hepatoma cell lines Hep 3B (Faquin et al., 1992) and Hep G2 (Fandrey and Jelkman, 1991), have demonstrated a reduced Epo production when hypoxic cells are exposed to the inflammatory cytokines IL-1, TGF- β and TNF- α . Interestingly, IL-6 was synergistic with hypoxia in inducing Epo production. A reduced Epo production has also been shown in the hypoxic, isolated, perfused rat kidney treated with IL-1 β (Jelkman et al., 1992). A definite correlation has yet to be made between the effect of inflammatory cytokines on Epo production in an *in vivo* animal model or in humans. Inflammatory cytokines and plasma Epo concentration were measured in each study of the present investigation to determine if a suppressed Epo response, secondary to continuous presence of inflammatory cytokines contributes to the decreased red blood cell concentration observed during an inflammatory response.

Inflammatory cytokines may suppress erythropoiesis

Suppressors of erythropoiesis are less well defined. The block to erythropoiesis may be caused by any of a number of direct or indirect mechanisms such as arresting of erythroid progenitors in the G₁ stage of the cell cycle by interferons and IL-6 (Resnitzky et al., 1992) and TGF- β (Axelrad, 1990) or by repressing the expression of the SCF gene by bone marrow stromal cells, as in the *in vitro* effect of TGF- β (Harms et al, 1991; Heinrich et al., 1992) and TNF- ∞ (Andrews et al., 1992). IFN- γ , TNF- ∞ and IL-1 have been shown to inhibit erythropoiesis *in vivo* and *in vitro* and have been implicated in ACD. *In vitro*, TNF- ∞ has been reported to suppress erythroid colony formation (Abboud et al., 1991; Vreugdenhil, 1992b) and in another study, IL-1 stimulated early-stage progenitors (BFU-E) and had no effect on late-stage progenitors (CFU-E) (Zucali et al., 1987). Means et al., 1990 demonstrated that the inhibitory effect of TNF on CFU-E colony formation was indirect and mediated by a soluble factor released from marrow stromal cells, now identified as β interferon. *In vivo* studies (murine) have demonstrated that IL-1 ∞ and TNF- ∞ significantly suppress CFU-E formation, an effect reversed by treatment with exogenous Epo (Johnson et al., 1991). Clibon et al. (1990), however, found that Epo failed to reverse the anemia developed in mice continuously exposed to TNF- ∞ . IFN- γ inhibition of human erythroid colony-forming units *in vitro* can be corrected by addition of rhEpo (Means et al., 1989). There is as yet no data from human studies to show conclusively that anemia is caused by the direct suppression of erythroid precursors as a result of the continuous presence of inflammatory cytokines. Inflammatory cytokines and reticulocyte count, as an indicator of erythropoietic activity, were measured in each study of the present investigation .

Cytokines that disturb iron metabolism

Cytokines also affect erythropoiesis indirectly by decreasing iron availability to the developing erythrocyte. It has been hypothesized that the action of IL-1 leads to a cellular inability to utilize iron because of the release of competitive iron-binding proteins such as lactoferrin from activated neutrophils (Van Snik et al., 1974). Lactoferrin may then act preferentially to shuttle iron to a macrophage storage site (Abels and Rudnick, 1991). TNF- α is also implicated in the underutilization of iron secondary to its effect of increasing ferritin expression and transferrin receptor expression on monocytes, which leads to iron uptake (Silver and Hamilton, 1992). Reduced serum iron and a reduced proportion of marrow nucleated red blood cells (marrow cellularity increased) was noted in rhesus monkeys administered rhIL-6 (Srinivasiah et al., 1992; Winton et al., 1994). Markers of iron metabolism were measured in each study of the present investigation to determine if a disturbance in iron metabolism contributes to the decreased red blood cell concentration observed during an inflammatory response.

Treatment with IL-6 may cause sequestration of red blood cells leading to anemia

In preclinical studies, animals receiving IL-6 develop an anemia which reverses on cessation of treatment (Asano et al., 1990; Srinivasiah et al., 1992). In human trials of rhIL-6, used to increase the platelet count in cancer patients receiving chemotherapy, most patients experienced a mild to moderate fall in hemoglobin concentration (Chang et al., 1987; Demetri et al., 1992; Gordon et al., 1992; Weber et al., 1993). In a non-human primate chemotherapy model, Winton et al., 1994 found that the pattern of reticulocyte recovery was similar in rhIL-6-treated animals and controls, thus it is unlikely that suppressed erythropoiesis accounted for the reduced hemoglobin concentration observed in rhIL-6-treated animals. In the study of Srinivasiah et al. (1992), rhesus monkeys

administered a rhIL-6 injection twice daily showed a mean hemoglobin value significantly below that of control animals, while serum haptoglobin and bilirubin remained normal with no evidence of gastrointestinal blood loss. Serum iron was reduced, indicating a possible role for IL-6 in abnormal iron distribution. Within four days of cessation of rhIL-6 treatment, hemoglobin had returned to a control concentration and the authors suggested that early anemia associated with rhIL-6 treatment may be secondary to hemodilution and/or sequestration of red blood cells. This interpretation would explain the seeming paradox of the *in vitro* findings that IL-6 was synergistic with hypoxia in inducing Epo production (Faquin et al., 1992) and that addition of anti-IL-6 antibody to human bone marrow cultures impaired erythroid colony growth (Vreugdenhil et al., 1990). *In vivo* data supporting an increased erythroid progenitor number in response to rhIL-6 treatment (Hangoc et al., 1991; Ulrich et al., 1991), leads one to expect an increase in erythrocyte count during rhIL-6 treatment, yet the opposite is found. Hemodilution is expected as an acute response to infection or injury, as described above. Splenic sequestration of erythrocytes as a mechanism of reduced red cell concentration observed in response to inflammation has not been reported and is discussed below

The human spleen as a reservoir for red cells during inflammation

Capillaries in the spleen are highly porous and allow a large number of whole blood cells to pass into the cords of the red pulp. Although the human splenic capsule is non-muscular, dilatation of vessels within the spleen can lead to storage of several hundred milliliters of blood (Timens, 1990). Control of splenic storage of erythrocytes in humans is poorly understood. The hematocrit of blood stored in the spleen has been estimated to be between 50-70%, far exceeding the venous hematocrit (Flamm et al, 1990), so that blood released from or sequestered in the spleen may change the hematocrit of the circulating blood.

However, as the normal-sized human spleen pools only about 5% of the total erythrocyte population, its reservoir function is thought to be quantitatively unimportant (Allsop et al., 1992). While the reservoir function of the whole visceral circulation is important and can contribute an extra litre of blood to the arterial circulation in less than a minute (Guyton and Hall, 1996), the hematocrit of the circulating blood will not change as a result. A unique aspect of the present study is the monitoring of splenic and hepatic red blood cell content to determine if sequestration contributes to the decreased red blood cell concentration observed during an inflammatory response. The experimental groups investigated in this study are described below.

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic, disabling and painful disease of the synovial joints. Population studies suggest a prevalence of 1/100 and although peak incidence is in middle age, no age is exempt. RA has a prevalence of three to one in the female over the male. The type of arthritis varies according to geographic location but its frequency is approximately the same in every country of the world. The etiology of RA is largely unknown although, in the majority of patients, non-genetic factors such as the environment, sex hormones and dysfunction of the hypothalamic-pituitary-adrenal axis are important. A genetic factor may account for up to 60% of a person's susceptibility to the disease (Macgregor and Silman, 1994), and a second factor activates the predisposition of the disease to become manifest. The association of RA with an HLA-DR molecule, which is involved in antigen binding and its subsequent presentation to T-cells, together with the presence in rheumatoid joints of activated memory T cells and autoantibodies in the serum of patients, suggests an important role for an immune response in initiating and maintaining RA (Maini et al., 1995). The observation of a deficient secretion of IL-4 and a detectable

level of IFN- γ mRNA in rheumatoid joints is consistent with a predominance of the TH1 subset over the TH2 subset of T cells, suggesting a T-cell-driven chronic inflammatory disease (Maini et al., 1995).

The main pathological event in RA involves the synovial joint which is infiltrated by activated T-cells, neutrophils, monocytes and plasma cells. Inflammatory cytokines orchestrate an interaction between immigrant cells and resident connective tissue cells and matrix to produce an adherent pannus tissue, which erodes cartilage and bone (Jalkanen et al, 1986). Concomitantly there is an unsuccessful attempt at regeneration and repair of disrupted/degenerated tissue. IL-1, IL-6, TNF- α and TGF- β , mainly macrophage-derived cytokines, are usually detected in large quantities in synovial fluid (Ridderstad et al., 1991). IL-1 and TNF stimulate IL-6 secretion from lymphocytes, fibroblasts, synovial and endothelial cells to the synovial fluid. IL-6 which enters the circulation leads to production of acute phase proteins. An anemia is a common finding in patients with RA and the degree of anemia correlates with the severity of inflammatory activity. Continuous presence of circulating cytokines are thought to contribute to the development of anemia in Rheumatoid Arthritis and other chronic diseases. Thus, studies have focused on the cytokines produced during the inflammatory response to discern their role in the pathogenesis of the anemia accompanying RA. These studies are reviewed in the introduction to Chapter 4.

Runners with exercise-induced skeletal muscle disruption

The local, metabolic and systemic sequelae following sustained strenuous exercise are similar to a pathologically induced non-immune inflammatory response. A decrease in RBC concentration observed following intense exercise and a low-normal RBC concentration observed during continuous training were both originally attributed entirely to hemodilution and termed a "pseuoanemia" (Oscari et al., 1968; Akgun et al., 1974;

Convertino et al., 1980; Green et al., 1991). However, in recent years further observation suggests that a factor related to an inflammatory response, other than plasma volume expansion, could contribute to a decreased RBC concentration in response to sustained strenuous exercise. The plasma concentration of certain cytokines is increased following intense exercise to the level of that observed in an RA patient during the active disease stage (Sprenger et al., 1992; Northoff and Berg, 1991; Northoff et al., 1993) and there is evidence that strenuous exercise can initiate an acute phase response (Noakes et al., 1983; Taylor et al., 1987; Weight et al., 1991). Splenic sequestration of erythrocytes as a factor producing a reduced circulating red cell concentration in the response to strenuous exercise has not been previously reported and is investigated in the present study.

Hypotheses

It is hypothesized in the present study that there is a similar pathophysiology of reduced circulating red cell concentration accompanying an inflammatory response to acute and persistent strenuous exercise in runners and the active inflammatory phase in RA patients.

In runners, intense training sufficient to produce a persistent myogenic inflammatory response, and a marathon run, which produces an even greater inflammatory effect, will lead to an increase in plasma concentration of IL-1 β , IL-6 and TNF- α , which may produce:

- I. a decrease in plasma Epo concentration and/or
- II. a decrease in reticulocyte concentration and/or
- III. an increase in splenic red cell content. These factors will contribute (together with a decrease in reticulocyte concentration and/or plasma volume expansion) to a lower intravascular red cell concentration in the days following exercise

A period of rest (taper and post-marathon), during which the level of circulating inflammatory cytokines subsides will lead to release of RBCs from the spleen and normalization of the Epo and reticulocyte response, leading to subsequent normalization of the intravascular red cell concentration.

In RA subjects, active disease at the beginning of the study will correspond with an elevated plasma concentration of IL-1 β , IL-6 and IFN- γ , which may produce:

- I. a decrease in plasma Epo concentration and/or
- II. a decrease in reticulocyte concentration and/or
- III. an elevated splenic red cell content. These factors will contribute (together with a decrease in reticulocyte concentration and/or plasma volume expansion) to a subnormal intravascular red cell concentration in an RA subject with active disease.

As disease activity subsides following four weeks of methotrexate medication the level of circulating inflammatory cytokines will decrease, leading to a release of RBCs from the spleen and normalization of the Epo and reticulocyte response, contributing to normalization of the intravascular red cell concentration.

A second hypothesis adopted, which links the two studies, is that the pattern of response of cytokines, erythropoietin, reticulocyte count and splenic erythrocyte content to a change in level of inflammation in the exercise study and in the RA study will be the same. Finding a correspondence between cytokines and erythropoietin, reticulocyte count and splenic erythrocyte content in a non-immune inflammatory response to skeletal muscle disruption would further strengthen the hypothesis of a causal link between the continuous presence of circulating cytokines and anemia in a pathologically-induced chronic disorder.

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Chapter 2. General Methods

Radiolabelling of erythrocytes

The procedure followed was a modification of that described by the International Committee for Standardization in Haematology (1981). Fifteen ml of whole blood was withdrawn from a subject's vein into a heparinized syringe then added to a sterile vial containing three ml of acid citrate dextrose. Approximately 8 Mbq (200 μ Ci) of ^{51}Cr in the form of chromate ion was added to the vial with a resultant prompt transport of ^{51}Cr across the erythrocyte membrane. After incubation at room temperature for 20 minutes, 50 mg of ascorbic acid was added to reduce the free chromate to the chromic ion, disabling the latter from crossing the erythrocyte membrane. At this point, therefore, the tagging procedure was halted and the intracellular ^{51}Cr , bound to hemoglobin, remained within the erythrocyte. A standard was prepared from the sodium chromate- ^{51}Cr solution to enable correction of an organ count for radioactive decay and to adjust for machine variability at various staged time intervals of repeat ^{51}Cr -RBC count in each subject. Ten ml of a subject's tagged cells were reinjected and the remainder of the tagged blood sample was used to determine the subject's blood volume.

Determination of blood volume

Volume of red cells (RCV) was measured on the day of the red cell label. Whole blood volume (TBV) and plasma volume (PV) were calculated using RCV and hematocrit (Hct). Briefly, red cells were labelled with ^{51}Cr as described above. Thirty minutes after reinjection of the labelled cells a blood sample was drawn from the opposite arm and was analysed for radioactivity.

Following correction for radioactive decay of the second sample, RCV was determined as follows:

$$\text{RCV} = \frac{\text{Total activity in RBCs injected}}{\text{Activity in 1ml of RBCs (30 min)}}$$

$$\text{TBV} = \frac{\text{RCV}}{\text{Hct} \times 0.915}$$

$$\text{PV} = \text{TBV} - \text{RCV}$$

RCV was measured directly only once. A subject's weight and height were used to determine a normal range for TBV, RCV and PV, based on the Wennesland formulae (Wennesland et al., 1959).

Organ counts of ^{51}Cr -RBC

Figure 2.1 illustrates the experimental set-up showing the Nuclear Data Inc. 62 gamma counter (NaI crystal), the placement of the subject, and the placement of the probe used to measure the ^{51}Cr -RBC organ counts. Before each recount, the machine was calibrated using a standard ^{137}Cs source. The spectral range setting of the gamma counter (0.290-0.370 million electron volts) was centered on either side of the ^{51}Cr energy peak (0.320 million electron volts). Each count (standard, background and organs of the subject) was measured during a five minute period. A background count was established by placing the detector over the front of the thigh midway between the hip joint and the patella (Figure 2.1A). Liver activity was recorded by placing the detector over the ninth and tenth ribs on the right side of the body between the midclavicular and anterior axillary line with the subject in a seated position. Splenic activity was determined by placing the detector over the ninth and tenth ribs on the left side at the posterior axillary line, with the subject seated (Figure 2.1B). Heart activity was determined by placing the detector over the third costal cartilage on the left side. After initial positioning of the detector was established at each

site, the outer circumference of the detector was traced with permanent ink on the skin of the subject to ensure reproducibility of detector placement. Each subject returned for an external organ count one and three days after their ^{51}Cr -RBC label, then once (RA subjects and controls) or twice (runners) a week for four weeks. During each visit the standard was recounted. A normalizing factor was calculated by dividing the count obtained on the first day by the count recorded on each return visit. The background count (thigh) was subtracted from each of the heart, liver and spleen counts to establish a corrected count, which was then multiplied by the normalizing factor to give a decay-corrected count. From the external organ counting of the liver and spleen, their respective accumulation of ^{51}Cr -labeled red cells may be evaluated and the change in distribution during the period of the study could be detected (Loevinger and Berman, 1968). The normal spleen-to-liver ratio when an equal amount of tissue is counted is 1.0 and the half-life of ^{51}Cr -labeled red cells is 26.5 days. A decay-corrected organ count was used in statistical analysis of the results, but for graphical representation these data were expressed as percentage change from the first count measured.

Limitation of the ^{51}Cr -RBC method for organ counting

The estimation of organ radioactivity by external monitoring of gamma emission is complicated by several factors such as the distance of the geometrical centre of the organ from the counter, radioactivity in surrounding structures or interposing tissue, size and shape of the organ and accurate positioning of the counter. If these factors remain relatively constant then the radioactivity measured over an organ such as the spleen represents the summation of three main sources; first, the blood flow through the organ, secondly, the deposition of ^{51}Cr bound to hemoglobin, and thirdly, irradiation from other



Figure 2.1. Organ Count of ^{51}Cr -RBC: the experimental set-up showing a Nuclear Data Inc. 62 gamma counter (NaI crystal), placement of the subject, and placement of the gamma counter probe. Figure 2.1A shows the Nuclear Data Inc. 62 gamma counter connected to a computer and the subject set up for a count over the thigh (background radiation). Figure 2.1B shows the subject set up for a count over the spleen.

sites. Careful positioning of the gamma probe is required in order to ensure that the spleen count does not include counts from the liver. Ideally, the red cells should be labelled with ^{99m}Tc on the first day of organ counting so that each organ may be imaged, rather than relying totally on anatomical placement, as was the case in the present study. It is not possible to quantify the number of red cells in an organ from an external measurement of ^{51}Cr activity, nor is it possible to determine what proportion of the ^{51}Cr -RBC count is due to viable and non-viable cells using the present method. However, the choice of ^{51}Cr as the red cell label allowed a serial weekly organ count during a four week period, which was the minimum time required for detection of reduced inflammation in response to methotrexate treatment in RA subjects.

Analysis of IL-6, IL-18, TNF- α , IFN- γ , and Epo concentration in plasma

EDTA plasma samples were assayed for content of an inflammatory cytokine or Epo using commercially available immunoassay kits (Quantikine) from R & D Systems (Illinois). The Quantikine kits are solid phase immunoassay kits which are based on the double-antibody sandwich method. In this method microtiter wells, precoated with monoclonal antibody specific for the factor to be tested (Figure 2.2a), are incubated with the plasma sample or a standard. Any of a specified factor present in the sample will be bound by the immobilized monoclonal antibody in the microtitre well (Figure 2.2b). Excess plasma or standard is then removed, and each well is incubated with a conjugate of anti-factor polyclonal antibody and horseradish peroxidase, which binds to the factor captured by the immobilized monoclonal antibody (Figure 2.2c). Excess antibody-enzyme conjugate is then washed away and a substrate solution added to the wells (Figure 2.2d). The subsequent reaction is stopped by the addition of sulfuric acid. Colour is developed in proportion to the amount of factor present in the sample tested, which is then quantitated based on a standard curve (Figure 2.2e). Epo, IL-6 and TNF- α and IFN- γ values were

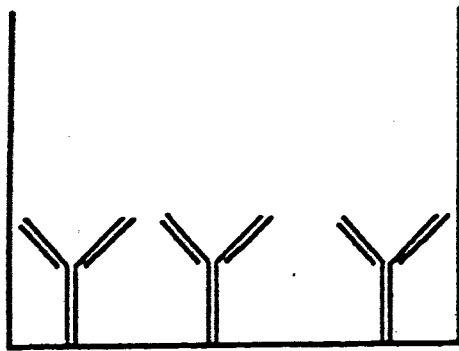


Figure 2.2a

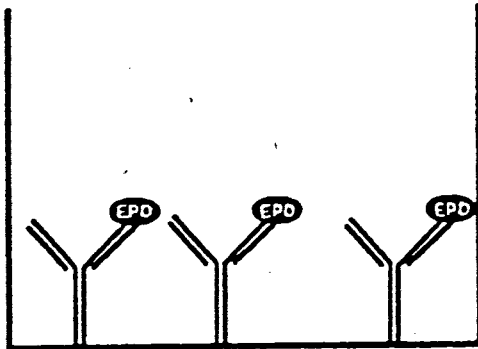


Figure 2.2b

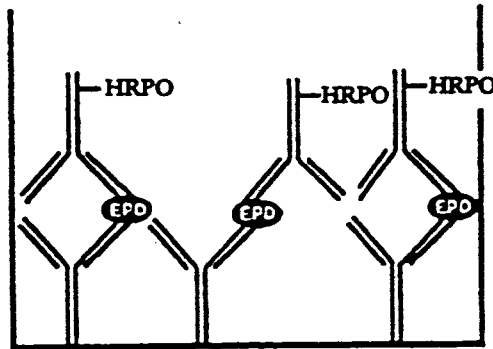


Figure 2.2c

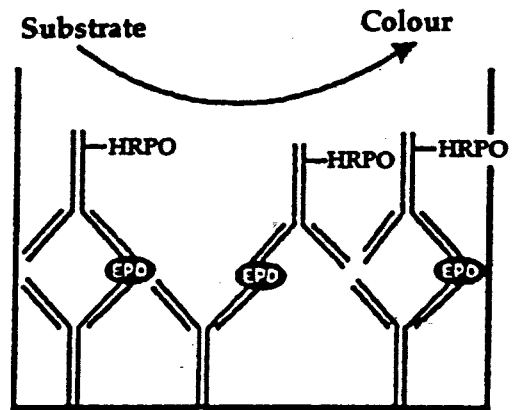


Figure 2.2d

EPO Standard Curve

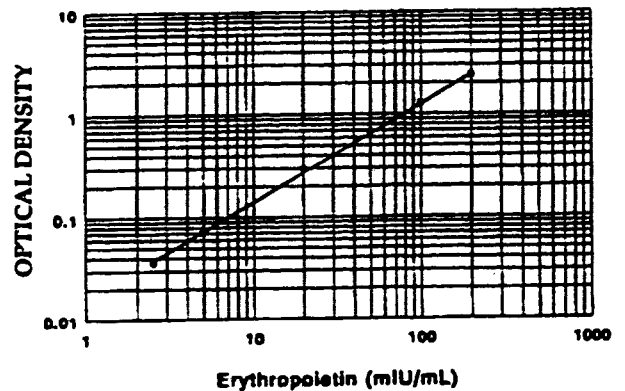


Figure 2.2e

Figure 2.2. Quantikine solid phase immunoassay kits: principles of the test.

Figures 2.2a-e above represent diagrammatically the steps of an assay of plasma for erythropoietin concentration. Figure 2.2a shows immobilized anti-Epo monoclonal antibody in a microtitre well. Figure 2.2b shows Epo bound to the immobilized antibody. Figure 2.2c shows horseradish peroxidase (HRPO)-conjugated anti-Epo polyclonal antibody capturing the bound Epo. Figure 2.2d shows the addition of a substrate which leads to development of colour in proportion to the amount of bound Epo in the microtitre well. Figure 2.2e shows a standard curve prepared from assay of an erythropoietin sample of a known concentration.

determined within 6 months and IL-1B was determined within two years following collection of the samples. Assays were performed in duplicate and intra-assay variation was <10%. Interassay comparisons were not performed although when samples were tested at a later date, all gave results within 10% of the initial measurement, except for the IL-6 values of the runners and two RA subjects. A second and third repeat assay of two plasma samples for each RA subject and a second assay of one plasma sample for each of four runners returned a value on average 50% higher than the value obtained in the first ELISA. As most laboratories report that IL-6 degrades rapidly, even at -70° C, the best interpretation of the above data is that the values obtained from the first ELISA for IL-6 were erroneously low. No blood variable concentration was corrected for hemodilution since one premise of the study is that the spleen and/or liver plays a role in the removal of red cells from the intravascular space during an inflammatory response.

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Chapter 3. Exercise-induced disruption of skeletal muscle

Abstract

The decrease in red blood cell (RBC) concentration observed following intense exercise has previously been attributed entirely to hemodilution. In the present study a role is proposed for the spleen, which may enlarge or sequester red cells during an inflammatory response. In addition, a role is proposed for the continuous presence of circulating inflammatory cytokines, which may alter the functioning of the erythron. Training and laboratory data were serially measured in one female and five male subjects throughout a four-week period of training and a two-week taper for a marathon, and subsequently in a seven-day period of recovery following the competition itself. Serum creatine kinase (CK) and lactate dehydrogenase (LDH) concentration were serially measured as indicators of disruption of skeletal muscle. Plasma interleukin (IL)-1 β , IL-6, and tumour necrosis factor alpha (TNF- α) were serially measured in order to determine their relation to the reticulocyte count and erythrocyte content of the spleen ($^{51}\text{Cr-RBC}$), either of which may contribute to a variation in RBC concentration in the days following an inflammatory response. The training completed by the runners failed to induce a continuing inflammatory response and RBC concentration was unchanged during training with respect to a baseline level measured at the end of a two week taper, 24 hours prior to a marathon run. Thus, further studies employing a more strenuous training regiment are required in order to evaluate the contribution of a non-hemodilutional component to the low-normal RBC concentration observed in endurance-trained athletes, especially runners. In contrast, both the mean CK and LDH concentration increased markedly from baseline following the marathon run, indicating disruption of skeletal muscle. IL-6 was detected in plasma two hours post-marathon (mean 16 pg/ml \pm 5 SD). The transient increase in plasma IL-6 concentration

may have mediated an acute phase response, evidenced by a decrease in serum iron concentration and saturation of transferrin and an increase in serum ferritin concentration from their respective baseline measure and an increase in serum haptoglobin concentration from a reduced concentration noted two-hours following the marathon. The mean ^{51}Cr -RBC liver count was increased at the time of the maximum decrease in RBC concentration, 24 or 48 hours after the marathon. Splenic ^{51}Cr -RBC count tended to increase concomitantly with the decrease in RBC concentration, then to decrease again when the RBC concentration returned to baseline. These observations support the notion that red cells were sequestered at 24 or 48 hours following the marathon and were then released seven days after the marathon. The inconsistent correlation of ^{51}Cr -RBC organ content with RBC concentration during 48 hours following the marathon and the limitations of the method, however, leave unclear the exact role splenic sequestration of RBCs may play in the reduction of intravascular RBC concentration. A reduction in hemoglobin concentration may have stimulated the increase in plasma erythropoietin concentration observed 24 or 48 hours following the marathon, which in turn may have led to the increase in reticulocyte count seven days following the marathon. Further studies are required to clarify the proportional contribution of splenic sequestration/release of erythrocytes and variation in rate of erythropoiesis to the variation in RBC concentration in the days following an intense bout of exercise.

Introduction

Exercise-induced inflammation

When muscular exercise is unaccustomed and especially when it requires repetitive, forceful, eccentric muscle effort (lengthening of muscle as it develops tension), the active muscle fibres exhibit a generalized or focal cellular disruption (Armstrong et al., 1983; Friden et al., 1983; Newham, 1988). Since inflammation is a nonspecific response of tissue to diverse stimuli, it is not surprising that the time course of exercise-induced muscle injury and regeneration parallels, in general detail, the morphological events in muscle traumatized either by a direct blow (Fisher et al., 1990; Hurme et al, 1991) or ischemia/reperfusion injury (Suval et al., 1987). The cellular infiltrates predominating at the site of tissue disruption are macrophages (24 to 48 hours) (Tullson and Armstrong, 1981; Armstrong et al., 1983; Jones et al., 1986; Round et al., 1987), neutrophils (0-12 hours) (Kuipers et al., 1983; Friden et al., 1983). Concomitantly with these events there is a loss of cellular enzyme (Armstrong et al., 1983), protein (Ekblom, 1969) and myoglobin (Roxin et al., 1986) first to the extracellular space and subsequently to the vascular space. This finding is attributed to cellular membrane disruption and increased vascular permeability. Quantitative estimation of damage to cardiac muscle following interruption of the blood supply to the tissue was first made by Sobel et al., 1972 and Roberts et al., 1975. Apple and Rhodes (1988) applied the same method in a preliminary way to the estimation of skeletal muscle disruption in runners after a marathon race. Recently this model has been extended to define the complete, developing time course of elevated serum enzyme activity (ESEA) (Banister et al., 1992). Thus ESEA was used in the present study to indicate disruption of skeletal muscle during a 4 week period of intense training and following a marathon run. Signs of healing are typically observed 72 hours following

exercise-induced skeletal muscle disruption (Armstrong et al., 1983; Friden et al., 1983) although the severity of damage probably explains a variation in this timing observed by others (Jones et al., 1986; Gleeson et al., 1991).

The exercise-induced acute phase response

There is evidence that strenuous exercise can initiate an acute phase response. One of the earliest observations was a significant, transient increase in white blood cell count following a marathon run (Larrabee, 1902), mainly due to an increase in neutrophil granulocytes. This observation has been confirmed many times (eg. Wells et al., 1982; Nieman et al., 1989). Noakes et al, 1983 reported an increase in ceruloplasmin and C-reactive protein (CRP) concentration in runners following an ultra marathon. Taylor et al., 1987 reported an increase in CRP concentration of nearly 300% 24 hours following a 160 km triathlon involving competitors in canoeing, cycling and running. Weight et al., 1991 reported an increase in plasma CRP concentration in runners 24 and 48 hours after a marathon. Subject serum haptoglobin concentration decreased 24 hours then increased 48 hours after their completion of a marathon run. Gleeson et al., 1995 reported an increase of more than 200% in CRP, but no significant change in serum concentration of alpha-1-antitrypsin and alpha-1-acid glycoprotein, 24 hours after a 40 minute bout of bench stepping exercise in healthy untrained subjects. Other investigators have reported a change in plasma fibrinogen concentration (Osterund et al., 1989; Weight et al., 1991), and serum ferritin concentration (Vidnes and Opstad, 1981; Dickson et al., 1982; Taylor et al., 1987) consistent with an acute phase response. In contrast, the increase observed in globulin and glycoprotein concentration (Liesen et al., 1977; Noakes et al., 1982) specifically, transferrin (Haralambie and Keul, 1970; Liesen et al., 1977) following an exercise challenge is inconsistent with the classical pattern of their decrease, although non-exercise

exceptions do exist (Grau, 1990). Other evidence of an acute phase response to a severe exercise stimulus includes the detection of a hypoferrremia and hypozincemia (Ross and Attwood, 1984; Van Rensburg et al., 1986; Taylor et al., 1987; Gleeson et al., 1995), although this is not a consistent observation (Dressendorfer et al., 1981; Campanini et al., 1988; Seiler et al., 1989; Weight et al., 1991).

There have been few long term studies in which the acute phase proteins have been monitored during continuous training. The mean haptoglobin concentration resulting from training is often below the value found in control subjects (e.g. Magnussen et al., 1984) due to an ongoing increase in intravascular hemolysis. Banister and Hamilton, 1985 reported a decrease in total iron binding capacity (TIBC) during times of heavy training in two of five female distance runners. However, Hamilton, 1986 (MSc thesis) measured TIBC and serum transferrin (TF) concentration throughout six weeks of cycle training in previously sedentary males and found that while TIBC decreased in 6 of 11 subjects, TF increased slightly in 7 of 11 subjects. Thus, whereas TIBC is generally considered to be closely correlated with TF (control subjects: $r^2=0.82$) there was a significant dissociation of TIBC and SF in subjects in training ($r^2=0.29$), indicating that a conclusion regarding serum transferrin concentration may not be extrapolated from the TIBC concentration measured in a training study. Banister and Hamilton, 1985 reported a higher serum iron concentration during heavy training in female distance runners. Dressendorfer et al., 1981 reported a significantly elevated serum iron concentration in subjects during a 20-day, 312 mile running race. Seiler et al., 1989 studied male and female ultramarathon runners during a 1000 km run covered in 20 days. During the first three days of the competition serum iron concentration increased coincidentally with an increase in serum bilirubin concentration and a decreased haptoglobin concentration, indicative of hemolysis. Serum iron concentration decreased between day three and six, then remained below a pre-race concentration for the

remainder of the race. Serum ferritin concentration increased almost two-fold within the first days, then decreased without reaching a pre-race level. Campanini et al., 1988 reported no change in serum iron, TIBC or serum ferritin concentration, respectively, in professional male cyclists during a 20-day race. The difference in various study findings may be due to a variation in exercise regime and timing of the blood samples (e.g., Banister and Hamilton 1985, at least 12 hours post-exercise compared with Dressendorfer et al. 1981, at least 20 hours after the last bout of exercise, Seiler et al. 1989, immediately after a daily 50 km run, Campanini et al. 1988, on day 1 before beginning exercise followed by days 10 and 20 in the afternoon and Weight et al. 1991 10 minutes and 24 hours after a marathon run). Most acute phase proteins which have been serially measured during continuous training are associated with iron status. These proteins may be uniquely altered in an acute phase response which is exercise-induced.

Presence of circulating cytokines in response to exercise

Cytokines mediate the biological effect of an acute phase response. Many reports describe the presence of cytokines in the serum or plasma in response to exercise (Cannon and Kluger, 1983; Cannon et al., 1986; Evans et al., 1986; Viti et al., 1985; Dufaux and Order, 1989; Espersen et al., 1990; Northoff and Berg, 1991; Northoff et al., 1993; Northoff et al., 1994; Cannon et al., 1991; Sprenger et al., 1992). The presence of serum inflammatory cytokines indicates their significant role linking the local and systemic changes usually observed following sustained strenuous exercise to those of the pathologically induced, non-immune, inflammatory response. The most consistent finding is of an increased IL-6 concentration in subjects zero to six hours following a run of at least two hours in length (Sprenger et al., 1992; Northoff and Berg, 1991; Northoff et al., 1993). Continuous cytokine production during the time course of routine training is

hypothesized from their presence found at rest prior to engagement in a single bout of exercise. Evans et al., 1986 reported the presence of IL-1 activity (as measured by the ability of sephadex G-50 chromatographed plasma fractions to increase thymocyte proliferation above a basal rate) in the plasma of highly trained endurance runners who had been inactive for 2 days. Sprenger et al., 1992 detected cytokines in the urine (but not the plasma) of well-trained runners, whereas the cytokine concentration is usually undetectable in the urine of an untrained person. A high cytokine concentration in the urine probably reflects an enhanced rate of new cytokine synthesis in the body, which indicates a persistent leukocyte stimulation in the well-trained individual. The runners in the study of Sprenger et al., 1992 trained over a distance of 15-20 km two or three times weekly. At the time of the baseline measurement, when cytokines were detected in the urine, the mean CK concentration was only 68 U/L. In the present study runners were asked to double this workrate (approximately), which would presumably elevate the cytokine level to the point of detection in the plasma using the sensitive Quantikine ELISA kits for their detection.

The hypervolemic response to exercise

An expansion of blood volume (hypervolemia) in response to endurance exercise is well studied (Holmgren et al., 1960; Oscai et al., 1968; Convertino et al., 1980; Green et al., 1991). The acute increase in plasma volume following exercise may continue for 48 hours (Costill et al., 1976; Lijnen et al., 1985). Chronic training typically produces an 8 to 10% expansion in blood volume. One of the mechanisms that accounts for an exercise-induced acute plasma volume expansion is activation the renin-angiotensin-aldosterone (RAS) system. During an acute bout of vigorous exercise, plasma renin activity (Wade and Claybaugh, 1980; Convertino et al., 1980), and vasopressin concentration, respectively (Convertino et al., 1980) rise, but quickly return to a pre-exercise level following exercise,

whereas the elevation in plasma aldosterone concentration and the associated reduction in sodium excretion rate continues for up to 6 hours (Costill et al., 1976; Wade et al., 1989) and 20 hours post-exercise (Wade et al., 1981). Another mechanism which accounts for an exercise-induced acute plasma volume expansion is entry of protein to the intravascular space. Several studies have demonstrated that exercise-induced plasma volume expansion is accompanied by a proportionate increase in total circulating protein (Senay, 1972; Koch and Rucker, 1977; Convertino et al., 1980; Edwards and Harrison, 1984; Freund et al., 1987). For every gram increase in plasma protein, 14-15 ml of water may be added to the intravascular space, due to an increase in the vascular colloidal osmotic pressure (Scatchard et al., 1944). The relative contribution of an increase in total osmolar content and total protein content to the observed exercise-induced plasma volume expansion is currently unresolved. Luetkemeier et al., 1994 reported the effect of spironolactone treatment (an aldosterone inhibitor) on males cycling for 120 minutes at a relative intensity of 65% of VO_2 max for three consecutive days. Although total serum protein content increased to the same extent in Spironolactone-treated and non-treated subjects, there was no significant increase in resting plasma volume in spironolactone-treated subjects. However, at the end of seven days of training, both plasma volume and total circulating protein content of the drug and control groups were similar (Luetkemeier, 1988).

Maintenance of an expanded blood volume with exercise

An increase in fluid volume within a given vascular space would normally stimulate volume excretion via the effect of volume/pressure receptors. Schrier and Niederberger, 1993 present a hypothesis to explain excessive sodium and water retention during pregnancy and in various disease states, including sepsis. They suggest that release of endothelium-derived nitric oxide leads to arterial underfilling due to peripheral arterial vasodilation which

then activates the renin-angiotensin-aldosterone axis. Peripheral arterial vasodilation activates arterial baroreceptors which signal nonosmotic vasopressin stimulation. Gilligan et al., 1994 demonstrated in human forearm studies of intermittent handgrip exercise that endothelium-derived nitric oxide contributes to exercise-induced vasodilation, since inhibition of nitric oxide synthesis reduces exercise-induced vasodilation. It is not known if nitric oxide concentration in tissue remains elevated after cessation of exercise. A possible stimulus for such an increase in nitric oxide synthesis beyond the end of an exercise session could result from endotoxin release from the intestine, which has been documented following an ultradistance triathlon (Bosenberg et al., 1988). However, although the blood vasopressin concentration increases during exercise, its half-life is no more than 5 minutes, and no change from a normal resting concentration has been reported in this variable in the individual undertaking training (Convertino et al., 1980; Wade et al., 1981; Convertino et al., 1991).

Accommodation of an expanded blood volume to exercise

The expansion of blood volume with training raises an important issue regarding the distribution of the fluid. Convertino et al., 1991 found an increase in central venous pressure was linearly related to the percent change in blood volume following 10 weeks of endurance training in healthy untrained males. Total effective vascular capacitance, defined as the ratio of volume to pressure in the vascular space, was unchanged. In order for blood volume to remain chronically expanded with an increase in central venous pressure, the sensitivity of volume reflex control must become attenuated with exercise training (Convertino, 1991), a notion supported by Mack et al., 1987. A possibility, yet unexplored, is that an increase in spleen size assists in accommodating the expanded blood volume attendant upon long term training.

Response of red cell mass to exercise: is it optimal?

In response to exercise, plasma volume expands to a greater extent than does red cell mass, leading to an apparent dilutional "pseudoanemia" (Oscai et al., 1968; Akgun et al., 1974; Convertino et al., 1980; Green et al., 1991).

The additional plasma provides the advantage of:

- a lower blood viscosity
- a greater body fluid available for heat dissipation
- a larger vascular volume and filling pressure for greater cardiac output during exercise.

However, the effect of induced erythrocythemia (commonly termed blood doping) on both athletic performance and related physiological parameters (Ekblom et al., 1972; Buick et al., 1980; Brien and Simon, 1987; Brien et al., 1989) is one of improvement, leading to the conclusion that the "native" hematocrit is suboptimal. Thus, although the advantage of an appropriate increase in total blood volume is indisputable, it seems that there would also be an advantage in maintaining the original hematocrit by increasing red cell (RBC) production to balance the increase in plasma volume. This latter effect has not been reported, however.

Red cell destruction with exercise

Schmidt et al., 1988 reported that the rate of RBC destruction increased 2-fold during a 3 week period of moderate endurance training (cycle ergometer) by previously untrained males. Gastrointestinal (Stroehlein et al., 1976; McMahon et al., 1984; McCabe et al., 1986) and urinary tract bleeding (Davidson, 1964; Fred and Natelson, 1977), intravascular hemolysis (Gilligan et al., 1943; Lindemann et al., 1978; Magnusson et al., 1984), osmotic (Davis and Brewer, 1935; Puhl and Runyan, 1980) and oxidative (Duthie et al., 1990;

Smith et al., 1995) damage to red blood cells have all been a documented response to exercise and may explain the above result. Regular endurance training is reported to result in:

- a decreased percentage of high density RBCs (Mairbaurl et al., 1983)
- an elevated glutamate oxaloacetate transaminase activity concentration in the RBC population (Schmidt et al. 1988)
- an elevated creatine concentration in the RBC population (Schmidt et al. 1988)
- a lower mean corpuscular hemoglobin concentration (Schmidt et al. 1988)
- an increased mean cell volume without a change in RBC mass (Green et al., 1991).

These observations are consistent with an increase in the ratio of young to old RBCs, as a result of an accelerated removal of the oldest red cells and replacement by new red cells.

Weight et al. (1991) found that the mean (\pm SD) erythrocyte lifespan of male and female distance runners training 50 to 129 km/week was 67.2 ± 22.2 and 72.4 ± 26.0 days respectively compared with 113.4 ± 31.0 and 114.1 ± 29.0 days respectively in sedentary male and female subjects. However, in man a 14-fold increase in erythropoiesis has been found to completely compensate for the reduction of a mean erythron life span of up to ten days (Pollycove and Tono, 1975), so that an increased turnover of RBCs need not lead to anemia if RBC replacement keeps pace.

Erythropoietin response to exercise

Erythropoietin (Epo) is the principal regulator of red blood cell production and plasma Epo activity is roughly an inverse exponential function of the blood hematocrit and hemoglobin concentration in anemic humans with intact kidney function (Erslev et al., 1987).

Comparison of the endurance athlete at rest and a sedentary control has generally shown no

difference in their serum Epo concentration (Berglund et al., 1988, Weight et al., 1992). Hallberg and Magnusson (1984) and others have suggested that an increase in red blood cell 2,3-diphosphoglycerate concentration (2,3-DPG), which improves oxygen delivery to the tissues by inducing a right shift in the oxygen dissociation curve, may cause a lower "set point" for hemoglobin (Hb) concentration in athletes. Thus, the cells in the kidney responsible for regulation of the Epo level will sense an adequate oxygen delivery at a lower concentration of hemoglobin. While Schmidt et al. (1988) did detect an increase in 2,3-DPG concentration per mol Hb, the position of the oxygen dissociation curve expressed as half saturation pressure (P_{50}) did not correlate with 2,3-DPG concentration but rather corresponded with an increase in number and proportion of young erythrocytes. In other studies measurement of the P_{50} of the oxygen dissociation curve has not revealed the expected right shift (Shappell et al., 1971; Ricci et al., 1984), thus the basis for maintenance of a lower hematocrit in athletes is currently unexplained. While the Epo value at rest may not differ between sedentary and athletic populations, exercise may exert an acute effect on Epo production, which should be evident about 90 minutes after the beginning of the stimulus (Kurtz et al., 1988, Eckart et al., 1989). Results to date have shown both an increased (Schwandt et al., 1991, Klausen et al., 1991) and unchanged (Schmidt et al., 1991, Weight et al., 1992) concentration of Epo in the blood in the hours following exercise. The variability in hematologic status, the exercise regimen practiced, timing of blood samples for analysis and the large intra-individual diurnal variation in Epo (Klausen et al., 1993) may explain the varying results. *In vitro* studies show that cytokines produced during an inflammatory response (eg. IL-1, IL-6, TNF- α) influence the production of Epo (Fandrey & Jelkman, 1991, Faquin et al., 1992). Schmidt et al. (1991) reported that under hypoxic conditions both submaximal exercise and rest itself lead to an increase in Epo value, as expected, but maximal exercise has no effect on Epo concentration. One explanation for the lack of increase in Epo following maximal exercise

is that certain circulating cytokines suppress Epo production. As mentioned previously, the presence of cytokines in the serum or plasma is an oft reported response to exercise (Cannon and Kluger, 1983; Cannon et al., 1986; Evans et al., 1986; Viti et al., 1985; Dufaux and Order, 1989; Espersen et al., 1990; Northoff and Berg, 1991; Northoff et al., 1994; Cannon et al., 1991; Sprenger et al., 1992). Cytokines may also affect red cell production by inhibiting red cell precursors from responding to Epo (eg. Resnitzky et al., 1992). Thus cytokines produced both in response to an acute bout of exercise and to extended endurance training may play a role in disturbance of normal red cell production.

Erythropoiesis in response to exercise

The reticulocyte count is a simple test used to detect the amount of effective red blood cell production taking place in bone marrow. Immediately after an exercise session of sufficient intensity, an increase in reticulocyte number is noted due to the release of immature reticulocytes from the bone marrow into the peripheral blood (Seip, 1953; Imelik, 1988). The peripheral reticulocyte count has been shown to increase within a few days following different types of exercise (Schmidt et al., 1988; Schmidt et al., 1989; Yoshimura et al., 1980). Generally an increase in Epo concentration in the blood leads to reticulocytosis after about two days (Kurtz et al., 1988), however, in exercise studies, a correlation between Epo concentration and reticulocyte count is not always found (Klausen et al., 1991; Schmidt et al., 1991). Other hormones released during exercise, which may obscure the relationship between peripheral reticulocyte count and Epo, are cortisol, growth hormone, adrenaline, testosterone and thyroxine (Jelkmann, 1986). While there have been many investigations into the cause of plasma volume expansion, there have been few longitudinal studies of the dynamics and mechanisms involved in adaptation of the erythron itself to endurance training. In the experiment described below, plasma Epo, IL-1 β , IL-6,

TNF- α , reticulocyte count and related blood indices were measured throughout a period of training and taper for a marathon competition and subsequently during recovery from a marathon run in an attempt to identify their possible role in the RBC variation.

The *in vivo* effect of recombinant human IL-6

The availability of recombinant human cytokines has permitted the study of their *in vivo* effect in the whole animal. Anemia associated with recombinant human IL-6 treatment has been observed in chemotherapy-treated rhesus macaques (Winton et al., 1994), hematologically unperturbed cynomolgus monkeys (Asano et al., 1990), rhesus monkeys (Srinivasiah et al., 1992) and humans (Ulrich et al., 1991; Weber et al., 1993). As discussed in the general introduction, an increased plasma volume and/or sequestration of red cells during rhIL-6 treatment could account for reduced hemoglobin concentration in rhIL-6-treated animals and humans.

The role of the spleen in variation of RBC concentration with exercise

As mentioned previously, an increase in IL-6 concentration 0-6 hours following a run of at least 2 hours in length is a consistent finding (Sprenger et al., 1992; Northoff and Berg, 1991; Northoff et al., 1993). A unique aspect of the present study is the measurement of the time course of splenic red blood cell content to determine its role in the decreased red cell concentration observed following intense exercise, which is generally attributed entirely to hemodilution. It has been recognized for many years that the spleen provides storage for erythrocytes, particularly in species like the dog (Barcroft and Stephens, 1927; Carneiro and Donald, 1977) and horse (Boucher et al., 1985) whose splenic capsules actively contract in response to sympathetic stimulation. In man the splenic capsule is nonmuscular

but the visceral circulation is known to contract in response to adrenergic stimulation, decreasing blood flow to the spleen (Froelich et al., 1988; Flamm et al., 1990; Allsop et al., 1992) so that more blood is available to the general circulation. However, the average splenic volume in healthy adults is only about 220 cm³ (Hendersen et al., 1981) and is thought to contain no more than 5% of the total erythrocyte population in an equal volume of plasma at rest. In a subject with a blood cell disease an enlarged spleen may contain up to 66% of the total red cell mass (Hegde et al., 1973). While there is little evidence to substantiate a reservoir function for the human spleen, some studies using red cells labelled with ⁵¹Cr have suggested that a pooling of abnormal cells by a normal spleen and pooling of normal cells by an abnormal spleen both may occur (Harris, McAlister and Pranker, 1957 and 1958; Bowdler, 1962; Pranker, 1963; Toghil, 1964; Richards and Toghil, 1967 and Ayers et al., 1972). Oxidative, mechanical and osmotic stress during exercise may lead to RBCs with reduced deformability (Reinhardt & Chien 1985). Thus, the passage of such damaged cells through the spleen is impeded (Smith et al., 1995). In pathologic conditions there is a highly significant and as yet unexplained relationship between plasma volume and spleen size. This relationship is uninvestigated in the endurance athlete, so that the possibility of a larger than normal spleen in an athlete with hypervolemia cannot be ignored.

Study of the relationship of the spleen to the change in hematocrit with exercise has largely been made to explain the increase in hematocrit observed during and immediately after exercise. Sandler et al., (1984), Flamm et al., (1990), Allsop et al., (1992) and Laub et al., (1993) observed splenic shrinkage during exercise which paralleled an increase in hematocrit and they hypothesized that red cells which normally reside within the spleen contribute to the increase in intravascular red cell concentration during exercise. In a more detailed analysis, Wolski et al., 1996 measured plasma volume and total red cell volume

pre-exercise then following 30 minutes of graded exercise (cycling). Splenic volume was determined after each 10 minute exercise load. A 54% reduction in spleen size corresponded with an expulsion of 139 ml of red cells. A 12% reduction in plasma volume, in addition to the release of red cells from the spleen, contributed to the observed increase in hematocrit. In the present study it is hypothesized that intense training, sufficient to produce a persistent myogenic inflammatory response, and a marathon run, which produces an even greater inflammatory effect, will lead to an increase in splenic red cell content. This will contribute (together with a plasma volume expansion and suppressed erythropoiesis) to a lower intravascular red cell concentration in the days following exercise.

