ASPECTS OF IRON METABOLISM IN INFECTION, INFLAMMATION AND NEOPLASIA

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SECTION 3

Chapter Twelve

THE NON-IMMUNE INFLAMMATORY RESPONSE: SERIAL CHANGES IN PLASMA IRON, IRON-BINDING CAPACITY, LACTOFERRIN, FERRITIN AND C-REACTIVE PROTEIN

12.1 INTRODUCTION

The non-immune inflammatory response was first documented by Bennett and Beeson (1953), who showed that cell free extracts of sterile inflammatory exudates caused fever when injected into experimental animals. The results of subsequent studies have demonstrated that there are a number of other components in this response. These include a fall in the plasma iron concentration (Kampschmidt and Upchurch, 1969), alterations in the tissue distribution of iron and zinc (Pekarek et al. 1972), mobilisation of bone marrow neutrophils (Kampschmidt and Upchurch, 1977) with release of lactoferrin from them (Klempner et al. 1978), and a rise in the plasma concentrations of acute phase reactants, including C-reactive protein (Kushner, 1982; Lee, 1983). In addition to the fall in the plasma iron concentration, there occurs a rise in the plasma concentration of ferritin (Bentley and Williams, 1974; Elin et al. 1977; Birgegard et al. 1978; Lee, 1983). It has been shown that the rise in ferritin is associated with increased synthesis of the protein by reticuloendothelial cells (Konijn and Hershko, 1977; Birgegard and Caro, 1984; Lee, 1983). These changes appear to result from the elaboration and release of a closely related series of small molecular weight proteins (Bornstein, 1982; Kampschmidt et al. 1983; Dinarello, 1984a), which have been collectively termed as interleukin-1.

Currently there is controversy as to the precise mechanism of the hypoferraemic, hyperferritinaemic response to inflammation. On the one hand, it has been suggested that

stimulation of ferritin synthesis in reticuloendothelial cells is an integral part of the inflammatory response and that as a result iron is deviated into this storage compound and is therefore not released into the plasma (Konijn and Hershko, 1977; Roeser, 1980; Lee, 1983). According to the other hypothesis, lactoferrin binds iron avidly at the low pHs which occur in inflammatory lesions and is subsequently taken up by macrophages (Van Snick et al. 1974; 1975). The iron then stimulates ferritin synthesis.

The present clinical investigation was undertaken in order to study various components of the non-immune inflammatory response in both acute and chronic inflammatory states.

12.2 PATIENTS AND METHODS

12.2.1 Patients

The conditions studied included acute infection (acute lobar pneumonia), acute infection in the face of chemotherapy induced marrow-suppression, chronic infection (pulmonary tuberculosis) and chronic non-infectious inflammation (rheumatoid arthritis).

The 21 patients with pneumonia all had a history of acute illness, no prior treatment and radiological features of lobar or multilobar consolidation. Bacteria isolated included Streptococcus pneumoniae (3), Klebsiella pneumoniae (3), Pseudomonas pyocyaneus (1) and Haemophilus influenzae (1). All were treated with appropriate antibiotics after baseline investigations had been done and all were followed until

discharge from hospital or death. Blood samples were taken prior to therapy and were then repeated on alternate days.

The 7 patients with active pulmonary tuberculosis had all been diagnosed on the basis of positive sputa examinations and appropriate radiological findings. Most had received 1 to 2 weeks antituberculous therapy prior to entering the study but all still had clinical evidence of active infection, including fever.

The 6 patients with rheumatoid arthritis had previously been treated only with non-steroidal anti-inflammatory drugs. Base-line values were taken prior to the start of intramuscular gold therapy and the patients were followed at weekly intervals thereafter.

Nine patients, all of whom had been rendered neutropenic by chemotherapy, were studied during episodes of infection. Six had acute leukaemia, two had carcinoma and one a lymphoblastic lymphoma. All these patients were sampled on alternate days. Neutrophil counts were not obtained in this group because of the marked neutropenia. One other patient with refractory leukaemia was studied longitudinally for 44 days after he had received chemotherapy followed by bone marrow transplantation.