Summary of study aims

The aim of this study is to determine if factors other than hemodilution contribute to the observed decrease in red blood cell concentration following intense exercise . A role is proposed for the spleen, which may enlarge or sequester red cells during an inflammatory response. In addition, a role is proposed for continuing presence of circulating inflammatory cytokines, which may alter functioning of the erythron.

Methods

Subjects

Runners were recruited from a university student population and from local running clubs. Of the six subjects who completed the study, all were moderately trained and all but one

had completed at least one marathon prior to entering the study. Control subjects (n=4), not in training, were recruited from the staff of the Lions Gate Hospital.

Ethical Considerations

The study was approved by the Committee for Research in Human Subjects of Simon Fraser University and of the Lions Gate Hospital. Informed consent was obtained from all participants after the nature of the investigation had been explained to them.

Training

The training pattern of each runner was determined during a four week training phase, a two week recovery phase leading up to and including a marathon competition and then during a two week recovery following the marathon. Initially a runner was asked to complete two one-hour training sessions each day during the training phase, however, one subject dropped out, two subjects were lost to injury before formal data collection began and the remaining six subjects were not able to complete the prescribed amount of training. Thus, the exercise prescription was changed to a single one-hour training session five days per week with a 2-3 hour run once per week and an optional day of rest on the seventh day. Even this amount of training represented a substantial increase in each subject's previous training dose (based on recall) except in subject NW. The runners were asked to begin a taper schedule two weeks prior to the marathon run such that TRIMPS (Morton et al., 1990; Banister et al., 1992b; described below) would decrease to 50%, then to 25% of the average during the intense four weeks of training in the first and second week of taper respectively. During all runs a runner wore a heart rate monitor to record heart rate (HR)

elevation. These data were logged in a computer and used to define the "dose" of training (TRIMPS) undertaken each day from the following formula:

$$\text{TRIMP SCORE} = \frac{\text{(Duration of exercise)}}{\text{(arbitrary units)}} \times \frac{\text{(\Delta HR ratio)}}{\text{(minutes)}} \times \frac{\text{(Y)}}{\text{(mM)}}$$

where duration of exercise is in minutes, Δ HR ratio = Heart rate during exercise less heart rate at rest divided by maximum heart rate less heart rate at rest and Y is a metabolic arousal factor based on the relationship between blood lactate concentration and heart rate during exercise (Green et al., 1983) thus:

$$Y = e^{1.92x} \text{ (male)}$$

$$Y = e^{1.67x} \text{ (female)}$$

where $x = \Delta$ HR ratio

Maximum heart rate was determined during a subject's graded run to exhaustion on a treadmill. After a five minute warm-up period, one minute ramp increment in work rate, produced from an increasing treadmill speed on a level grade, was maintained to the point of voluntary exhaustion by a subject, which usually occurred between 10 to 15 minutes.

Blood Samples

A blood sample was collected from the antecubital vein of a sitting subject. In order to minimize the effect of diurnal variation, a sample was normally collected between 10 am and 2 pm except on the day of the marathon when a second sample was taken between 5 and 7 pm. Subjects were asked to report to the Nuclear Medicine department at the Lions

Gate Hospital within 1 hour of finishing the marathon run. However, collection of the first sample varied between 80 and 110 minutes post-marathon, due to variation in the time of arrival at the test site and the number of procedures to perform. Blood for immunoassays was drawn into a tube containing EDTA, centrifuged, separated into aliquots and stored in a freezer within 30 minutes of collection to avoid both *ex vivo* secretion and degradation of cytokines. Analysis of iron status, hematology, basic chemistry and enzymes were performed using standard methods in a professional laboratory at the Lions Gate Hospital, North Vancouver, Canada. EDTA-plasma samples were collected and frozen (-70 °C) for batch analysis of IL-6, IL-1 β , TNF- α and Erythropoietin (Epo) using commercially available immunoassay kits (Quantikine) from R&D Systems (Illinois). The Quantikine kits are solid phase immunoassay kits which are based on the double-antibody sandwich method. The details of this method are included in the general methods section (pp 23-25). Epo, IL-6 and TNF- α values were determined within 6 months and IL-1 was determined within 2 years following collection of the samples. Assays were performed in duplicate and intra-assay variation was <10%.

Radiolabelling of erythrocytes

The procedure followed is described in detail in the general methods section (p 19).

⁵¹Cr-RBC Organ Counts

⁵¹Cr-RBC organ counts were measured twice a week beginning in the final week of intense training, continuing through the two week taper period and measured at the same time as the routine blood tests in the two week period following the marathon run. Details of the experimental set-up are described in the general methods section (pp 20-23).

Determination of blood volume

The volume of red cells (RCV) was measured on the day of the red cell label described above. Whole blood volume (TBV) and plasma volume (PV) were calculated using RCV and hematocrit (Hct) as described in the general methods section, p 19.

Calculated change in plasma volume

The percentage change in plasma volume was calculated according to the method of Dill and Costill (1974) from:

$$\% \Delta PV \cong 100[(Hb_{pre} \times Hb_{post}^{-1}) \times (1 - Hct_{post} \times 1 - Hct_{pre}^{-1})] - 100$$

A second method based on change in total protein (prot) (Greenleaf and Hinghofer-Szalkay, 1985) was used as a comparison :

$$\% \Delta PV = (prot_{pre} \times prot_{post}^{-1})100$$

Analysis of data

Blood and organ count data were analysed by analysis of variance for repeated measures. Pairwise comparison of each variable (using the Bonferoni adjustment) revealed which measurement showed a significant difference from the baseline value. A Student's t-test was used to determine any difference in the initial spleen-to-liver ratio between the runners and the controls and to compare the change in organ counts between specified time points for the runners and the controls. A level of significance of $p \leq 0.05$ was used to determine the significance of a variable's different value between two measures. Unless otherwise stated, experimental comparisons are made with a measurement taken 24 hours before the marathon and designated a "baseline" measure.

Results

Characteristics of subjects at study entry

Table 3.1 shows demographic and blood volume data of the runners and control subjects at study entry. Total blood volume (TBV), volume of red cells (RCV) and plasma volume (PV) for each runner were evaluated, based on the Wennesland formulae, which use a subject's weight and height to determine a normal range of variability for each blood volume measure (Wennesland et al., 1959). One of four control subjects demonstrated blood volumes within the normal range of variability while one or more measures were elevated in three others. However, the group mean TBV and RCV were significantly higher in the runners than in control subjects.

Training

Figure 3.1 shows training data for individual subjects and Table 3.2 summarizes the data in a numerical form. It is apparent that the volume of training differs between subjects, partly due to an individual's response to the workrate attempted and partly due to various minor bouts of injury and illness encountered throughout the period of training, competition and recovery from the marathon run. The taper schedule was adhered to successfully by 4 out of 6 runners who managed a reduction of 50-60% of the average TRIMPS recorded during the training phase in the first week of the taper. In addition, 4 out of 6 subjects recorded a reduction to 30% or less in the second week of the taper (Table 3.2).

Table 3.1 Demographic and blood volume data of runners and control subjects.

| Subject runner | Sex | Age, yr | Wt, kg | Ht, cm | TBV, ml/kg | RCV, ml/kg | PV, ml/kg | a | Prior Marathons |
|----------------|-----|---------|--------|----------|----------------|------------|----------------|----------|-----------------|
| RT | F | 36 | 55.8 | 167 | 75.6 | 29.5 | 46.1 | * | 9 |
| NW | M | 46 | 67.5 | 171 | 88.9 | 34.7 | 54.2 | 22.6 | 48 |
| JT | M | 54 | 80.0 | 187 | 86.2 | 31.0 | 55.2 | 22.2 | 9 |
| RG | M | 36 | 80.5 | 173 | 84.3 | 32.0 | 52.2 | 28.5 | |
| MC | M | 43 | 68.6 | 172 | 82.6 | 33.9 | 48.7 | 10.3 | 1 |
| AY | M | 25 | 80.0 | 180 | 76.8 | 29.9 | 46.8 | 9.0 | 0 |
| average (M)= | | 40.8 | 75.3 | 176.6 | 83.8 | 32.3 | 51.4 | 18.5 | |
| SD = | | 10.9 | 6.7 | 6.8 | 4.5 | 2.0 | 3.6 | 8.5 | |
| | | | | <i>b</i> | <i>p</i> =0.03 | <i>b</i> | <i>p</i> =0.04 | <i>b</i> | |
| <u>control</u> | | | | | | | | | |
| JM | M | 50 | 67.8 | 172 | 79.7 | 32.8 | 46.9 | 8.8 | |
| PC | M | 43 | 80.0 | 174 | 65.2 | 24.1 | 41.1 | * * | |
| MB | F | 53 | 73.5 | 180 | 79.4 | 27.7 | 51.7 | 12.3 | |
| CK | F | 33 | 54.3 | 162 | 72.8 | 28.4 | 44.4 | * | |
| average (M)= | | 46.5 | 73.9 | 173.0 | 72.5 | 28.5 | 44.0 | | |
| SD = | | 4.9 | 8.6 | 1.4 | 10.3 | 6.2 | 4.1 | | |
| average (F)= | | 43.0 | 63.9 | 171.0 | 76.1 | 28.1 | 48.1 | | |
| SD = | | 14.1 | 13.6 | 12.7 | 4.7 | 0.5 | 5.2 | | |

a the percentage above the upper limit of normal (based on height and weight, Wennesland et al., 1959).

b results of an unpaired T-test comparing blood volume values for runners and control subjects.

* within the normal range.

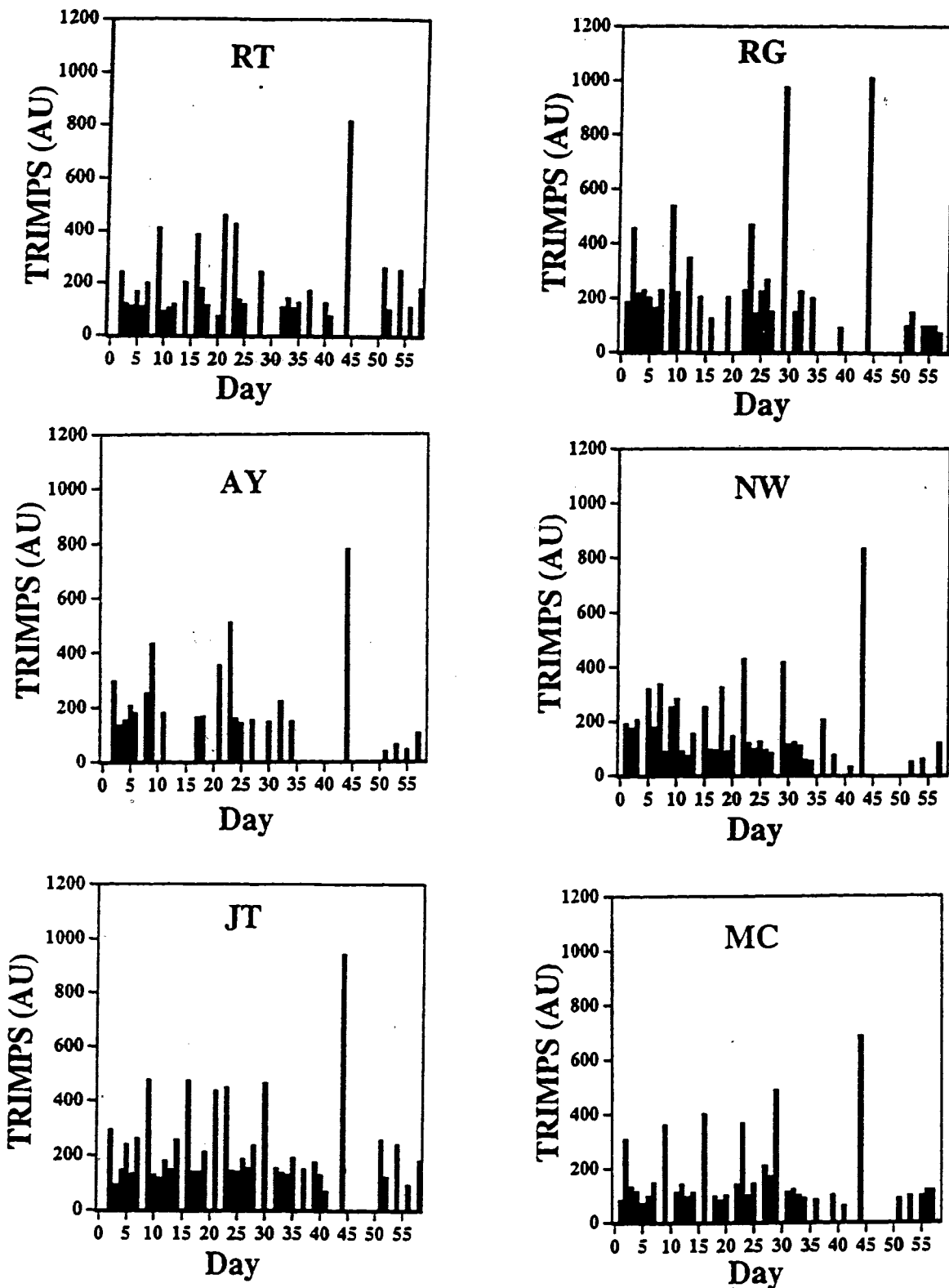


Figure 3.1 Training profile in arbitrary training units (AU) during preparation and recovery from a marathon. Training data for subjects RT, RG, AY, NW, JT and MC are presented individually. Days of training, taper, the marathon run and recovery from the marathon are respectively, 1-29, 30-43, 44 and 45-58.

Table 3.2 Runners: Comparison of training data.

| | AY | JT | MC | NW | RT | RG | Average ± SD |
|--|-----------|------------|-----------|-----------|-----------|-----------|--------------|
| Total TRIMPS during training (4 weeks) | 3471 | 5160 | 4020 | 4299 | 4008 | 4928 | 4314 ± 630 |
| average TRIMPS/week | 868 | 1290 | 1005 | 1075 | 1002 | 1232 | 1078 ± 157 |
| Total TRIMPS in week 1 of taper | 511 (59%) | 1070 (83%) | 510 (51%) | 874 (81%) | 478 (48%) | 727 (59%) | 695 ± 240 |
| Total TRIMPS in week 2 of taper | 0 | 518 (40%) | 162 (16%) | 311 (29%) | 362 (36%) | 0 | 226 ± 208 |
| TRIMPS for marathon run | 778 | 943 | 685 | 832 | 815 | 878 | 822 ± 88 |
| total TRIMPS in week 1 of recovery | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| total TRIMPS in week 2 of recovery | 238 | 707 | 530 | 230 | 720 | 625 | 508 ± 223 |

Explanatory note: TRIMPS

A method for quantifying the size of a training stimulus has been developed and is described in the methods section. Briefly, training value, expressed in arbitrary units called a training impulse (TRIMP) is calculated based on the duration of a training session and the relative heart rate produced during the session, to which is applied a metabolic intensity factor. Thus: $TRIMP\ SCORE = (Duration\ of\ exercise) \times (\Delta\ HR\ ratio) \times (Y)$. Duration of exercise is in minutes, $\Delta\ HR\ ratio = \text{heart rate during exercise} - \text{heart rate at rest}$ divided by maximum heart rate less heart rate at rest and Y is a metabolic factor derived from the relationship between blood lactate concentration and heart rate.

Marathon

Table 3.3 shows each subject's marathon completion time, maximum elevation after the marathon of creatine kinase and plasma IL-6 concentration, respectively and plasma volume change calculated by the method of Dill and Costill (1974). Each runner in this study reported a classical delayed onset of muscle soreness 24-48 hours following the marathon run. Marathon completion time ranged from 168-254 minutes. Details of the response of blood variables to the marathon run and to training are reported below.

Serum Enzymes

At no time during the training period was any enzyme concentration (group mean) outside of the limit of normal variation or significantly elevated above the baseline value (measured at the end of a two week taper, 24 hours prior to the marathon competition). However, in the first measurement following the marathon (two hours post), lactate dehydrogenase (LDH; Figure 3.2) and creatine kinase (CK; Figure 3.3) were elevated beyond the baseline value and outside of the limit of normal variation. Serum concentration of CK continued to rise, averaging 1427% (range 524-2790) of baseline 24 hours after completion of the marathon, returning to a baseline level four days after the marathon. Although LDH reached its highest value in the first measurement after the marathon, averaging 196% of baseline (range 168-259) it did not return to rested level until seven days after the marathon.

Table 3.3 Response of plasma volume, CK and IL-6 to a marathon run.

| | marathon time(min) | post-marathon CK, U/L | post-marathon Δ PV, % (max) | post-marathon IL-6, pg/ml |
|----|-----------------------|--------------------------|---------------------------------------|------------------------------|
| RT | 207 | 1140 | 3.0 (+24 hrs) | 11 |
| NW | 168 | 666 | no increase | 25 |
| JT | 208 | 1358 | 17.5*(+48 hrs) | 27 |
| RG | 202 | 977 | 18.7*(+48 hrs) | 8 |
| MC | 185 | 2148 | 14.7*(+24 hrs) | 19 |
| AY | 254 | 2420 | 12.1*(+2 hrs) 9.8 (+24 hrs) | 8 |

Column 1 gives the marathon finish time for each runner. The maximum serum creatine kinase (CK) value measured for each runner occurred 24 hours post-marathon is shown in column 2. Post-marathon change in plasma volume, based on the method of Dill and Costill (1974) is shown in column 3. Column 4 shows the 2 hour post-marathon value for IL-6 in the plasma sample.

* Significant change ($p \leq 0.05$) in plasma volume from the baseline measure to the designated post-marathon value.

Hematologic and iron status variables

Specific changes in laboratory measures were predicted to occur by the end of training and in response to a marathon run with respect to a rested ("baseline") value measured from a blood sample taken at the end of a two week taper, 24 hours prior to the marathon competition. Variables measured were red blood cell concentration (RBC), hemoglobin concentration (Hb), hematocrit (Hct), ferritin (Fer), haptoglobin (Hap) and serum iron (SI) concentration, percentage saturation of transferrin (%sat), serum transferrin concentration (SF) and serum albumin concentration (Alb). A large change was observed also in these variables during illness in training for subjects RT and RG and following the marathon in subject NW, respectively (see appendix A, Table A1). In an attempt to isolate the effect of the training and the marathon event *per se*, these clinical data were eliminated from the main analysis. Subject MC was referred to a Hematologist with suspected hemochromatosis

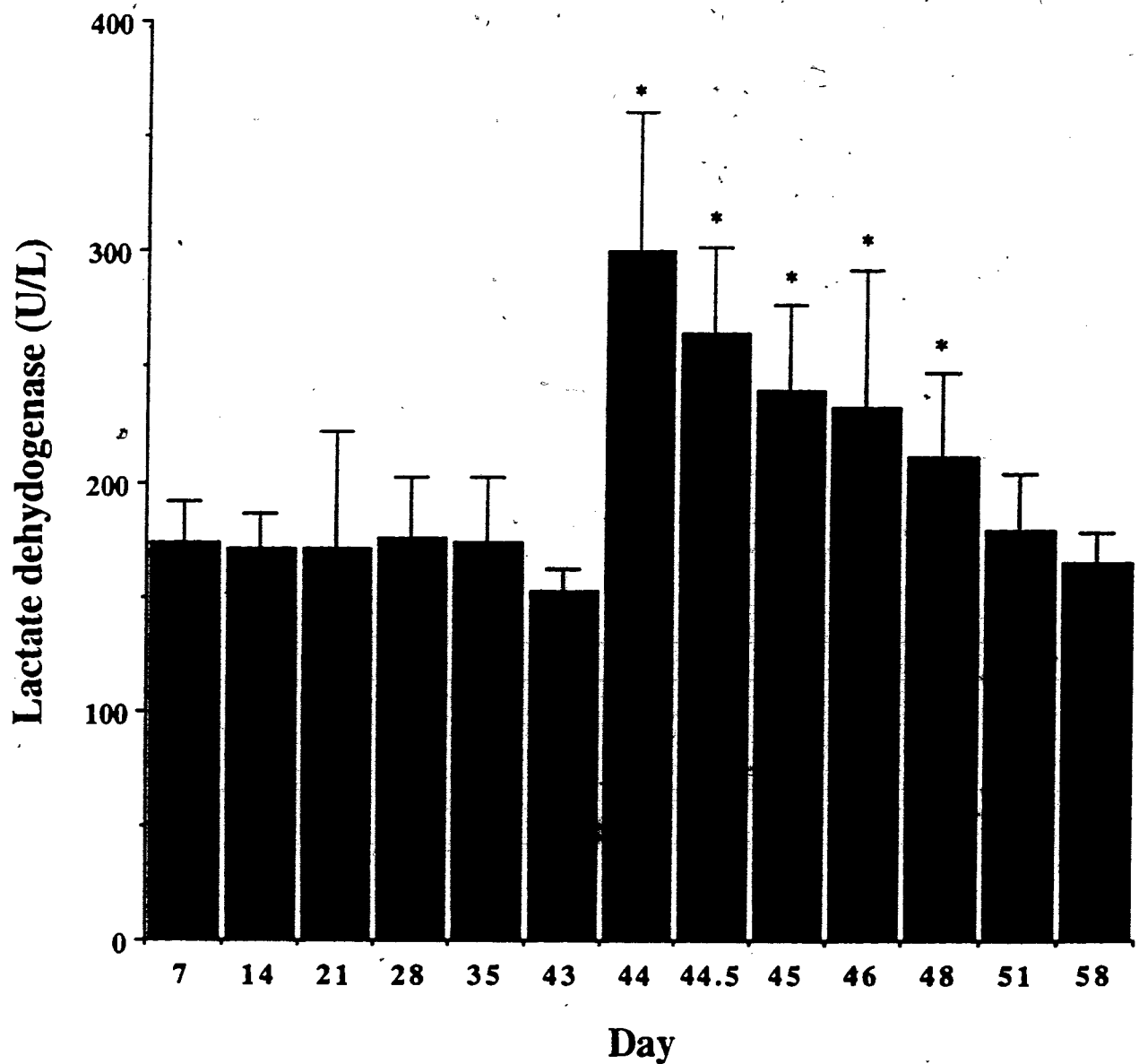


Figure 3.2 Serial change in mean (\pm SD) serum lactate dehydrogenase concentration. Training days 1-29, taper days 30-43, post-marathon days 44-58.

* significantly different ($p \leq 0.05$) from day 43 (baseline) value.

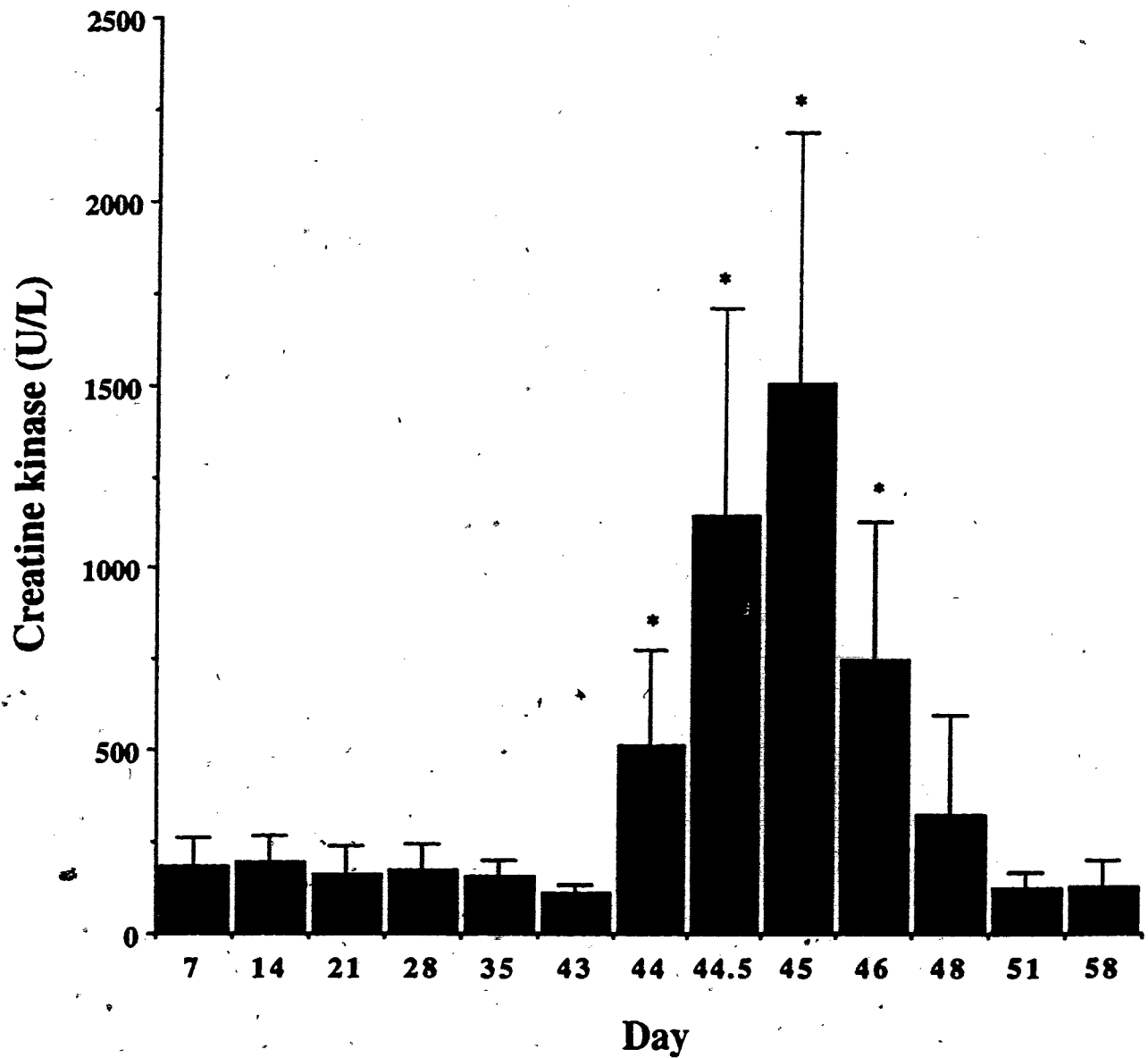


Figure 3.3 Serial change in mean (\pm SD) serum creatine kinase concentration. Training days 1-29, taper days 30-43, post-marathon days 44-58.

significantly different ($p \leq 0.05$) from day 43 (baseline) value.

after his Fer, SI, %sat and SF values were consistently outside normal range of variability for each measure. Data for the above named variables for subject MC demonstrated the same pattern of change as in other subjects and were included in the the analysis of variance for repeated measures (ANOVA) but were not included in the group mean calculation. RBC, Hct, Hb and Fer data from subject RT were included in the ANOVA but were not included in the group mean calculation. RBC, Hct and Hb values are consistently lower in a female subject. In addition, Fer values for subject RT were consistently below two standard deviations of the group mean (excluding her data). In the ANOVA, the day of measurement was significant for all variables. Thus pairwise comparison of each variable was used to identify which measurement (pre-planned) showed a significant difference from the baseline value. The result of this analysis is shown in Table 3.4 and is graphically illustrated in Figures A.1 to A.9. SI and %sat were increased significantly in the fourth week of training (from a mean 15.3 to 22.5 $\mu\text{g/L}$ and from a mean 28.8 to 43.0%, respectively). Following the marathon run, SI and %sat were significantly reduced in the six hour post-marathon sample (from a mean 15.3 to 8.8 $\mu\text{g/L}$ and from a mean 28.8 to 15.5%, respectively). Hb and Hct were significantly reduced 48 hours post-marathon (from a mean 145.3 to 138.5 g/L and a mean 42.6 to 40.4% respectively). Hap was reduced (from a mean 1.35 to 0.87 and 0.91), while Fer was elevated in the two and six hour post-marathon sample (from a mean 99.8 to 126.0 and 122.0 $\mu\text{g/L}$, respectively). Hap at 48 hours post-marathon was increased from its 2 hour post-marathon measure (from a mean 0.87 to 1.70 g/L).

Plasma IL-6, IL-1 β and TNF- α concentration

IL-6 was undetectable in the plasma of any runner during training except on one occasion in subject RT, who was ill with a viral infection (presumed) at that time. Table 3.3 shows

the maximum level of IL-6 detected in the plasma of a runner, which in all cases was reached in the measurement taken two hours after the marathon (mean 16 pg/ml range 8-27). At no time were IL-1 β or TNF- α detected in the plasma of a runner.

Erythropoietin concentration and reticulocyte count

Figures 3.4 and 3.5 show the time course of change in erythropoietin concentration (Epo) and reticulocyte count respectively during the training, marathon and recovery period. As illness may have had an effect on the aforementioned variables (Table A1), data collected on a day when a subject was ill was not included in the analyses. There was no consistent change in Epo concentration throughout the training period. In the first two measures following the marathon Epo response was variable, which is reflected in the large standard deviation (Figure 3.4). A change in the group mean EPO concentration from 10.1 mIU/mL (baseline) to 15.4 and 14.4 mIU/mL respectively 24 and 48 hours post-marathon did not reach significance. However, the Epo value corresponding to the minimum post-marathon RBC concentration (either 24 or 48 hours post-marathon) was significantly greater than the pre-marathon (baseline) value (10.1 to 15.6 mIU/ml). The group mean reticulocyte count seven days following the marathon was significantly higher than baseline value (an increase of 74%) (Figure 3.5).

⁵¹Cr-RBC organ counts

A non-training group was included in the present study as a control measure of normal intra-individual variability in the ⁵¹Cr-RBC organ count throughout the four week measurement period. When decay-corrected values were adjusted for the loss over 30 days of ⁵¹Cr-RBC from an organ, intra-individual variation in organ count averaged $4.4 \pm 3.1\%$

Table 3.4 Change in selected laboratory measures during training, and following a taper and a marathon run.

| | <u>Training</u> | <u>Taper</u> | <u>post-marathon</u> → | | | | | |
|-----------------------------|-----------------|--------------|------------------------|--------|---------|---------|---------|---------|
| | Wk4 | baseline | +2 hrs | +6 hrs | +24 hrs | +48 hrs | +4 days | +7 days |
| IL-6 | | | ↑ | | | | | |
| SI | ↑ | | | ↓ | | | | |
| %sat | ↑ | | | ↓ | | | | |
| Fer | | | ↑ | ↑ | | | | |
| Hap | | | ↓ | ↓ | | ①↑ | | |
| Hb | | | | | | ↓ | | |
| Epo | | | | | ②↑ | ②↑ | | |
| Retic | | | | | | | | ↑ |
| ⁵¹ Cr-RBC-spleen | | | | | | | | ③↓ |
| ⁵¹ Cr-RBC-liver | | ④↓ | | | ②↑ | ②↑ | | |

↑, ↓ denote a significant increase or decrease, respectively from the baseline value.

- ① 48 hour post-marathon measure is compared with the 2 hour post-marathon measure.
- ② Epo or ⁵¹Cr-RBC organ count value corresponding with the minimum post-marathon RBC concentration (either 24 or 48 hours post-marathon) compared to the baseline value.
- ③ ⁵¹Cr-RBC organ count value corresponding with the minimum post-marathon RBC concentration compared to the 7 day post-marathon value.
- ④ ⁵¹Cr-RBC-liver count at the end of a 14 day taper is reduced compared to its value during training.

IL-6-plasma interleukin-6 concentration; **SI**-serum iron concentration; **%sat**-percentage saturation of transferrin; **Fer**-serum ferritin concentration; **Hap**-serum haptoglobin concentration; **Hb**-hemoglobin concentration; **Epo**-plasma erythropoietin concentration; **⁵¹Cr-RBC**-organ count.

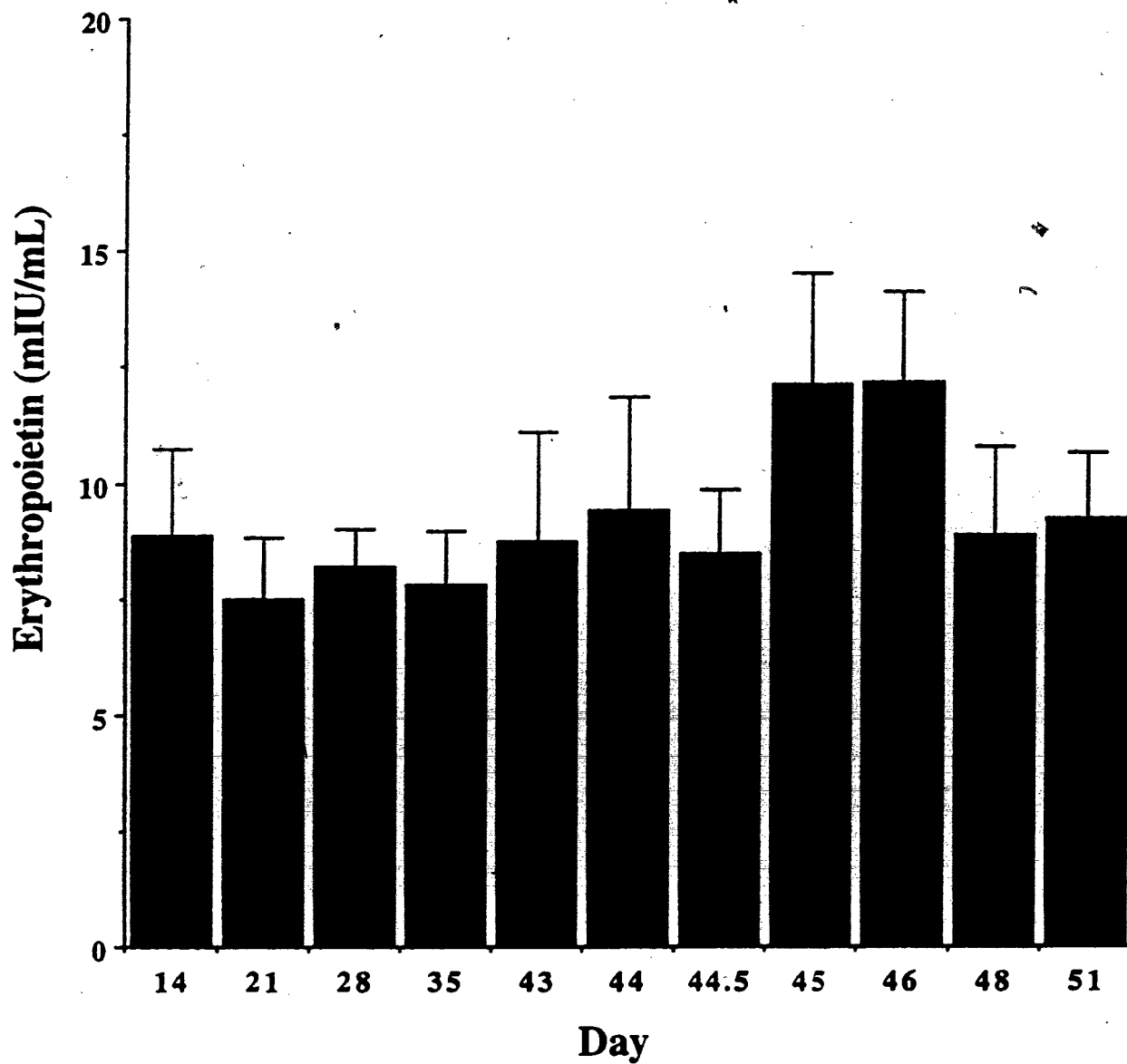


Figure 3.4 Serial change in mean (\pm SD) plasma erythropoietin concentration. Training days 1-29, taper days 30-43, post-marathon days 44-58.

* significantly different ($p \leq 0.05$) from day 43 (baseline) value.

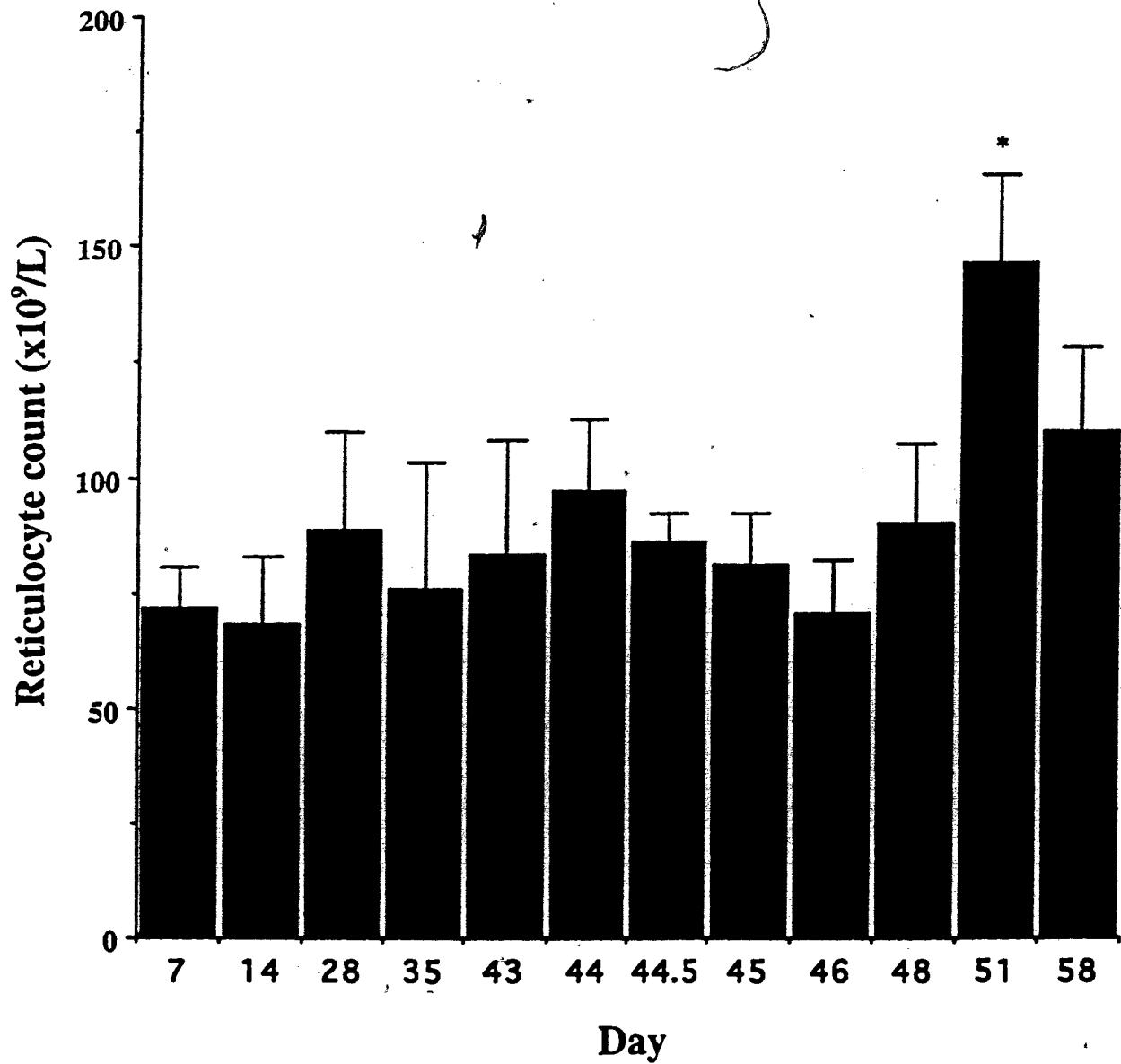


Figure 3.5 Serial change in mean (\pm SD) reticulocyte count. Training days 1-29, taper days 30-43, post-marathon days 44-58.

* significantly different ($p < 0.05$) from day 43 (baseline) value.

for the heart, $6.6 \pm 4.3\%$ for the liver and $5.7 \pm 3.5\%$ for the spleen. It was not possible to test the ^{51}Cr -RBC organ count data of the runners and control subjects in a 2-way ANOVA, due to the lack of matching data both within the experimental group and between the runners and controls. In each case where two organ counts are compared in the runners, the difference between organ counts for a similar time period is compared in the control group (see Tables 3.5, 3.6 and 3.7). Data from any subject at the time of an illness (Table A1) were not included in the group mean calculation, nor were data from subject MC, due to the abnormal pattern of organ counts, possibly secondary to hemochromatosis (see Figure A16). Figure A11 shows the group mean ^{51}Cr -RBC heart, liver and spleen counts during a one month period for runners compared with the control subjects. Organ count data for each individual runner is compared with a control count in Figures A12-A17. The spleen-to-liver organ count ratio of a runner during training (mean 0.94 ± 0.37) was comparable with that of the control average for the same measure (1.05 ± 0.22).

^{51}Cr -RBC organ count following a 14-day taper in runners

The runners in the present study were in training for a marathon competition and were prescribed an intense training schedule for four weeks followed by a two week taper leading to a marathon run. In order to test the hypothesis that a period of rest will result in a reduction of red cell content of the spleen of a runner in training, the difference between the ^{51}Cr -RBC spleen count from the final day of the 14 day taper and the ^{51}Cr -RBC spleen count at the end of a period of intense training in each runner was calculated. These data were then compared with the difference found between two ^{51}Cr -RBC spleen counts measured during a similar time period in the controls, using a t-test. Heart and liver counts were evaluated in the same manner. Data from subject RT were not included as she was ill during this period (see Figure A12). Data from subject MC were not included, due to an

abnormal pattern of organ counts, possibly secondary to hemochromatosis (see Figure A16). The mean difference in ^{51}Cr -RBC counts from the liver was significantly greater in runners than in the control group (Table 3.5). The mean difference in ^{51}Cr -RBC count from the spleen was 15.7% in runners and 5.6% in controls, a difference that did not reach significance ($p=0.09$), possibly due to the small number of subjects.

^{51}Cr -RBC organ counts following a marathon run

In order to test the hypothesis that the inflammatory effect of a marathon run leads to an increase in splenic red cell content, ^{51}Cr -RBC organ counts pre- and post-marathon were compared as described above (Table 3.6). In subject NW there was no decrease in RBC concentration 24 or 48 hours after the marathon, therefore these data were not included in the analysis. The maximum change from baseline in intravascular RBC concentration occurred between 24 and 48 hours post-marathon in the remaining subjects. Therefore, the baseline value of a ^{51}Cr -RBC organ count was paired with a value corresponding to the time of maximum change in circulating RBC concentration after the marathon, and the difference between the two values was calculated and compared with the difference found between two ^{51}Cr -RBC organ counts measured during a similar time period in the controls, using a t-test. The mean difference in ^{51}Cr -RBC heart and liver counts of the runners were significantly different from the control. However, as liver and heart counts decreased continuously throughout the 4 week period in the control group, the shorter interval between measures in runners (2-4 days vs 7 days in the control group) affects the validity of this comparison. The mean difference in ^{51}Cr -RBC spleen count of the runners (10.7%) was not significantly different from the control (3.7%), possibly due to the small number of subjects compared. ~

Table 3.5 The effect of a period of rest on the mean ⁵¹Cr-RBC organ count in runners compared with the mean organ count for control subjects during a similar time period.

| <u>training followed by a 14 day taper</u> | | | | | | |
|--|---------|---------|----------|---------|---------|---------|
| | heart | | liver | | spleen | |
| | runners | control | runners | control | runners | control |
| n= | 4 | 4 | 4 | 4 | 4 | 4 |
| measure 1 | 94.4 | 99.1 | 108.0 | 103.9 | 111.9 | 104.1 |
| measure 2 | 68.3 | 75.5 | 76.1 | 85.8 | 96.2 | 98.5 |
| mean difference | -26.1 | -23.6 | -31.9 | -18.1 | -15.7 | -5.6 |
| | (NS) | | (p=0.04) | | (NS) | |

⁵¹Cr-RBC organ counts are expressed as a percentage of the day 1 count. In runners, measures 1 and 2 refer to group mean ⁵¹Cr-RBC organ counts measured during training (day 3 after label) and at the end of a 14 day taper in preparation for a marathon run (day 17 or 18 after label), respectively. Data from subject RT is not included as she was ill during this period (see Figure A11). Data from subject MC is not included, due to an abnormal pattern of organ counts, possibly secondary to hemochromatosis (see Figure A15). In the control group measures 1 and 2 refer to ⁵¹Cr-RBC organ counts measured on days 3 and 16, respectively. A t-test was used to determine if there was a difference in the mean change in organ count between the runners and control subjects (mean difference).

⁵¹Cr-RBC organ counts after a seven-day recovery period following the marathon

Following the marathon, runners refrained from any exercise for seven days, allowing a second test of the hypothesis that a period of rest will result in a reduction of splenic red cell content concomitantly with an increase in RBC concentration. Table 3.7 shows the group mean ⁵¹Cr-RBC organ count measured at the minimum RBC concentration 24-48 hours after the marathon and the group mean ⁵¹Cr-RBC organ count at the end of a seven-day rest following the marathon, when RBC concentration had returned to its baseline value. The mean difference in ⁵¹Cr-RBC spleen count of -16.7% in runners was significantly greater than that of controls (3.7%).

Table 3.6 Mean ⁵¹Cr-RBC organ count in runners after a marathon run at a time corresponding with the maximum decrease in intravascular RBC concentration.

| | heart | | liver | | spleen | |
|-----------------|-----------|---------|-----------|---------|---------|---------|
| | runners | control | runners | control | runners | control |
| n= | 4 | 4 | 4 | 4 | 4 | 4 |
| measure 1 | 67.1 | 75.5 | 79.5 | 85.8 * | 92.7 | 98.5 |
| measure 2 | 67.5 | 62.2 | 82.6 | 76.2 | 103.3 | 102.4 |
| mean difference | -0.8 | -13.3 | 4.7 | -9.6 | 10.7 | 3.7 |
| | *(p=0.03) | | *(p=0.01) | | (NS) | |

* As liver and heart counts decreased continuously throughout the 4 week period in the control group, the shorter interval between measures in runners (2-4 days vs 7 days) affects the validity of this comparison.

⁵¹Cr-RBC organ counts are expressed as a percentage of the day 1 count. In runners, measures 1 and 2 refer respectively to a group mean baseline measure (24 hour pre-marathon; at the end of a 14 day taper) ⁵¹Cr-RBC organ counts and to a post-marathon measure. The post-marathon value selected corresponded with the maximum decrease in RBC concentration in the blood, which varied in individuals of the present study between 24 and 48 hours after completion of the marathon run. There was no decrease in RBC concentration from its baseline value either 24 or 48 hours after the marathon in subject NW, therefore his data is not included with that of the rest of the group. As previously stated, data from subject MC is not included, due to an abnormal pattern of organ counts possibly secondary to hemochromatosis. In the control group measures 1 and 2 refer to ⁵¹Cr-RBC organ counts measured on days 16 and 23, respectively. A t-test was used to determine if there was a difference in mean change between the two organ counts in runners compared with mean change in organ counts measured in control subjects during a similar time period (mean difference).

Blood Volume

Measurement of the volume of red cells (RCV) was made once during the study in each subject on the day of the red cell label (between week three and four of training). From this measure and the hematocrit, whole blood volume (TBV) and plasma volume (PV) were

calculated. These results are reported in Table 3.1. The group mean value of all three measures were significantly higher in the runners than in the control group. The change in plasma volume (ΔPV) throughout the training period was calculated by the method of Dill and Costill (1974). Figure 3.6 shows the time course of ΔPV calculated from the equations described in the methods: from Dill and Costill (1974), based on change in Hb concentration and Hct, and secondly using the change in total protein concentration to calculate ΔPV (Greenleaf and Hinghofer-Szalkay, 1985). There was no significant change in plasma volume during the training period or following the marathon using either method of calculation.

Table 3.7 The effect of a period of rest following the marathon on the mean ^{51}Cr -RBC organ count in runners compared with the mean organ count for control subjects during a similar time period.

| <u>seven days of rest following a marathon competition</u> | | | | | | | |
|--|---------|---------------|---------|--------------|---------|-----------------|--|
| | heart | | liver | | spleen | | |
| | runners | control | runners | control | runners | control | |
| n= | 4 | 4 | 4 | 4 | 4 | 4 | |
| measure 1 | 66.1 | 75.5 | 85.8 | 85.8 | 105.1 | 98.5 | |
| measure 2 | 59.2 | 62.2 | 78.5 | 76.2 | 88.5 | 102.4 | |
| mean difference | -8.3 | -13.3 (NS) | -7.4 | -9.6 (NS) | -16.7 | 3.7 (p=0.03) | |

^{51}Cr -RBC organ counts are expressed as a percentage of the day 1 count. In runners measures 1 and 2 refer to ^{51}Cr -RBC organ counts at a time corresponding with the minimum Hb concentration following the marathon (day 19 or 20 after label) and at the end of seven days of rest following the marathon, when Hb concentration had returned to baseline value (day 25 or 26 after label), respectively. In the control group measures 1 and 2 refer to ^{51}Cr -RBC organ counts measured on days 16 and 23, respectively. As previously stated, data from subject MC is not included, due to an abnormal pattern of organ counts possibly secondary to hemochromatosis. Data from subject NW is not included with that of the other subjects as there was no decrease in Hb concentration either 24 or 48 hours after the marathon. A t-test was used to determine if there was a difference in mean change between the two organ counts in runners compared with mean change in organ counts measured in control subjects during a similar time period (mean difference).