12.2.2 Methods

Measurement of plasma ferritin, iron, total iron-binding capacity, percentage saturation of transferrin and serum C-reactive protein concentrations were by the methods outlined in Section 1 (Chapters 2 and 3). Plasma lactoferrin concentration was measured by the enzyme linked immunosorbent assay described

in Section 2 (Chapter 6). Full blood counts were performed as described in Section 1. The statistical and ethical considerations were as described in Section 1 (Chapters 2, 3 and 4).

12.3 RESULTS

12.3.1 Relation Between Plasma Lactoferrin and White Cell Count

The initial peak concentrations of plasma lactoferrin were compared with white cell and neutrophil counts (Table 16). In addition, the initial lactoferrin:leucocyte ratios were compared for the various conditions studied. The geometric mean plasma lactoferrin was 4 times normal in pneumonia, 5 times normal in tuberculosis, twice normal in rheumatoid arthritis and one tenth normal in neutropenic sepsis. The lactoferrin:leucocyte ratios were normal in rheumatoid arthritis and neutropenia but 2-3 times normal in pneumonia and tuberculosis. The patterns of change in lactoferrin concentrations and in white cell counts that occurred with time in the 4 groups were also followed (Figure 23). These changes were noted to run in parallel with each other in each group. Patients with pneumonia showed an initial rapid fall in lactoferrin concentration and white cell counts over the first 3 days. Thereafter there was a secondary rise at about 7 days followed by a gradual decline towards normal. Values in the tuberculotic group declined steadily from their initial high values to normal over a period of 11 weeks. In contrast, the

Table 16. Initial peak concentrations (geometric means and SD of the plasma lactoferrin concentrations and white cell and neutrophil counts in 21 patients with acute pneumonia, 7 with active pulmonary tuberculosis, 6 with active rheumatoid arthritis and initial trough concentrations in 9 with acute infection superimposed on chemotherapy induced marrow suppression. In addition, similar values for 48 normal subjects and the mean initial lactoferrin:leucocyte:ratio for each condition are shown.

	Lactoferrin* (/ug/l)		Leucocyte count ($x10^9$/l)	Neutrophil count ($x10^9$/l)	Lactoferrin** leucocyte ratio
Lobar pneumonia	1182	(604-2313)	12.1 (± 7.1)	9.7 (± 4.7)	164 (± 183)
Tuberculosis	1784	(1485-2145)	12.1 (± 4.3)	9.8 (± 4.5)	139 (± 58)
Rheumatoid arthritis	721	(461-1126)	8.5 (± 2.2)	6.4 (± 2.2)	62 (± 25)
Neutropenic infection	23	(7-74)	0.8 (± 0.2)	-	57 (± 47)
Normal	293	(191-448)	7.0 (± 1.8)	-	51 (± 26)

* Lactoferrin concentrations in all groups were significantly different from normal as determined by an unpaired Student's t test ($p<0.0005$). Lactoferrin concentrations in pneumonia and tuberculosis were similar (t 1.5850; $p>0.1$). Lactoferrin concentrations in pneumonia were not different from those in rheumatoid arthritis (t 1.6893; $p>0.1$). Lactoferrin concentrations were significantly higher in tuberculosis than in rheumatoid arthritis (t 4.9404; $p<0.0005$). The lactoferrin concentration in the neutropenic group was significantly lower than in all other groups ($p<0.0005$).

** The lactoferrin:leucocyte ratio was significantly greater than normal in pneumonia (t 3.8848; $p<0.0005$) and tuberculosis (t 6.6517; $p<0.0005$) but similar to normal in rheumatoid arthritis (t 0.9873; $p>0.1$) and neutropenic sepsis (t 0.5031; $p>0.1$)