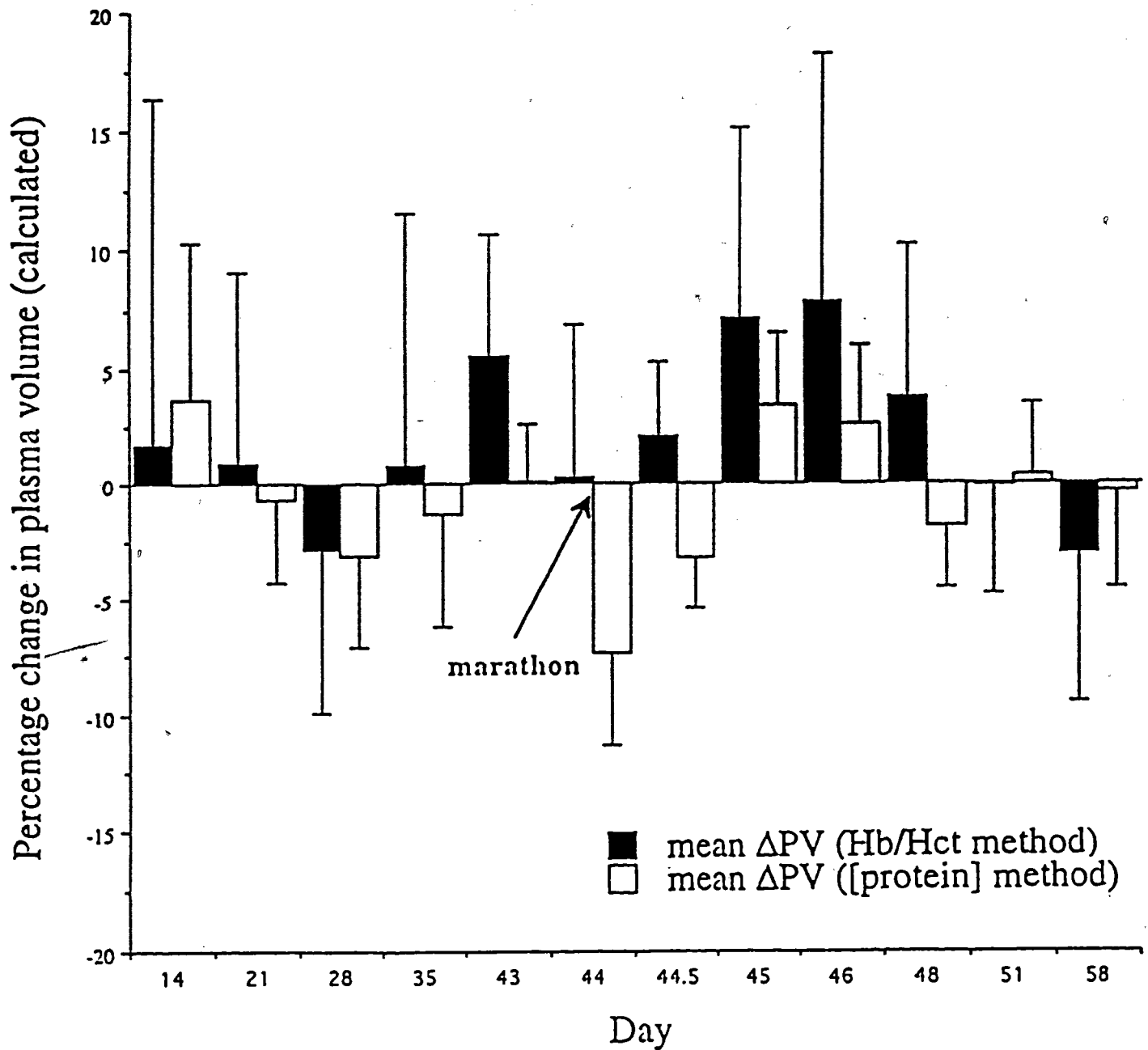


Figure 3.6 Sequential change in plasma volume comparing two methods of calculation. Each measure of change in plasma volume (ΔPV) prior to the marathon was determined by the previous week's hemoglobin (Hb) and hematocrit (Hct) or total protein concentration ([protein]) value, as a plasma volume change was expected in response to the intense training schedule and subsequent taper. Post-marathon ΔPV measures were determined by the baseline value (24 hrs prior to the marathon) of Hb and Hct or [protein].

DISCUSSION

The response to training

Detection of disruption of skeletal muscle

During four weeks of training in the runners an unsuccessful attempt was made to induce a continuing inflammatory response. In a healthy individual, presence in the serum of certain enzymes, such as creatine kinase (CK) and lactate dehydrogenase (LDH) indicate disruption of skeletal muscle. Group mean CK and LDH concentrations were not significantly different from their respective baseline value during the training period.

Detection of plasma cytokines

During the training phase of the study plasma cytokines were undetectable at the time measured. This finding contrasts with the results of Evans et al., 1986 who observed a persistent low level of IL-1 activity in the serum of trained runners, but agrees with the results of other investigators who have used a more specific assay system and who report an undetectable level of cytokines in a trained subject at rest (Cannon et al., 1986; Evans et al., 1986; Viti et al., 1985; Dufaux and Order, 1989; Espersen et al., 1990; Northoff and Berg, 1991; Northoff et al., 1994; Cannon et al., 1991; Sprenger et al., 1992). However, in the above mentioned studies, which were not training studies, the "at rest" value was a pre-activity measure taken before a single exercise challenge and not when the subject was engaged in an extended period of exhausting, defined training, such as was the case in the present study. For example, in the study of Sprenger et al. (1992), the group mean creatine kinase (CK) concentration at rest was merely 68 U/liter (range 42-133), however

the group mean CK value increased to 174 U/liter (range 90-303 U/liter) after a 20 km run, which matches more closely the concentration reported in the present study during training (mean 131, range 61-239 U/liter). In the study of Sprenger et al. (1992) plasma IL-6 was detectable for at least 5 hours following the 20 km run. Perhaps the failure to detect plasma cytokines in the present study during training was related to timing, i.e., in the present study a plasma sample was collected only 12 hours following the last bout of exercise.

Detection of an acute phase response

While plasma cytokine concentration may not be detected 12 hours after a training session which induces an acute phase response, acute phase reactants are detectable beyond 12 hours. During the training phase of the present study there was no change in the group mean concentration of any acute phase reactant measured (haptoglobin, albumin, transferrin, ferritin). Tissue injury rapidly leads to increased synthesis of tissue ferritin (Konijn and Hershko, 1977). Serum ferritin concentration tended to decrease at the end of the taper period (Figure A9), which suggests its original elevation throughout the training period, perhaps due to a modest elevation in rate of synthesis (Dickson et al., 1982).

An increase in the production of haptoglobin, of course, could be masked during training if there was a corresponding increase in the rate of red cell hemolysis. Support for this view include the many reports of intravascular hemolysis in response to exercise (e.g. Gilligan et al., 1943; Lindemann et al., 1978; Magnusson et al., 1984), the shorter lifespan of the red cell during training (Weight et al., 1991) and the increase in the rate of RBC destruction during moderate endurance training (Schmidt et al., 1988). Evidence of an increased rate of RBC destruction during training in the present study is the increase in the group mean serum iron concentration (SI) and percentage saturation of transferrin (%sat) in the fourth

week of training. This result agrees with Dressendorfer et al., (1981) and Banister and Hamilton (1985), who found that SI was high during a period of strenuous distance running. Seiler and colleagues (1989) reported that SI increased coincidentally with an increase in serum bilirubin concentration and a decreased haptoglobin concentration, during the first 3 days of a 1000 km run covered in 20 days. These investigators concluded that SI and %sat were increased as a result of hemolysis. In the present study there was no significant change in group mean concentration of haptoglobin (Figure A6) or bilirubin (Figure A10), respectively during training, although bilirubin concentration tended to decrease during the taper and post-marathon recovery period.

Variation in RBC concentration

During the training phase of the study mean reticulocyte count and plasma erythropoietin (Epo) and RBC concentration did not differ from their respective baseline value.

Furthermore, the hypothesized increase in RBC concentration at the end of the two week taper was not observed. These observations indicate that the level of activity maintained during training in a runner was insufficient to induce a change in their hematological profile. If a higher daily level of activity had been maintained by the subject group in the present study, the results might have revealed a pattern of response more similar to that of an ongoing inflammatory response such as in the study of Seiler et al., (1989) where runners who covered 1000 km in 20 days experienced a significant reduction in mean RBC concentration accompanied by elevated serum ferritin concentration and reduced serum iron concentration (day 6-20).

Sequestration of RBCs

One of the main purposes of the present study was to determine the role of the spleen and/or liver in the reduction of intravascular RBC count normally observed during an extended period of intense exercise training. There was a significant decrease in the ^{51}Cr -RBC liver count after two weeks of taper compared with its level at the end of a four week period of training and a concomitant tendency of the group mean splenic ^{51}Cr -RBC count to decrease during the same period of time (Table 3.5). A reduction in the ^{51}Cr -RBC content of an organ may be interpreted several ways. It could indicate a "demargination" of red cells, a reduced overall size of the organ or a reduced rate of uptake of damaged red cells. Since there was no detectable corresponding increase in RBC concentration at the end of the taper period, and in the absence of an increase in plasma volume masking an absolute increase in circulating RBC, the best interpretation is probably that there was a reduced rate of uptake of damaged red cells by the liver and the spleen. Support for this premise is the trend towards a decrease in mean bilirubin concentration and an increase in mean haptoglobin concentration at the end of the two week taper from their respective values recorded during training, which reflects a reduction in intravascular hemolysis accompanying reduced training.

The response to the marathon run

Detection of disruption of skeletal muscle

In contrast to the lack of evidence of an inflammatory response to an exercise session during the training period, there is evidence of disruption of active muscle fibers in response to the marathon run. Serum CK concentration peaked 24 hours post-marathon

and continued to be elevated above a baseline level in the 48 and 96 hour post-marathon measures. The unaccustomed distance and the long stretches of downhill running probably led to the large increase in serum CK concentration and muscle soreness. There is a large body of evidence in the exercise literature that relates delayed onset of muscle soreness (DOMS) to disruption of contractile and/or connective tissue of the exercised muscle. Each runner in the present study reported DOMS 24-48 hours following the marathon run. There is evidence in subjects with myocardial infarction that cardiac muscle damage *per se* can initiate an acute phase response (Griffiths et al., 1985) and, paradoxically, there is also evidence that the acute phase response may itself worsen muscle injury by precipitating a continuing proteolysis (Baracos et al., 1983). However, it is unlikely that muscle injury was extended in the present study since the group mean serum CK concentration peaked 24 hours following the marathon run and returned to a baseline concentration 96 hours after the marathon.

Detection of plasma cytokines

Following the marathon run, plasma IL-6 was detected but IL-1 β and TNF- α were undetectable at the time points sampled. This disagrees with the results of Cannon and Kluger (1983) who reported detectable plasma pyrogen activity immediately after one hour of exercise at 60% VO_{2max} and Cannon et al. (1986) who confirmed, using the same exercise protocol, that the plasma concentration of IL-1 (radioimmunoassay) did increase. However, Smith et al. (1992) using the same exercise challenge found no change in plasma concentration respectively in IL-1 β , IL-6 or TNF- α . Dufaux and Order (1989) found an increase in TNF- α after 2.5 hours of running at an intensity which elicited a blood lactate concentration of 4 mM, an exercise protocol similar to that of the present study.

The reason why in the present study neither IL-1 β nor TNF- α were detected in the plasma of a runner following the marathon is unclear, but agrees with Sprenger et al. (1992). The underlying mechanism of enhanced cytokine production in response to intense exercise remains unknown at present, making it difficult to determine the reason for the selective production of cytokines in response to an inflammatory stimulus. Endotoxin is a potent inducer of TNF- α and endotoxin (released from the intestine) has been detected in the blood following an ultradistance triathlon and an ultradistance run, respectively (Bosenberg et al., 1988; Brock-Utne et al., 1988). In the present study it is unlikely that endotoxin was released into circulation in response to the marathon run, since TNF- α was undetectable following the marathon run. Northoff and Berg, (1991) found IL-1 inhibitor present in 1:40-1:100 dilution and failed to detect IL-1 from serum of marathon participants in a thymocyte assay or IL-1 β in an ELISA with a detection limit of 15 pg/ml. The effect of IL-1 inhibitor(s) on the IL-1 β ELISA was not analyzed in the present study and is of yet unavailable by the supplier of the ELISA. The rate of production and final volume of each cytokine produced may vary. The presence of any one cytokine in plasma could be reduced in the face of an equal rate of production if more of one cytokine than another was taken up by cells expressing the appropriate receptor. Local production of a cytokine may not be detectable in an assay of plasma. For instance, Cannon et al. (1989) found IL-1 β in muscle tissue up to five days after an eccentric exercise challenge, whereas its detection in plasma is normally limited to a few hours. Another accounting for the differing plasma concentration of the various cytokines could be their differing rate of clearance.

Detection of an acute phase response

Acute phase reactants

As IL-6 was detected in the plasma of a runner after the marathon in the present study, an alteration in the concentration of acute phase reactants was expected. Mean serum ferritin concentration was elevated in the two and six hour post-marathon samples of runners in the present study, in agreement with Vidnes and Opstad, (1981), Dickson et al., (1982) and Taylor et al., (1986). Haptoglobin concentration was reduced in the two and six hour post-marathon samples of all runners, presumably in response to intravascular hemolysis. Its return back to pre-marathon level 48 hours following the marathon represents an increase in production, which is expected as part of an acute phase response.

Two findings in the present study which deviate from the classical acute phase response are a significant increase in serum transferrin and serum albumin concentration two and six hours post-marathon. When comparing measures made at different times of the day, it is important to consider diurnal variation. An increase from morning to afternoon in TIBC has been observed in many, but not all chronobiological studies (Casale et al., 1981). In the present study, the two and six hour post-marathon samples occurred in the early afternoon and late afternoon, respectively, when no change due to diurnal variation is expected. In the classical acute phase response, an early decline in plasma albumin and transferrin concentration is thought to be due primarily to increased vascular permeability (Gordon and Koj, 1985), shifting albumin from the intravascular to the extravascular pool. Injury to active muscle fibres has been defined by Armstrong, 1990 as microinjury because the initial lesion is usually subcellular and occurs in a relatively small proportion of muscle fibres. Injury occurring in a small proportion of muscle may not stimulate an increase in

vascular permeability to the same extent as blunt trauma, the method used to experimentally-induce injury in animal studies. An increase in the rate of lymphatic drainage to the vascular space is a common effect of an acute phase response, whatever its origin, and is one hypothesized mechanism for the observed early increase in protein content following exercise (Senay, 1972). If this indeed occurs, addition of protein from the lymphatic system during the marathon may have masked any acute loss due to increase in microvascular permeability to albumin. Part of the increase in serum albumin (and transferrin) concentration could also be explained by a lingering hemoconcentration, due to failure of complete rehydration at the two hour post-marathon measurement. Although it is apparent that the expression of an acute phase response in this and other studies (Haralambie and Keul, 1970; Liesen et al., 1977; Noakes et al., 1982) varies from the classical pattern, it is recognized that the kinetics of a particular acute phase protein varies with the type of injury or disease (Gordon and Koj, 1985) and that the specific response to exercise-induced focal disruption of skeletal muscle will be superimposed upon the general response. For example, when hemolysis plays no part in the condition inducing an acute phase response, an increase in serum haptoglobin concentration will be more pronounced than observed in the present study.

Hypoferremia

In response to the marathon run, a decrease in mean serum iron concentration (SI) and percentage saturation of transferrin (%sat) were detected six hours post-marathon, in agreement with the results of Ross and Attwood, (1984) and van Rensburg et al., (1986), but not Dressendorfer et al., (1981); Campanini et al., (1988) and Weight et al., (1991). The timing of the blood sample may explain the conflicting study findings, as there was no sample taken at six hours post-exercise in those of the above studies not reporting a

reduction in SI or %sat. Without the six hour post-marathon sample, the fall in SI and %sat in the present study would have also been undetected. In chronobiological studies, a characteristic circadian rhythm of plasma iron has been observed (Hamilton et al., 1950; Halberg et al., 1977). However, the reduction from a mean of 15.3 to 8.8 $\mu\text{mol/L}$ in the present study exceeds the 20-30% fall observed between mid-morning and evening in chronobiological data. Thus it may be concluded that the marathon stimulated an hypoferremic response in the runners.

Variation in RBC concentration

Hemoglobin (Hb) concentration and hematocrit were significantly reduced 48 hours post-marathon, while a reduction in RBC concentration failed to reach significance ($p=0.069$), probably due to the small number of subjects. Interestingly, in the only subject in which serum iron and %sat values were not below baseline at six hours, indicating a lack of an acute phase response, there was no decrease in hematocrit, Hb or RBC concentration 24 or 48 hours after the marathon. The mechanism(s) which may mediate the observed reduction in hematocrit and RBC and Hb concentration are discussed below.

Sequestration of RBCs

As mentioned in the introduction, in human studies the net result of treatment with rhIL-6 is anemia (Ulrich et al., 1991; Weber et al., 1993). Winton et al., 1994 made several observations which led them to the conclusion that rhIL-6-induced anemia in primates was secondary to erythrocyte sequestration and/or hemodilution secondary to a shift of fluid to the intravascular space. In the present study the group mean hepatic ^{51}Cr -RBC count was increased at a time corresponding with the lowest red cell count measured 24 or 48 hours

after the marathon. However, as the liver count decreased continuously throughout the four week period in the control group, the shorter interval between measures in runners (2-4 days vs 7 days in controls) affects the validity of this comparison. Furthermore, there was no corresponding increase in intravascular RBC concentration when the ^{51}Cr -RBC liver count decreased after two weeks of taper from its level at the end of a four week period of training. In addition, ^{51}Cr -RBC liver count increased from baseline in a single runner with no decrease in RBC concentration up to 48 hours after the marathon (Table A2). Taken together, these latter two observations demonstrate the inconsistent relationship of variation in the ^{51}Cr -RBC liver count with variation in RBC concentration. Splenic ^{51}Cr -RBC count tended to increase concomitantly with a decrease in RBC concentration, 24 or 48 hours after the marathon run, then decreased from this level by seven days post-marathon, when RBC concentration had returned to baseline level. This supports the notion that red cells were sequestered at the 24 or 48 hour time points and released seven days after the marathon. An increase in the ^{51}Cr -RBC content of an organ may be interpreted in several ways. One interpretation is that there is an increase in splenic or hepatic uptake of damaged red cells. However, red cells damaged during the marathon would likely be sequestered in the spleen within the first few hours. Another interpretation of an increase in ^{51}Cr -RBC count is that the spleen or liver enlarge with sequestered red cells in response to the onset of inflammation and/or to an increase in blood volume. Several observations complicate the interpretation of the significance of the change in ^{51}Cr -RBC organ content in a single group mean measure after the marathon. Although an individual's post-marathon RBC concentration in the present study was always lower than baseline when splenic ^{51}Cr -RBC count was higher than baseline, peak ^{51}Cr -RBC count was not always reached at the same time as the minimum RBC measurement. Furthermore, splenic ^{51}Cr -RBC count was not always higher than baseline when intravascular RBC concentration was lower than baseline, which may indicate that another

mechanism, such as hemodilution accounted entirely for the reduced RBC concentration at that time. Thus the role that splenic or hepatic uptake of red cells plays in the reduction of RBC concentration remains unclear.

Hemodilution

Using the method of Dill and Costill (1974) and Greenleaf and Hinghofer-Szalkay (1985), a trend of increasing plasma volume up to 48 hours following the marathon run was calculated in the present study. Although an increase in plasma volume and total protein content in response to an acute bout of strenuous exercise has been frequently reported, the source of the additional protein has not been established. A shift of protein from the interstitial space via the lymphatic system and *de novo* synthesis have both been proposed. As IL-6 concentration increased in every runner following the marathon in the present study, it is reasonable to assume that newly synthesized acute phase proteins contributed to an increase in total intravascular protein content. The possibility that newly synthesized acute phase protein contributes to an increase in plasma volume, which in turn provides a proportional contribution to an increase in plasma volume and a reduction in RBC concentration, all in response to an exercise-induced disruption of skeletal muscle, are topics for further study.

Post-marathon increase in erythropoiesis

Variation in Erythropoietin concentration

An increase in the rate of production of new red cells may have contributed to the return of Hb and RBC concentration to their respective baseline level seven days after the marathon.

Epo concentration was increased from its baseline level at the time of a maximal decrease in RBC and Hb concentration, either 24 or 48 hrs post-marathon, an observation also noted by Schmidt et al., 1991. Schwandt et al., 1991 found a small increase in Epo concentration at three hours and a larger increase 31 hours after a run lasting two and a half hours. Unfortunately, in this latter study the change in testing time the day after the run meant that diurnal variation may have played a role in the change in Epo concentration. As subjects were retested at the same time each day in the present study, diurnal variation in Epo concentration was not a factor in any measurement except for the one taken six hours after the marathon. The most simple explanation for the observed increase in plasma Epo concentration 24 or 48 hrs post-marathon is that the kidney was producing more Epo in response to the reduced hemoglobin concentration during the same time period.

Reticulocyte count

An increased Epo concentration in the blood normally leads to an increase in reticulocyte count after 2 days (Kurtz et al., 1988). In the present study an increase in the group mean reticulocyte count was detected seven days, rather than four days after the marathon, thus it is uncertain what role, if any, the increase in Epo concentration played in reticulocytosis. Although their study was complicated by altitude, Klausen et al. (1991) also found that although Epo concentration was significantly increased above its pre-altitude level by day two of exercise at altitude, the reticulocyte count did not increase until four days after completing a seven day training session. Inflammatory cytokines have been shown to suppress red cell precursors from responding to Epo (e.g. Johnson et al., 1991), thus the lag between the increase in reticulocyte count and the increase in Epo concentration in the present study may have been due to a residual inflammatory effect following the marathon.

Problems encountered in the study

The small number of subjects that completed the study limits its statistical power. There is little data in the literature regarding normal intra-individual variability in the ^{51}Cr -RBC organ counts throughout a four week measurement period and no data on the percentage of change in counts to be expected accompanying variation in inflammatory activity, thus it was not possible to determine beforehand the appropriate number of subjects using a power calculation. However, the present study will aid future investigators in this regard. The values obtained in the ELISA for IL-6 may have been underestimated, as a repeat assay 2 years later showed an increase in activity. Because there were six runners and it was only possible to schedule three ^{51}Cr -RBC labelling procedures each day, i.e. at the first rest period at the end of the taper (24 hours before the marathon competition) which corresponded to day 17 or day 18 after the label, there was no exact matching day for the control subjects so that an analysis of variance test could not be used to compare the pattern of change in organ counts of ^{51}Cr -RBC in a runner with that in the controls. Therefore, the paired ^{51}Cr -RBC organ count data from day 3-17 and day 3-18 (a 14-15 day interval) for runners was pooled and compared with the data from day 3-16 (a 13 day interval) in the control subjects using a t-test for paired data (Table 3.6). Similarly, pre and post-marathon ^{51}Cr -RBC organ count data from day 17-22 (a 2-4 day interval) in runners was compared with data from day 16-23 (a 7 day interval) in the control subjects (Table 3.7) and post-marathon ^{51}Cr -RBC organ count data from day 19 or 20 to day 25 or 26 in runners (a 5-6 day interval) was compared with data from day 16 to 23 (a seven day interval) in control subjects (Table 3.8).

Some of the limitations of using ^{51}Cr -RBC to follow the distribution of erythrocytes are discussed on page 23. A major drawback of this method is that the percentage change in

the ^{51}Cr -RBC organ count from baseline cannot be converted into an actual number of red cells gained or lost. In addition, it is not possible to determine the proportion of viable to non-viable cells in any one count. The importance of a change in percentage of red cells lost and gained during induction of and recovery from an exercise-induced inflammatory response depends upon a spleen substantially larger than that reported in the literature. Although a spleen-to-liver ratio of 0.94 would normally indicate an average-sized spleen, an increased ^{51}Cr -RBC liver count, due to hemolysis would mask an increased spleen size in the runners. Further studies are required to resolve these issues, especially studies to quantitate the size of the spleen at rest and following various levels of an exercise stimulus in the endurance trained individual.

Conclusion

The decrease in red blood cell (RBC) concentration observed following intense exercise has previously been attributed entirely to hemodilution. In the present study a role is proposed for the spleen, which may enlarge or sequester red cells during an inflammatory response. In addition, a role is proposed for the continuous presence of circulating inflammatory cytokines, which may alter the functioning of the erythron. The failure to induce a continuing inflammatory response during the training phase of the study precludes evaluation of a non-hemodilutional factor's contribution to the low-normal RBC concentration observed in endurance-trained athletes, especially runners. Following the marathon run, however, mean serum CK and LDH concentrations, respectively, were increased, indicating disruption of skeletal muscle. Evidence of an exercise-induced acute phase response, possibly mediated by the observed transient increase in plasma IL-6 concentration following the marathon, includes a decrease in serum iron concentration and saturation of transferrin and an increase in serum ferritin concentration from their respective

baseline measure together with an increase in serum haptoglobin concentration from a previously reduced level two hours following the marathon. Thus, it was possible to identify a non-hemodilutional contributor to the decrease in RBC concentration observed following the marathon. The mean ^{51}Cr -RBC liver count increased and splenic ^{51}Cr -RBC count tended to increase at a time corresponding with a minimum intravascular RBC concentration 24 or 48 hours after the marathon run. The inconsistent correlation of ^{51}Cr -RBC organ content with RBC concentration during 48 hours following the marathon and the limitations of the method, however, leave unclear the exact role splenic or liver sequestration of the RBC may play in the reduction of intravascular RBC concentration.

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Chapter 4. Disease-linked inflammation of synovial joints in Rheumatoid Arthritis

Abstract

The purpose of this study was to define further the pathophysiology of anemia in Rheumatoid Arthritis (RA). A role is proposed for the spleen, which may enlarge or sequester red cells during an inflammatory response. In addition, a role is proposed for continuous presence of circulating inflammatory cytokines, which may alter the function of the erythron. Plasma interleukin (IL)-1 β , IL-6, interferon gamma (IFN- γ), erythropoietin (Epo), reticulocyte count and other related indices were serially measured during a period of active disease followed by four weeks of methotrexate treatment, in five anemic and five non-anemic RA subjects. These data were examined in order to determine the relation of variation in the concentration of circulating cytokines to erythrocyte content of the spleen ($^{51}\text{Cr-RBC}$) and to reticulocyte count, either of which may contribute to an increase in RBC concentration as inflammation subsides with methotrexate treatment. Mean plasma IL-6 concentration and serum haptoglobin concentration were higher in the anemic RA subgroup than in the non-anemic subgroup prior to commencement of methotrexate treatment. During methotrexate treatment, inflammatory activity declined as shown by a reduction in the mean plasma IL-1 β , serum ferritin and serum haptoglobin concentration, respectively, in the non-anemic subgroup and a reduction in mean erythrocyte sedimentation rate and mean serum concentration of ferritin and haptoglobin, respectively, in the anemic RA subgroup. The mean reticulocyte count was increased and mean plasma Epo concentration was reduced after three weeks of methotrexate treatment in the anemic RA subgroup. While the group mean RBC concentration was unchanged after four weeks of methotrexate treatment, RBC concentration was increased by the third or fourth week of methotrexate treatment in four of five anemic subjects. There was no pattern of decreasing $^{51}\text{Cr-RBC}$

spleen count, indicating that release of sequestered RBCs did not contribute to the observed improvement in RBC concentration in any of the four anemic subjects. An increase in reticulocyte count and a decrease in plasma Epo concentration corresponded to an increase in RBC concentration in three of four anemic subjects, suggesting that erythropoiesis was stimulated as disease activity declined, but that Epo played no role. The baseline level of cytokines differed in the three anemic RA subjects who each showed an increased reticulocyte count above normal, however in all three cases, plasma IFN- γ concentration decreased from its baseline value following methotrexate treatment. These data provide evidence for a reduced suppression of erythropoiesis, unrelated to an increase in Epo concentration, acting to improve the reticulocyte count and RBC concentration in an anemic RA subject treated with methotrexate. Further studies using a larger subject population are required to confirm the finding of a decrease in Epo concentration and an increase in reticulocyte count during methotrexate treatment and to determine the mechanism responsible for the observed increase in reticulocyte count.

Introduction

Anemia in Rheumatoid Arthritis

Anemia is the most common extra-articular manifestation of rheumatoid arthritis (RA) (Baer et al., 1990). Many causes of anemia are associated with RA. Gastric mucosal atrophy and antibodies occur in a higher than normal frequency in RA (Couchman et al., 1968) which may explain the increased frequency of vitamin B₁₂ deficiency sometimes reported (Bieder and Wigley, 1964, Vreugdenhil et al., 1990a). Folic acid deficiency, possibly caused by anorexia and its increased utilisation by proliferating synovial cells is reported to be more frequent in RA (Cough et al., 1964, Vreugdenhil et al., 1990a). Autoimmune phenomena can occur in RA leading to destruction of erythrocytes or thrombocytes, producing an increased tendency to bleed. Cases of pure red cell aplasia have been described during the course of RA (Dessypris et al., 1984). Antirheumatic treatment such as with gold, penicillamine and cytostatic drugs may lead to anemia (Kay, 1976, Adachi 1985, Balme and Huskisson 1975, Putte 1988). A higher frequency of gastrointestinal blood loss may also be observed, probably due to non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroid treatment (Graham and Smith 1986, Jick and Porter 1978). In a recent study, however, a mean blood loss of only 28 ml during 44 days was estimated by counting stool samples in patients on anti-inflammatory medication (Lotter et al, 1991).

Anemia of chronic disorders in RA

A specific type of anemia is associated with chronic infection, inflammation, malignancy and autoimmune disorders (Means and Krantz, 1992). The precise pathogenesis of this

"anemia of chronic disorders" (ACD) remains unclear, but is most likely multifactorial. ACD is usually normocytic and normochromic, although mean corpuscular hemoglobin and mean corpuscular volume (MCV) can be low, even without iron deficiency (Chernow and Wallner, 1978). ACD is further distinguished from iron deficiency by a reduced transferrin concentration. It is clear that in RA, more than one cause of anemia may be simultaneously present, complicating the interpretation of investigations into the pathogenesis of ACD. In active RA, a concomitant iron deficiency was reported in 30-70% of patients (McCrea 1958, Rajapakse et al., 1980, Hansen et al., 1983, Vreugdenhil et al., 1990c). According to Cartwright (1966) three main factors contribute to ACD: a reduced red cell lifespan, depressed erythropoiesis and a disturbance in iron metabolism. The relative importance and origin of these processes in the ACD of RA has been investigated and is described below.

Red cell life span in RA

A moderate reduction in red cell life span of RA patients has been reported e.g., 90 days in anemic RA patients and 114 days in normal control subjects (Dinant and Maat, 1978). However, an increase in erythropoiesis, sufficient to compensate for a modest reduction in red cell survival should be within the capacity of normal marrow (Pollycove and Tono, 1975).

Erythropoiesis in RA

Reticulocyte count is often low for the degree of anemia observed in ACD (Vreugdenhil et al., 1992a), suggesting suppression of erythropoiesis. Studies using a methylcellulose culture of bone marrow cells from RA patients have produced conflicting data regarding the

activity of erythroid progenitors. In the RA patient both with and without anemia, Reid and colleagues (1984) found no difference between growth of burst forming units (BFU-E) and colony forming units (CFU-E) (erythroid progenitors cells at an early and late stage of their respective development). Harvey et al., 1983 found a trend towards a lower number of BFU-Es from the peripheral blood of the anemic RA patient than from a non-anemic RA and control subject, respectively. Vreugdenhil et al., 1992b found the bone-marrow derived BFU-E count was lower in RA patients with ACD. However, *in vitro* findings may not translate to defining the *in vivo* condition. Investigation of the soluble transferrin receptor (TfR) concentration in serum is a new method for evaluating total erythropoiesis in the bone marrow. Noe et al. (1995) reported a mean TfR level of 4.1-times normal in patients with hemolytic anemia whereas the mean TfR value of iron-replete and iron-deficient anemic RA patients was 1.6- and 2.2-times normal, respectively, indicating a modest degree of hypoproliferation in RA patients. Similar results were shown by Skikne et al. (1990) in 41 patients with ACD and by Vreugdenhil et al. (1992a) in 10 RA patients.

Iron metabolism in RA

The distinctive pattern in ACD of a low serum iron and transferrin concentration together with a low percent saturation of transferrin in the presence of a normal to increased iron store suggests disturbance of iron metabolism. As mentioned above, iron deficiency in RA is a common and frequent complication of ACD. An early finding of disturbed iron absorption in the anemic RA patient (Roberts et al., 1963) was suggested as a cause of iron deficiency. However, data are conflicting. Boddy and Will (1969) and Weber et al. (1988) found a smaller rise of mean iron absorption in the iron deficient RA patient group compared with the iron deficient control group, but Benn et al. (1988) found no difference. Vreugdenhil et al., 1990c found that iron absorption correlated negatively with iron stores,

which were higher in the RA patient with ACD. Thus these authors concluded that a decreased iron absorption is the result of active RA rather than a cause of iron deficiency. Additional factors which operate during ACD are evident in studies which show a correction of iron deficiency does not correct anemia (Bentley and Williams, 1982; Richmond et al., 1985; Pincus et al., 1990). In fact, iron deficiency may be protective in RA. In support of this premise are the studies which report a lower disease activity in an iron deficient subgroup of anemic RA patients (Baer et al., 1987; Vreugdenhil et al., 1990a; Vreugdenhil et al., 1991; Noe et al., 1995). Parenteral iron treatment may actually lead to an increase in disease activity (Reddy and Lewis, 1969; Blake et al., 1985). Both ferritin (Biernond et al., 1984) and iron content (Niedermeier and Griggs, 1971) are elevated in the synovial fluid of the RA patient and the degree of elevation correlates with a prognosis of disease progression (Blake et al., 1985). Theoretically, excess iron could contribute to the persistence of synovitis by augmenting local free radical damage in joints.

However, in ACD, by definition, iron stores range from normal to elevated, in spite of their low circulating concentration. Thus, many studies have investigated the release of iron from the mononuclear phagocyte system (MPS), formerly called the reticuloendothelial system, in a model of ACD since impaired release could result indirectly in suppressed erythropoiesis. Ferrokinetic studies using intravenous ^{59}Fe -dextran show normal erythrocyte incorporation in patients with RA and anemia (Freireich et al., 1957; Beamish et al., 1971). However, the second and most important source of iron for erythroblasts is transferrin-bound iron recovered from the hemoglobin of senescent erythrocytes and subsequently released by the MPS. Freireich et al., 1957 reported diminished reutilization of this iron after administration of ^{59}Fe -hemoglobin. This observation was confirmed by Beamish et al. (1971). However, other investigators, using lower doses of radiolabeled iron found no block in iron reutilization or mobilization, suggesting that a normal to

increased MPS iron store with hypoferremia is a secondary phenomenon caused by decreased erythropoiesis (Williams et al., 1974; Konijn and Hershko, 1977; Williams et al., 1982; Zarrabi et al., 1977).

It is likely that both an impaired iron metabolism and suppressed erythropoiesis are operating simultaneously, as shown in studies which use disease modifying agents to reduce inflammation. For instance, it is postulated that iron-chelating agents may effect iron release from the MPS and/or increase transferrin receptor expression on erythroid precursors (Louache et al., 1984; Testa et al., 1985). Vreugdenhil et al., 1990 and others (Giordano et al., 1984; Giordano et al., 1986) found that iron chelation correlated with an increase in hemoglobin concentration, while Polson et al., 1986 in a study of longer duration (6 months) found that hemoglobin concentration declined. Altered iron metabolism has been reported in response to treatment with inflammatory cytokines (Sun et al., 1993; Jongen-Lavrencic et al., 1996). In the present study serum iron, transferrin concentration and percentage saturation of transferrin with iron and erythrocyte corpuscular volume were measured throughout a period of change in disease activity as an indicator of any change in ferrokinetics.

The role of cytokines in the pathogenesis of ACD in RA

ACD occurs frequently in patients with rheumatoid arthritis and its severity is correlated with disease activity (Shore et al., 1986; Swaak et al., 1988; Dasgupta et al., 1992).

Increased disease activity is associated with an elevated concentration of circulating IL-6 (Vreugdenhil et al., 1990; Barrera et al., 1993; Manicourt et al., 1993), TNF- α (Teppo and Maury, 1987; Vreugdenhil et al., 1992b; Manicourt et al., 1993) and IL-1 (Eastgate et al., 1988; Maury et al., 1988). Improvement of the disease by antirheumatic drug treatment is

often accompanied by a reduction in serum cytokine concentration (Dasgupta et al., 1992; Barrera et al., 1993). Thus, one line of study has been to investigate the role of the cytokines produced during the inflammatory response to discern their role in the pathogenesis of ACD in RA.

Cytokines and suppressed erythropoiesis in RA

Insufficient red cell production can result from direct suppression of erythropoiesis in the bone marrow by mediators of the inflammatory response. Experimental evidence supports the effect of a humoral factor in suppressed erythropoiesis. Serum of an anemic patient with RA (Dainiak et al., 1980; Reid et al., 1984) and juvenile chronic arthritis (Prouse et al., 1987) inhibits colony formation by normal erythropoietic progenitor cells cultured *in vitro*. Baer et al., 1990 found suppression of erythroid colony-forming units (CFU-E) *in vitro* not only with serum from two of five anemic patients with RA but also by serum from one of four non-anemic RA patients. Maury et al. (1988) demonstrated a raised IL-1 β concentration in the anemic RA patient. rhIL-1 in a concentration similar to that found in the arthritic patient suppressed normal erythropoiesis and proliferation of human erythroleukemia (HEL) cells *in vitro*. Several other studies using purified cytokine in an *in vitro* colony forming assay, support the identification of IL-1 and TNF- α as suppressors of erythropoiesis (Schooley et al., 1987; Lu et al., 1986; Roodman 1987). The presence in patient serum of IL-1 (or other factors) detectable by ELISA assay does not, however, prove its role in ACD or that it is biologically active. For example, the synovial fluid mononuclear cells from RA patients also produce a potent IL-1 inhibitor (Ridderstad et al., 1991). As stated by Vreugdenhil and Swaak (1990c), proving the role of inflammatory cytokines in reduced erythropoiesis may be possible by making measurement of the

cytokine concentration and erythropoiesis before and after anti-rheumatic treatment. This is the approach taken in the present study.

Another line of evidence which supports the role of an inflammatory cytokine in altered erythropoiesis results from studies which have followed erythropoiesis after administration of a cytokine, or its inhibitor, to a patient. The rationale for these experiments has evolved from consideration of the pathogenesis of certain conditions which suggests that a cytokine or its inhibitor represent a beneficial therapeutic approach. The effect of over-expression of TNF- α in transgenic mice; its effects on cartilage metabolism, including the inhibition of collagen synthesis and the stimulation of collagenase production by fibroblast and synovial cells combined with its direct and indirect effect on proteoglycan metabolism suggest that TNF- α plays a critical role in the pathogenesis of RA (reviewed in Maini et al. 1995). Trials of a neutralizing dose of monoclonal anti-TNF- α antibody (cA2) in RA patients have been promising. In a multi-center, randomized, double-blind study which compared the use of cA2 to a placebo, hemoglobin concentration increased in the experimental group while it decreased in the control group after 4 weeks (Elliott et al., 1994). The authors acknowledge that improvement of hemoglobin concentration in cA2 treated patients might simply reflect improvement in the overall disease state, but cannot rule out direct interference of TNF- α mediated suppression of erythropoiesis by cA2. Serum IL-6 concentration decreased followed by a reduction in acute phase proteins. In RA synovial cell cultures blocking the activity of TNF- α by its a polyclonal antibody resulted in inhibition of IL-1, GM-CSF, IL-6 and IL-8 production, the cytokine cascade (reviewed in Maini et al., 1995). Thus experimental results focused on a single cytokine must be interpreted with caution. The therapeutic effect with cA2 administration may be due to interruption of the cytokine cascade via an altered recruitment and trafficking of blood cells to the joint.

Recombinant TNF was administered to patients with metastatic cancer in a phase I trial (Blick et al., 1987). The patients who completed the 4 weeks of treatment became anemic and the mean hemoglobin concentration decreased although no significant decrease in granulocyte or platelet count was observed. It is important to recognize, however that hemodilution and/or sequestration can lead to a change in concentration of the peripheral cell count and that hemoglobin concentration can change independent of red cell count.

In a chemotherapy model, Winton et al., 1994 observed a significantly greater decrease in hemoglobin concentration in recombinant human (rh) IL-6-treated rhesus macaques than in controls. rhIL-6-related anemia has been observed in hematologically unperturbed cynomolgus (Asano et al., 1990) and rhesus monkeys (Srinivasiah et al., 1992) and in humans (Weber et al., 1993). In a human study in which rIL-6 was administered daily, Ulrich et al. (1991) found a transient early reticulocytosis and erythroid hyperplasia of the bone marrow, however, the net result of treatment with rhIL-6 was anemia. Wendling et al., 1993 found an increase in hemoglobin concentration in RA patients treated with anti-IL-6 monoclonal antibodies. Interestingly, the serum concentration of IL-6 increased after treatment.

Mechanisms other than suppression of erythropoiesis in response to cytokine treatment

Winton et al., 1994 made several observations which led them to the conclusion that anemia was secondary to erythrocyte sequestration and/or hemodilution. The pattern of reticulocyte recovery was similar in rhIL-6-treated animals and controls, thus it is unlikely that suppressed erythrocyte production accounted for the reduced hemoglobin concentration in rhIL-6-treated animals. There was no evidence of hemolysis. A greater decrease in hemoglobin concentration in rhIL-6-treated animals increased to the control level within

two days of discontinuing treatment. Thus, a mechanism which would be fast acting is indicated to account for the rapidity of onset and resolution of the rhIL-6-induced anemia. In these latter experiments the body weight of all animals decreased throughout the study period. Thus, if there was an increase in plasma volume, it was likely due to a fluid shift from either the intracellular or interstitial space and not the result of fluid expansion. Splenic and/or liver sequestration of red cells during rhIL-6 treatment could account for the reduced hemoglobin count in rhIL-6-treated animals and humans and was investigated in RA patients in the present study.

Erythropoietin response to anemia in RA

Erythropoietin (Epo) is the hormone primarily responsible for the regulation of erythropoiesis (Krantz and Jacobson, 1970). The supply of Epo to erythroid precursors might be the limiting factor in the impaired marrow response in ACD (Cartwright and Lee, 1971). Furthermore, Epo may directly regulate the transferrin receptor concentration per cell (Sawyer and Krantz, 1988) and thus play a role in iron uptake by the erythroblast. The role of erythropoietin in the pathogenesis of anemia in RA is controversial. Some investigators have demonstrated the Epo concentration in RA patients to be inappropriately low for the degree of anemia encountered (Baer et al., 1987; Hochberg et al., 1988, Boyd and Lappin, 1991; Noe et al., 1995) while others have found a normal Epo response (Birgegard et al., 1987; Erslev et al., 1987; Nielsen et al., 1990). In a longitudinal study, Graudal and colleagues (1992) found a consistently elevated concentration of Epo throughout a 10-year period in patients with RA who were anemic compared with RA patients with a normal hemoglobin concentration. Vreugdenhil and colleagues (1991) found an increased Epo concentration in non-anemic RA patients and speculated that their increased Epo may concurrently prevent anemia developing. The hypothesized role of a

deficiency in Epo response in ACD has led to the use of recombinant human Epo (rhEpo) in clinical trials. Most RA patients treated with a pharmacologic concentration of rhEpo demonstrate an increase in hematocrit and hemoglobin concentration (Means et al., 1989b; Pincus et al., 1990; Gudbjornsson et al., 1992; Vreugdenhil et al., 1992a) and inhibition of erythroid colony-forming units by IFN- γ *in vitro* can be corrected by rhEPO (Means and Krantz, 1991), which argues for the use of rhEpo in the treatment of ACD.

Cytokines and Epo response in RA

Disease activity may modulate Epo production. Noe et al. (1995) and Vreugdenhil et al. (1990a) found that the Epo response to anemia was higher in an iron-deficient subgroup than in the iron-replete anemic RA patient. However, disease activity was lower in the iron deficient group. *In vitro* studies show that cytokines produced during an inflammatory response (eg. IL-1, IL-6, TNF- α) influence the production of Epo (Fandrey & Jelkman, 1991, Faquin et al., 1992). Thus in the present study several measures of disease activity were made serially throughout a period of change in disease activity to determine the role of either the modulating effect of erythropoiesis or Epo production as peripheral red cell and hemoglobin count improved.

Since rhEpo treatment can correct ACD in RA patients, impaired erythropoiesis may be the most important contributor to the observed anemia. However, while a decrease in the incremental response of Epo to anemia may contribute to the reduced erythropoiesis in ACD, the Epo concentration is still higher than in the non-anemic subject. It is unclear why the bone marrow fails to respond to the higher Epo level and why the dose of rhEpo required to achieve a meaningful increase in hematocrit varies considerably from patient to patient. Since it has been shown that treatment with rhEpo results in an increased

expression of the transferrin receptor that facilitates iron uptake by erythroblasts (Sawyer and Krantz, 1988), a large Epo concentration may overcome a blockade of iron-uptake acting in the unperturbed condition via a mechanism unrelated to Epo concentration.

Hypervolemia in the anemia of RA

In a recent study, Nieken et al., 1995 found that an increase in plasma volume (hypervolemia) could completely account for the rapid development of anemia in cancer patients treated with rhIL-6. Robinson (1943) used phenol-tetra-brom-phthalein-sodium sulphate dye and reported an increase in blood volume of arthritic patients compared with normal subjects. Dixon et al., 1955 concluded that the chief cause of the anemia in the arthritic patient in their study was a group mean increase (20.4 per cent above control level) in the plasma volume. Red cell mass averaged a non-significant 7.1 per cent below the control level. Dixon et al., 1955 also observed a reduction in hemoglobin and erythrocyte concentration in the peripheral blood and an increase in plasma volume of 14.9% 24 hours after withdrawal of a cortisone treatment, that had been used to manage rheumatoid spondylitis in a single patient. In the present study, plasma volume was measured at the commencement of the study, when the RA subject was in an active disease state, in order to determine its possible involvement in their anemia.

Splenomegaly and anemia in RA

Splenomegaly is frequently associated with an increased plasma volume as well as increased splenic red cell pool and both factors can contribute to peripheral anemia. There have been several reports of RA patients with elevated splenic activity (Zhang and Lewis, 1987; Rutland, 1992) supporting the theoretical possibility of splenic sequestration during

active disease in the anemia of the RA patient. In the present study, ^{51}Cr -RBC content of spleen and liver was serially measured throughout a period of change in disease activity to determine if release of sequestered cells contributes to any improvement in the peripheral red cell count observed.

Summary of study aims

The aim of this study was to use a pre-, post- anti-rheumatic drug treatment design to define the pathophysiology of anemia in Rheumatoid Arthritis. A role is proposed for the spleen, which may enlarge or sequester red cells during an inflammatory response, and then release the sequestered red cells as inflammation subsides. In addition, a role is proposed for continuous presence of circulating inflammatory cytokines, which may alter the functioning of the erythron.

Methods

Subjects

Ten subjects, 8 females and 2 males, who were 27-77 years of age (mean 52.1 SD 16.1) and who fulfilled the American Rheumatism Association criteria for rheumatoid arthritis (RA) (Arnett et al., 1988) were recruited for the study. A subject was a candidate for the study if there was evidence of active RA-associated disease and if she/he was considered a candidate for methotrexate treatment. Subjects with active disease met at least 3 out of 4 of the following criteria: >3 swollen joints, >6 painful joints, >45 minutes of morning stiffness and a Westergren erythrocyte sedimentation rate (ESR) >28 mm/h. Subjects were classified as anemic if hemoglobin concentration was less than 115 g/L (female) or 135 g/L

(male). Demographic data are shown in Table 4.1. Control subjects were recruited from the staff of the Lions Gate Hospital.

Ethical Considerations

The study was approved by the Committee for Research in Human Subjects of the Lions Gate Hospital. Informed consent was obtained from all participants after the nature of the investigation had been explained to them.

Study Design

Subjects were asked to report to the Nuclear Medicine Department of the Lions Gate Hospital on a Tuesday for the red cell label procedure. The following day and then again on Friday subjects returned for a blood test and organ counts, described below. Following the testing procedures on a Friday, subjects began methotrexate treatment; a single dose of 7.5 mg taken once per week. The two pre-drug measures served to test for day-to-day variation within a subject while in an active disease state and also for possible effect of the label procedure itself. After methotrexate treatment began, a subject reported back for four weekly retests of blood and organ counts. Appointments were generally scheduled at the same time each Thursday between 1 and 4 pm, to control for diurnal variation. In the first two subjects of the study, more frequent testing showed no significant variation in laboratory values (excluding cytokines and erythropoietin concentration) between days three and six post-medication. Thus, six days post-medication was chosen, based on the results of Segal et al., 1989, who measured serum C-reactive protein daily for a week after a single injection of methotrexate and found maximum decrease from baseline to minimum value between day 5 and 7 in 8 out of 9 subjects.

Radiolabelling of erythrocytes

The procedure followed is described in detail in the general methods section (p 19).

⁵¹Cr-RBC Organ Counts

An external count of the gamma activity of the liver and spleen will determine their accumulation of ⁵¹Cr-labeled red cells (⁵¹Cr-RBC) from which the change in distribution of red cells in the organs during an elapsed time may be detected (Loevinger and Berman, 1968). ⁵¹Cr-RBC organ counts were measured twice (two days apart) prior to commencement of methotrexate treatment and weekly thereafter. Details of the experimental set-up are described in the general methods section (pp 20-23).

Determination of blood volume

The volume of red cells (RCV) was measured on the day of the red cell label described above. Whole blood volume (TBV) and plasma volume (PV) were calculated using RCV and hematocrit (Hct) as described in the general methods section, p 19.

Blood Samples

A blood sample was collected from the antecubital vein of a seated subject. In order to minimize the effect of diurnal variation, a sample was normally collected between 1 pm and 4 pm. Blood for immunoassays was drawn into a tube containing EDTA, was centrifuged, aliquoted and moved into a freezer within 30 minutes of collection to avoid both *ex vivo* secretion and degradation of cytokine within the sample. Analysis of iron status,

hematology, basic chemistry and enzymes were performed using standard methods in a professional laboratory at the Lions Gate Hospital, North Vancouver, Canada. EDTA-plasma samples were collected and frozen (-70°C) for batch analysis of IL-6, IL-1 β , IFN- γ and Erythropoietin (Epo) using commercially available immunoassay kits (Quantikine) from R&D Systems (Illinois). The Quantikine kits are solid phase immunoassay kits which are based on the double-antibody sandwich method. The details of this method are included in the general methods section (pp 23-25). Epo, IL-6 and IFN- γ values were determined within 6 months and IL-1 β was determined within 2 years following collection of the samples. Interassay comparisons were not performed although when samples were tested at a later date, all gave results within 10% of the initial measurement, except for the IL-6 values of two RA subjects. A second and third repeat assay of two plasma samples for each RA subject returned a value on average 50% higher than the value obtained in the first ELISA. As most laboratories report that IL-6 degrades rapidly, even at -70° C; the best interpretation of the above data is that the values obtained from the first ELISA for IL-6 were erroneously low.

Analysis of data

Laboratory data and spleen-to-liver ratios were analysed by analysis of variance for repeated measures. Pairwise comparison of each variable (using the Bonferoni adjustment) revealed which measurement showed a significant difference from the pre-methotrexate value. Serial measurement of ⁵¹Cr-RBC content in the heart, liver and spleen was analysed by a repeated measures analysis of variance to test for any difference between the pattern of change in organ counts between the anemic and non-anemic RA subject subgroups and the control group. A Student's t-test was used to test for the difference in blood volume and

selected laboratory measures between respective anemic and non-anemic sub-groups of RA subjects and control subjects. A level of significance of $p \leq 0.05$ was used to determine the significance of a variable's different value between two measures. Unless otherwise stated, each comparison was made with the first pre-methotrexate measurement, which was termed "baseline".

Results

Characteristics of subjects at study entry

Tables 4.1 and 4.2 show demographic data of the rheumatoid arthritis subjects at the time of their study entry. The number of subjects is small but equally distributed between anemic and non-anemic subgroups with a male subject in each group. The group mean volume of red cells (RCV), measured at the time of the red cell label with ^{51}Cr , was significantly lower in the anemic RA subgroup than in the non-anemic RA subgroup. Mean hemoglobin (Hb) and serum iron concentration was lower and mean plasma IL-6 concentration was higher in the anemic than non-anemic subgroup of RA subjects. Mean serum haptoglobin concentration was higher in the anemic than non-anemic subgroup. Mean serum ferritin concentration was less than $60 \mu\text{g/L}$ and the mean reticulocyte count was similar, respectively, in both RA subgroups.

Correspondence between log Epo and Hb concentration in RA subjects and controls

When the log Epo concentration versus hemoglobin (Hb) concentration from anemic and non-anemic RA subjects and controls are compared several patterns emerge. Figure 4.1 shows that the mean Epo concentration of the anemic RA subgroup was higher than the

non-anemic subgroup and control value respectively and the mean Epo concentration of non-anemic subjects is higher than the control value. There was no difference in the mean Hb concentration between the control and non-anemic RA subgroup.

Table 4.1 Characteristics of Rheumatoid Arthritis subjects.

| | | |
|-------------------------------|---------------------------------|-----------|
| Total number | | 10 |
| Female: male | | 8:2 |
| Age (yr) mean±SD | | 52.1±16.1 |
| Disease duration (yr) mean±SD | | 9.5±7.7 |
| Rheumatoid factor positive | | 6 |
| <u>Concurrent drugs:</u> | prednisone or its equivalent | 2 |
| | Non-steroidal anti-inflammatory | 8 |
| | salazopyrine | 1 |
| | cardizem & lozide | 1 |
| <u>Concurrent diseases:</u> | hypertension | 1 |
| | pacemaker | 1 |
| | lupus (suspected) | 4 |
| | diabetes (type I) | 1 |
| | diabetes (type II) | 1 |
| | psoriasis (suspected) | 3 |

Table 4.2 Pre-methotrexate blood volume data and selected laboratory measures.

| RA Subject | Sex | Wt (kg) | Ht (cm) | TBV (ml/kg) | RCV (ml/kg) | PV (ml/kg) | Hb (g/L) | Retic count x 10 ⁹ /L | IL-6 (pg/ml) | Serum Iron (µM) | Ferritin (µg/L) | Haptoglobin (g/L) |
|-------------------|-----|-----------------|---------|-------------|-------------|------------|----------|----------------------------------|--------------|-----------------|-----------------|-------------------|
| anemic | | | | | | | | | | | | |
| DG | M | 79.0 | 178 | 75.2 | 23.4 | 51.8 | 113.5 | 189 | 28 | 2 | 419 | 8.51 |
| LT | F | 52.0 | 159 | 49.5 | 16.6 | 32.8 | 98 | 130 | 11 | 3 | 5 | 2.62 |
| MM | F | 63.8 | 160 | 62.6 | 19.2 | 43.4 | 97 | 46# | 21 | 6 | 38 | 6.23 |
| BC | F | 65.4 | 160 | 57.1 | 15.6 | 41.5 | 105 | 73# | 51 | 1 | 127 | 6.77 |
| EU | F | 78.0 | 168 | 54.7 | 18.2 | 36.6 | 114.5 | 43# | 20 | 4 | 22 | 4.47 |
| | | F average (n=4) | 64.8 | 161.8 | 56.0 | 17.4* | 103.6* | average (n=5) | 26.2* | 3.2* | 48.0 | 5.72* |
| | | ±SD | 10.6 | 4.2 | 5.4 | 1.6 | 8.1 | ±SD | 15.1 | 1.9 | 54.4 | 2.25 |
| non-anemic | | | | | | | | | | | | |
| LB | M | 73.0 | 172 | 70.4 | 27.7 | 42.7 | 147.5 | 195 | 29 | 8 | 28 | 4.86 |
| BM | F | 56.6 | 165 | 60.9 | 20.6 | 40.2 | 126.5 | 30 | 11 | 15 | 34 | 3.18 |
| AC | F | 80.0 | 165.5 | 57.1 | 21.6 | 35.5 | 133 | 148 | 6 | 14 | 106 | 4.39 |
| IS | F | 78.4 | 156 | 61.7 | 23.3 | 38.4 | 143 | 357 | 6 | 10 | 26 | 1.92 |
| EM | F | 70.4 | 160 | 47 | 18.7 | 28.3 | 147.5 | 42 | 2 | 15 | 28 | 3.10 |
| | | F average (n=4) | 71.4 | 161.6 | 56.7 | 21.1 | 137.5 | average (n=5) | 10.8 | 12.4 | 48.5 | 3.49 |
| | | SD= | 10.7 | 4.5 | 6.8 | 1.9 | 9.5 | SD= | 10.7 | 3.2 | 38.5 | 1.16 |

* significantly different (p<0.05) from non-anemic RA group mean value.

reticulocyte count inappropriately low for the degree of anemia (Berliner and Duffy, 1991).

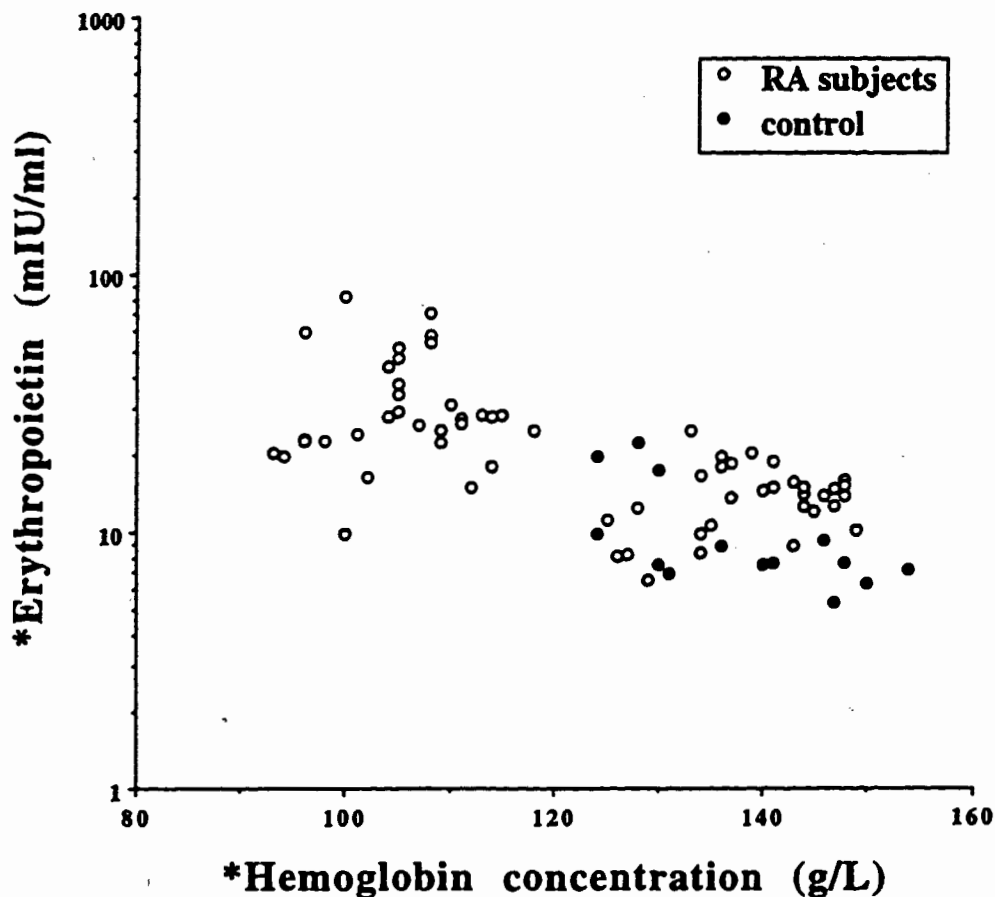


Figure 4.1 Plasma erythropoietin concentration versus hemoglobin concentration: a comparison between anemic and non-anemic RA subjects and non-anemic controls. All data for each subject is reported as a negative correlation between hemoglobin concentration and Epo concentration was maintained during methotrexate treatment.

| | | |
|--|----------------------|----------------------|
| * Erythropoietin concentration (mIU/ml): | control mean | 10.2 |
| | RA (non-anemic) mean | 13.6 ^① |
| | RA (anemic) mean | 34.0 ^{① ②} |
| Hemoglobin concentration (g/L): | control mean | 137.8 |
| | RA (non-anemic) mean | 138.9 |
| | RA (anemic) mean | 105.4 ^{① ②} |

① significantly different ($p \leq 0.05$) from control.

② significant difference ($p \leq 0.05$) between anemic and non-anemic RA subject groups.

Effect of methotrexate treatment on laboratory measures

Table 4.3 shows a summary of the change from a pre-methotrexate measure in various laboratory variables in anemic and non-anemic subgroups during four weeks of methotrexate treatment and laboratory data are presented graphically in Figures B1-B17. Data of subject DG (anemic) were skewed for certain variables, possibly due to a concurrent viral illness, thus group means are compared with and without these data. There was a trend ($p \leq 0.2$) towards an increase from day one to day three (the second pre-drug measure) in plasma IL-6 concentration (17.5-27.0 pg/ml) in the pooled data of anemic and non-anemic subgroups (subject DG omitted). Pairwise comparison of group means from before (day 1, unless otherwise indicated) and during methotrexate treatment were significantly different for the following groups and variables:

White blood cell concentration (Figure B1)

for the non-anemic subgroup: a reduction on day 23 ($8.2-7.14 \times 10^9/L$)

for the anemic subgroup: a reduction on days 16 and 23 ($9.0-7.1; 7.06 \times 10^9/L$)

Hemoglobin concentration (Figure B2)

for the non-anemic subgroup: a reduction on day 9 (141-136 g/L).

Red blood cell concentration (Figure B3)

for the non-anemic subgroup: a reduction on day 9 ($4.62-4.45 \times 10^{12}/L$)

Hematocrit (Figure B4)

for the non-anemic subgroup: a reduction on day 9 (41.3-40.1%)

Mean corpuscular volume (erythrocyte) (Figure B5)

for the non-anemic subgroup: an increase on days 9, 16, 23 and 30 (89.6-90.3, 90.5, 90.4, 90.66 fL)

Serum iron concentration (Figure B6)

for the non-anemic subgroup: a reduction on day 9 (13.0-9.0 $\mu\text{mol/L}$)

Percentage saturation of transferrin (Figure B8)

for the non-anemic subgroup: a reduction on day 9 (23-15%)

Serum ferritin concentration (Figure B9)

for the non-anemic subgroup: a reduction on days 23 and 30 (44-34; 34 $\mu\text{g/L}$)

for the anemic subgroup with subject DG omitted: a reduction on day 23 (48-36 $\mu\text{g/L}$)

Reticulocyte count (Figure B10)

for the anemic subgroup with subject DG omitted: an increase on day 23 (73-160 $\times 10^9/\text{L}$)

Plasma erythropoietin concentration (Figure B11)

for the anemic subgroup: a reduction on days 23 and 30 (42-31; 26 mIU/ml)

Serum-haptoglobin concentration (Figure B12)

for the non-anemic subgroup: a reduction on days 23 and 30 (3.49-2.40; 2.69 g/L)

for the anemic subgroup with subject DG omitted: a reduction on day 30 (5.02-4.33 g/L)

Erythrocyte sedimentation rate (Figure B13)

for the anemic subgroup (no data for subject DG): a reduction on day 30 (73-62 mm/hr)

Plasma IL-1 β concentration (Figure B14)

for the non-anemic subgroup: a reduction on day 30 from day 3(20-9.5 pg/ml)

Plasma IFN- γ and platelet concentration (Figures B16 and B17)

There were insufficient plasma IFN- γ data (2 non-anemic and 4 anemic RA subjects) to make a group comparison (Figure B16). There was no significant change in the mean platelet count throughout the methotrexate trial (Figure B17). Group mean platelet count was higher in the anemic than the non-anemic subgroup prior to methotrexate treatment and this difference was maintained throughout the study. Table 4.3 summarizes the above pairwise comparison data.

Effect of 4 weeks of methotrexate treatment on ^{51}Cr -RBC organ counts

Figure 4.2 shows the pattern (group mean) of ^{51}Cr -RBC counts in anemic and non-anemic RA subjects over the heart, liver and spleen respectively, compared with the control value. Data for subject DG are not included with those of the other anemic subjects or the whole group. The former subject presented with flu-like symptoms which resolved within the four week period, whereas other aspects related to the Rheumatoid Arthritis did not improve. Thus, a viral infection present in in this subject at the beginning of the study may account for the pattern of ^{51}Cr -RBC spleen count which deviated significantly from that of the remainder of the anemic RA subjects. Data for this subject is presented in Figure B19 and individual data for each RA subject is presented in Figures B18 to B27. A 2-way analysis of variance revealed no significant difference in serial pattern of any organ count of the anemic and non-anemic RA subgroups compared with the relevant control value (data not shown).

Table 4.3 Change in selected laboratory measures during methotrexate treatment.

| | <u>Day</u> | 3 | 9 | 16 | 23 | 30 |
|-------|------------|--------|-----|----|-------------|-------------|
| WBC | | | | ↓A | ↓A↓NA | |
| IL-1β | | | | | | ①↓NA |
| IL-6 | | ②p≤0.2 | | | | |
| ESR | | | | | | ↓A |
| SI | | | ↓NA | | | |
| %sat | | | ↓NA | | | |
| Fer | | | | | ↓NA, A(-DG) | ↓NA |
| Hap | | | | | ↓NA | ↓NA, A(-DG) |
| Hb | | | ↓NA | | | |
| Hct | | | ↓NA | | | |
| RBC | | | ↓NA | | | |
| Epo | | | | | ↓A | ↓A |
| Retic | | | | | ↑A(-DG) | |

↑, ↓ denote a significant increase or decrease, respectively from the day 1 value.

①↓ denotes a significant decrease from the day 3 value.

② pooled data from anemic and non-anemic subgroups (subject DG deleted).

A-anemic RA subgroup; -DG-subject DG omitted; NA-non-anemic RA subgroup; WBC-white blood cell concentration; IL-1β-interleukin-1β concentration; IL-6-plasma interleukin-6 concentration; ESR-erythrocyte sedimentation rate; SI-serum iron concentration; %sat-percentage saturation of transferrin; Fer-serum ferritin concentration; Hap-serum haptoglobin concentration; Hb-hemoglobin concentration; Hct-hematocrit; RBC-red blood cell concentration; Epo-plasma erythropoietin concentration; Retic-reticulocyte count.

Effect of 4 weeks of methotrexate treatment on spleen-to-liver ratio

In an analysis of variance for repeated measures, there was no difference in the pattern of change of the spleen-to-liver ratio between the anemic and non-anemic RA subject or control (data not shown). A comparison of the initial pre-methotrexate spleen-to-liver ratio for anemic and non-anemic RA subgroups revealed no difference and neither RA subgroup mean was different from the control (anemic RA group mean 1.24 ± 0.13 ; control 1.05 ± 0.22 , $p=0.09$; non-anemic RA group mean 1.30 ± 0.37 ; control 1.05 ± 0.22 , $p=0.14$). However, in the final measure taken during methotrexate treatment, the group mean spleen-to-liver ratio for anemic and non-anemic RA subjects was significantly higher than that of the control (1.75 ± 0.33 and 1.90 ± 0.5 versus 1.34 ± 0.14 , $p=0.03$ and $p=0.04$ respectively).

Legend to Figure 4.2

In the top panel, group mean data for the anemic and non-anemic subgroup is plotted with control data (\pm SD) for ^{51}Cr -RBC count from the heart; the center panel shows ^{51}Cr -RBC count from the liver and the bottom panel shows ^{51}Cr -RBC count from the spleen. ^{51}Cr -RBC organ count data are expressed as a percentage of day one count and are plotted against day of measurement. Data for day 3 corresponds with the the ^{51}Cr -RBC organ count prior to methotrexate treatment. Organ count data from subject DG is not included in the anemic group mean due to concurrent viral illness (see Figure B18).

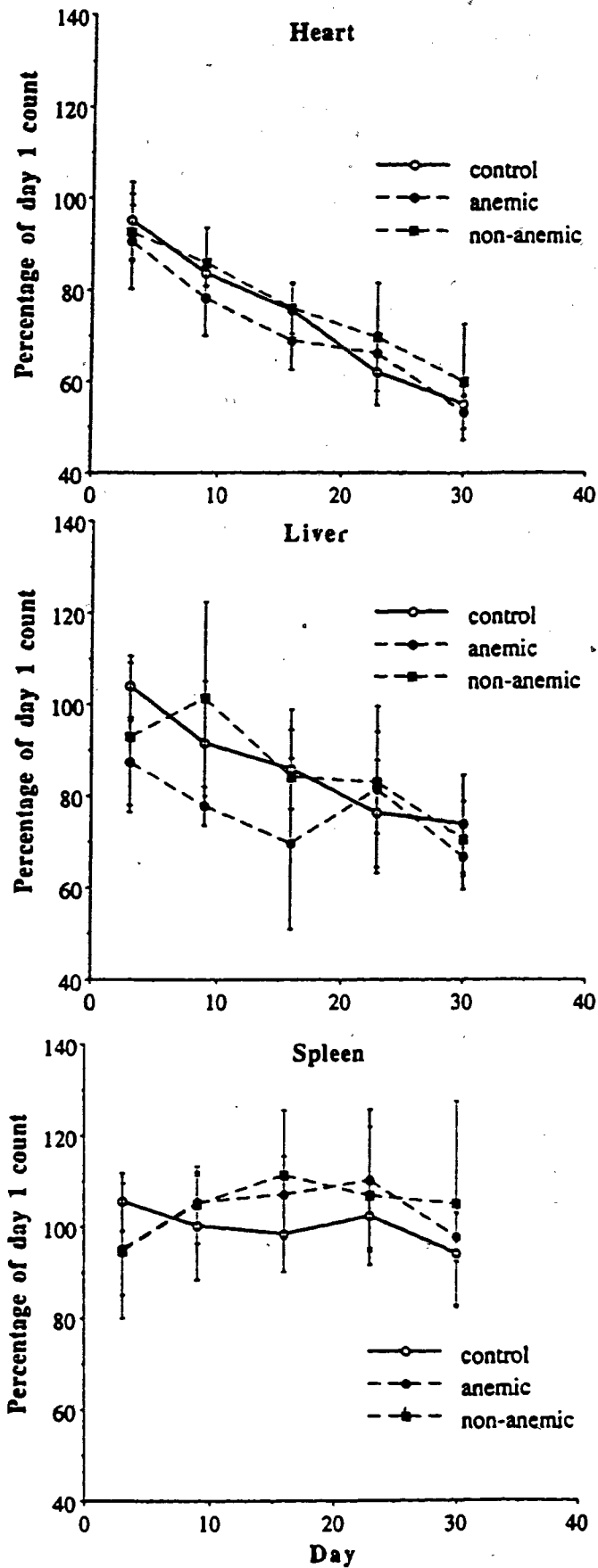


Figure 4.2 Group mean ^{51}Cr -RBC organ count for anemic and non-anemic RA subgroups compared with a control count.

Individual subject observations

Although there was no significant increase in the mean RBC concentration of the anemic subgroup after four weeks of methotrexate treatment, all four of the anemic subjects who demonstrated improvement in more than one laboratory measure showed a corresponding increase in RBC concentration after three or four weeks of methotrexate treatment (Table B1). Thus, the hypothesized correspondences were tested using "quadrant diagrams", which compared the difference between the pre- to post-methotrexate value of two variables for each subject.

Correspondence between change in ^{51}Cr -RBC organ count and change in intravascular red blood cell (RBC) concentration

A major aim of the present study was to investigate the role of sequestration in any reduced RBC concentration noted in response to inflammation. If sequestration does play a role, one would expect to observe a reduction in ^{51}Cr -RBC count from the spleen and possibly the liver as any inflammatory activity subsided as methotrexate treatment proceeded. This decrement would be accompanied by a corresponding increase in circulating RBCs.

Figures 4.3 and 4.4 show the change in ^{51}Cr -RBC count over spleen and liver with respect to change in RBC concentration in the RA subjects compared with controls. Figure 4.3, illustrates a test of the hypothesis that the release of erythrocytes sequestered in the spleen, contribute to an increased intravascular RBC concentration during methotrexate treatment.

Data points which fall in the lower-right quadrant demonstrate the above relationship.

Figure 4.3 shows that although there was a slight increase in RBC concentration from day 1 to day 30 in certain subjects, there was no corresponding decrease in ^{51}Cr -RBC count over the spleen, except in subject DG, whose RBC concentration decreased. In Figure 4.4, if the hypothesis that the release of erythrocytes sequestered in the liver contribute to an increased intravascular RBC concentration during methotrexate treatment is correct, data

points should fall in the lower half (below control points) of the lower-right quadrant.

Figure 4.4 shows that the decrease in ^{51}Cr -RBC count over the liver was within the range of the control subjects for each of the subjects with rheumatoid arthritis except for subject DG, whose RBC concentration also decreased, as mentioned previously and subject LT, whose RBC concentration increased.

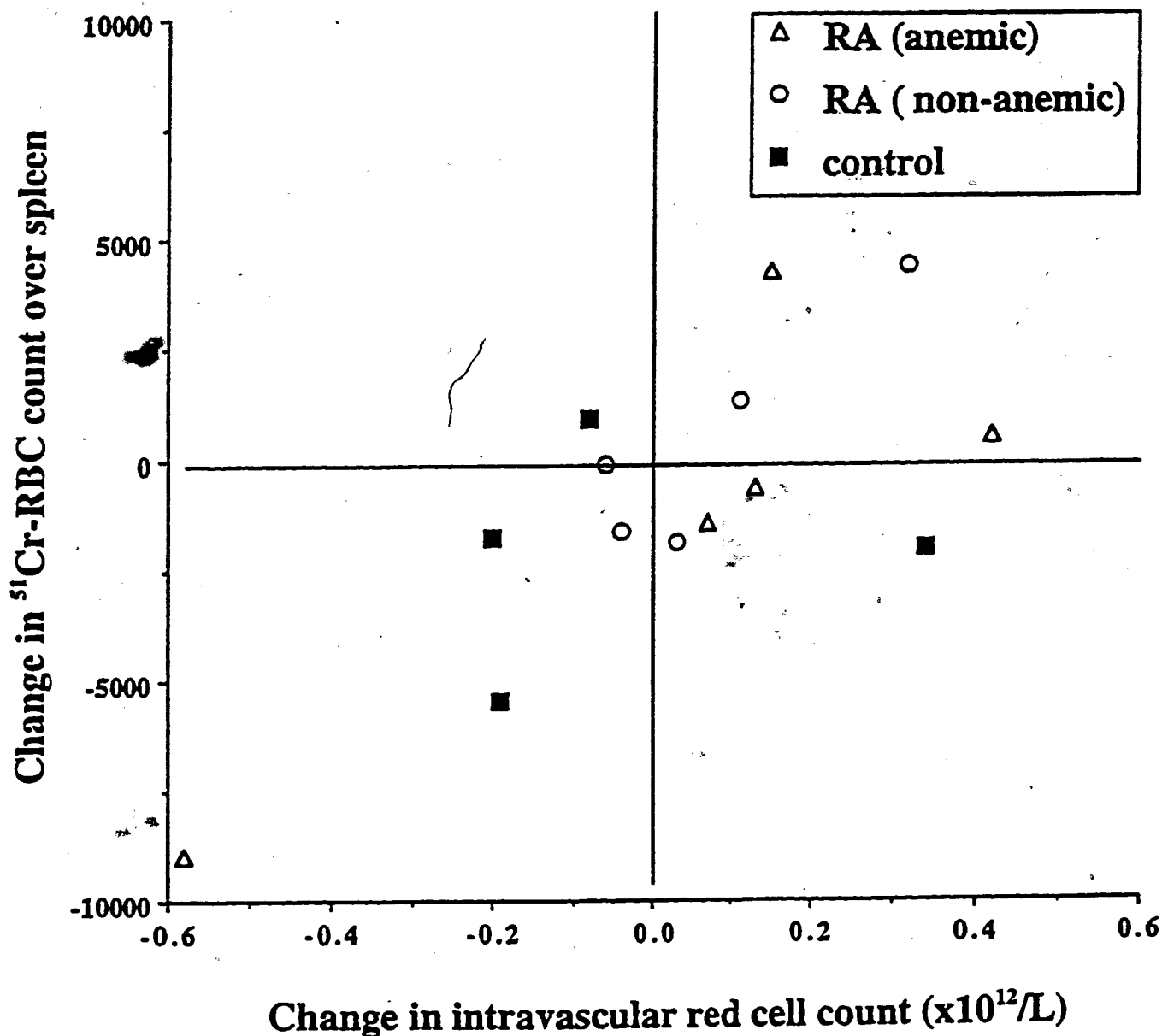


Figure 4.3 Comparison of change in ^{51}Cr -RBC count over the spleen with change in intravascular red blood cell concentration in individual anemic and non-anemic RA subjects and controls.

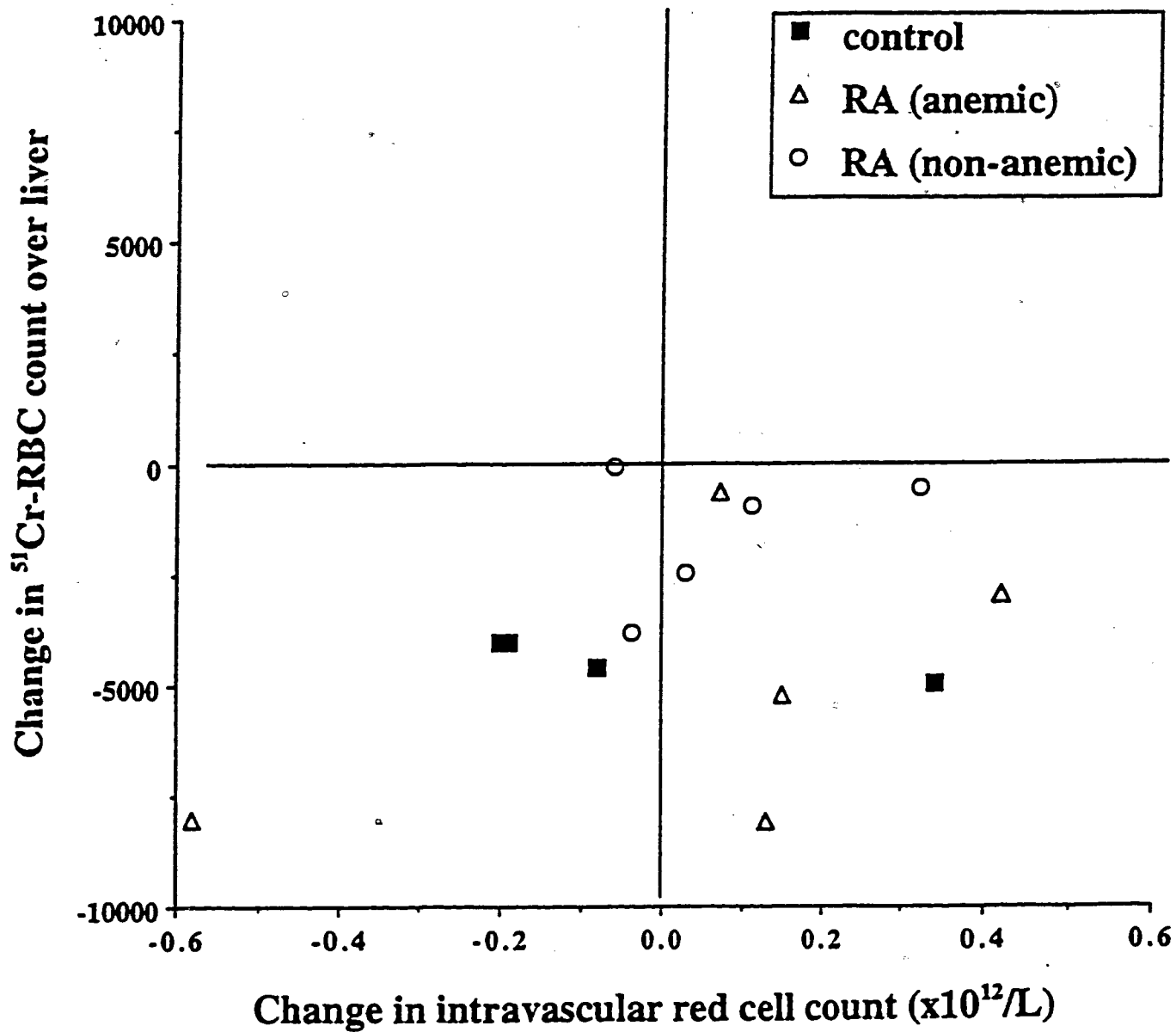


Figure 4.4 Comparison of change in ^{51}Cr -RBC count over the liver with change in intravascular red blood cell concentration in individual anemic and non-anemic RA subjects and controls.

Change in plasma concentration of cytokines compared with change in plasma Epo concentration, reticulocyte count and RBC concentration

As shown in Table B1, there was a decrease in the plasma concentration of one or more cytokines by the end of the study in all five anemic and 3 out of 5 non-anemic subjects. In Figures 4.5 to 4.7, the change in plasma concentration of cytokine is plotted against the change in plasma Epo concentration, reticulocyte count and RBC concentration respectively, in order to determine their correspondence. If the hypothesis that a reduction in plasma cytokine concentration corresponds with an increase in plasma Epo concentration, reticulocyte count, and RBC concentration, respectively is correct, data should fall in the upper-left quadrant of each diagram in Figures 4.5 to 4.7. Figure 4.5 fails to show such a pattern, as Plasma Epo concentration decreased from day 1 to day 30 in 7 out of 10 RA subjects, while plasma concentration of IL-6 and IL-1 β , respectively, decreased in 8 out of 10 RA subjects and plasma concentration of IFN- γ decreased in 4 out of 5 RA subjects. In Figure 4.6, several data points fall within the upper-left quadrant of each diagram, indicating that reticulocyte count did increase as cytokine concentration decreased. In all three anemic RA subjects with an increase in reticulocyte count, there was a corresponding decrease in either or both of plasma IL-1 β and IFN- γ concentration. Similarly, in Figure 4.7, an increase in peripheral RBC concentration corresponded with a decrease in either or both of plasma IL-1 β and IFN- γ concentration in 3 out of 4 anemic RA subjects.

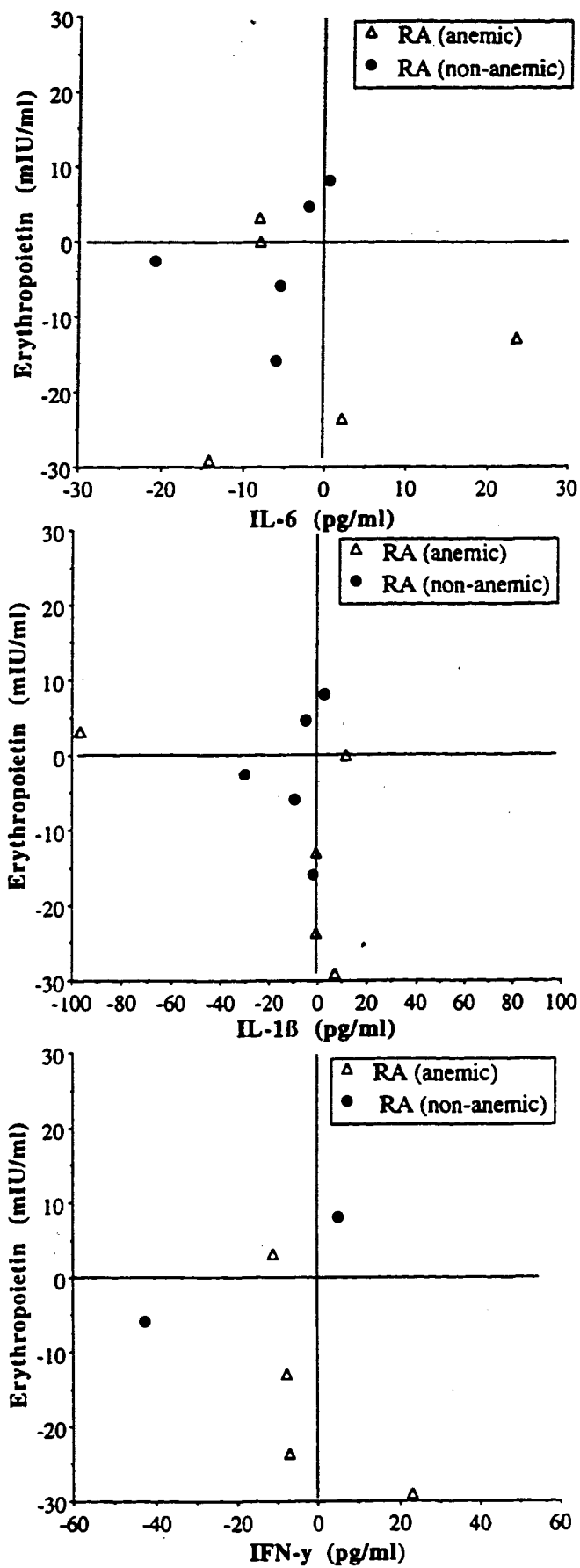


Figure 4.5 Change in plasma erythropoietin concentration with change in plasma cytokine concentration in individual subjects during four weeks of methotrexate treatment.

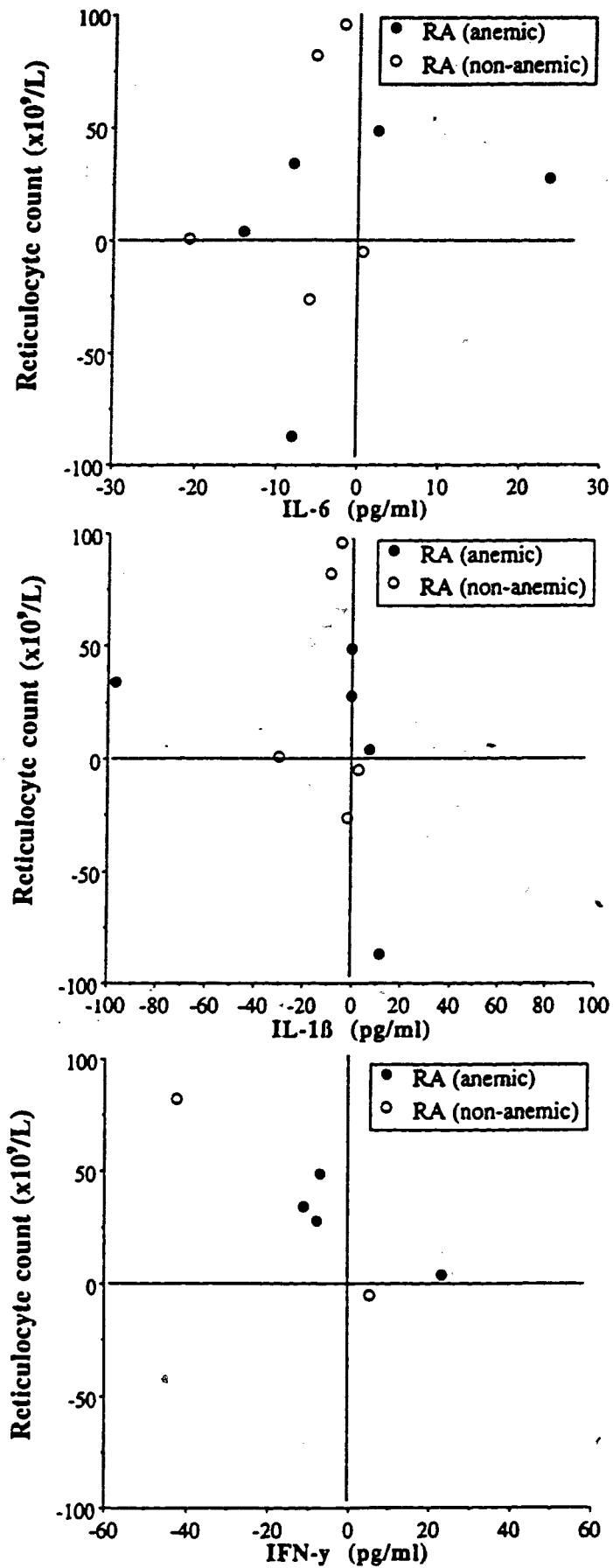


Figure 4.6 Change in reticulocyte count with change in plasma cytokine concentration in individual subjects during four weeks of methotrexate treatment.

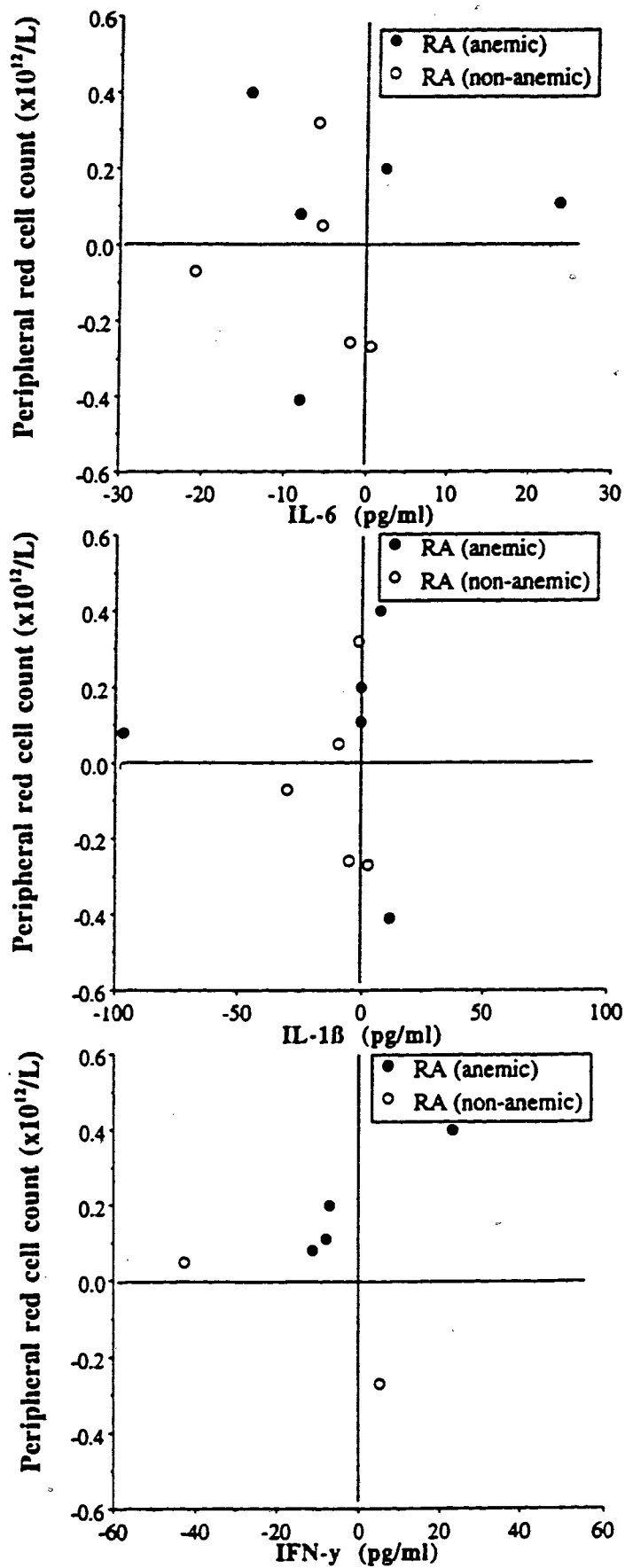


Figure 4.7 Change in intravascular red blood cell concentration with change in plasma cytokine concentration in individual subjects during four weeks of methotrexate treatment.

Change in RBC and Hb concentration compared with the change in Epo concentration and reticulocyte count

Figures 4.8 and 4.9 test for a correspondence between each individual's change in RBC concentration and change in plasma Epo concentration and reticulocyte count between day 1 (pre-methotrexate) and week three or four (during methotrexate treatment). Figure 4.8 shows that plasma Epo concentration decreased in an RA subject with an increase in RBC concentration and increased in an RA subject with a decrease in RBC concentration, except in one subject (DG). Figure 4.9 shows that of four anemic RA subjects with an increase in RBC concentration, reticulocyte count increased in three and remained constant in the fourth.

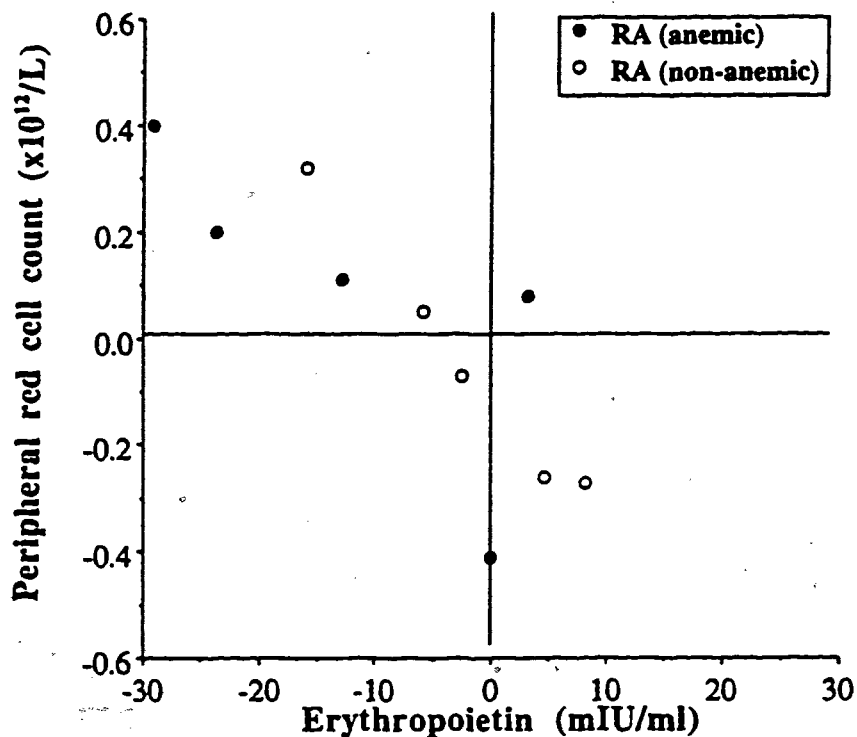


Figure 4.8 Change in intravascular red blood cell concentration compared with change in plasma erythropoietin concentration in individual subjects during four weeks of methotrexate treatment.

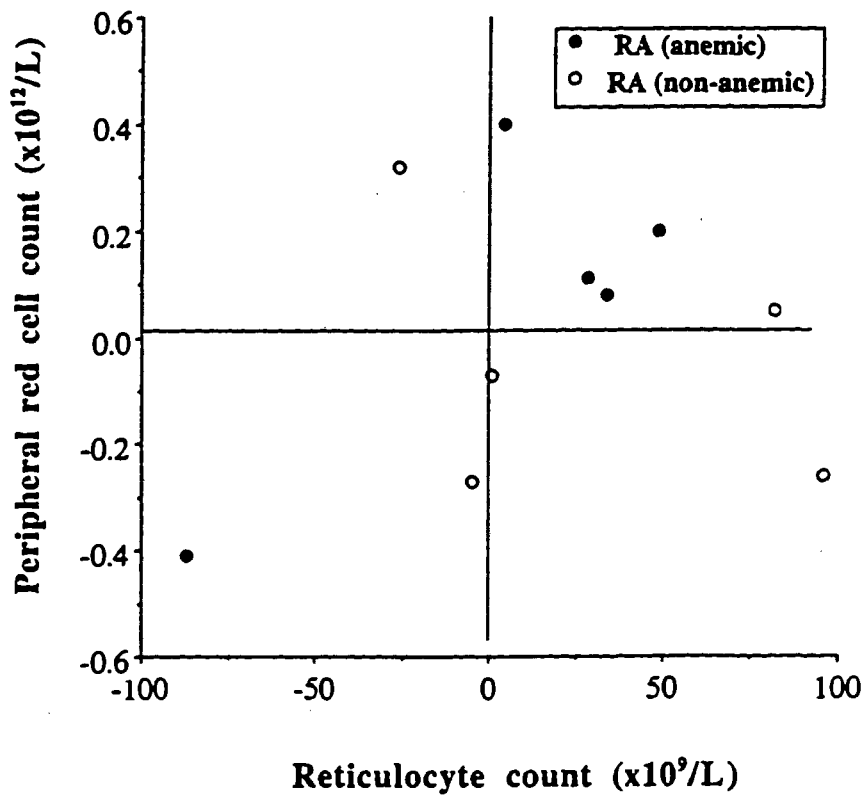


Figure 4.9 Change in intravascular red blood cell concentration compared with change in reticulocyte count in individual subjects during four weeks of methotrexate treatment.

Discussion

Contributing pathology to anemia in RA subjects at study entry

Disease activity

As has been previously reported (Cartwright and Lee, 1971; Birgegard et al., 1987; Vreugdenhil et al., 1991), disease activity, based on serum haptoglobin and plasma IL-6 concentration, was on average higher in the anemic than non-anemic RA subjects in the present study.

Erythropoiesis

The reticulocyte count gives an indication of the rate of erythropoiesis. If the erythron is responding normally, the rate of erythropoiesis should be higher in the anemic than in the non-anemic subject. However, in the present study there was no difference between the anemic and non-anemic subgroup in mean reticulocyte count. The reticulocyte count was inappropriately low for the degree of anemia in three of five RA subjects (Table 4.2), indicating that suppression of erythropoiesis may have contributed to their anemia at the commencement of the study.

Availability of iron to the developing erythron

The mean serum iron concentration in the anemic subgroup was lower than in the non-anemic subgroup. Furthermore, a recent report suggests that inflammatory cytokines may mediate a suppression of erythropoiesis by interfering with transferrin iron uptake via the

action of an acute phase protein alpha1-antitrypsin (A1A). Graziadei et al., 1994 found that A1A inhibited the growth and proliferation of human early erythroid progenitor cells (BFU) and human erythroleukemic cells *in vitro*. A concentration of A1A, found in the presence of anemia of chronic disease, completely prevented binding of transferrin to its receptor on BFUs, K526 cells and reticulocytes, while the cytokines IL-1, IL-6 and TNF- α showed no such effect. Although A1A was not measured in the present study, it is reasonable to assume that its concentration was at a level comparable to the haptoglobin concentration. As mentioned above, the group mean haptoglobin concentration (and presumably A1A) in non-anemic RA subjects was lower than that of the anemic RA subject group at the beginning of the study, which may contribute to their difference in hematologic status.

Erythropoietin (Epo)

The mean Epo concentration was higher in the anemic RA subgroup than in the non-anemic RA subgroup and control, but it is uncertain whether it was elevated to a degree appropriate to the level of anemia described, since there was no anemic control group in the study. Mean baseline Epo concentration for the non-anemic subgroup was higher than the control value. Although the number of subjects in the present study is small, this result corroborates that of Vreugdenhil and colleagues (1991), who found an increased Epo concentration in a non-anemic RA subgroup and speculated that the increased Epo was preventing the development of anemia.