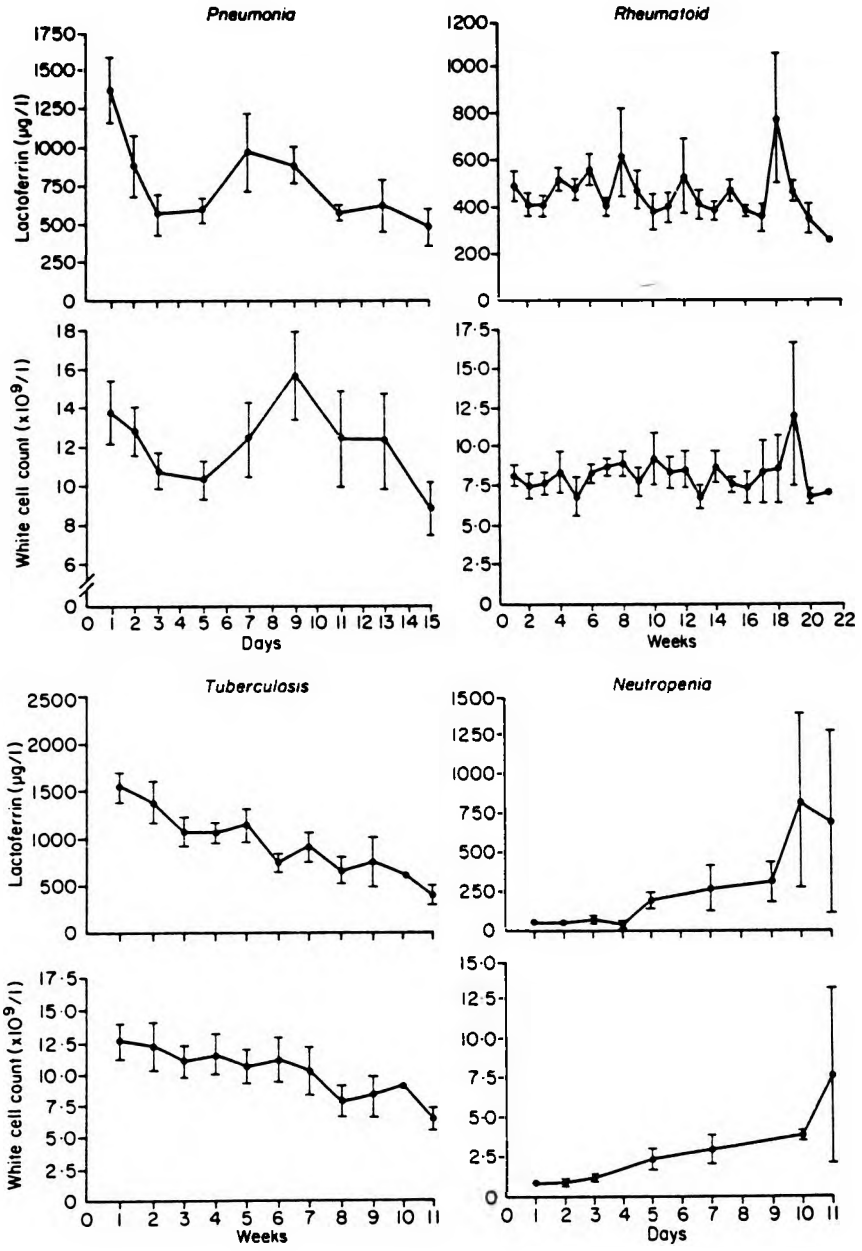
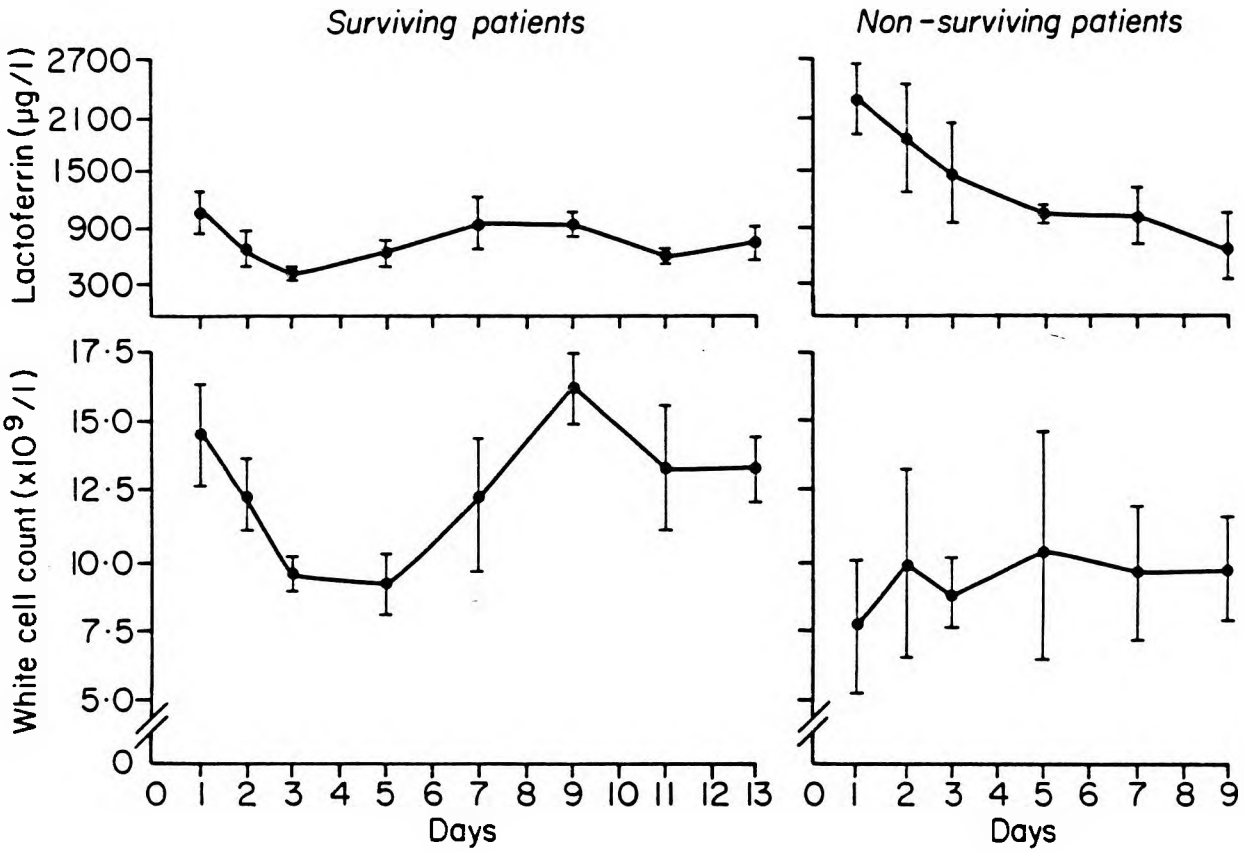