Sequestration of RBCs

An increased spleen-to-liver ratio normally indicates sequestration of red blood cells in the spleen. The trend of an increased spleen-to-liver ratio noted in RA subjects at their study

entry persisted into the final week of ^{51}Cr -RBC organ counting and was significant at that time, confirming an observation of elevated splenic size in RA subjects by Zhang and Lewis, 1987 and Rutland, 1992. However, the increased spleen-to-liver ratio was observed in both the anemic and the non-anemic RA subject, thus splenic sequestration was not a determining factor in the anemia of subjects in the present study.

The response to the red cell label procedure

The pre-methotrexate measures on days 1 and 3 were planned as a control for normal intraindividual variation and were to be pooled and compared with data collected during the methotrexate trial. However, the trend of an increase in plasma IL-6 concentration from day 1 to day 3 suggests that the red cell label procedure itself had an effect. Therefore, these data are treated separately; the day 1 value representing the condition of the RA subject experiencing active disease and the day 3 value representing an effect of the red cell label superimposed upon the RA condition.

Contributors to variation in RBC concentration during methotrexate treatment

Variation in inflammatory activity

The mechanism of action of a low dose of methotrexate in RA is poorly understood, although the medication is thought to act mainly as an anti-inflammatory agent (Segal et al., 1990). On day 9, following the first dose of methotrexate, serum iron concentration, saturation of transferrin, hematocrit, and hemoglobin and RBC concentrations were decreased in the non-anemic RA subgroup, indicating a possible superimposition of an acute phase response on the existing disease state in this subgroup. Evidence for a subsequent reduction in inflammatory activity includes a reduction from the day 3 value in

the group mean IL-1 β concentration of non-anemic subjects , a reduction from the day 1 value in the mean erythrocyte sedimentation rate in the anemic RA subgroup and a reduction in the mean ferritin and haptoglobin concentration, respectively in both RA subgroups during methotrexate treatment.

Reduced disease activity and RBC concentration

Although there was no significant increase in the mean RBC concentration of the anemic subgroup after four weeks of methotrexate treatment, all four of the anemic subjects who demonstrated improvement in more than one laboratory measure showed a corresponding increase in RBC concentration after three or four weeks of methotrexate treatment (Table B1). Thus it was possible to evaluate the rôle of sequestration, erythropoiesis, and ferrokinetics in the change observed in RBC concentration with reduced inflammatory activity in individual subjects.

Sequestration of RBCs

The hypothesized reduction in splenic ⁵¹Cr-RBC count from its baseline level was not found, except in subject DG (Figure B18), whose RBC concentration declined throughout the study period, instead of increasing as predicted. This result agrees with a recent report which demonstrated that splenic sequestration played no role in the early reduction in RBC concentration in cancer subjects treated with rhIL-6, rather an increase in plasma volume was responsible (Nieken et al., 1995).

The response of the erythron

An increase in the rate of erythropoiesis can lead to an increase in RBC concentration. The reticulocyte count had increased, concomitantly with an increase in RBC concentration after three or four weeks of methotrexate treatment, in three of four anemic RA subjects . In addition, the reticulocyte count declined as RBC decreased in the single anemic subject whose disease activity appeared to increase throughout the four weeks of methotrexate treatment, possibly due to a concurrent viral illness. These data support the correspondence between a change in the rate of erythropoiesis and a change in RBC concentration.

Mechanism of an increased rate of erythropoiesis during methotrexate treatment

Erythropoietin concentration

An increase in the concentration of Epo normally leads to an increased rate of erythropoiesis. However, as plasma Epo concentration was decreasing during methotrexate treatment, another mechanism must be responsible for the observed increase in reticulocyte count in the anemic RA subgroup.

Plasma concentration of cytokines

One hypothesis of the present study is that the continuous presence of circulating inflammatory cytokines are suppressing the response of erythroid precursors to growth signals in the anemic RA patient. In order to test the hypothesized correspondence between plasma cytokine concentration and reticulocyte count, the pattern of response was evaluated

in each subject individually. Of the three anemic RA subjects with an increase in reticulocyte count, the pattern of baseline cytokine expression and variation during four weeks of methotrexate treatment differed. However, in all three anemic RA subjects whose reticulocyte count and RBC concentration increased, the concentration of plasma IFN- γ decreased following methotrexate treatment. While controversy exists about the effect of IL-1 and IL-6 on erythropoiesis (Means and Krantz, 1992), the suppressive effect of IFN- γ on erythropoiesis is established (Mamus et al., 1985; Raefsky et al., 1985). However, in double-blind, placebo controlled studies of rhIFN- γ for treatment of RA, clinical improvement has been observed and laboratory measures, such as RBC concentration, have shown improvement (Sprekeler et al., 1990; Cannon et al., 1993). Thus, it is possible that factors related to reduction in inflammation are more important in increasing the reticulocyte count than the reduction in IFN- γ concentration.

Availability of iron to erythroid progenitor cells

A change in availability of iron to erythroid progenitor cells could account for the change in rate of erythropoiesis. While there was no significant change in any measure of iron status in anemic RA subjects following methotrexate treatment, it may be assumed that there must have been sufficient iron available to support the observed increase in reticulocyte count.

Problems encountered in the study

The small number of subjects and short period of time available for evaluation of an effect of the methotrexate treatment made it difficult to evaluate the data for a group effect and reduced the statistical power. The values obtained in the first ELISA for IL-6 may have been erroneously low, as a repeat assay two years later gave higher values. This affected

samples from subjects EM and DG. Another interpretation of this finding is that some of the IL-6 was bound to an inhibitor which interfered with its detection in the first assay and that subsequent release from the inhibitor led to a higher value in the second test. Most laboratories report that IL-6 degrades rapidly, even at -70° C, so that it is unusual to find an increase in level after two years. The limitation of the ⁵¹Cr-RBC method for organ counting is discussed in the general methods section (p 23). A problem unique to the RA subject group was that of repositioning the counter in the heavier subject, which probably explains the greater variation in count from measurement to measurement, especially for the heart and liver counts (Figures B23, B24, and B27). It was difficult to find hematologically normal subjects, that remained healthy for at least five weeks, as control subjects. Of the four subjects in the control group, one was iron-deficient, based on a low serum ferritin value (10 µg/L). This subject began oral iron treatment and his peripheral red cell concentration increased from 4.78-5.12 x10¹² cells/L after one month. In the remaining three control subjects, there was a small reduction in peripheral red cell concentration averaging 0.16 x10¹²±0.07 cells/L after one month. The data of three additional subjects who volunteered as controls, with no history of anemia or hematologic abnormality, is presented in appendix C. A change in plasma volume can contribute to an alteration in RBC count, since the latter is a concentration-dependent measure. The contribution of a change in plasma volume should be controlled for in future studies by simultaneous measurement of plasma volume and blood variables.

Conclusion

The aim of the present study was to use a pre-, post- anti-rheumatic drug treatment design to define the pathophysiology of anemia in Rheumatoid Arthritis. A role is proposed for the spleen, which may enlarge or sequester red cells during an inflammatory response, then

release the sequestered red cells as inflammation subsides. In addition, a role is proposed for the continuous presence of circulating inflammatory cytokines, which may alter the functioning of the erythron. At the commencement of the study, when each RA subject was experiencing active disease, the mean plasma IL-6 concentration was higher in the anemic than in the non-anemic RA subgroup, demonstrating the hypothesized negative correlation between cytokine activity and RBC concentration. Plasma erythropoietin (Epo) concentration was higher in the anemic subgroup, although the mean reticulocyte count of the anemic and non-anemic subgroups did not differ, suggesting that suppressed erythropoiesis was contributing to the observed anemia. There was no difference between the spleen-to-liver erythrocyte ratio, the only way of estimating sequestration at the beginning of the study in anemic and non-anemic subgroups. During methotrexate treatment, inflammatory activity declined as shown by a reduction in the mean plasma IL-1 β , serum ferritin and serum haptoglobin concentration, in the non-anemic subgroup and a reduction in the mean erythrocyte sedimentation rate and the mean serum concentration of ferritin and haptoglobin, respectively, in the anemic RA subgroup. An increase in reticulocyte count and a decrease in plasma Epo concentration corresponded to an increase in RBC concentration in three of four anemic subjects, suggesting that erythropoiesis was stimulated as disease activity declined, but that Epo played no role. There was no pattern of decreasing ⁵¹Cr-RBC spleen count, indicating that release of sequestered RBCs did not contribute to the observed improvement in RBC concentration in any of the four anemic subjects. A decrease in plasma IFN- γ concentration from its baseline value following methotrexate treatment corresponded to an increase in reticulocyte count in three of four anemic RA subjects. These data provide evidence for a reduced suppression of erythropoiesis, unrelated to an increase in Epo concentration, in the improvement of reticulocyte count and RBC concentration in the anemic RA subject treated with methotrexate.

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Chapter 5. General Conclusions

The aim of the present study was to investigate the effect of a change in level of inflammation on intravascular red blood cell (RBC) concentration. The following pattern was hypothesized to apply to a condition of continuing inflammation ("inflammation on") then a condition of reduced inflammation ("inflammation off") in both an exercise-induced inflammatory response to focal disruption of muscle and a disease-linked inflammation of synovial joints in rheumatoid arthritis:

| INFLAMMATION ON | INFLAMMATION OFF |
|--|---|
| ↑ plasma cytokine concentration | ↓ plasma cytokine concentration |
| ↓ plasma erythropoietin concentration | ↑ plasma erythropoietin concentration |
| ↓ reticulocyte count red blood cells (RBC) sequestered in the spleen | ↑ reticulocyte count sequestered RBCs released |
| ↓ intravascular RBC concentration | ↑ intravascular RBC concentration |

In the exercise study, an expected continuing inflammatory state was not elicited during training in preparation for a marathon run. Therefore, it was not possible to compare the two conditions directly. However, for up to two hours following the marathon, the plasma IL-6 concentration of a runner approached the level of plasma IL-6 continuously present in the RA subject. Thus it seemed possible to examine the effect of a transient increase in the plasma concentration of IL-6 in the exercise study. The following table summarizes the significant findings:

| INFLAMMATION ON (training and a marathon competition) | INFLAMMATION OFF (taper and post-marathon rest) |
|--|---|
| ↑ plasma cytokine concentration ✓ | ↓ plasma cytokine concentration ✓ |
| ↓ plasma erythropoietin concentration | ↑ plasma erythropoietin concentration ✓ |
| ↓ reticulocyte count red blood cells (RBC) sequestered in the spleen ? | ↑ reticulocyte count ✓ sequestered RBCs released ? |
| ↓ intravascular RBC concentration ✓ | ↑ intravascular RBC concentration ✓ |

The increase in IL-6 concentration following the marathon was not sustained for a sufficient period of time to expect that a decrease in plasma Epo concentration would lead to a decrease in reticulocyte count, nor was this observed. The mean liver ⁵¹Cr-RBC count increased and the splenic ⁵¹Cr-RBC count tended to increase concomitantly with a decrease in RBC concentration 24 or 48 hours after the marathon run. The inconsistent correlation of ⁵¹Cr-RBC organ content with RBC concentration during 48 hours following the marathon and the limitations of the method, however, leave unclear the exact role sequestration of RBCs may have played in the reduction of intravascular RBC concentration. Splenic ⁵¹Cr-RBC count decreased following a period of rest after the marathon, from the level 24 or 48 hours post-marathon, indicating the organ's possible role in the return of RBC concentration to its baseline value. An increase in plasma Epo concentration at 24 or 48 hours following the marathon preceded an increase in reticulocyte count seven days after the marathon run. Further studies are required to confirm this observation with a larger number of subjects, and also to determine the reason for the lag between the increase in Epo concentration and the increase in reticulocyte count.

The following table summarizes the findings in the RA study:

| INFLAMMATION ON | INFLAMMATION OFF |
|--|--|
| ↑ plasma cytokine concentration ✓ | ↓ plasma cytokine concentration ✓ |
| ↓ plasma erythropoietin concentration ? | ↑ plasma erythropoietin concentration |
| ↓ reticulocyte count ✓ red blood cells (RBC) sequestered in the spleen ? | ↑ reticulocyte count sequestered RBCs released |
| ↓ intravascular RBC concentration ✓ | ↑ intravascular RBC concentration ✓ |

At the commencement of the study, when each RA subject was experiencing active disease, the mean plasma IL-6 concentration was higher in the anemic than in the non-anemic RA subgroup, demonstrating the hypothesized negative correlation between cytokine activity

and RBC concentration. The mean Epo concentration was higher in the anemic RA subgroup than in the non-anemic RA subgroup and control, but it is uncertain whether it was elevated to a degree appropriate to the level of anemia described, since there was no anemic control group in the study. The mean reticulocyte count of the anemic and non-anemic subgroups did not differ, suggesting that suppressed erythropoiesis was contributing to the observed anemia. There was no difference between the spleen-to-liver erythrocyte ratio, the only way of estimating sequestration at the beginning of the study in anemic and non-anemic subgroups. During methotrexate treatment, the mean plasma IL-1 β concentration decreased in the non-anemic subgroup. A decrease in plasma IFN- γ concentration from its baseline value following methotrexate treatment corresponded with an increase in reticulocyte count in three of four anemic RA subjects. An increase in reticulocyte count and a decrease in plasma Epo concentration corresponded to an increase in RBC concentration in three of four anemic subjects, suggesting that erythropoiesis was stimulated as disease activity declined, but that Epo played no role. There was no pattern of decreasing ⁵¹Cr-RBC spleen count, indicating that release of sequestered RBCs did not contribute to the observed improvement in RBC concentration in any of the four anemic subjects. These data provide evidence for a reduced suppression of erythropoiesis, unrelated to an increase in Epo concentration, in the improvement of reticulocyte count and RBC concentration in the anemic RA subject treated with methotrexate. Further studies using a larger subject population are required to confirm the finding of a decrease in Epo concentration and an increase in reticulocyte count during methotrexate treatment and to determine the mechanism responsible for the observed increase in reticulocyte count.

Appendix A

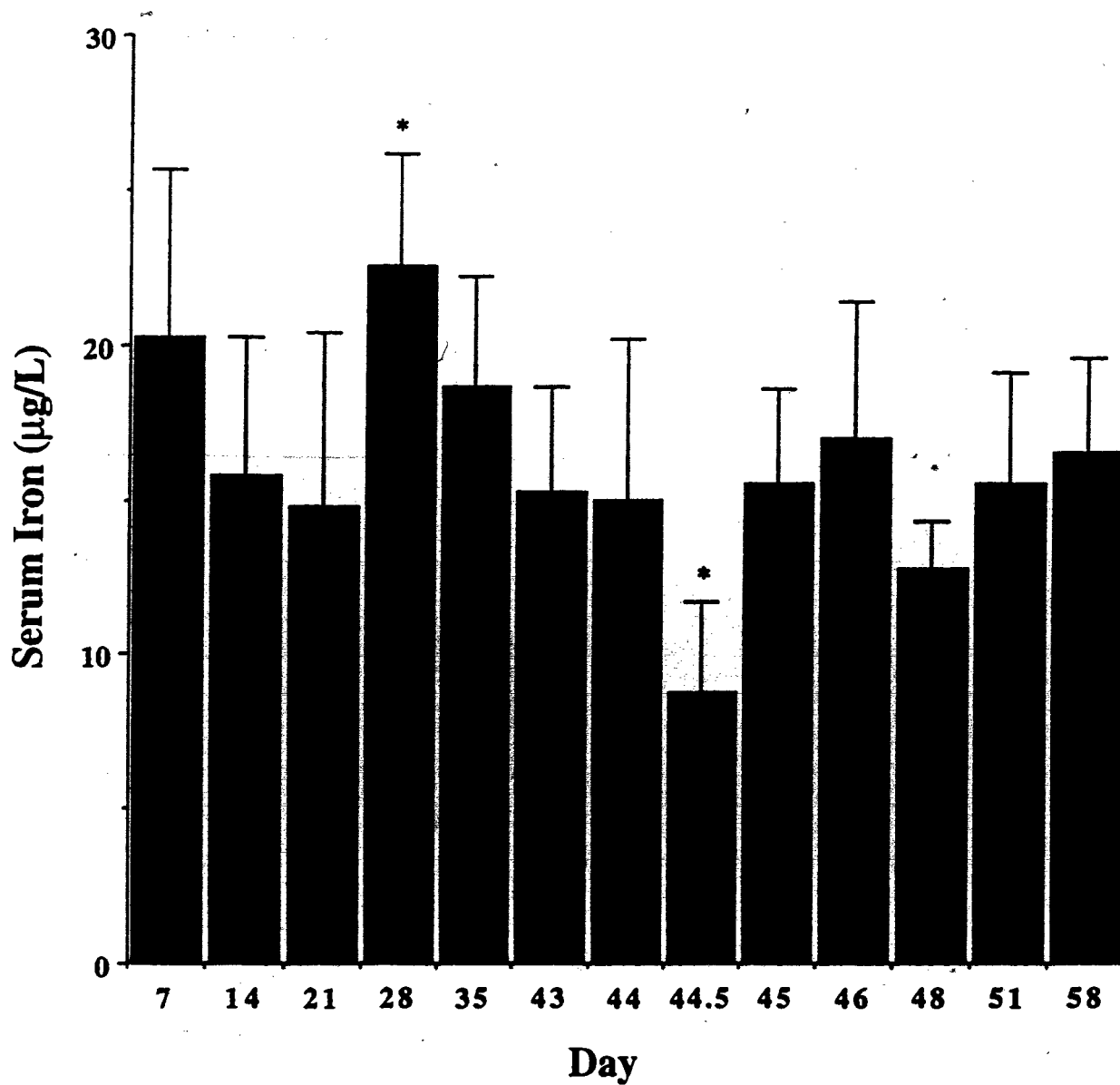


Figure A1 Serial change in mean (\pm SD) serum iron concentration. Training days 1-29, taper days 30-43, post-marathon days 44-58.

* significantly different ($p \leq 0.05$) from day 43 (baseline) value.

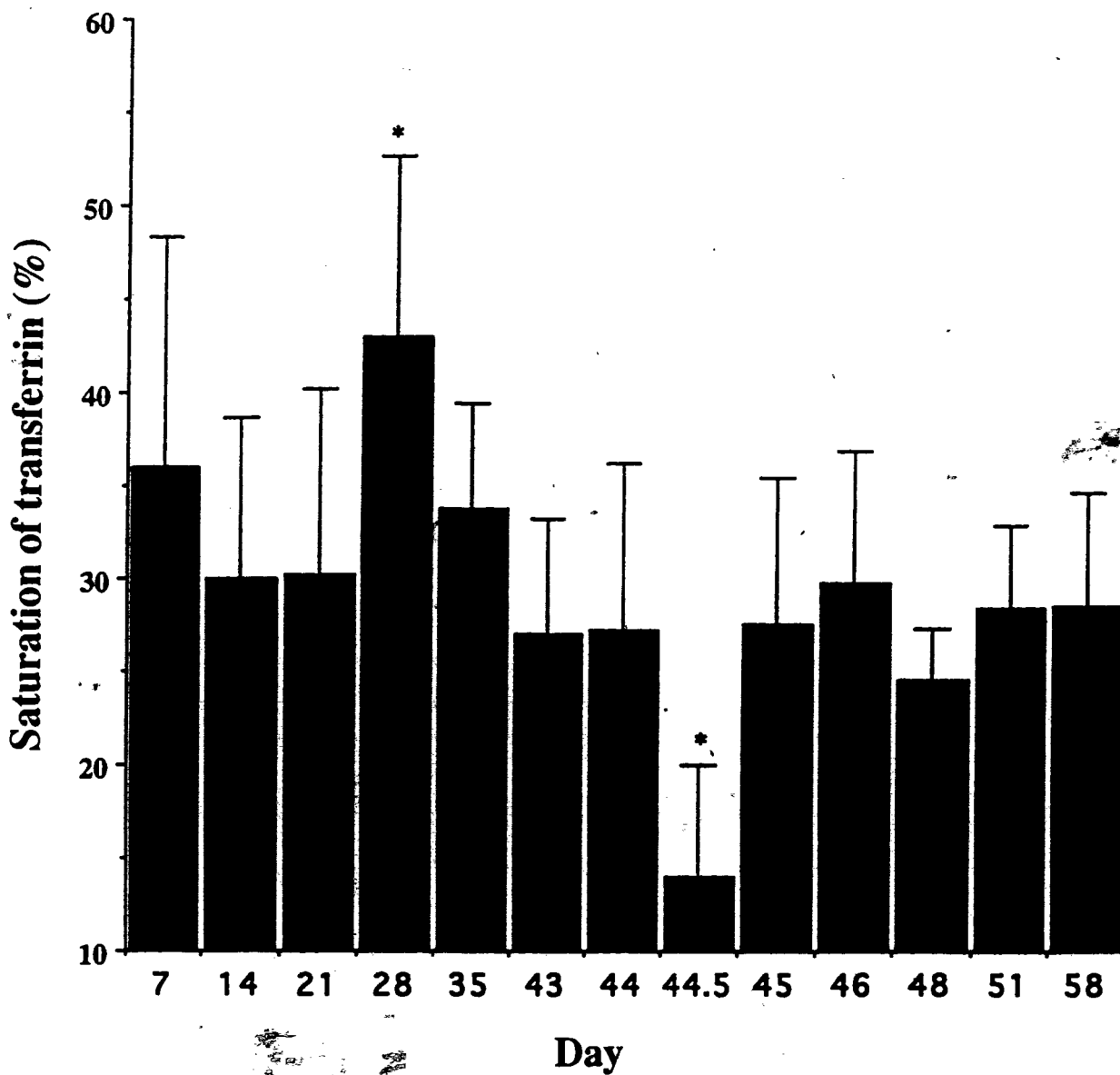


Figure A2 Serial change in mean (\pm SD) percentage saturation of transferrin. Training days 1-29, taper days 30-43, post-marathon days 44-58.

* significantly different ($p \leq 0.05$) from day 43 (baseline) value.

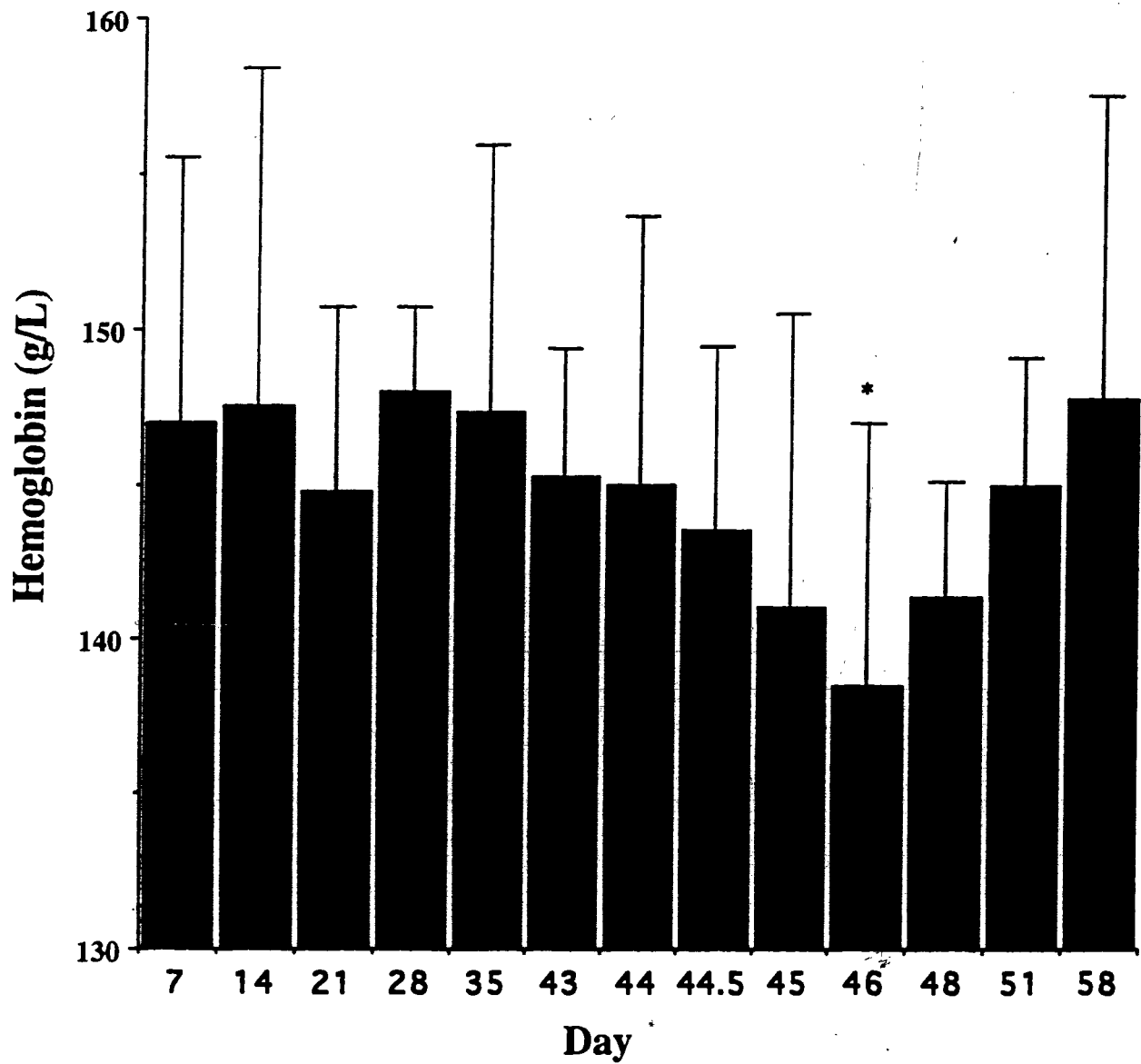


Figure A3 Serial change in mean (\pm SD) hemoglobin concentration. Training days 1-29, taper days 30-43, post-marathon days 44-58.

* significantly different ($p \leq 0.05$) from day 43 (baseline) value.

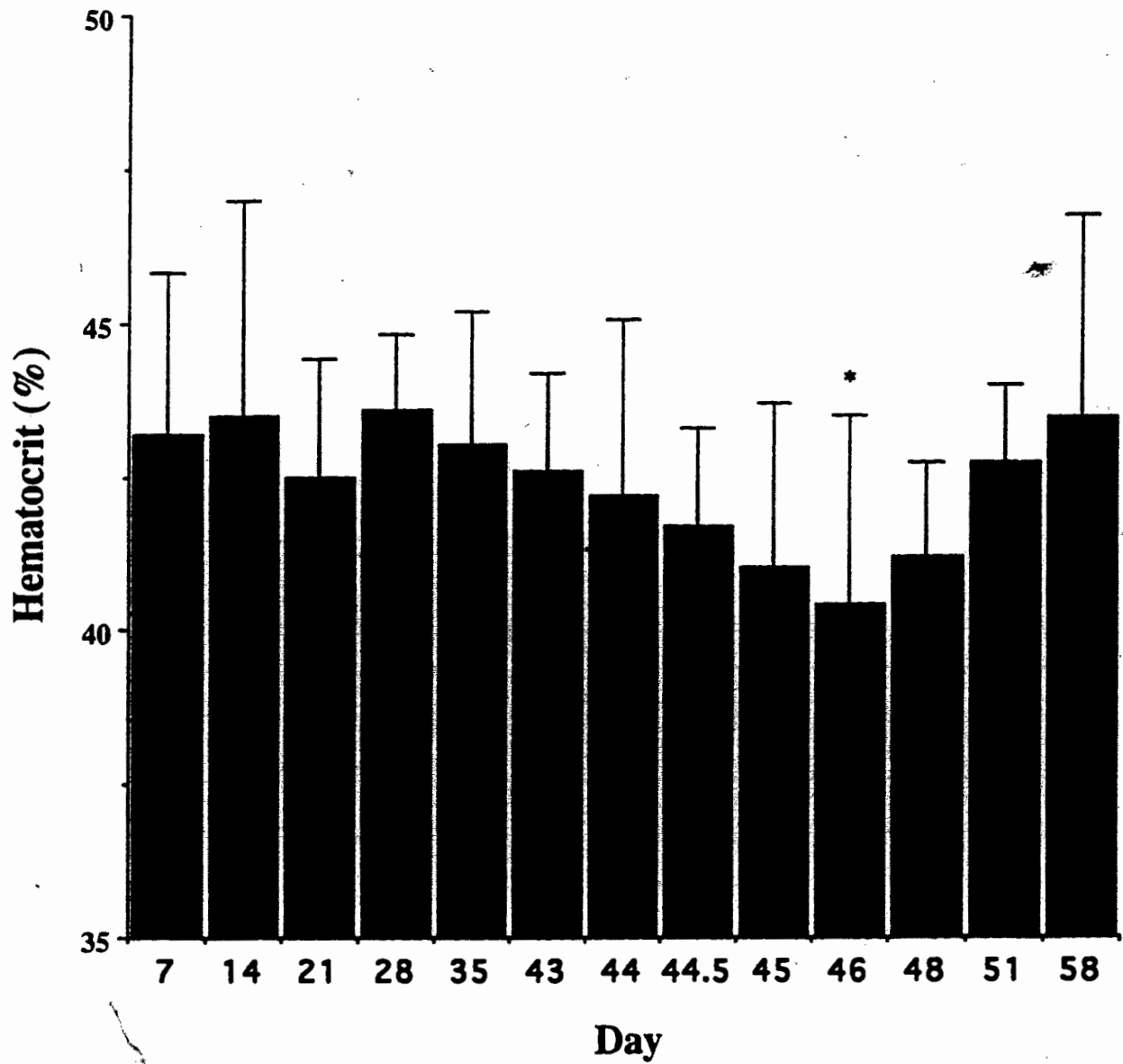


Figure A4 Serial change in mean (\pm SD) hematocrit. Training days 1-29, taper days 30-43, post-marathon days 44-58.

* significantly different ($p \leq 0.05$) from day 43 (baseline) value.

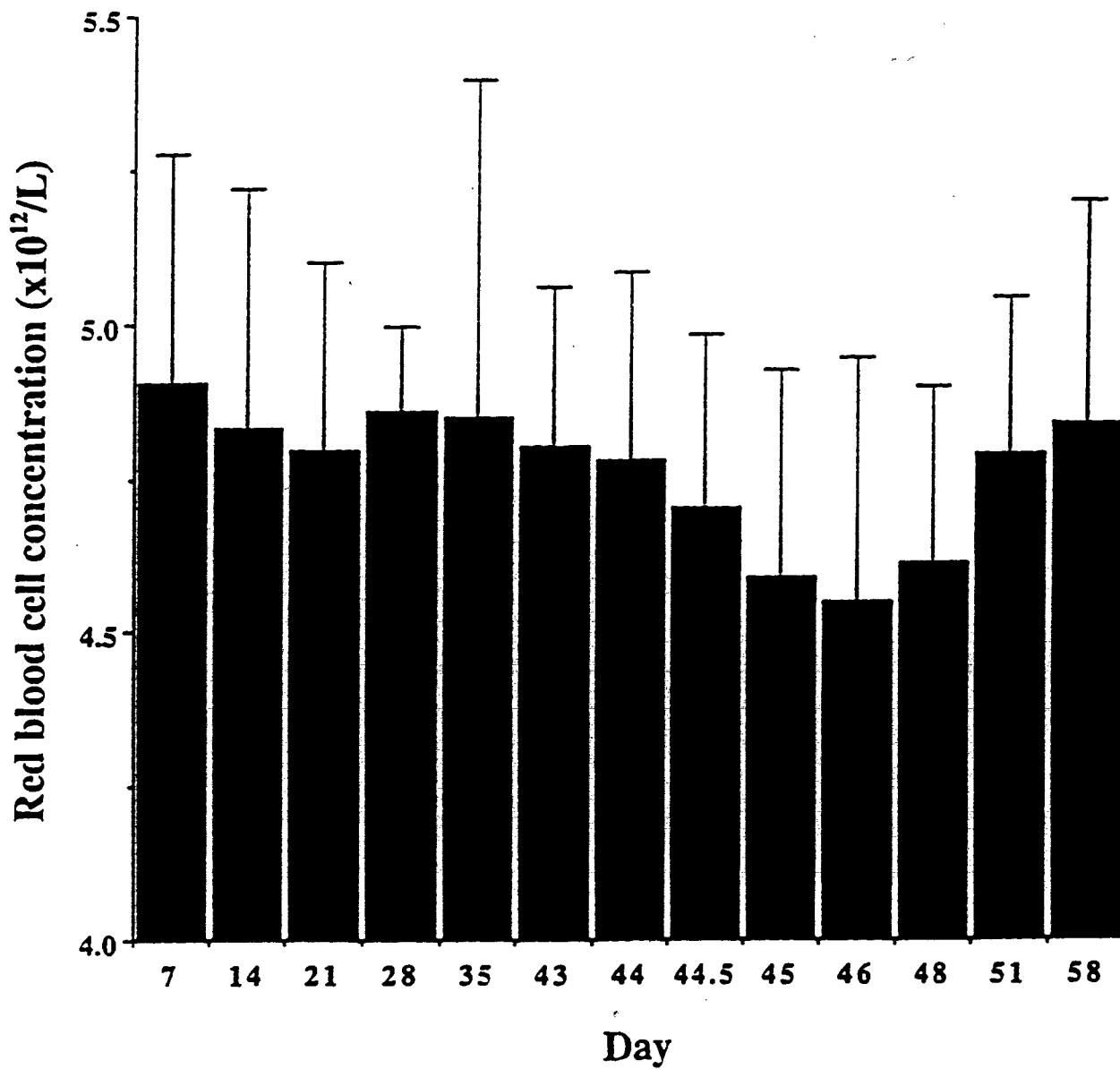


Figure A5 Serial change in mean (\pm SD) red blood cell (RBC) concentration. Training days 1-29, taper days 30-43, post-marathon days 44-58.

* significantly different ($p \leq 0.05$) from day 43 (baseline) value.

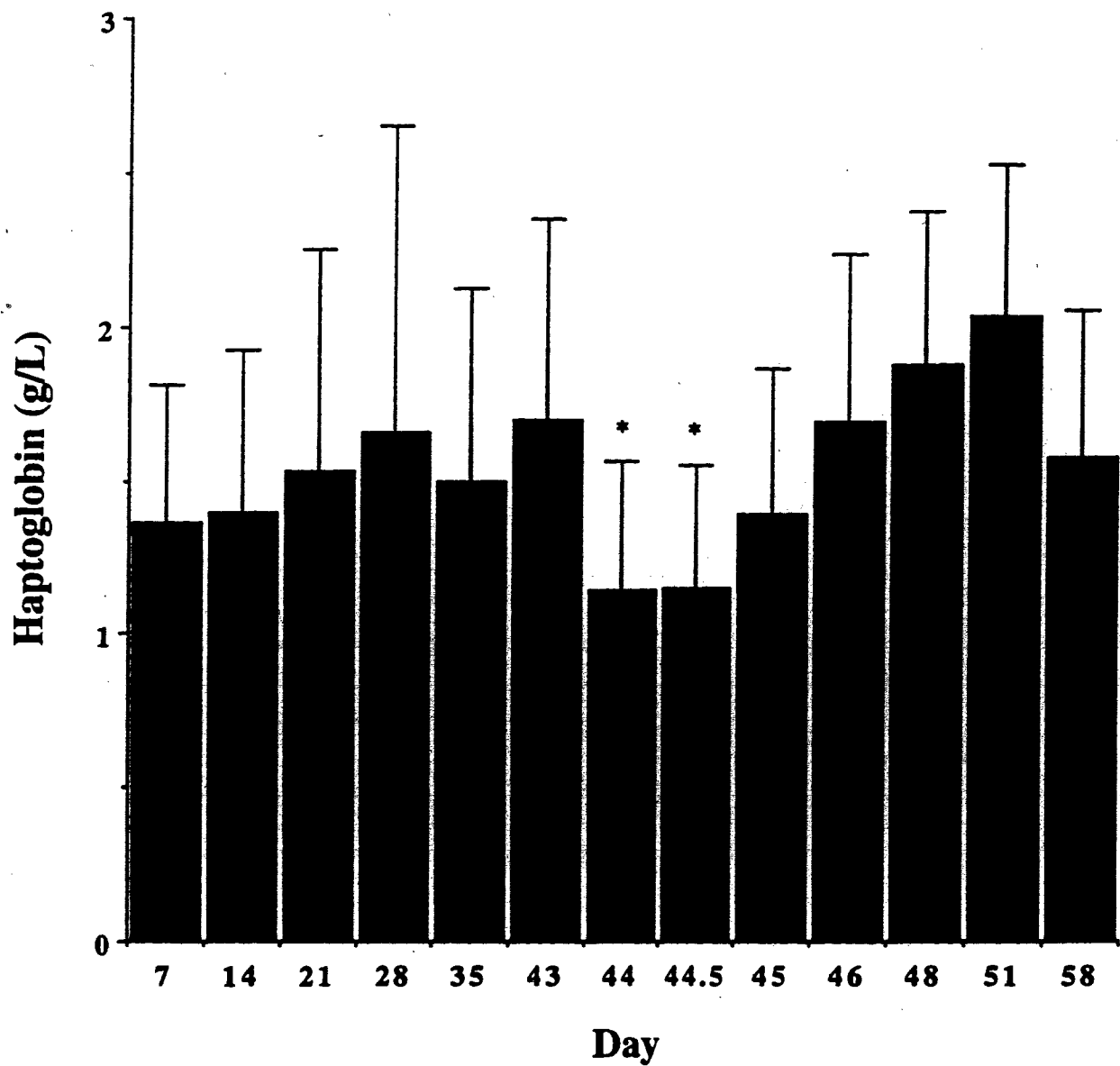


Figure A6 Serial change in mean (\pm SD) serum haptoglobin concentration. Training days 1-29, taper days 30-43, post-marathon days 44-58.

* significantly different ($p \leq 0.05$) from day 43 (baseline) value.

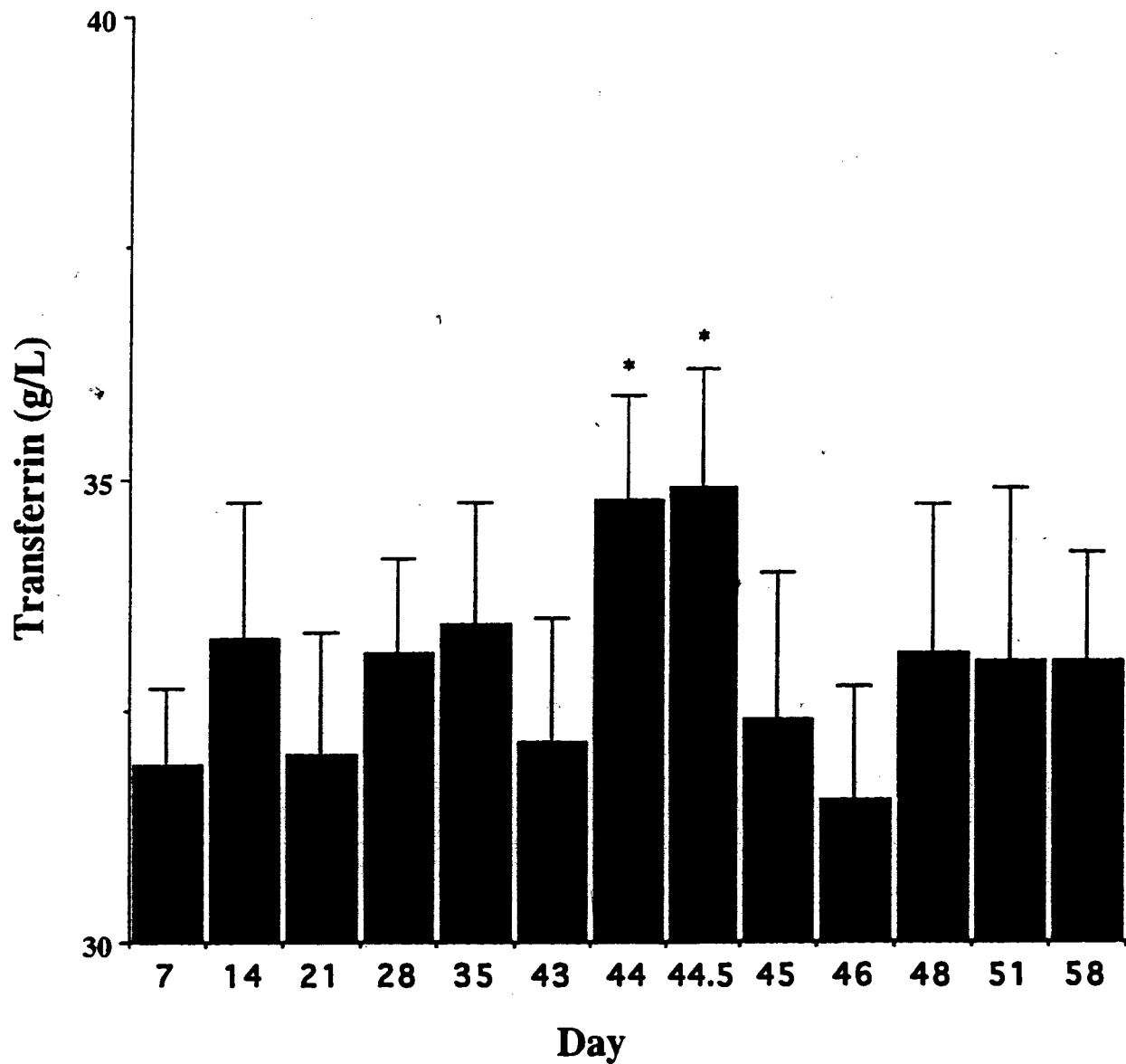


Figure A7 Serial change in mean (\pm SD) transferrin concentration. Training days 1-29, taper days 30-43, post-marathon days 44-58.

* significantly different ($p \leq 0.05$) from day 43 (baseline) value.

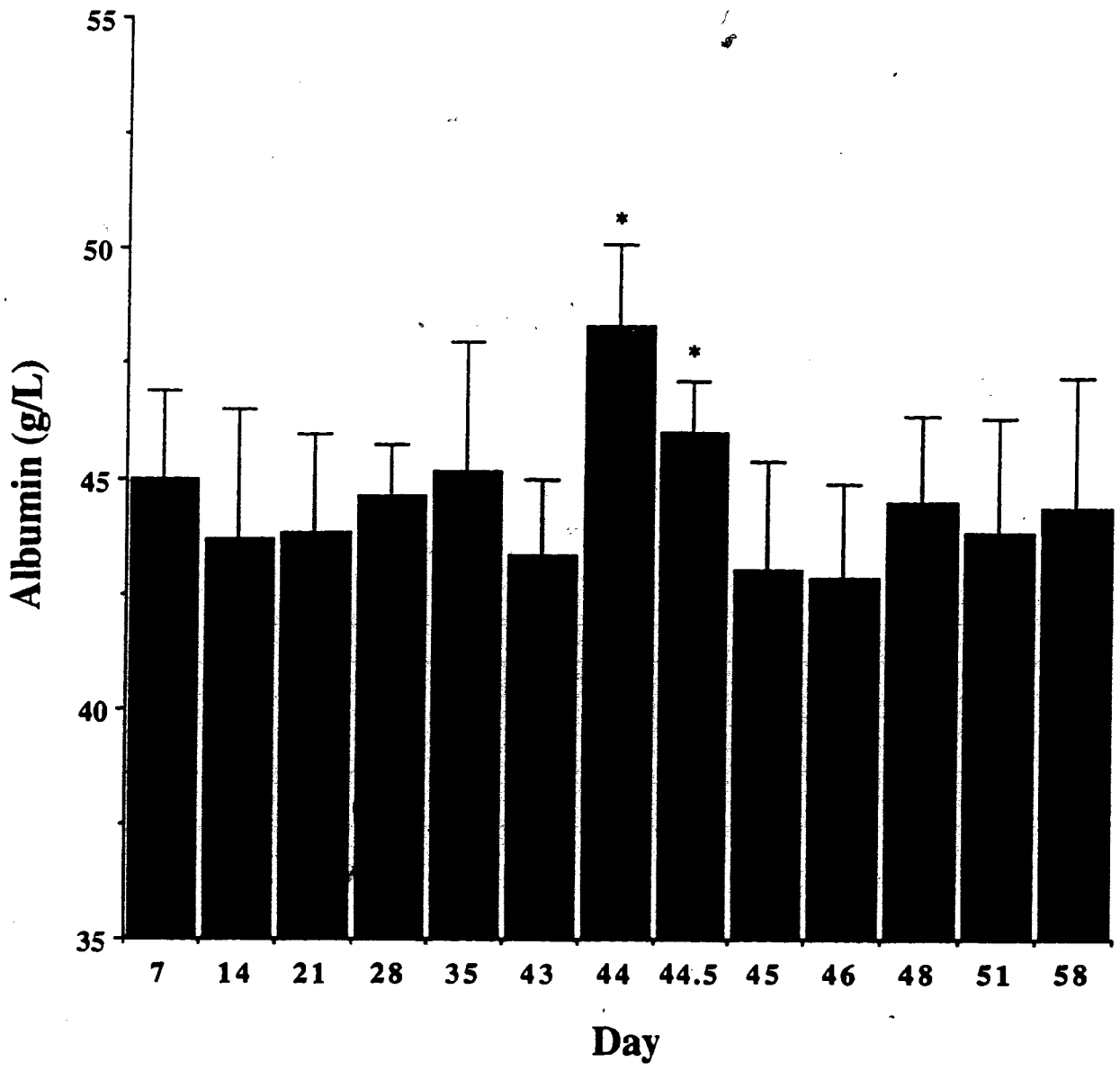


Figure A8 Serial change in mean (\pm SD) serum albumin concentration. Training days 1-29, taper days 30-43, post-marathon days 44-58.

* significantly different ($p \leq 0.05$) from day 43 (baseline) value.

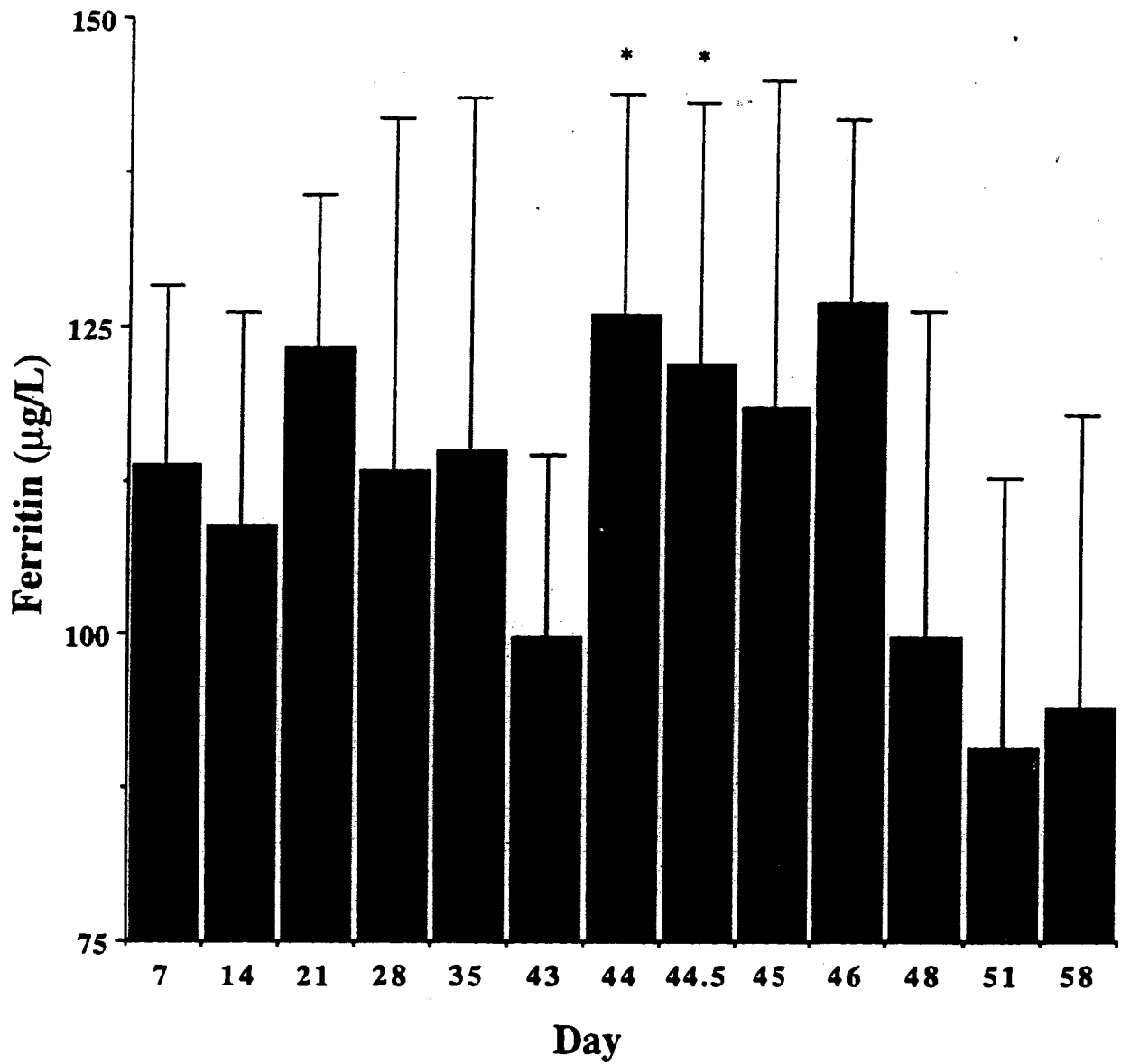


Figure A9 Serial change in mean (\pm SD) ferritin concentration. Training days 1-29, taper days 30-43, post-marathon days 44-58.

* significantly different ($p \leq 0.05$) from day 43 (baseline) value.

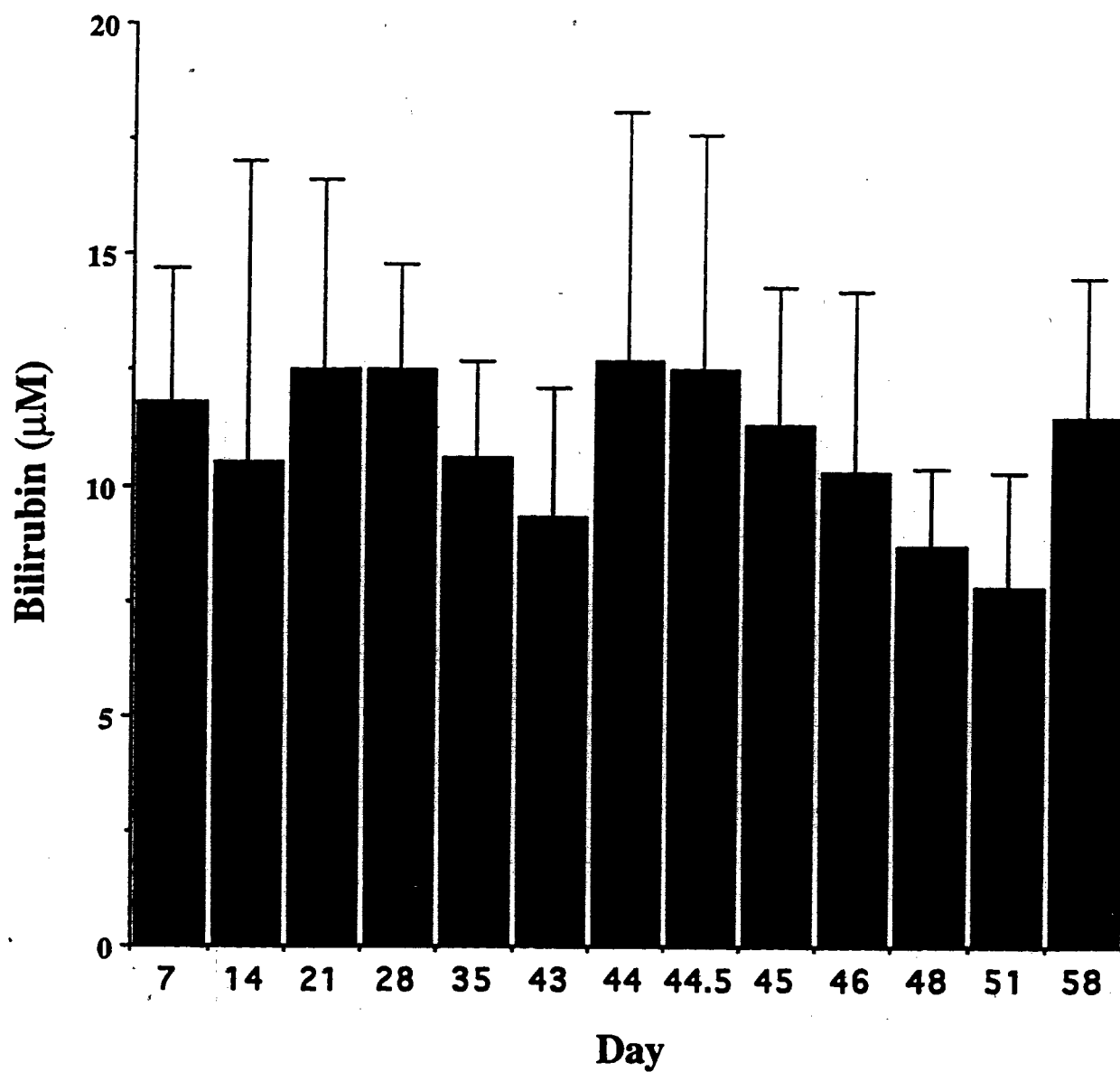


Figure A10 Serial change in mean (\pm SD) bilirubin concentration. Training days 1-29, taper days 30-43, post-marathon days 44-58.

* significantly different ($p \leq 0.05$) from day 43 (baseline) value.

Legend to Figures A11-A17

In the top panel of each figure subject data is plotted with control data (\pm SD) for ^{51}Cr -RBC count from the heart; the center panel shows ^{51}Cr -RBC count from the liver and the bottom panel shows ^{51}Cr -RBC count from the spleen. ^{51}Cr -RBC organ count data are expressed as a percentage of day 1 count and are plotted against day of measurement. Days are numbered from the first day of monitored training. Training days are 1-29, taper days are 30-43 and post-marathon days are 44-58. The "marathon" label indicates the first post-marathon value. In Figure A10, organ count data from any subject with an illness (see Table 4) were not included in the group mean calculation nor were data from subject MC, due to an abnormal pattern of organ counts, possibly secondary to hemochromatosis (see Figure 23). On the bottom panel of Figures 19-24 an RBC concentration value ($\times 10^{12}/\text{L}$) is indicated where a blood test corresponded with a ^{51}Cr -RBC spleen count. The first RBC value reported corresponds with the ^{51}Cr -RBC organ count in the final week of intense training, the second RBC value corresponds with the beginning of the taper period and the third RBC value corresponds with the end of the taper period, 24 hours prior to the marathon run (baseline). The remainder of the RBC values correspond with the respective ^{51}Cr -RBC measure taken in a non-training, recovery state two hours, six hours, 24 hours, 48 hours, four days and seven days after the marathon and following a week of light training, 14 days after the marathon.

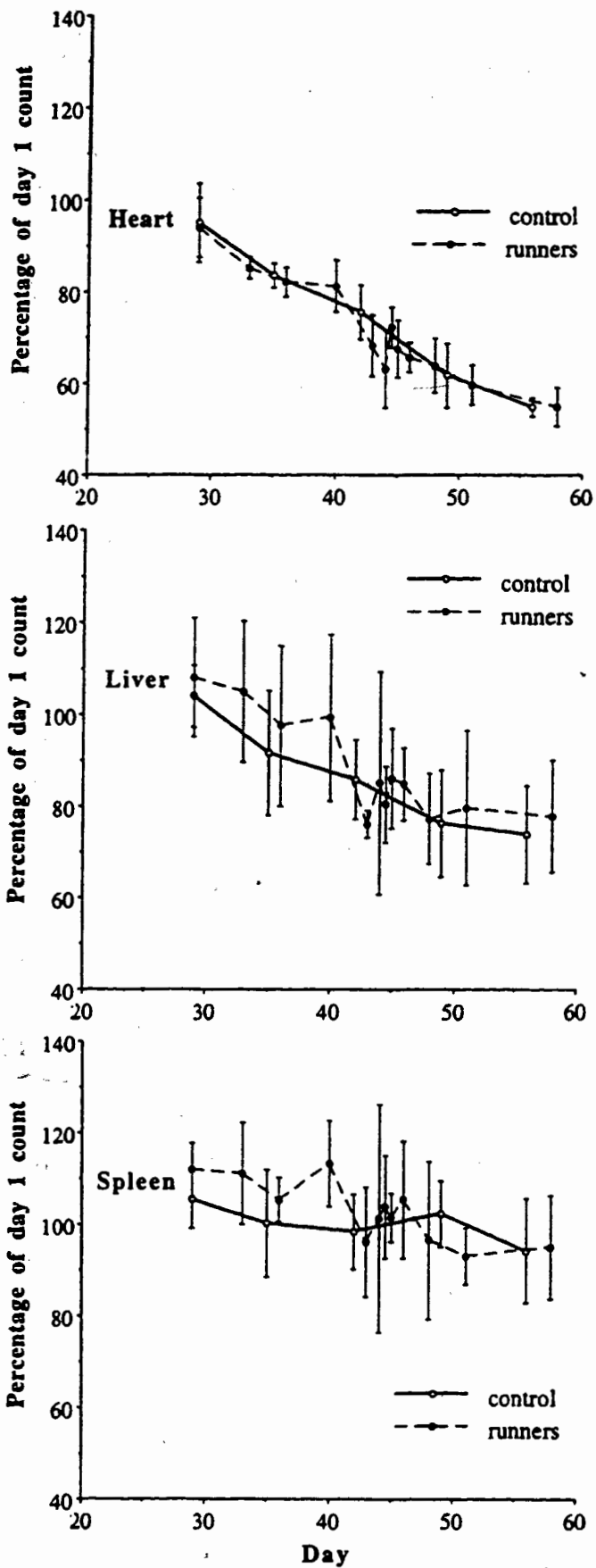


Figure A11 Group mean ^{51}Cr -RBC organ count for runners compared with a control count.

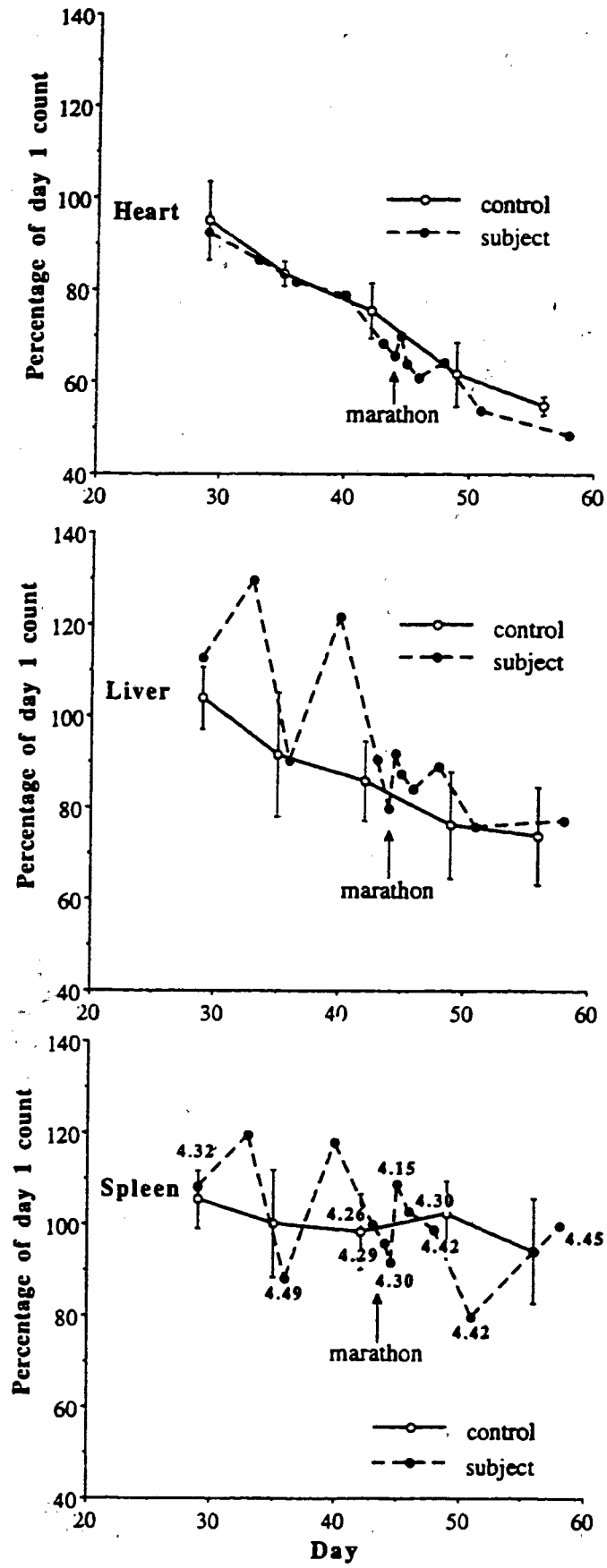


Figure A12 ⁵¹Cr-RBC organ count for subject RT compared with a control count .

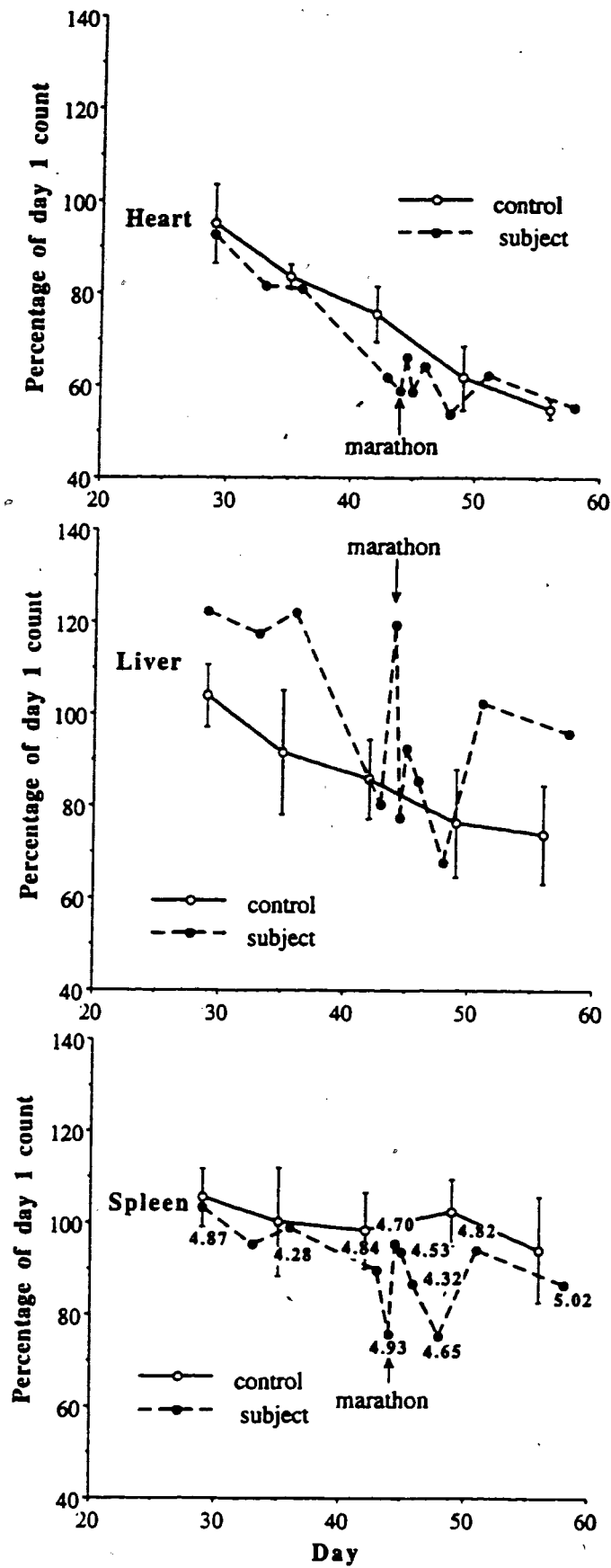


Figure A13 ⁵¹Cr-RBC organ count for subject RG compared with a control count .

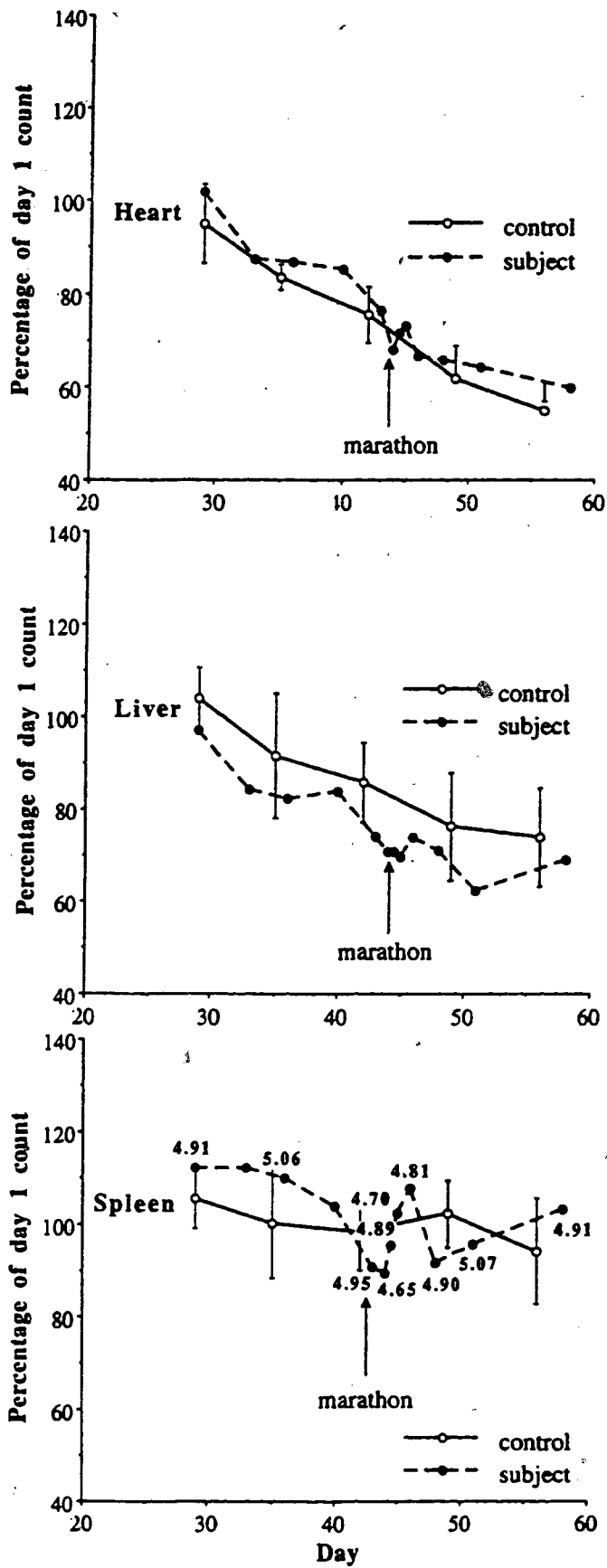


Figure A14 ^{51}Cr -RBC organ count for subject AY compared with a control count.

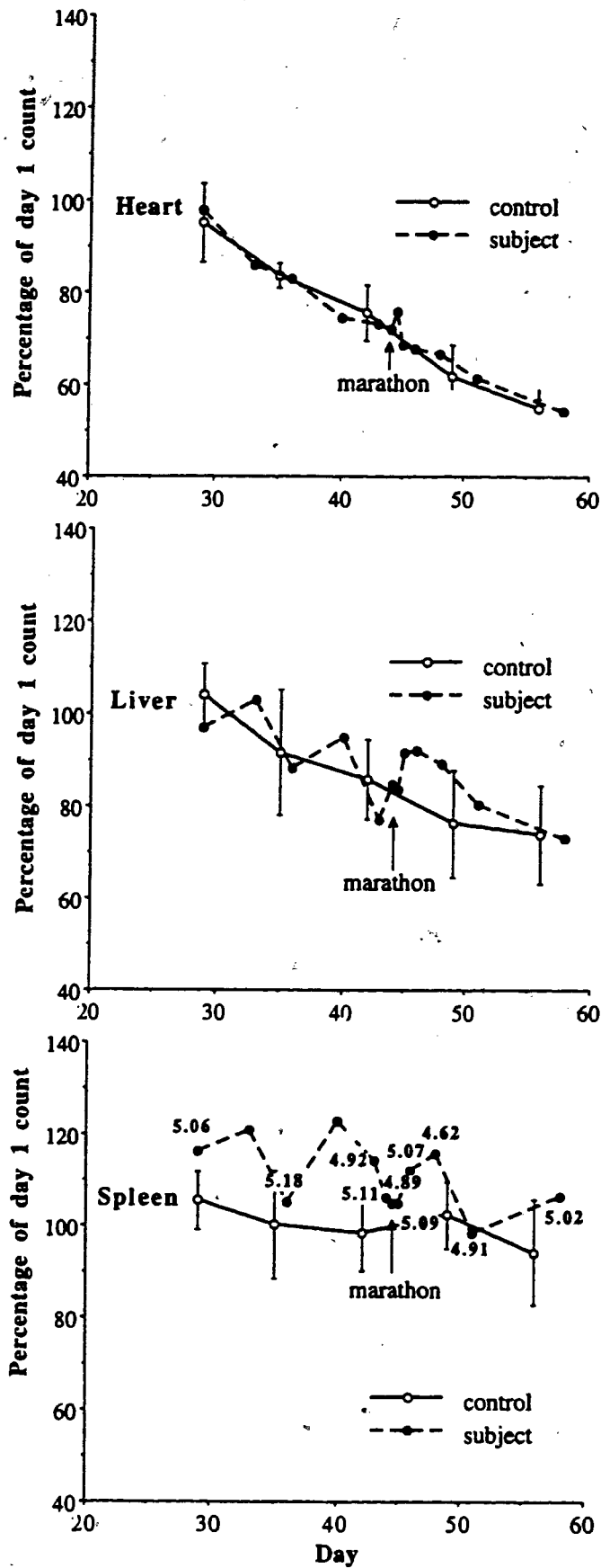


Figure A15 ⁵¹Cr-RBC organ count for subject NW compared with a control count.

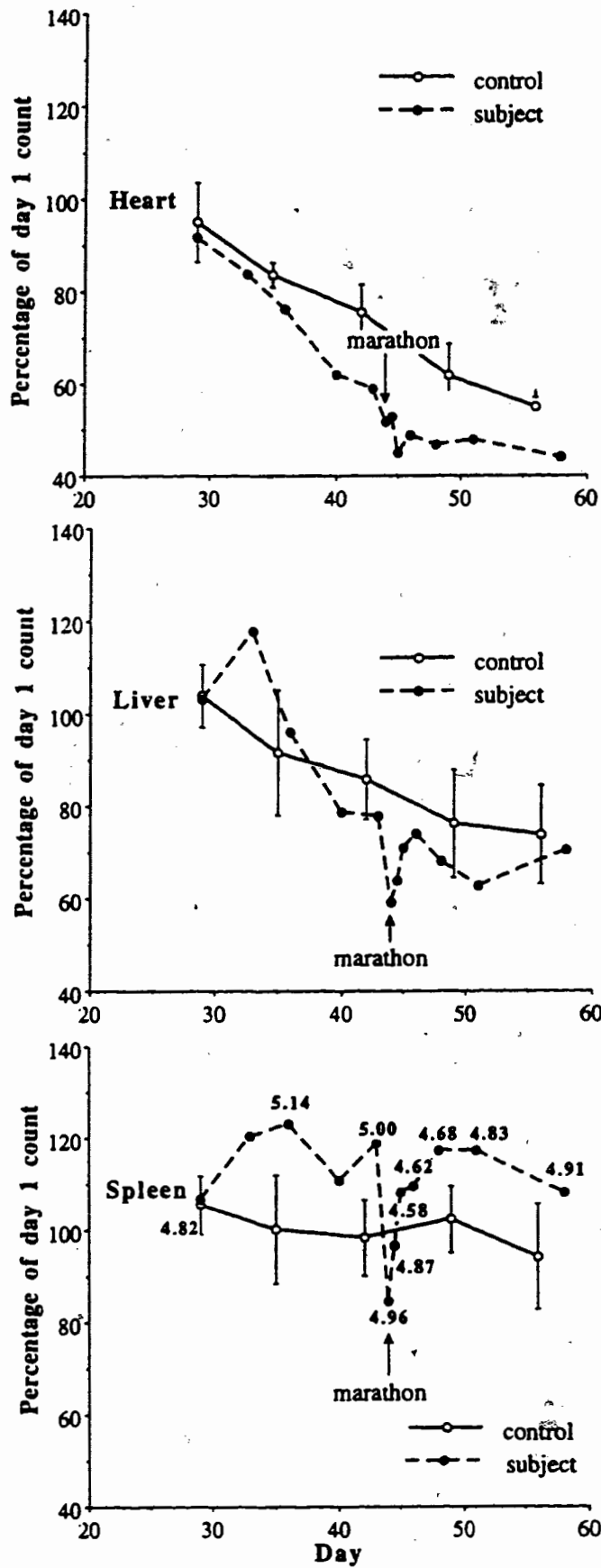


Figure A16 ⁵¹Cr-RBC organ count for subject MC compared with a control count.

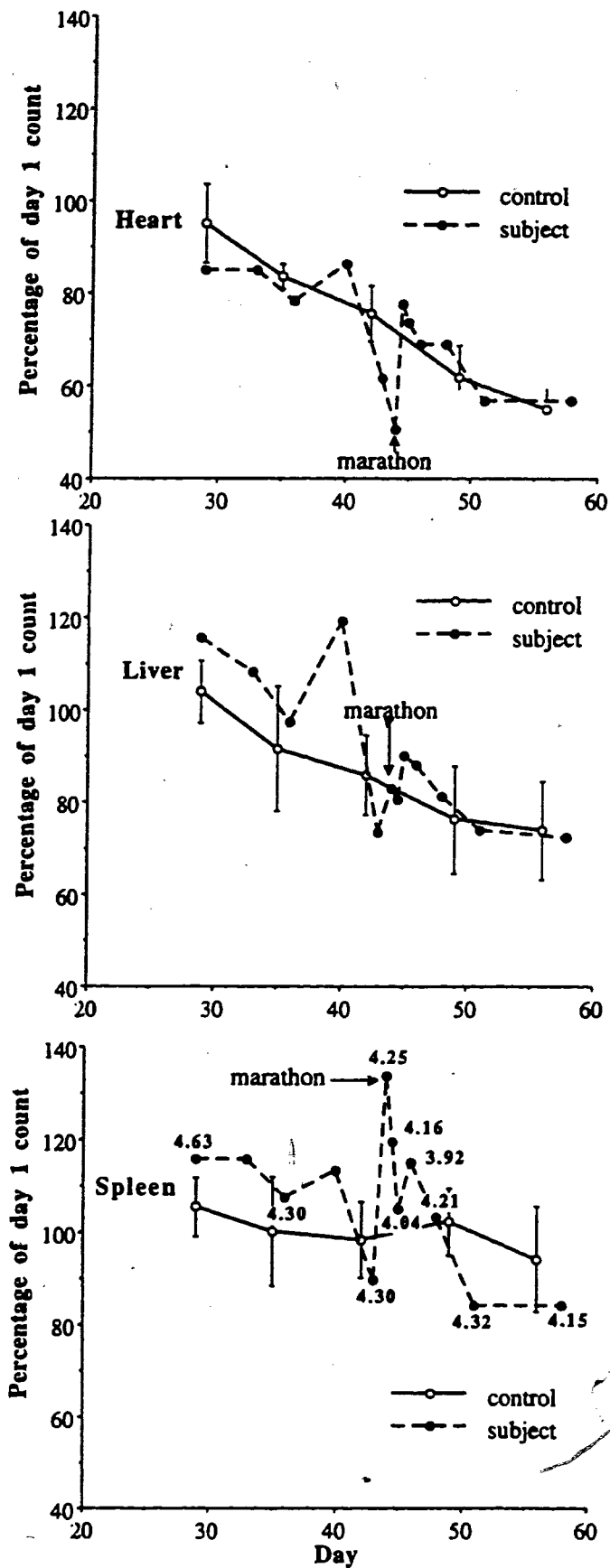


Figure A17 ^{51}Cr -RBC organ count for subject JT compared with a control count.

Table A1 Change in hematologic and iron status in response to an illness or an injury in runners.

Illness

| | Day | IL-6 | Hb | Δ PV | SI | SF | %sat | Fer | Hap | Retic | Epo |
|----|-----|------|-----|-------------|----|------|------|-----|------|-------|-----|
| RT | 21 | <1 | 151 | | 18 | 31.4 | 36 | 35 | 2.20 | 173 | 7 |
| | 28 | 10 | 133 | 24.0 | 2 | 27.7 | 5 | 53 | 2.98 | 65 | 12 |
| RG | 28 | <1 | 150 | | 19 | 32.0 | 37 | 102 | 0.94 | 97 | 10 |
| | 35 | <1 | 133 | 24.4 | 13 | 26.4 | 31 | 127 | 0.71 | 34 | 18 |
| NW | 45 | <1 | 153 | | 12 | 32.0 | 24 | 86 | 1.52 | 66 | 6 |
| | 46 | <1 | 149 | | 7 | 30.8 | 14 | 107 | 2.63 | 66 | 6 |
| | 48 | <1 | 137 | 12.2 | 11 | 28.9 | 24 | 61 | 2.63 | 65 | 9 |

Injury

| | Day | CK | Hb | Δ PV | Iron | SF | %sat | Fer | Hap | Retic | Epo |
|----|-----|-----|-----|-------------|------|------|------|-----|------|-------|-----|
| MC | 7 | 143 | 161 | | 36 | 25.2 | 90+ | 371 | 0.97 | 78 | M/D |
| | 14 | 207 | 144 | 22.0 | 22 | 24.0 | 58+ | 314 | 1.01 | 75 | 7 |
| AY | 14 | 142 | 151 | | 14 | 37.6 | 23 | 93 | 0.59 | 119 | 6 |
| | 7 | 303 | 144 | 9.2 | 22 | 34.5 | 40 | 110 | 0.50 | 103 | 7 |

IL-6-interleukin-6 concentration (pg/ml), Hb-hemoglobin concentration (g/L), Δ PV-change in plasma volume (%), based on the method of Dill and Costill (1974), SI-serum iron (μ M), SF-serum transferrin concentration (μ M), %sat-saturation of transferrin with iron (%), Fer-serum ferritin concentration (μ g/L), Hap-serum haptoglobin concentration (g/L), Retic-reticulocyte count ($\times 10^9/L$), Epo- plasma erythropoietin concentration (mIU/ml), CK-serum creatine kinase activity (U/L), M/D-missing data

Table A2 Mean ⁵¹Cr-RBC organ count and intravascular RBC concentration in subject NW after the marathon run.

| <u>Subject NW</u> | heart (%) | liver (%) | spleen (%) | RBC (x 10 ⁹ /L) |
|-----------------------|-----------|-----------|------------|----------------------------|
| baseline | 73.2 | 76.8 | 114.2 | 4.92 |
| 24 hrs. post-marathon | 68.6 | 91.5 | 104.8 | 5.09 |
| 48 hrs. post-marathon | 67.7 | 92.0 | 112.0 | 5.07 |
| 4 days post-marathon | 66.6 | 89.2 | 115.7 | 4.62 |
| 7th day post-marathon | 61.4 | 80.1 | 98.4 | 4.91 |

⁵¹Cr-RBC organ counts are expressed as a percentage of the day 1 count. The baseline measure was made 24 hour pre-marathon, at the end of a 14 day taper. While there was no evidence of an acute phase response or decrease in RBC concentration in the first 48 hours after the marathon run, an acute phase response was evident when subject NW became ill at 48 hours (Table A1), and RBC was decreased 2 days later (4 days post-marathon). RBC-intravascular red blood cell concentration.

Appendix B

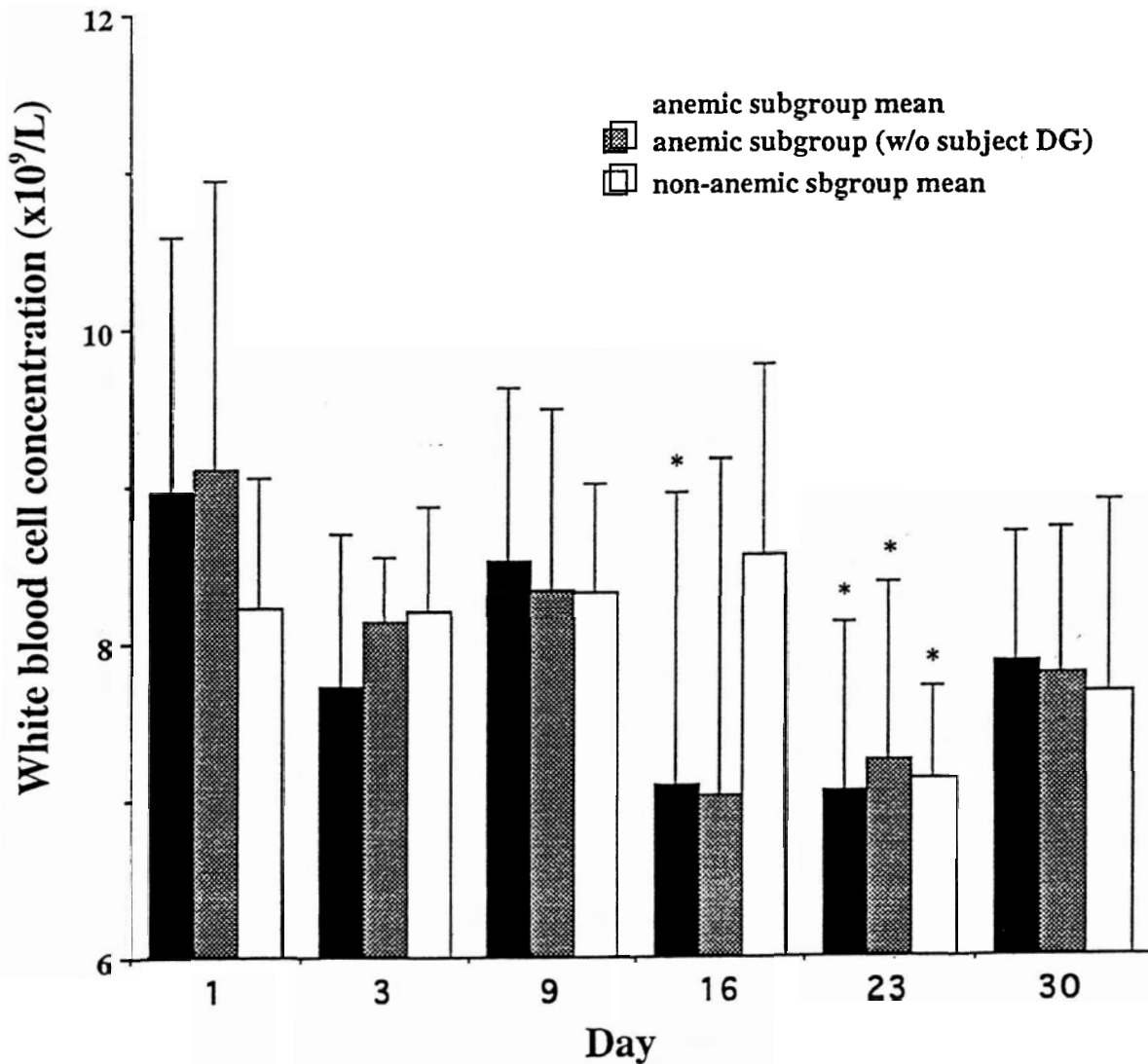


Figure B1 Serial change in mean (\pm SD) white blood cell concentration with methotrexate treatment in anemic and non-anemic RA subjects. Days 1 and 3 are pre-methotrexate; days 9-30 are during methotrexate treatment. Data are missing for non-anemic subject AC on day 3.

* significantly different ($p \leq 0.05$) from the pre-methotrexate measure on day 1.

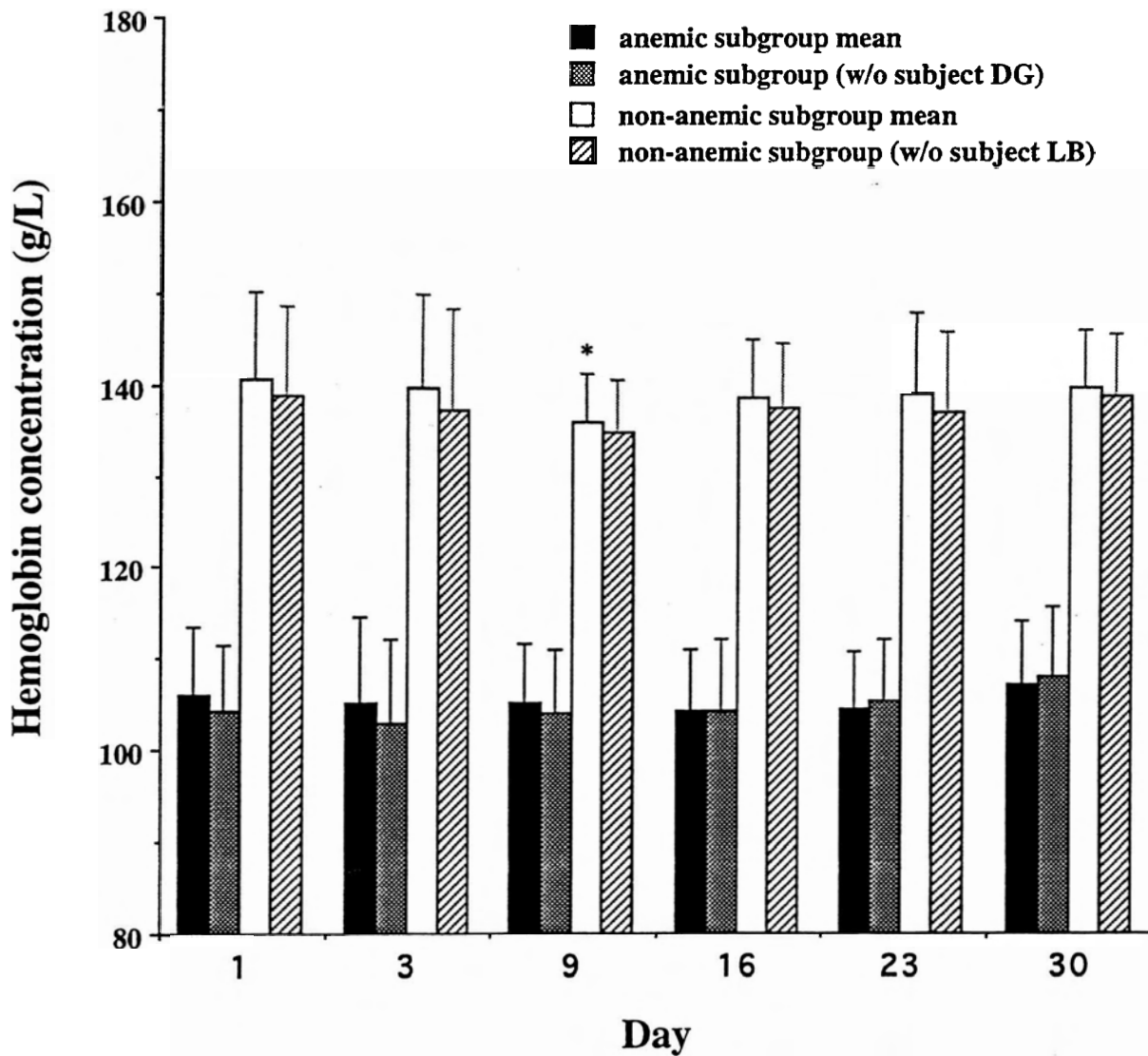


Figure B2 Serial change in mean (\pm SD) hemoglobin concentration with methotrexate treatment in anemic and non-anemic RA subjects. Days 1 and 3 are pre-methotrexate; days 9-30 are during methotrexate treatment. Data are missing for non-anemic subject AC on day 3.

* significantly different ($p \leq 0.05$) from the pre-methotrexate measure on day 1.

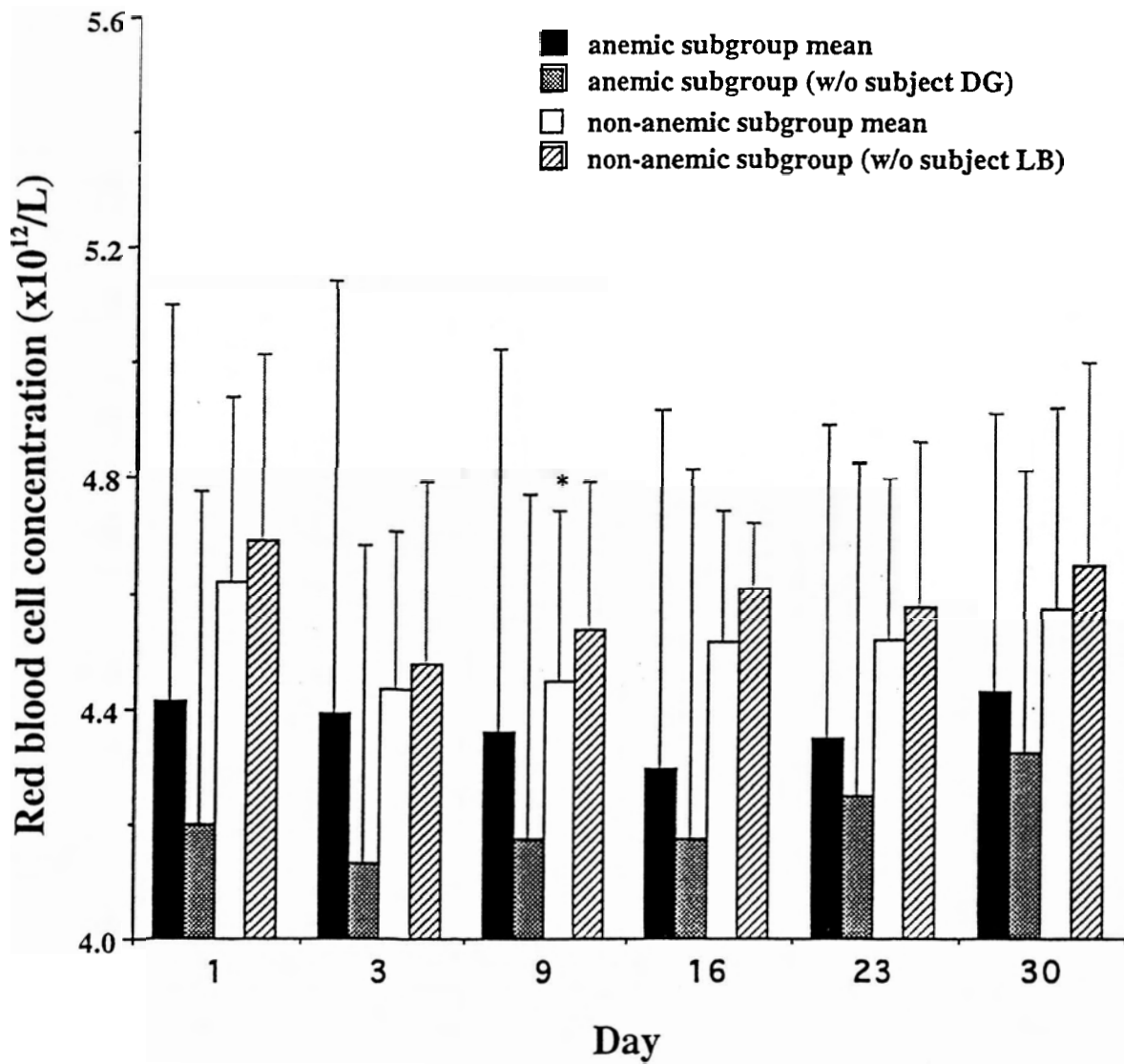


Figure B3 Serial change in mean (\pm SD) red blood cell concentration with methotrexate treatment in anemic and non-anemic RA subjects. Days 1 and 3 are pre-methotrexate; days 9-30 are during methotrexate treatment. Data are missing for non-anemic subject AC on day 3.

* significantly different ($p \leq 0.05$) from the pre-methotrexate measure on day 1.

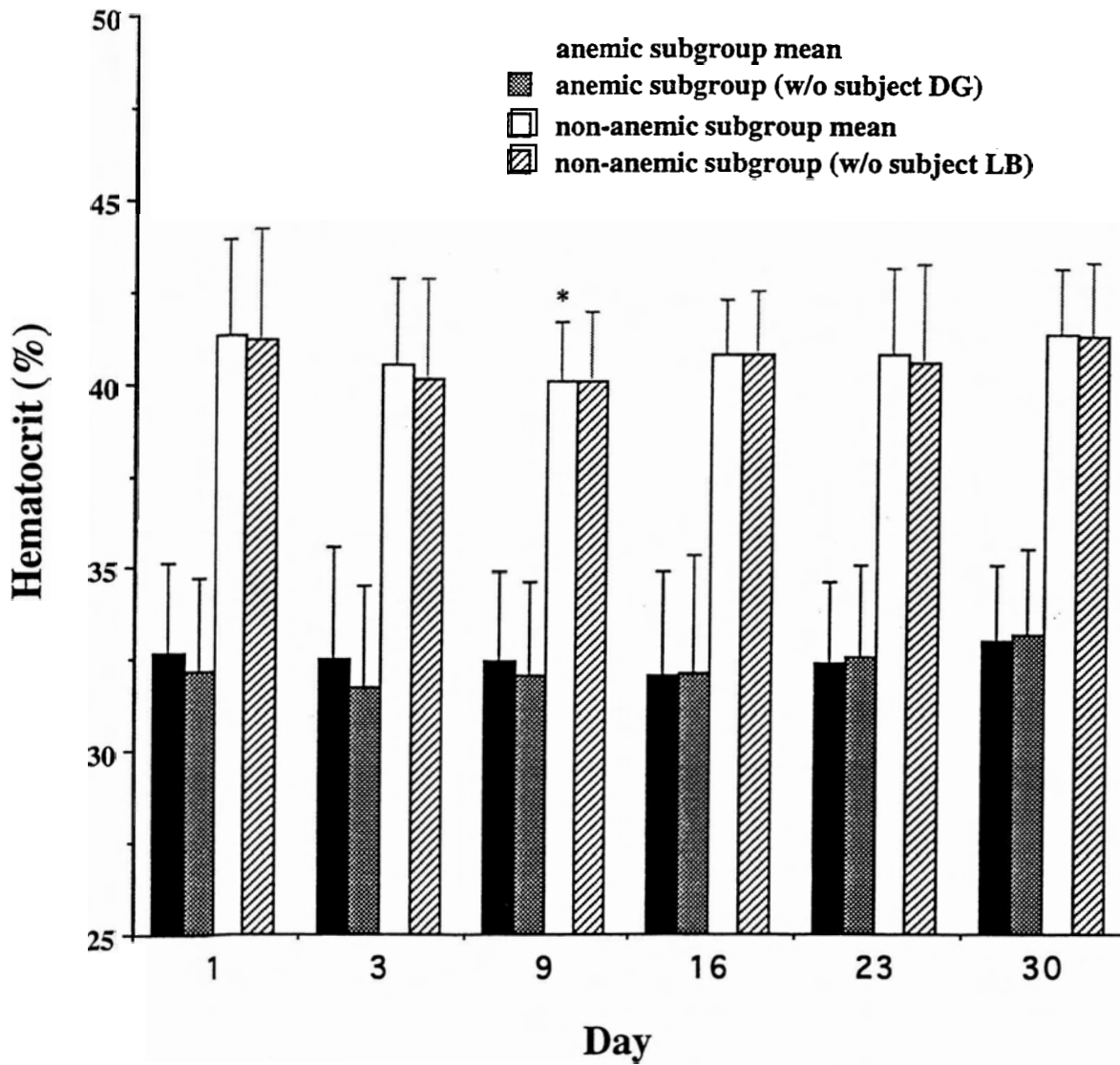


Figure B4 Serial change in mean (\pm SD) hematocrit with methotrexate treatment in anemic and non-anemic RA subjects. Days 1 and 3 are pre-methotrexate; days 9-30 are during methotrexate treatment. Data are missing for non-anemic subject AC on day 3.

* significantly different ($p \leq 0.05$) from the pre-methotrexate measure on day 1.

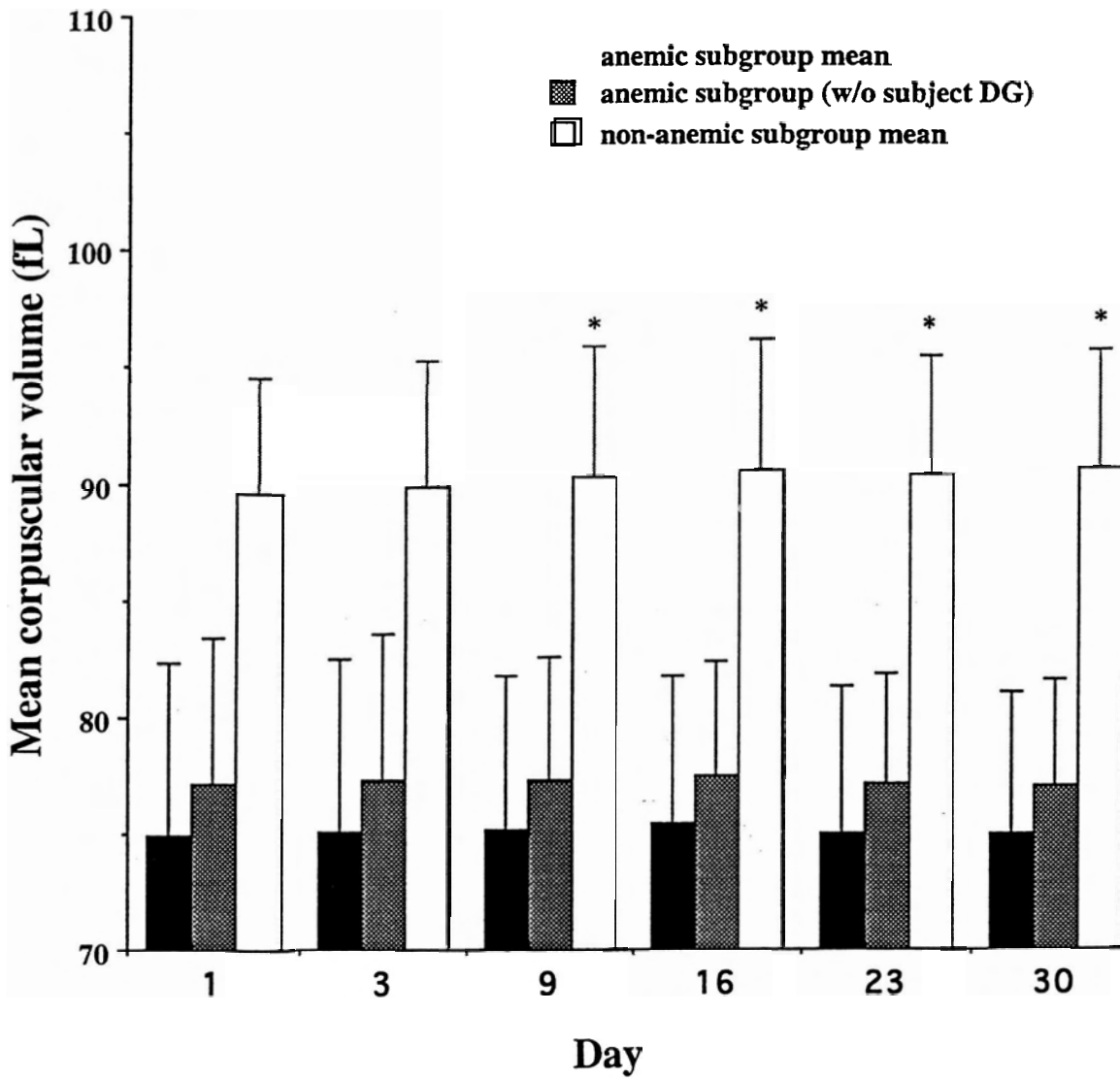


Figure B5 Serial change in mean (\pm SD) mean corpuscular volume (erythrocyte) with methotrexate treatment in anemic and non-anemic RA subjects. Days 1 and 3 are pre-methotrexate; days 9-30 are during methotrexate treatment. Data are missing for non-anemic subject AC on day 3.

* significantly different ($p \leq 0.05$) from the pre-methotrexate measure on day 1.

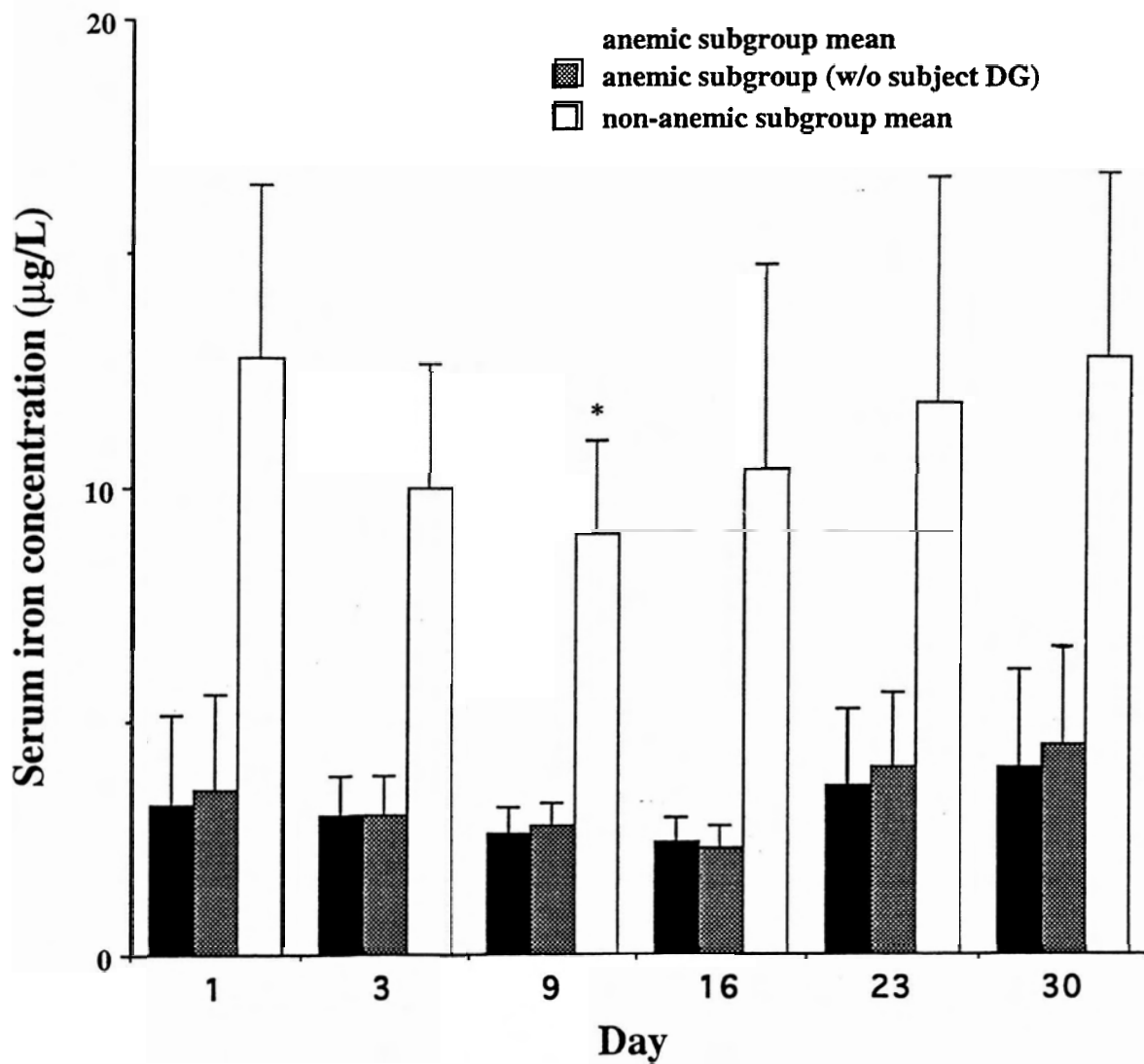


Figure B6 Serial change in mean (\pm SD) serum iron concentration with methotrexate treatment in anemic and non-anemic RA subjects. Days 1 and 3 are pre-methotrexate; days 9-30 are during methotrexate treatment. Data are missing for non-anemic subjects EM and AC and anemic subject DG on day 3.

* significantly different ($p \leq 0.05$) from the pre-methotrexate measure on day 1.

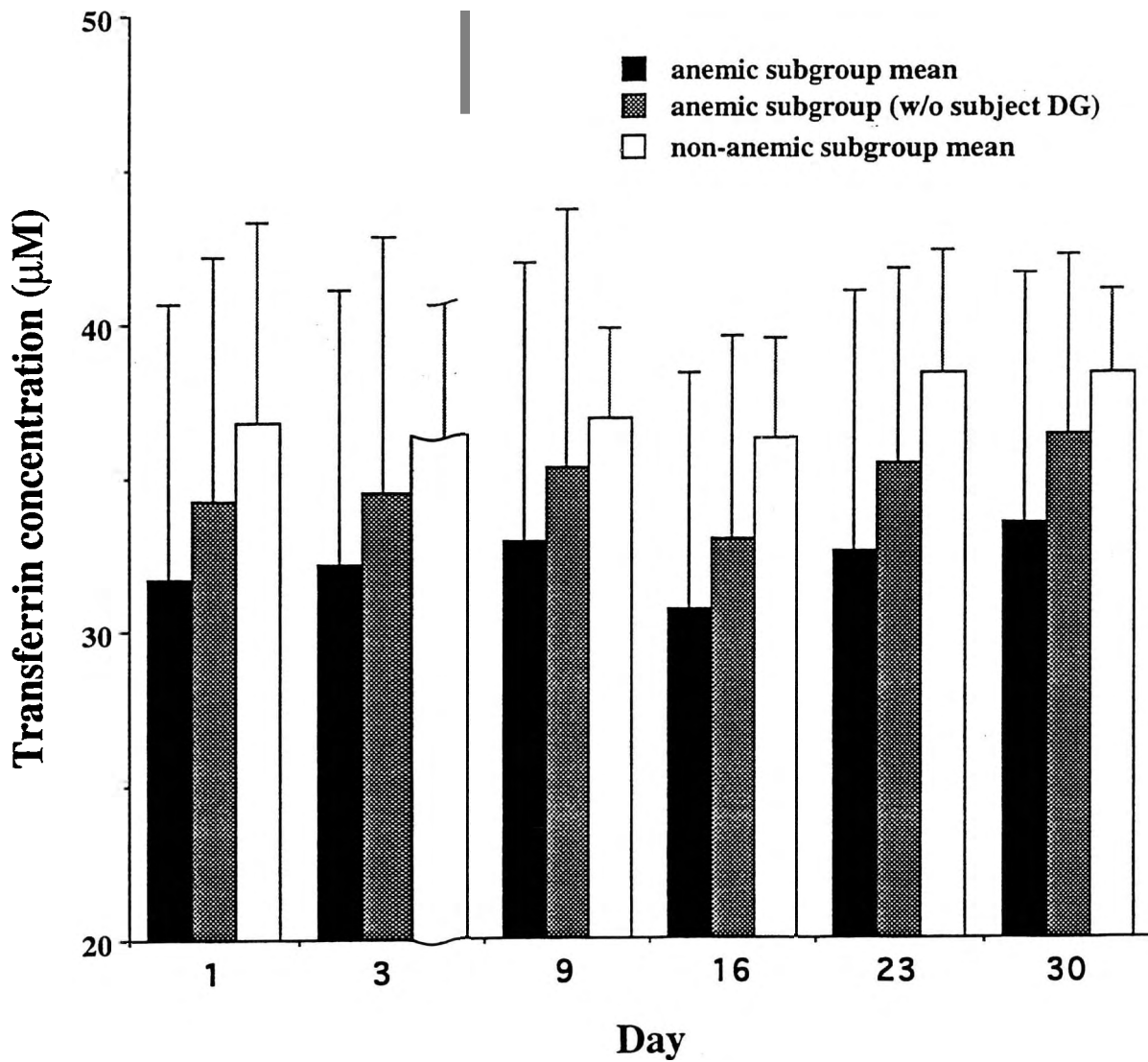


Figure B7 Serial change in mean (\pm SD) serum transferrin concentration with methotrexate treatment in anemic and non-anemic RA subjects. Days 1 and 3 are pre-methotrexate; days 9-30 are during methotrexate treatment. Data are missing for non-anemic subjects EM and AC and anemic subject DG on day 3.

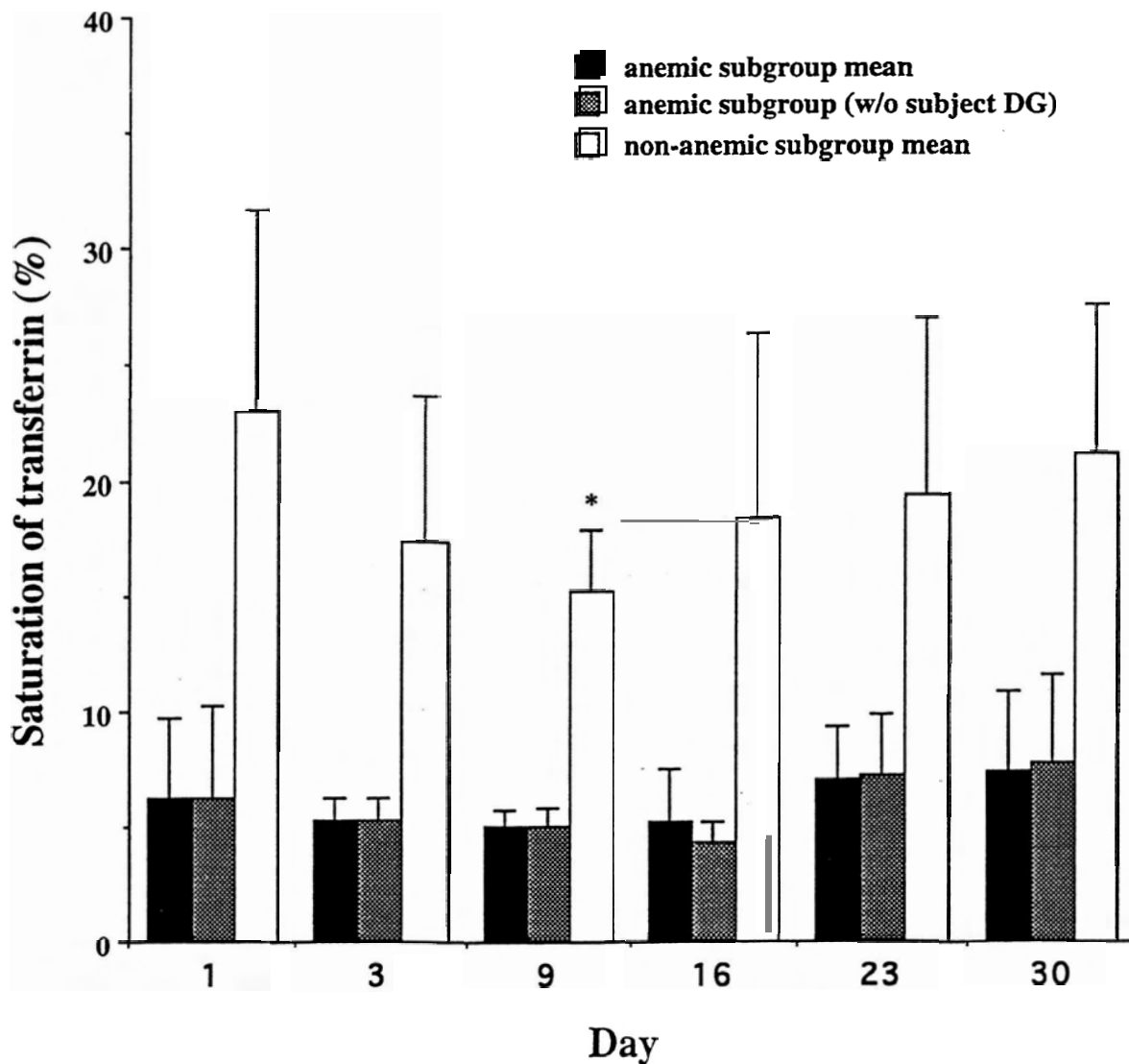


Figure B8 Serial change in mean (\pm SD) percentage saturation of transferrin with methotrexate treatment in anemic and non-anemic RA subjects. Days 1 and 3 are pre-methotrexate; days 9-30 are during methotrexate treatment. Data are missing for non-anemic subjects EM and AC and anemic subject DG on day 3.

* significantly different ($p \leq 0.05$) from the pre-methotrexate measure on day 1.

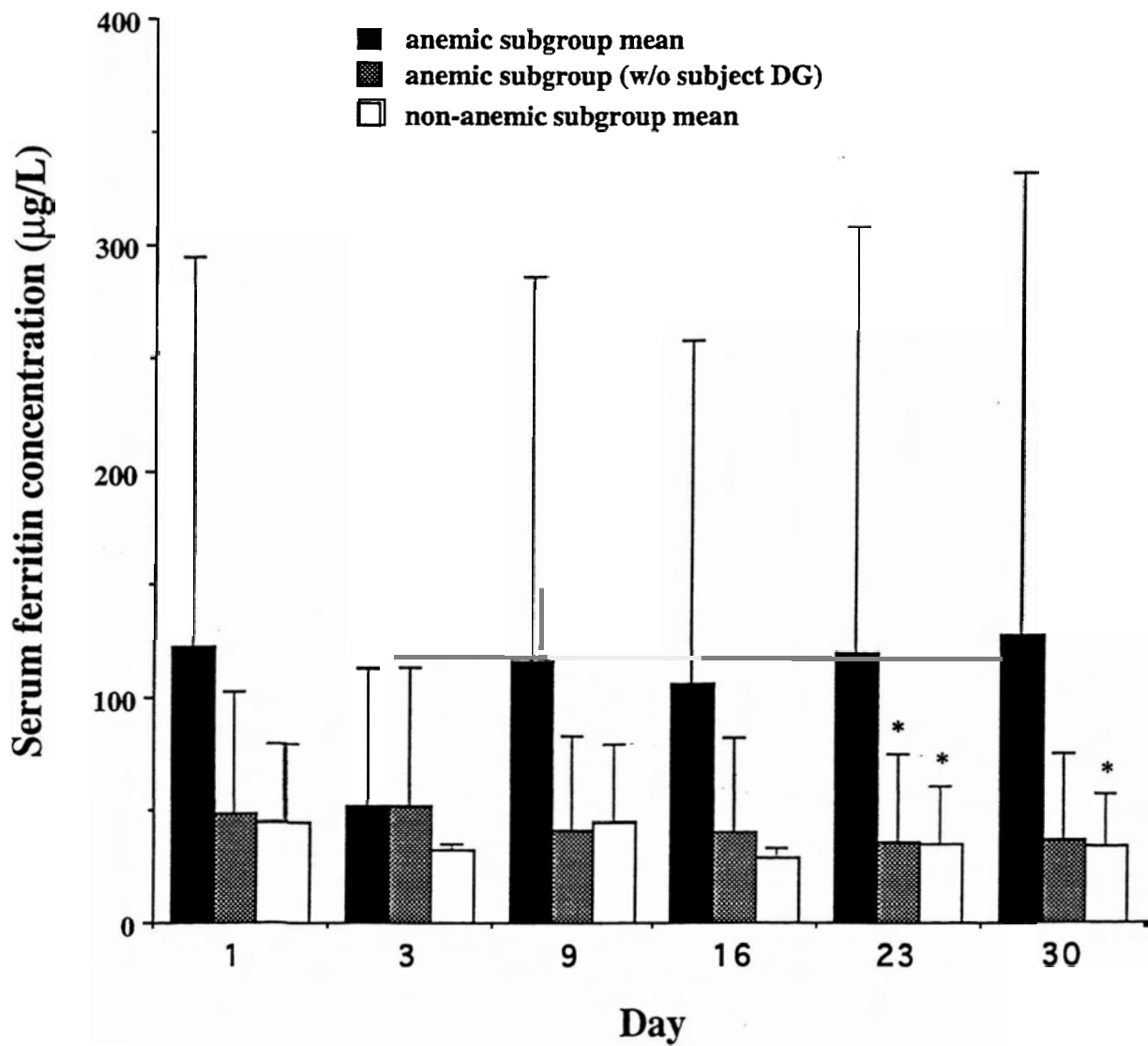


Figure B9 Serial change in mean (\pm SD) serum ferritin concentration with methotrexate treatment in anemic and non-anemic RA subjects. Days 1 and 3 are pre-methotrexate; days 9-30 are during methotrexate treatment. Data are missing for non-anemic AC and anemic subject DG on day 3 and non-anemic subject AC on day 23.

* significantly different ($p \leq 0.05$) from the pre-methotrexate measure on day 1.

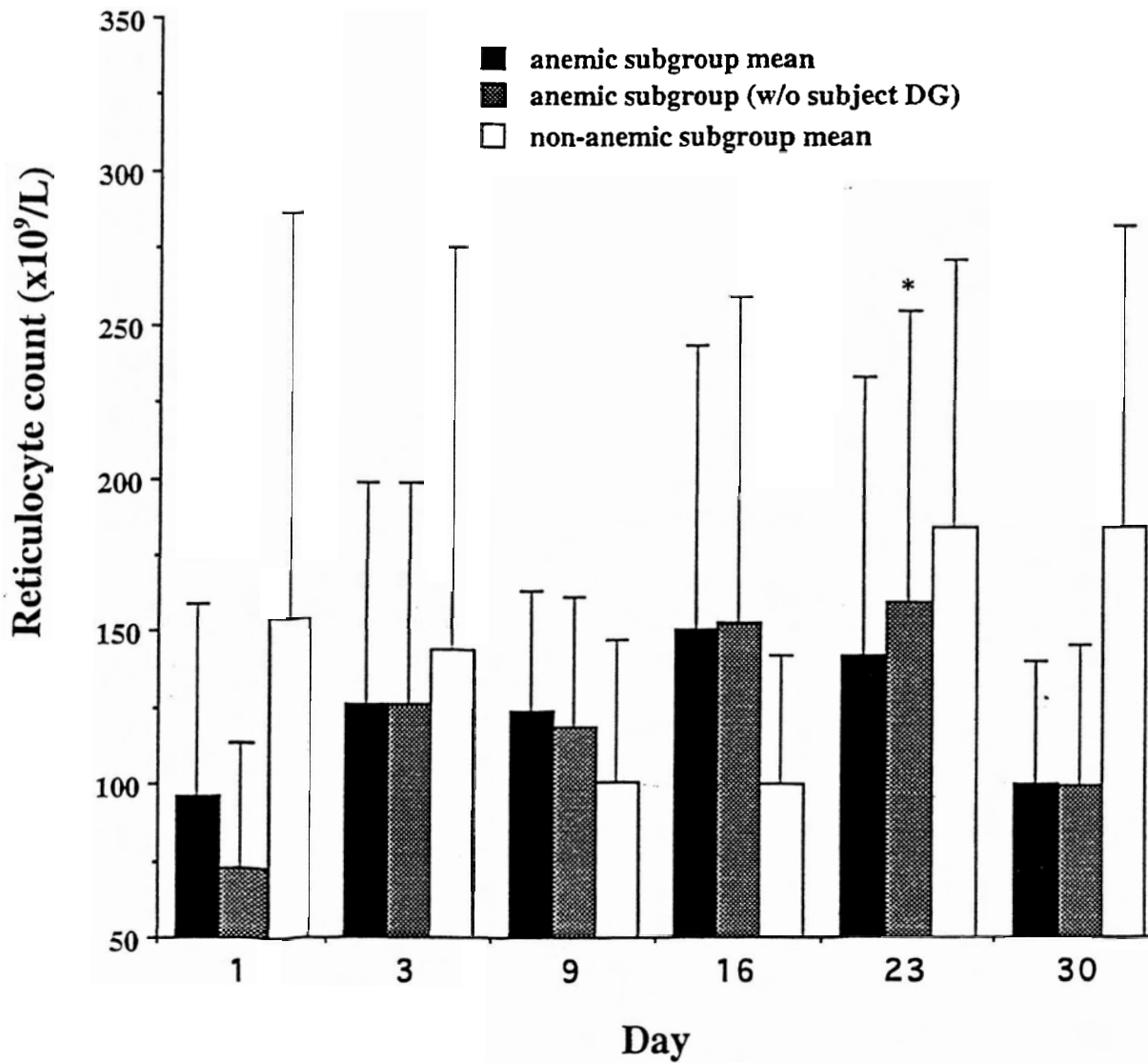


Figure B10 Serial change in mean (\pm SD) reticulocyte count with methotrexate treatment in anemic and non-anemic RA subjects. Days 1 and 3 are pre-methotrexate; days 9-30 are during methotrexate treatment. Data are missing for non-anemic subject AC on days 3 and 9.

* significantly different ($p \leq 0.05$) from the pre-methotrexate measure on day 1.

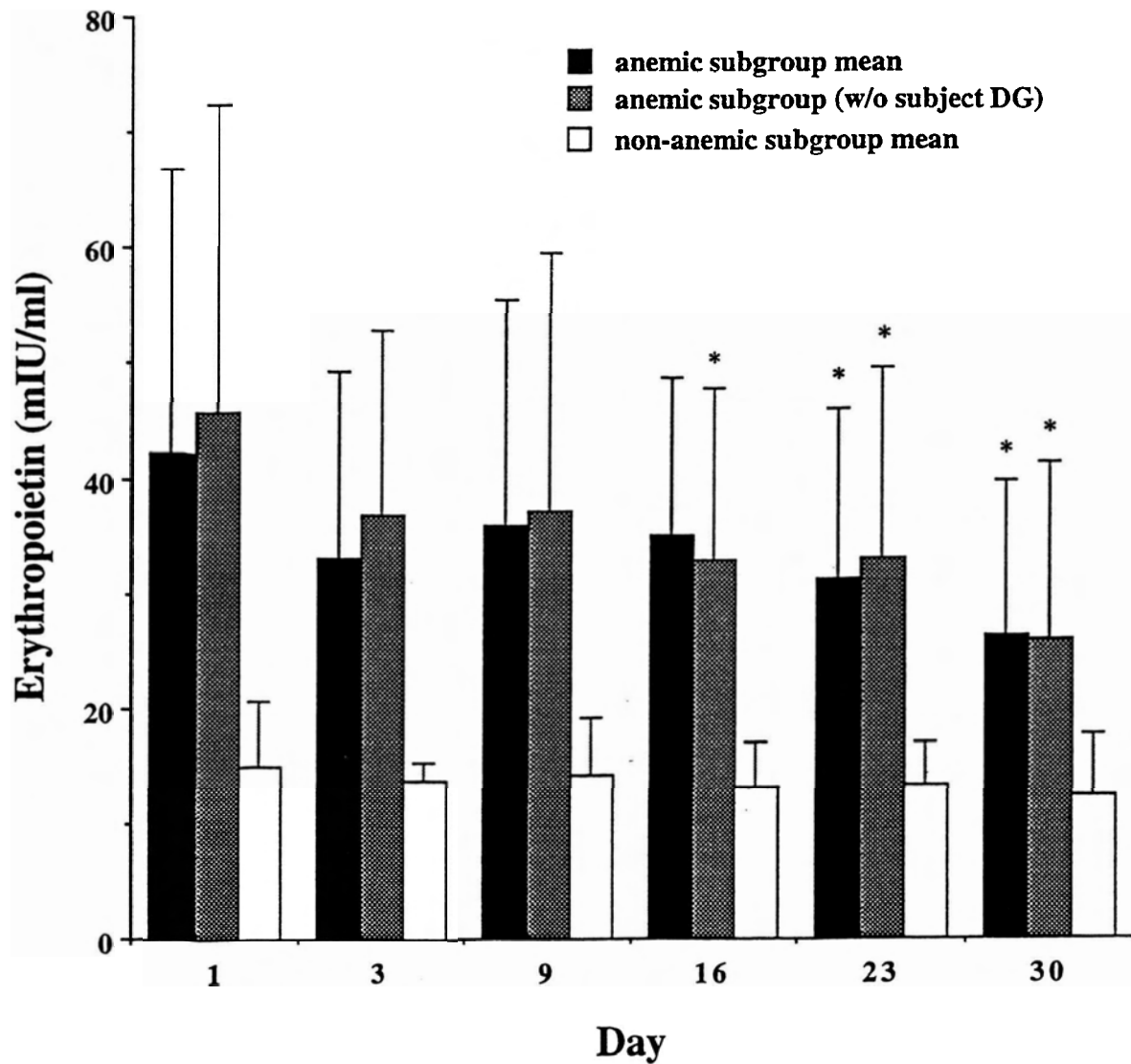


Figure B11 Serial change in mean (\pm SD) plasma erythropoietin concentration with methotrexate treatment in anemic and non-anemic RA subjects. Days 1 and 3 are pre-methotrexate; days 9-30 are during methotrexate treatment. Data are missing for non-anemic subject EM on day 3.

* significantly different ($p \leq 0.05$) from the pre-methotrexate measure on day 1.

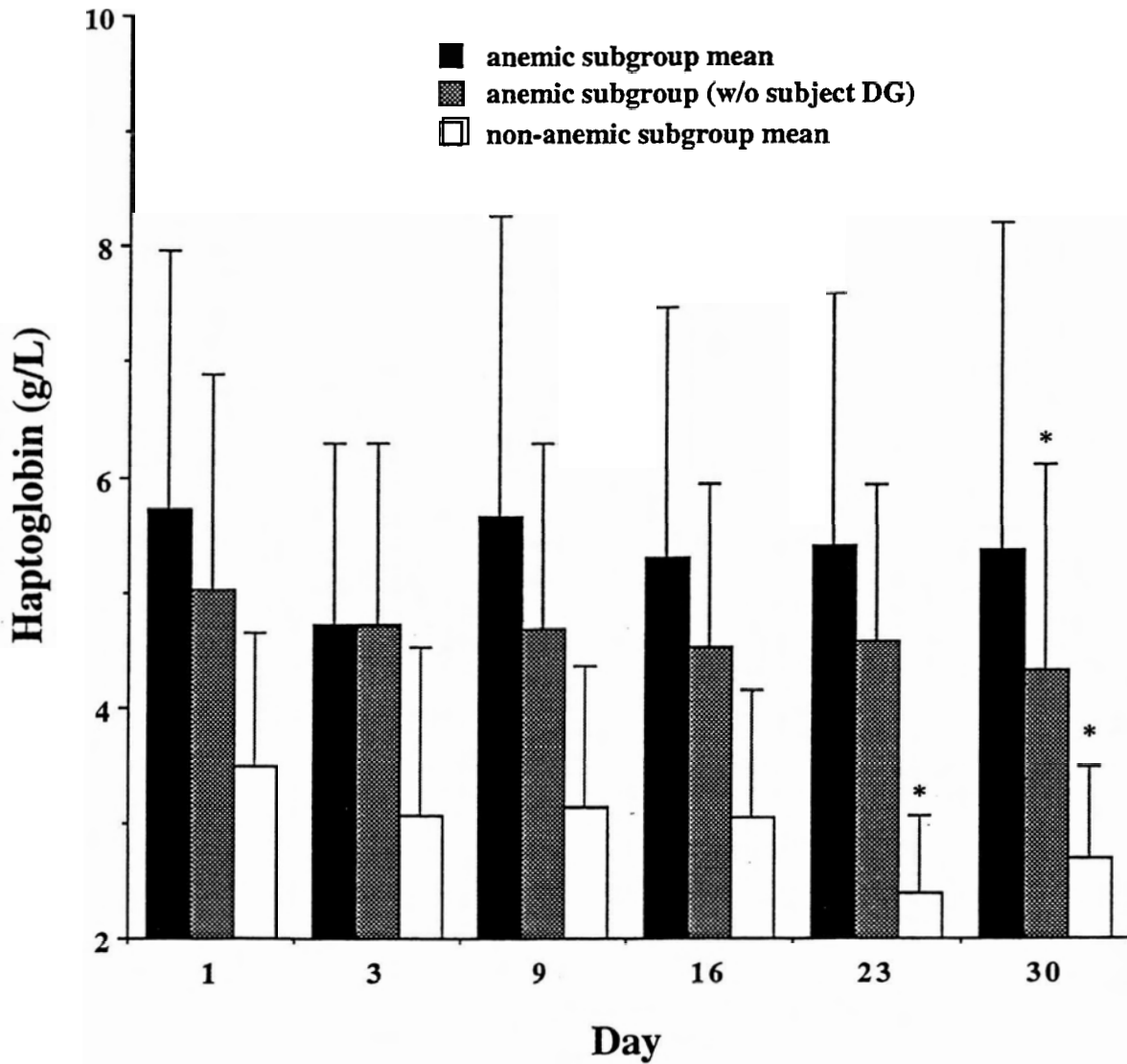


Figure B12 Serial change in mean (\pm SD) serum haptoglobin concentration with methotrexate treatment in anemic and non-anemic RA subjects. Days 1 and 3 are pre-methotrexate; days 9-30 are during methotrexate treatment. Data are missing for non-anemic subjects EM and AC and anemic subject DG on day 3 and non-anemic subject LB on day 23.

* significantly different ($p \leq 0.05$) from the pre-methotrexate measure on day 1.

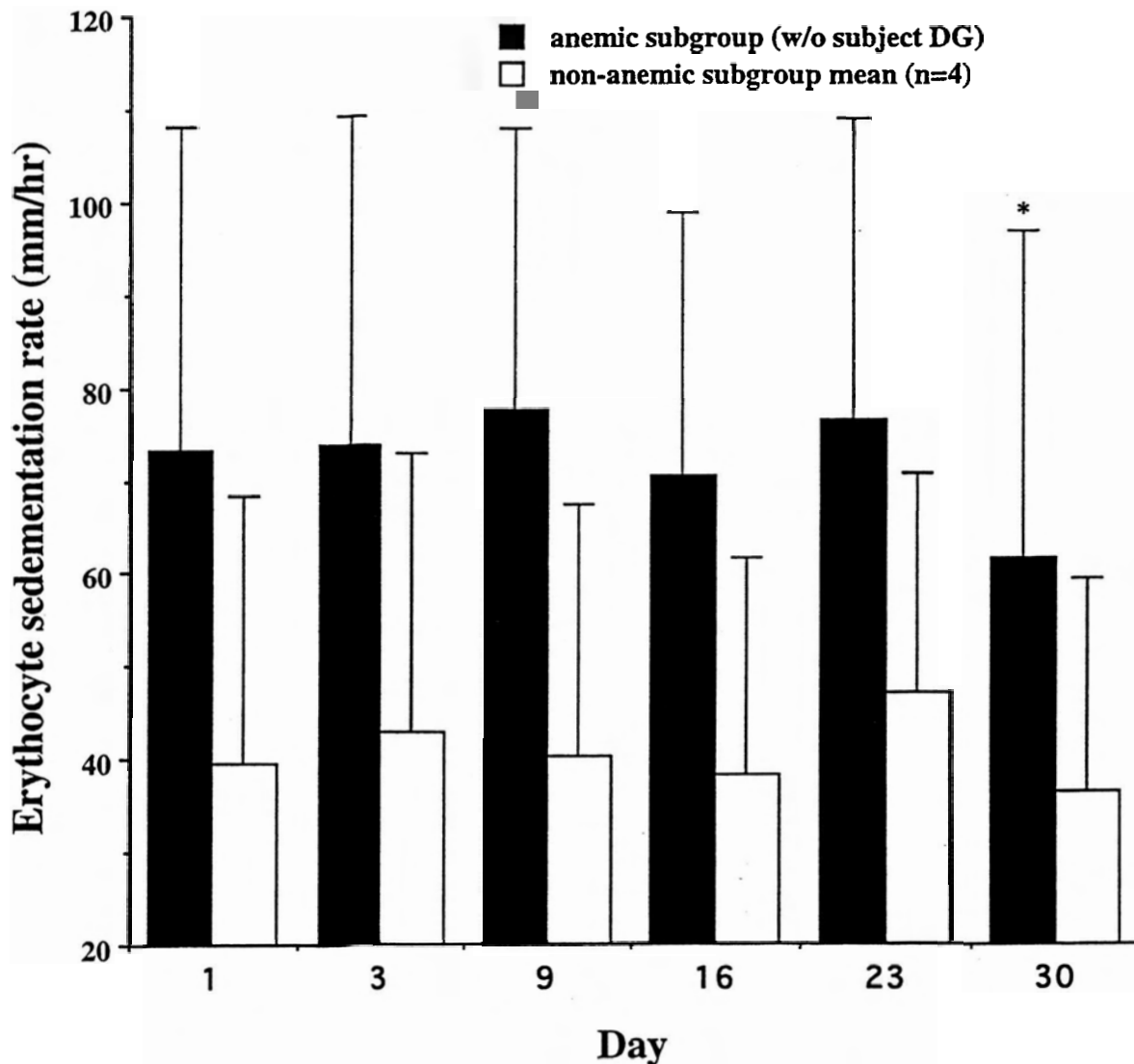


Figure B13 Serial change in mean (\pm SD) erythrocyte sedimentation rate with methotrexate treatment in anemic and non-anemic RA subjects. Days 1 and 3 are pre-methotrexate; days 9-30 are during methotrexate treatment. Data are missing for non-anemic subject AC on day 3 and anemic subject LT on day 23. There are no data for anemic subject DG and non-anemic subject EM.

* significantly different ($p \leq 0.05$) from the pre-methotrexate measure on day 1.

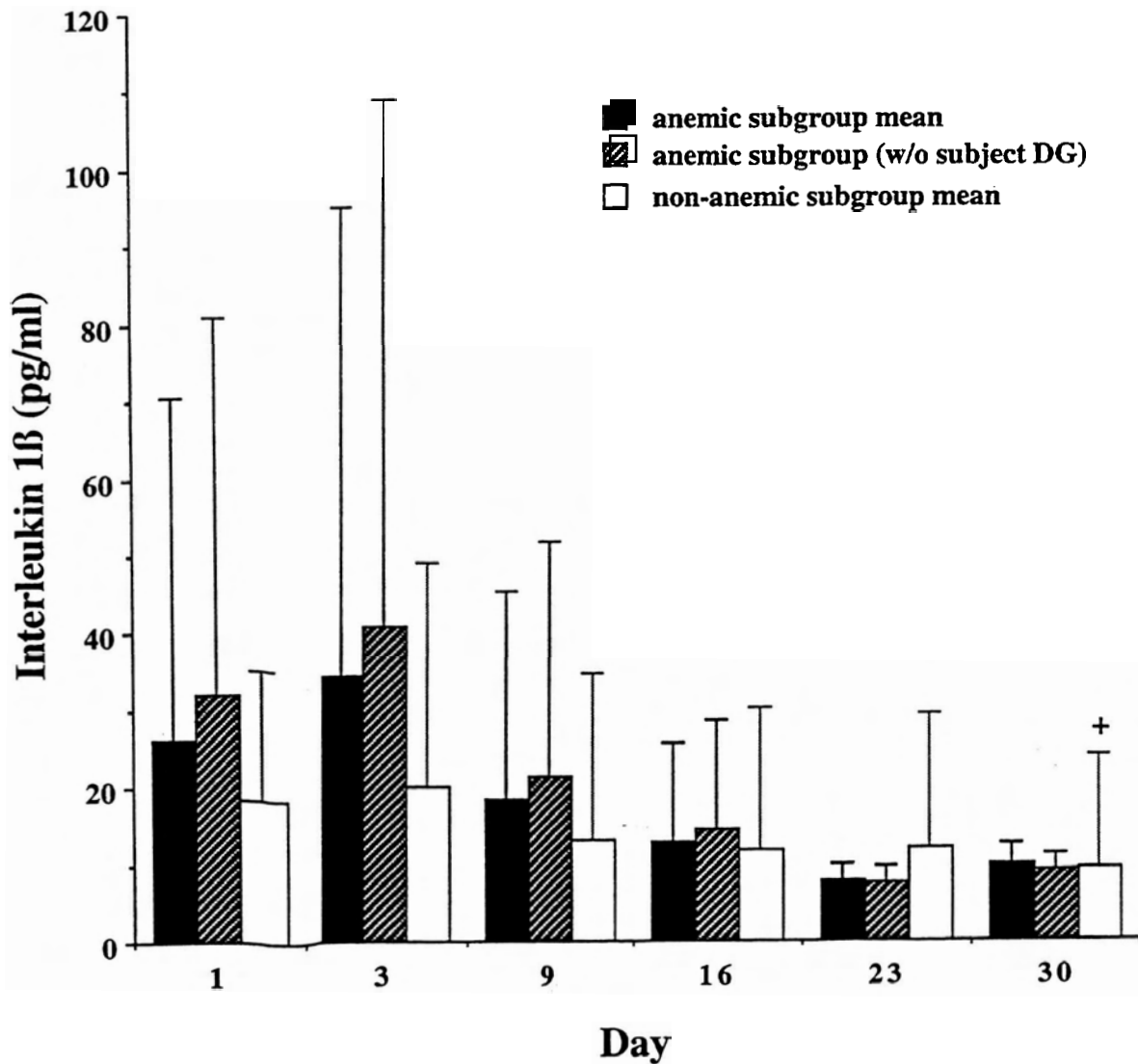


Figure B14. Serial change in mean (\pm SD) plasma interleukin-1 β concentration with methotrexate treatment in anemic and non-anemic RA subjects. Days 1 and 3 are pre-methotrexate; days 9-30 are during methotrexate treatment. Data are missing for non-anemic subject AC on day 3.

* significantly different ($p \leq 0.05$) from the pre-methotrexate measure on day 1.

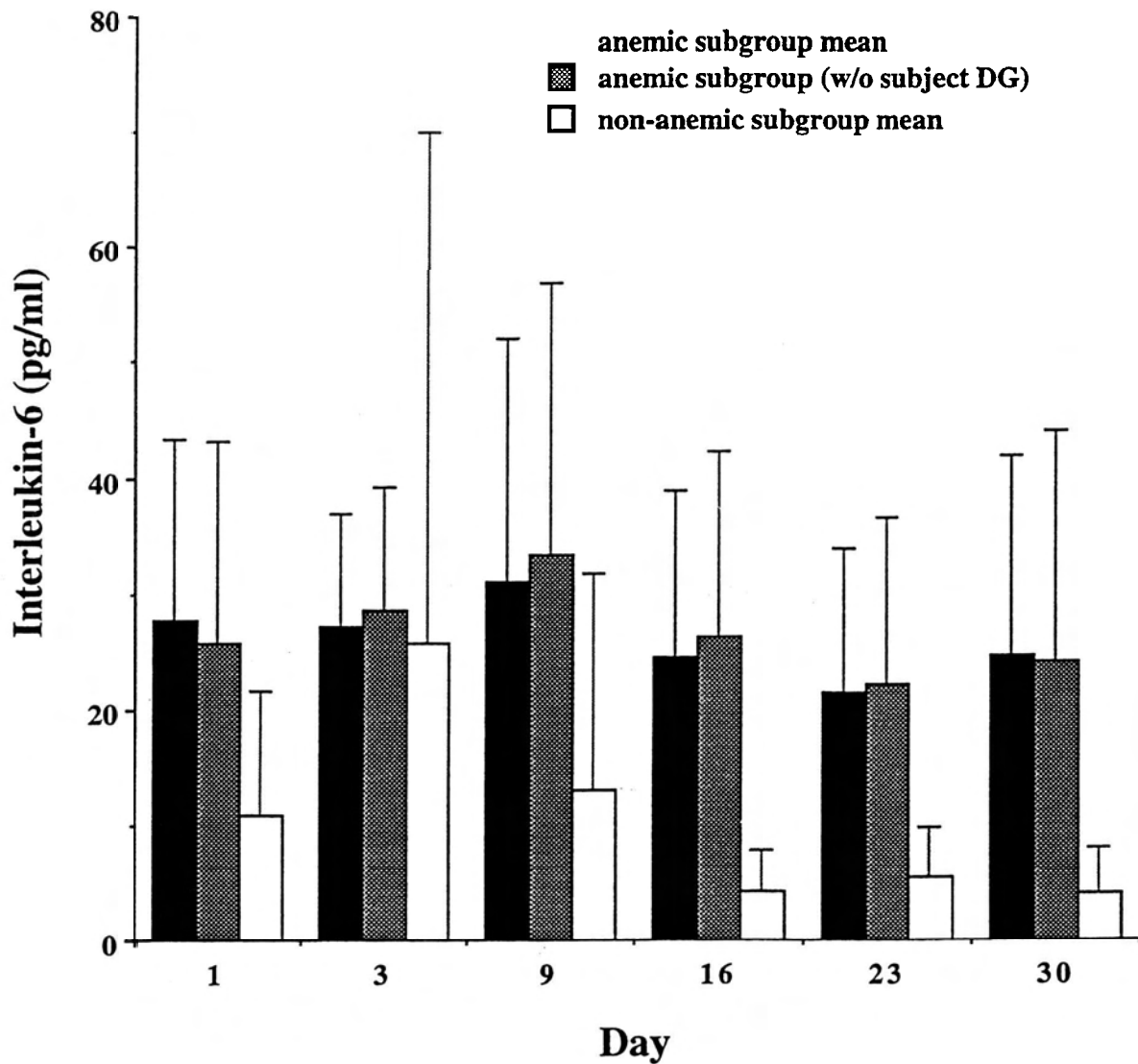


Figure B15. Serial change in mean (\pm SD) plasma interleukin-6 concentration with methotrexate treatment in anemic and non-anemic RA subjects. Days 1 and 3 are pre-methotrexate; days 9-30 are during methotrexate treatment. Data are missing for non-anemic subject AC on day 3.

+ significantly different ($p \leq 0.05$) from the pre-methotrexate measure on day 3.

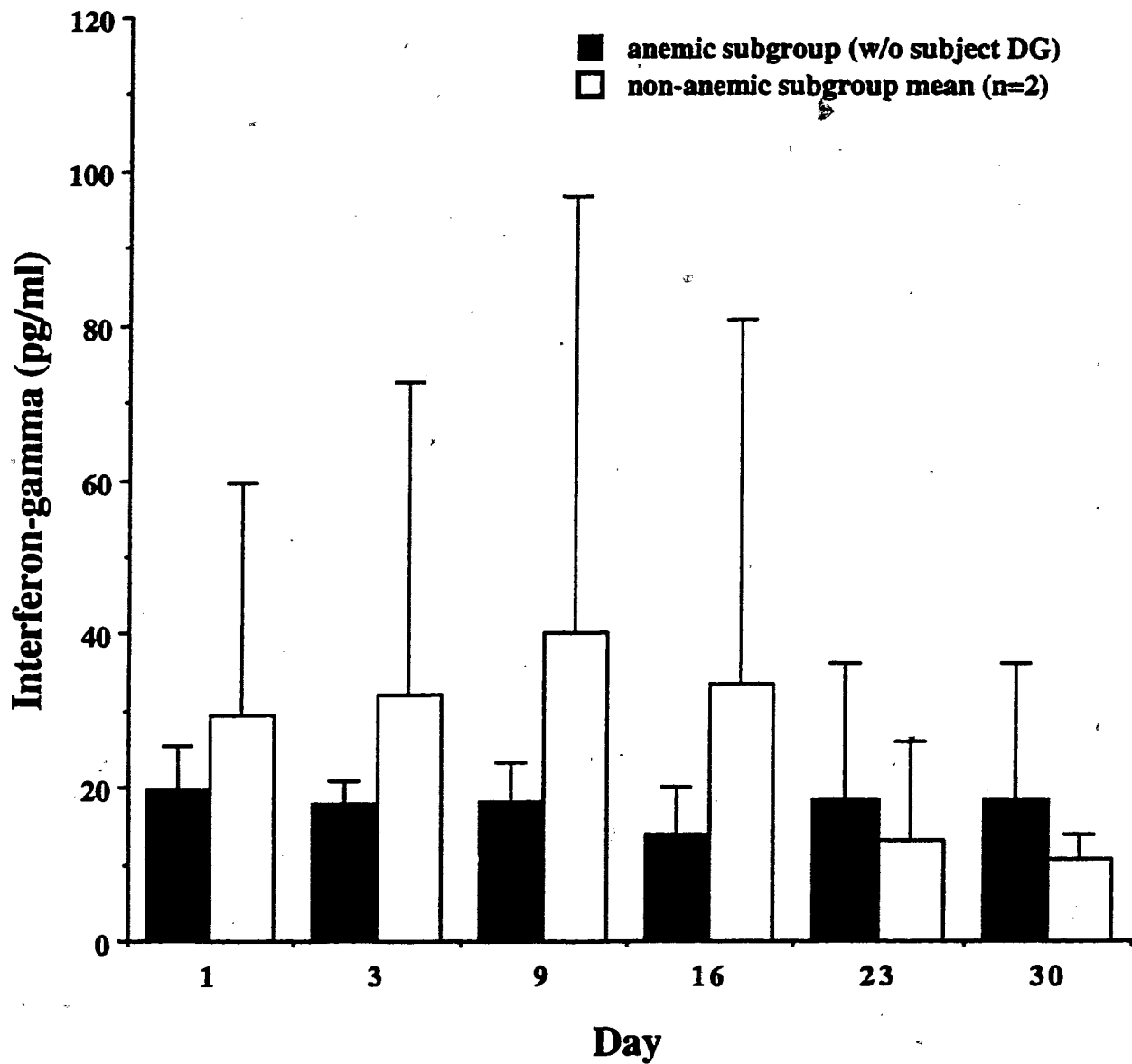


Figure B16 Serial change in mean (\pm SD) plasma interferon-gamma concentration with methotrexate treatment in anemic and non-anemic RA subjects. Days 1 and 3 are pre-methotrexate; days 9-30 are during methotrexate treatment. There are no data for anemic subject DG and non-anemic subjects LB, AC and EM.

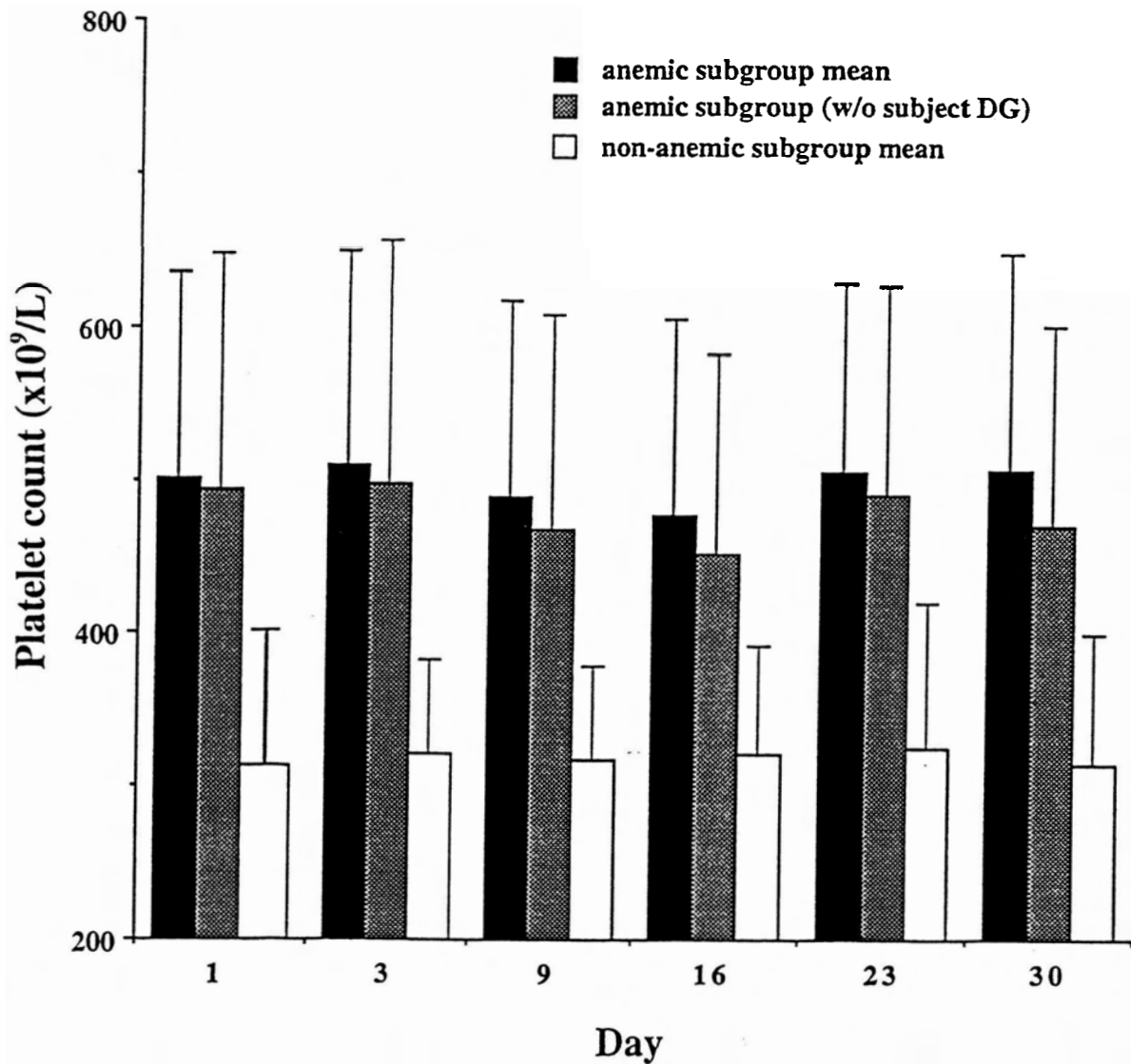


Figure B17 Serial change in mean (\pm SD) platelet count with methotrexate treatment in anemic and non-anemic RA subjects. Days 1 and 3 are pre-methotrexate; days 9-30 are during methotrexate treatment. Data are missing for non-anemic subject AC on day 3.

Legend to Figures B18-B27

In the top panel of each figure subject data is plotted with control data (\pm SD) for ^{51}Cr -RBC count from the heart; the center panel shows ^{51}Cr -RBC count from the liver and the bottom panel shows ^{51}Cr -RBC count from the spleen. ^{51}Cr -RBC organ count data are expressed as a percentage of day 1 count and are plotted against day of measurement. Data for day 3 corresponds with the ^{51}Cr -RBC organ count prior to methotrexate treatment. ^{51}Cr -RBC organ count data for anemic and non-anemic subjects respectively are presented in Figures B18-B22 and Figures B23-B27. On the bottom panel of Figures B18-B27, an RBC concentration value ($\times 10^{12}/\text{L}$) is indicated where a blood test corresponded with a ^{51}Cr -RBC spleen count. The first RBC value reported corresponds with the ^{51}Cr -RBC organ count prior to methotrexate treatment. The remainder of the RBC values correspond with the respective ^{51}Cr -RBC measure taken during methotrexate treatment.

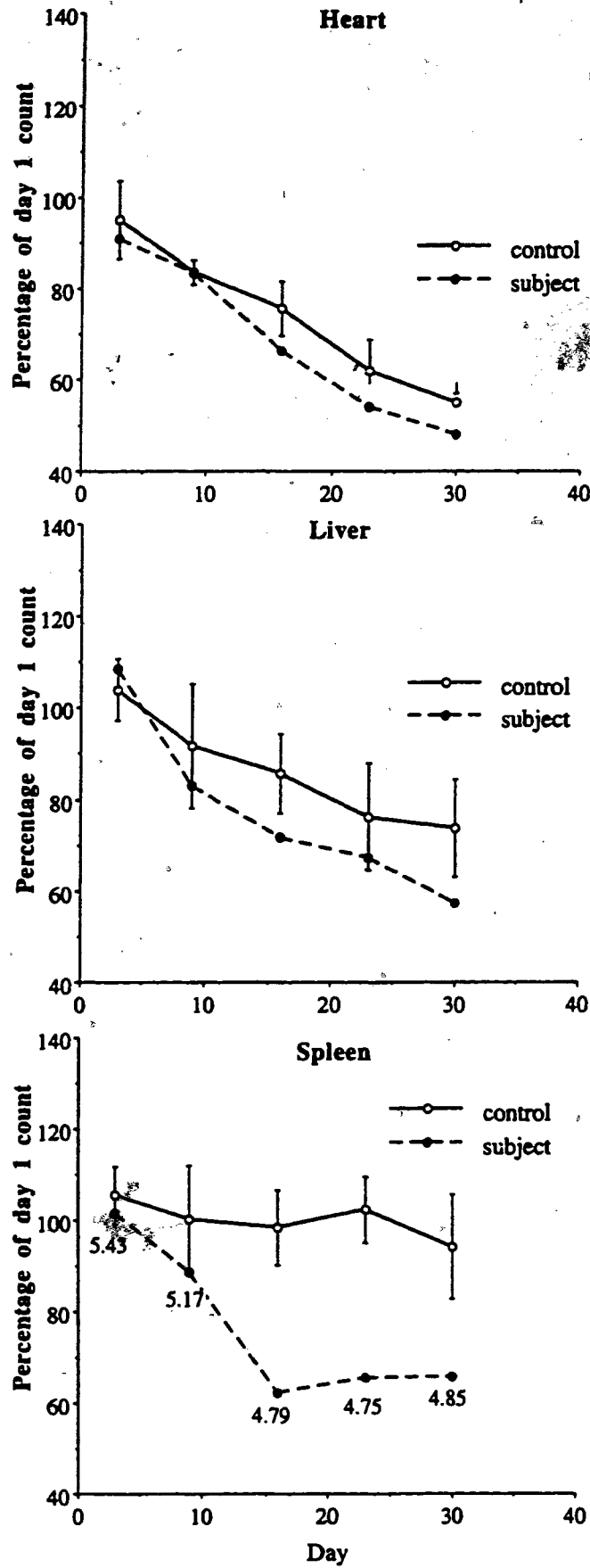


Figure B18 ⁵¹Cr-RBC organ count for subject DG compared with a control count.

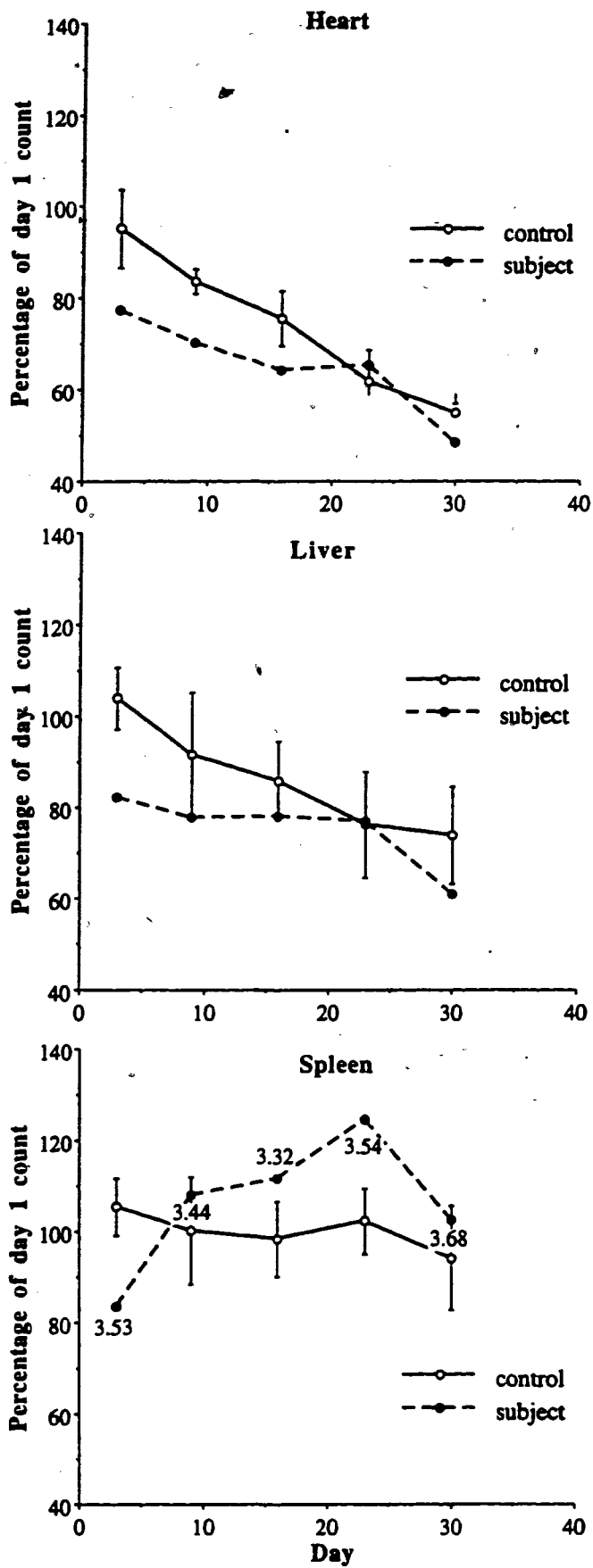


Figure B19 ^{51}Cr -RBC organ count for subject MM compared with a control count.

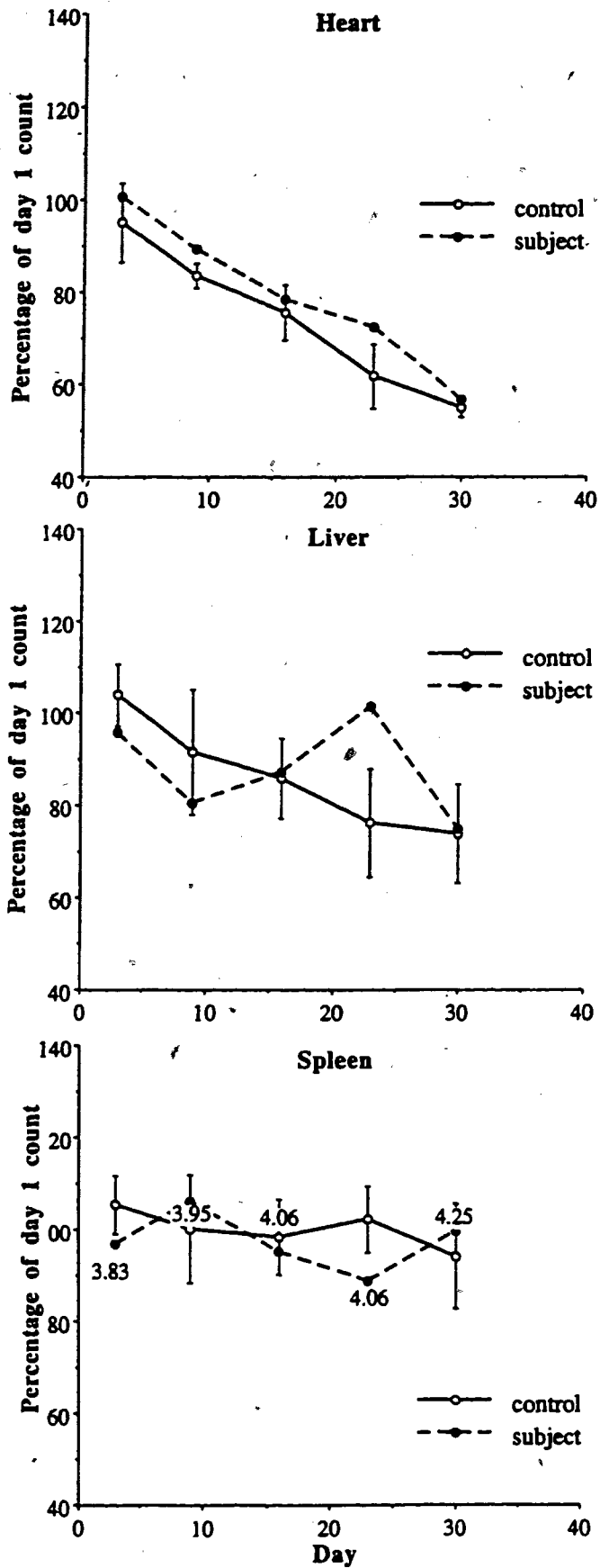


Figure B20 ⁵¹Cr-RBC organ count for subject BC compared with a control count.

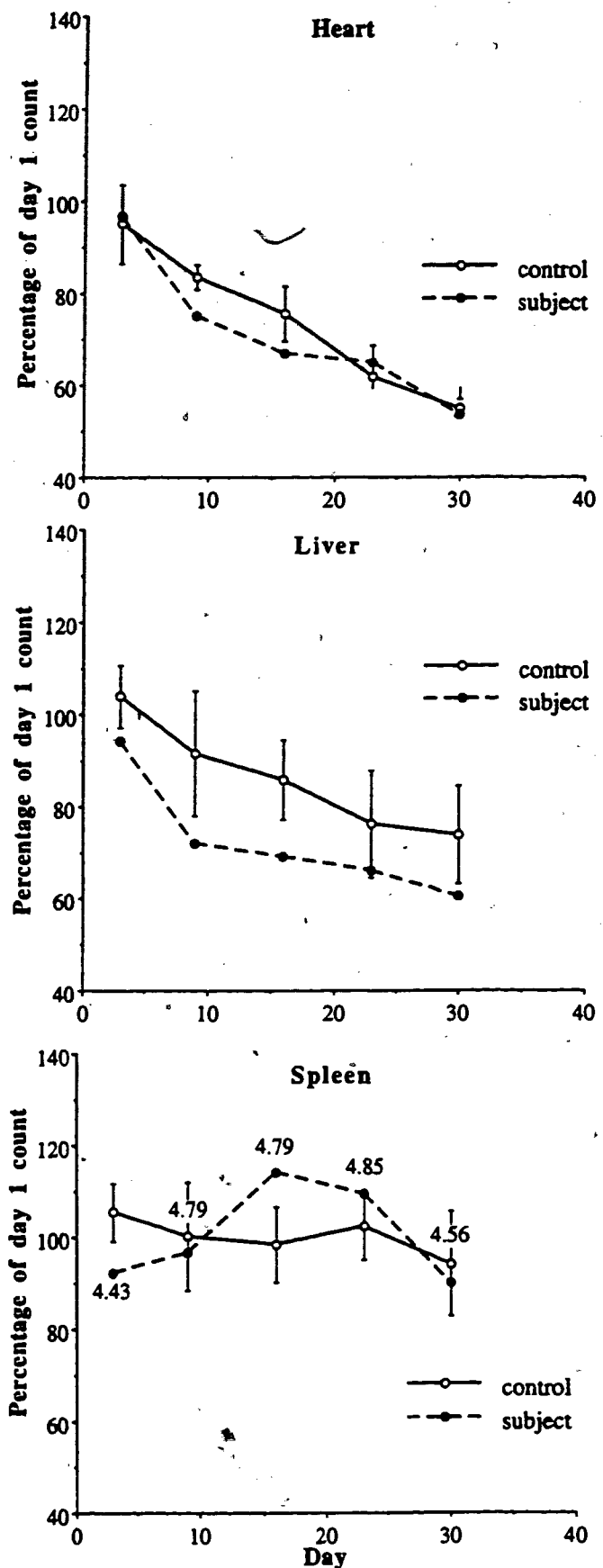


Figure B21 ⁵¹Cr-RBC organ count for subject LT compared with a control count.

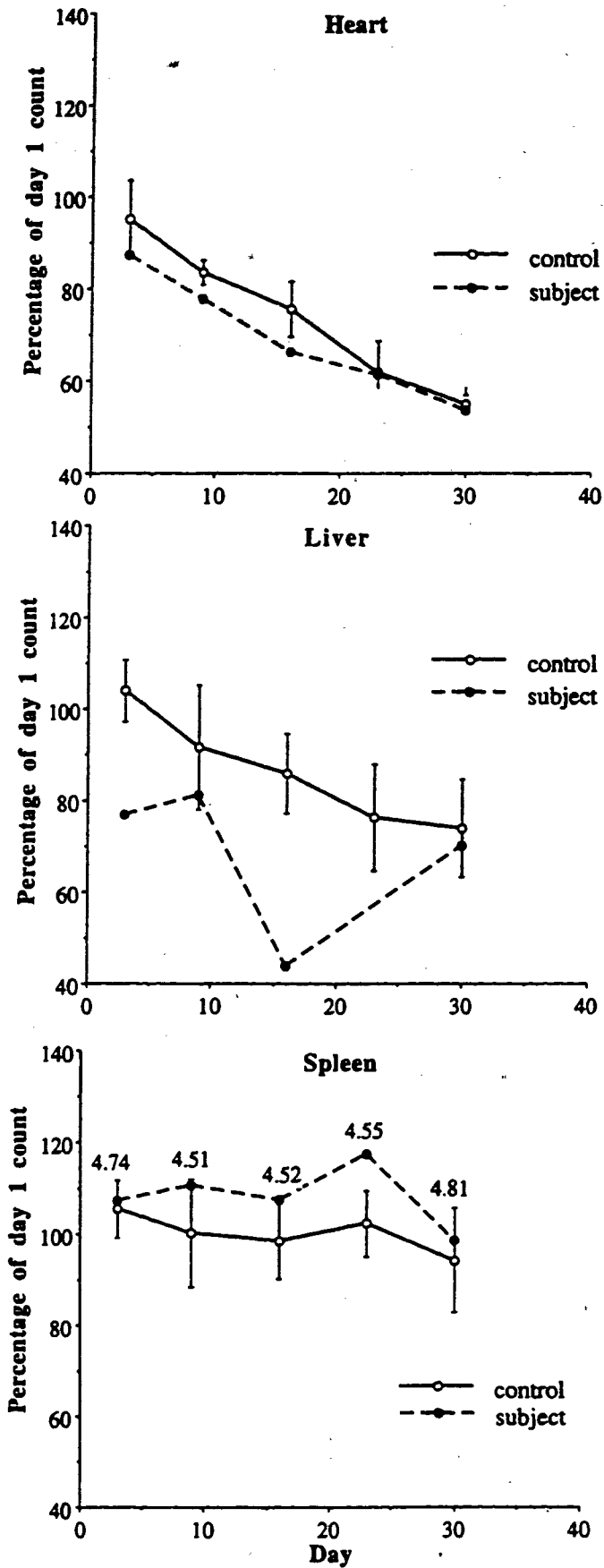


Figure B22 ⁵¹Cr-RBC organ count for subject EU compared with a control count.

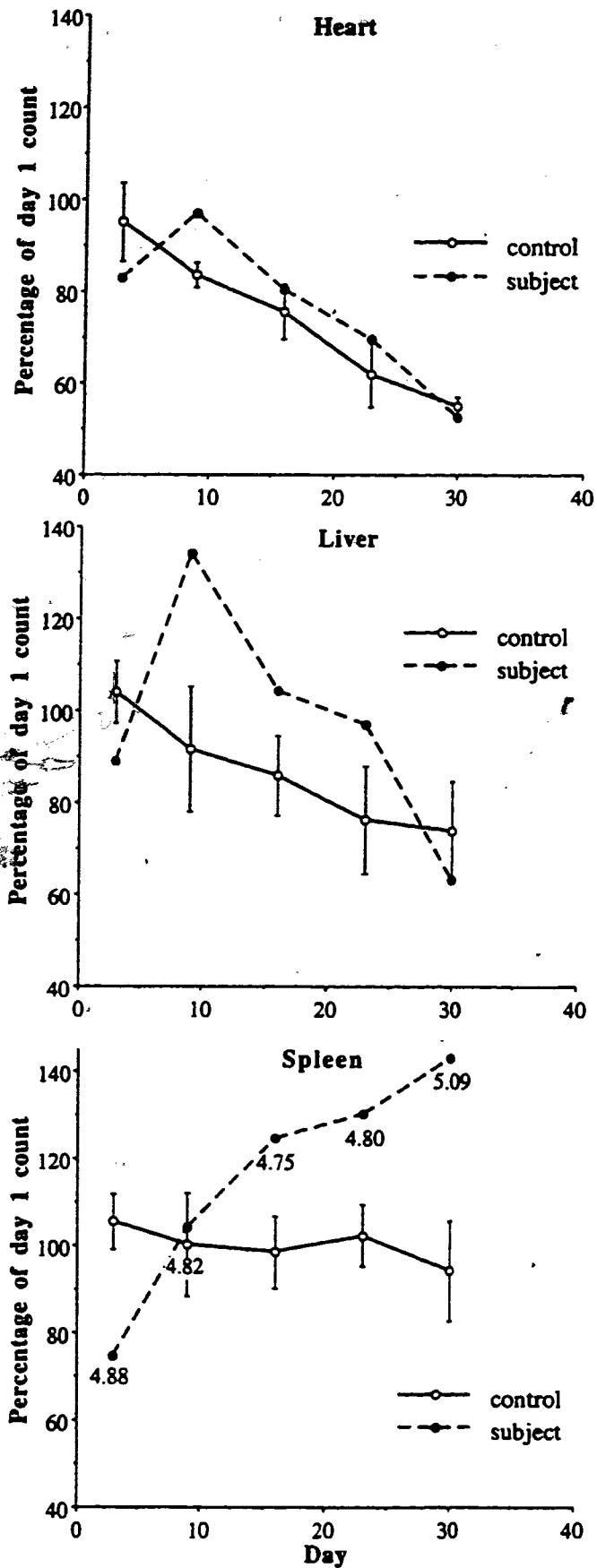


Figure B23 ⁵¹Cr-RBC organ count for subject AC compared with a control count.

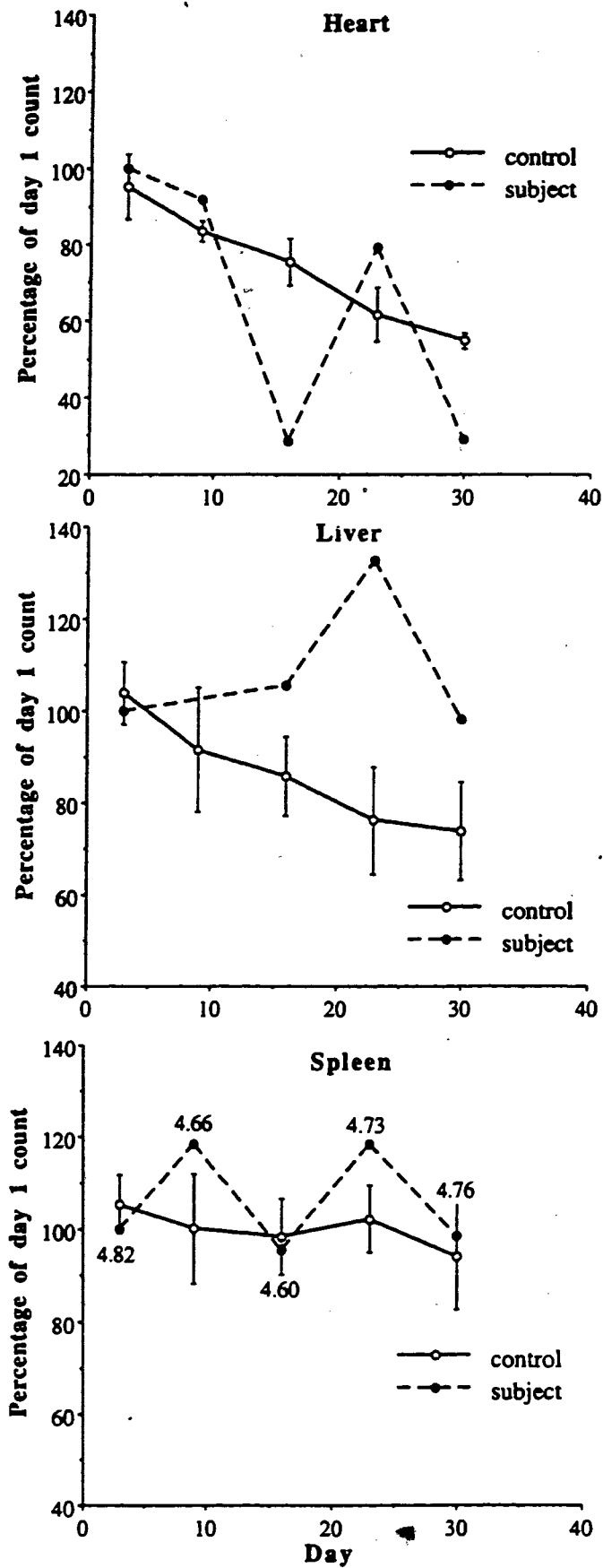


Figure B24 ⁵¹Cr-RBC organ count for subject IS compared with a control count.

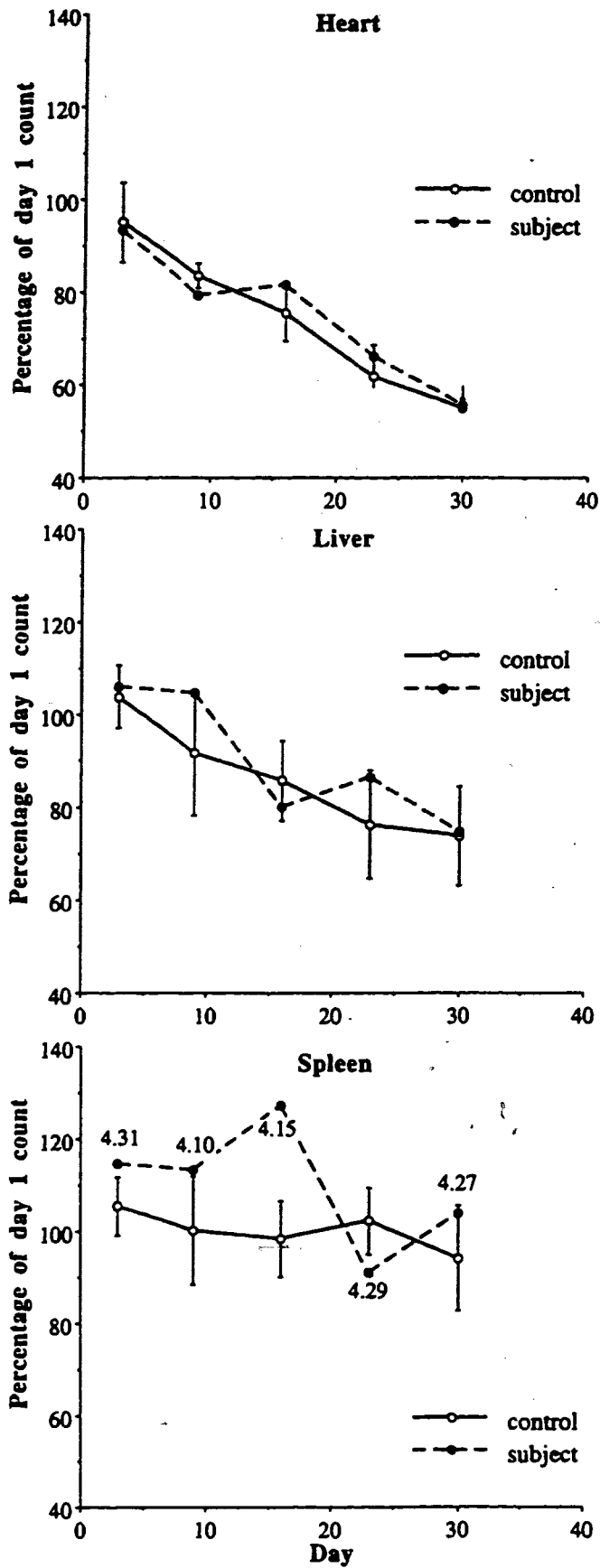


Figure B25 ⁵¹Cr-RBC organ count for subject LB compared with a control count.

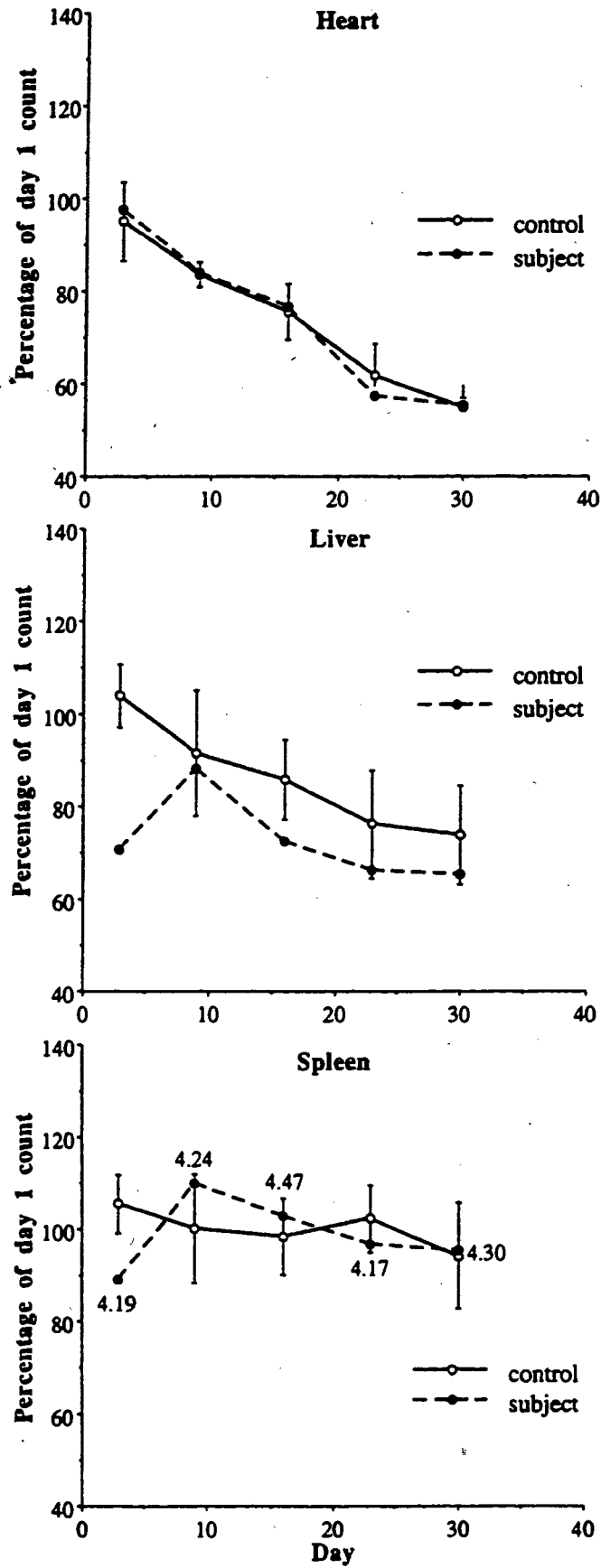


Figure B26 ⁵¹Cr-RBC organ count for subject BM compared with a control count.

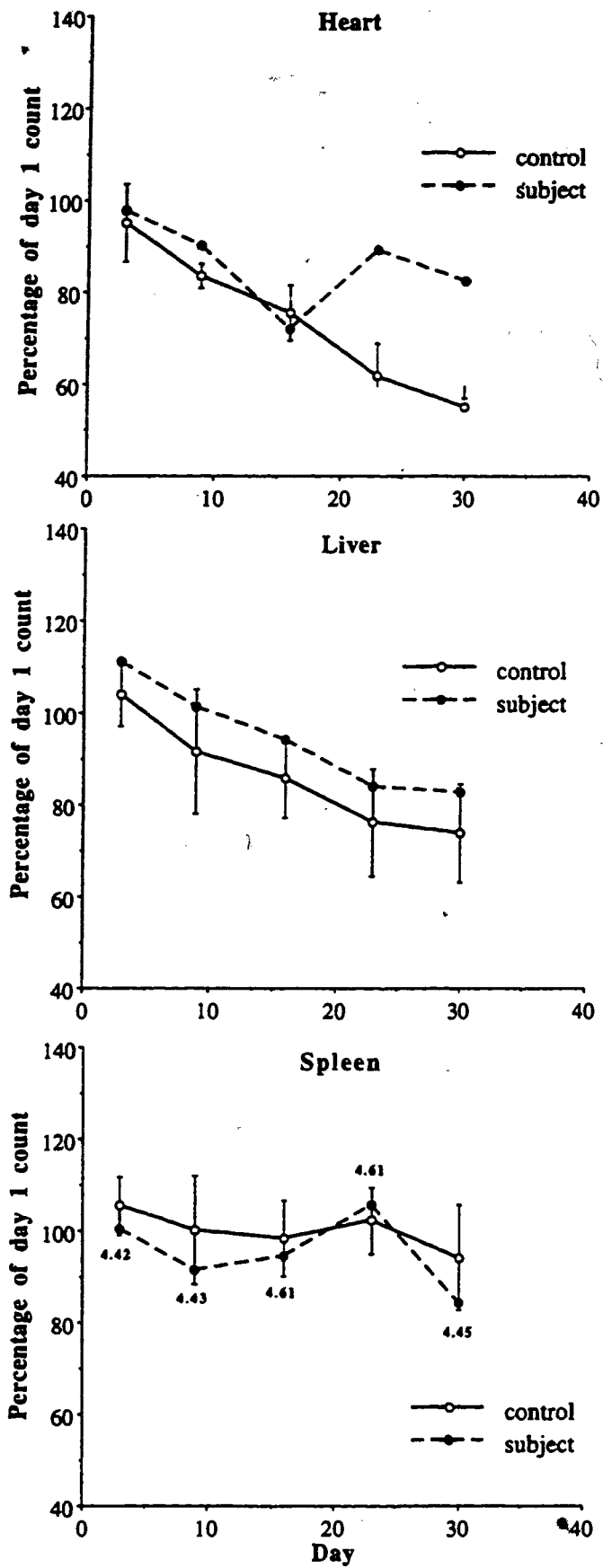


Figure B27 ⁵¹Cr-RBC organ count for subject EM compared with a control count.

Table B1 Change in selected clinical and laboratory measures after 4 weeks of methotrexate treatment in Rheumatoid Arthritic subjects.

| Subject | Clinical | Laboratory | RBC and reticulocyte count |
|-------------------|--|---|---|
| anemic | | | |
| DG | generally unchanged; improved energy and sense of well-being | haptoglobin: 8.51-9.63 g/L IL-1 β : 3-15 pg/ml IL-6: 35-27 pg/ml; (no IFN- γ measure) | 5.26-4.85 x10 ¹² /L 189-102 x10 ⁹ /L |
| MM | decreased number of swollen joints | haptoglobin: 6.23-4.94 g/L IL-6: 21-45 pg/ml IFN- γ : 25-17 pg/ml | 3.57-3.68 x10 ¹² /L 46-74 x10 ⁹ /L |
| BC | unchanged | ESR: 102-84 mm/hr IL-6: 51-37 pg/ml IFN- γ : 21-44 pg/ml | 3.85-4.25 x10 ¹² /L 73-77 x10 ⁹ /L MCV: 81.5-78.8 fL |
| EU | unchanged | ESR: 44-26 mm/hr haptoglobin: 4.47-3.67 g/L IL-18: 115-18 pg/ml IL-6: 20-12 pg/ml IFN- γ : 22-11 pg/ml | 4.73-4.81 x10 ¹² /L 43-77 x10 ⁹ /L |
| LT | unchanged | IFN- γ : 12-5 (wk 3) 3 (wk 4) pg/ml IL-6: 11-13 (wk 3) 3 (wk 4) pg/ml | 4.65-4.85 (wk 3) 4.56 (wk 4) x10 ¹² /L 130-179 (wk 3) 169 (wk 4) x10 ⁹ /L MCV: 69.2-71.3 fL (wk3) 72.1 fL (wk 4) |
| non-anemic | | | |
| EM | decreased number of painful and swollen joints | unchanged (no IFN- γ measure) (IL-18: 40-35 pg/ml, 2nd pre-drug: 71-35 (wk 4)) | 4.71-4.45 x10 ¹² /L 42-138 x10 ⁹ /L |
| AC | decreased morning stiffness and number of swollen joints; improved sense of well-being | ESR: 53-37 mm/hr haptoglobin: 4.35-2.84 g/L (no IFN- γ measure) | 4.77-5.09 x10 ¹² /L 148-122 x10 ⁹ /L |
| LB | decreased swelling in knees | haptoglobin: 4.86-3.79 g/L IL-18: 32-1 pg/ml IL-6: 29-8 pg/ml; (no IFN- γ measure) | 4.34-4.27 x10 ¹² /L 195-196 x10 ⁹ /L |
| BM | unchanged; improved sense of well-being | haptoglobin: 3.18-1.89 g/L IL-6: 11-6 pg/ml IL-18: 15-5 pg/ml IFN- γ : 51-8 | 4.25-4.30 x10 ¹² /L 30-112 x10 ⁹ /L |
| IS | decreased morning stiffness and number of swollen and painful joints, improved sense of well-being | ESR: 10-15 mm/hr IFN- γ : 8-13 pg/ml | 5.03-4.76 x10 ¹² /L 357-352 x10 ⁹ /L |

Clinical measures monitored were morning stiffness (hours), number of swollen joints and number of painful joints. Included in this section is a subject's report of improvement in energy level and/or sense of well-being. Laboratory measures are reported only with a response of >20%. MCV (mean corpuscular volume-erythrocyte) is reported where there was a significant change in its value. RBC: intravascular red blood cell concentration.

Appendix C

Table C1 Demographic, blood volume and laboratory measures in subjects who developed an abnormality after the red cell label.

| Subject | Sex | Age, yr | Wt, kg | Ht, cm | TBV, ml/kg | RCV, ml/kg | PV, ml/kg |
|---------|-----|---------|--------|--------|------------|------------|-----------|
| LH | F | 48 | 57.7 | 156 | 104.5 | 37.1 | 67.4 |
| LK | F | 64 | 49.8 | 159.5 | 66.5 | 24 | 42.5 |
| PT | F | 54 | 65.3 | 165 | 64.9 | 22.9 | 42 |

| Subject | Day | IL-6, pg/ml | TNF- α , pg/ml | IL-1 β , pg/ml | IFN- γ , pg/ml | Epo, mIU/ml | Hb, g/L |
|---------|-----|-------------|-----------------------|----------------------|-----------------------|-------------|---------|
| LH | 1 | 2.0 | ND | 5.5 | 1.2 | 32.7 | 126 |
| | 30 | 1.2 | ND | 5.8 | 6.4 | 6.6 | 122 |
| LK | 1 | UD | UD | ND | ND | 9.2 | 146 |
| | 30 | UD | UD | ND | ND | 17.3 | 130 |
| PT | 1 | 4.1 | UD | ND | ND | 6.9 | 131 |
| | 16 | ND | ND | ND | ND | ND | 121 |
| | 30 | 2.0 | UD | ND | ND | 9.9 | 124 |

| Subject | Day | RBC $\times 10^{12}/L$ | Hct, % | MCV, fL | Hapto, g/L | Bili, μM |
|---------|-----|------------------------|--------|---------|------------|---------------|
| LH | 1 | 4.19 | 36.6 | 87.4 | 2.36 | 6 |
| | 30 | 4.05 | 35.4 | 87.5 | 2.47 | 5 |
| LK | 1 | 4.79 | 42.2 | 88.0 | <0.43 | 41 |
| | 30 | 4.22 | 37.1 | 80.0 | ND | ND |
| PT | 1 | 4.14 | 38.6 | 93.3 | 1.3 | 8 |
| | 16 | 3.81 | 35.5 | 93.2 | ND | ND |
| | 30 | 3.92 | 36.5 | 93.0 | ND | ND |

Subject LH volunteered as a control subject but developed an inflamed knee soon after the data collection began. Therefore, these data were not included with those of the control group. Subject LK volunteered as a control subject, but a previously undiagnosed hemolytic condition (or a hemolytic condition precipitated by the red cell label procedure) was discovered soon after the data collection began. Therefore, these data were not included with those of the control group. Subject PT volunteered as a control subject but became ill with a viral infection soon after the data collection began. Therefore, these data were not included with those of the control group.

Abbreviations (not defined elsewhere): Hapto-haptoglobin, Bili-bilirubin, UD-undetected, ND-not done.

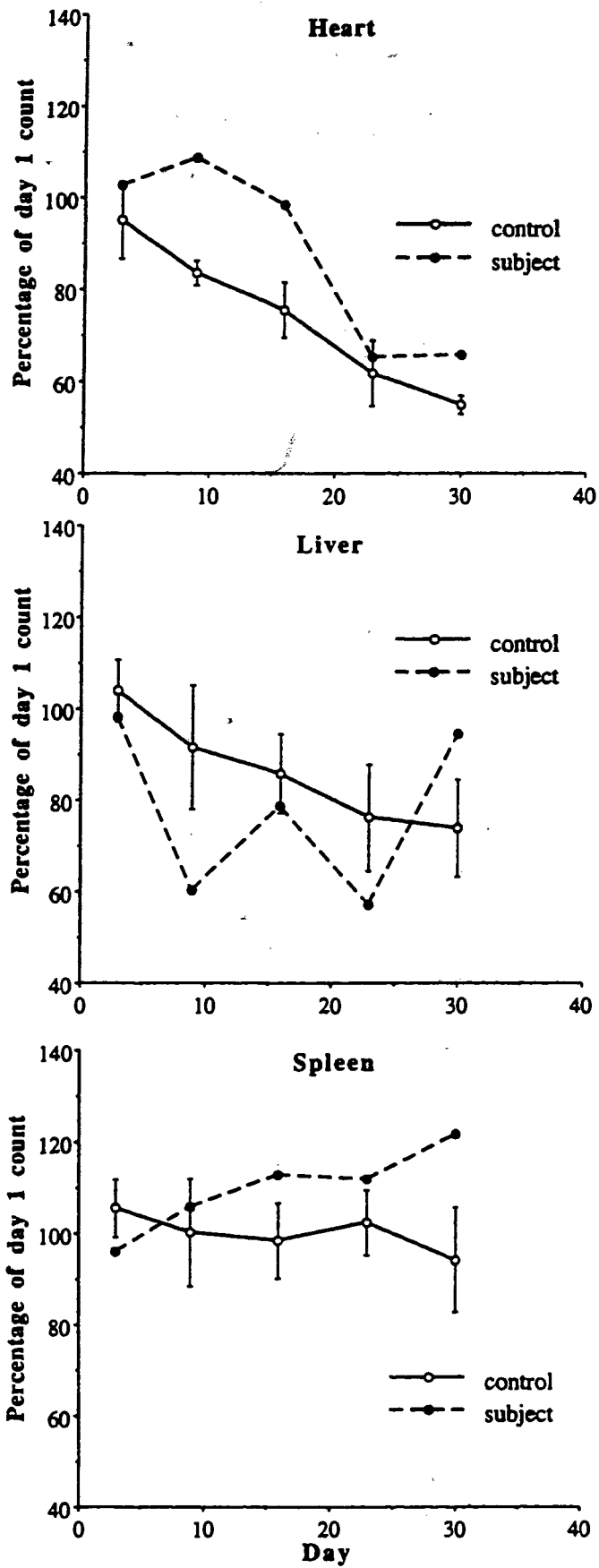


Figure C1 ^{51}Cr -RBC organ count for subject LH compared with a control count.

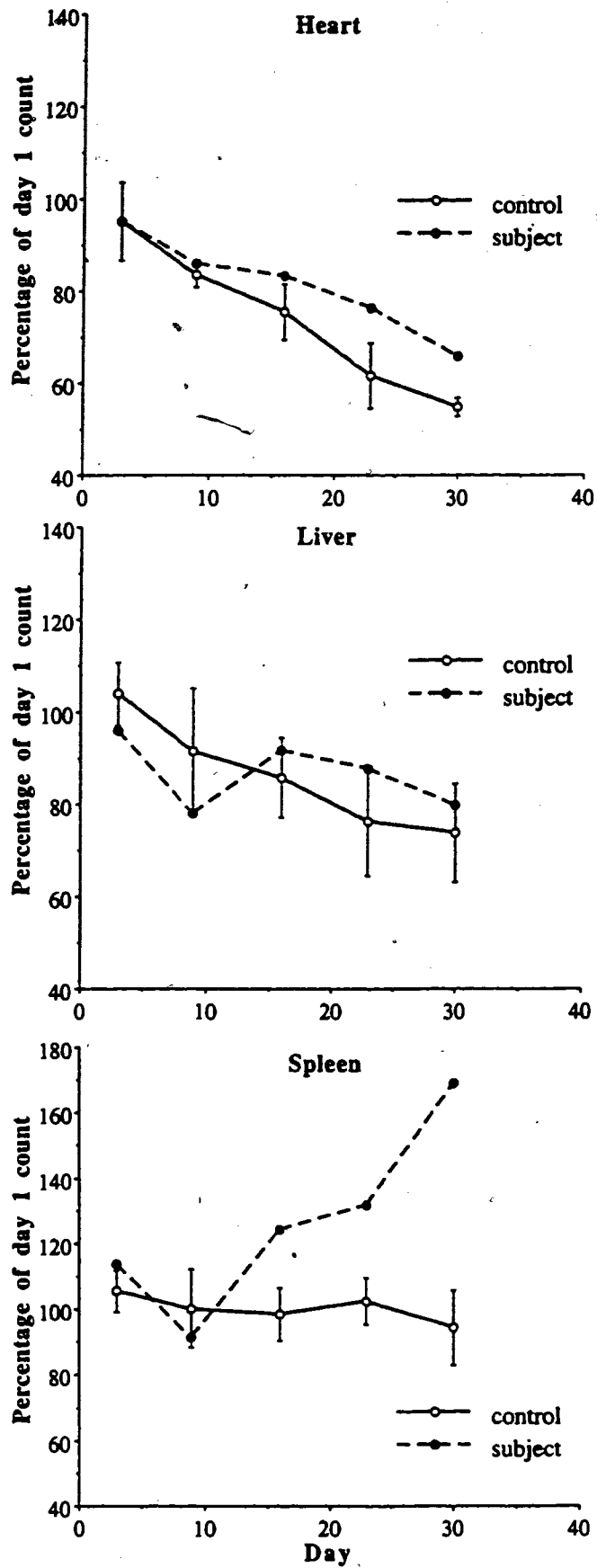


Figure C2 ^{51}Cr -RBC organ count for subject LK compared with a control count.

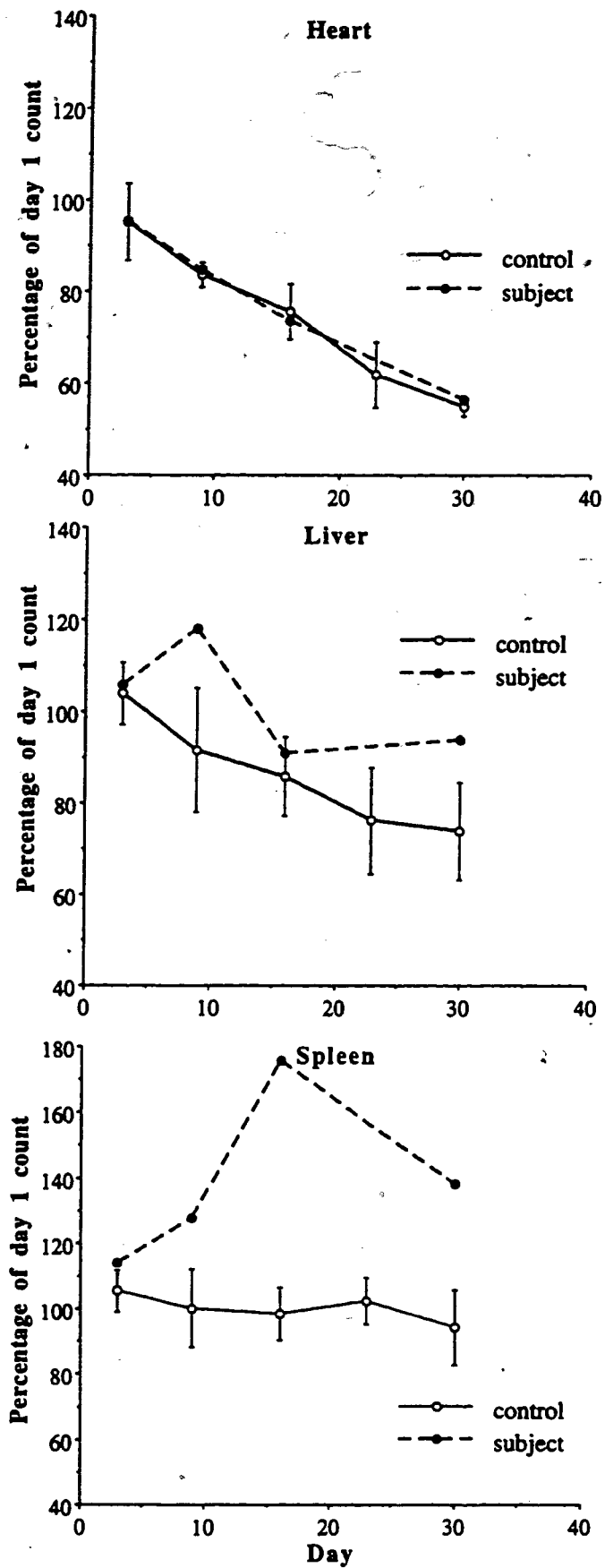


Figure C3 ^{51}Cr -RBC organ count for subject PT compared with a control count.