Figure 23. Patterns of change in mean (± S.E.) plasma lactoferrin concentrations (µg/l) and white cell counts (x 10^{9}/l) during the treatment of 21 patients with pneumonia, 7 with tuberculosis, 6 with rheumatoid arthritis and 9 with neutropenic sepsis.

mild elevation in lactoferrin concentrations that was noted in the patients with rheumatoid arthritis prior to therapy persisted despite clinical improvement on gold therapy. Finally, the low initial concentrations of lactoferrin in the patients with neutropenia and sepsis steadily increased in parallel with the recovery in the white cell count and were about twice normal by the 10th day.

An evaluation of the lactoferrin:leucocyte ratio in the various conditions revealed the following. In the group of pneumonia patients the initial lactoferrin:leucocyte ratio of 164, which was significantly different from the control figure of 51, returned to normal by day 3. In subjects with active pulmonary tuberculosis the lactoferrin:leucocyte ratio was significantly elevated above normal at the start (139) but then gradually declined so that it was normal by the 11th week of study. In those subjects with rheumatoid arthritis the lactoferrin:leucocyte ratio remained normal throughout the study. In the group with neutropenic sepsis the overall ratio remained normal except for an evanescent rise during the time the plasma concentration was rapidly increasing.

A comparison between the 5 patients who succumbed to acute lobar pneumonia and the 16 who survived showed that initial lactoferrin concentrations were significantly higher (t 2.8351, $p < 0.03$) in the group that died (geometric mean 2115 $/ug/l$ as compared with 1075 $/ug/l$) (Figure 24). In contrast, there were no differences in the initial white cell counts. During the ensuing days the surviving patients showed a secondary rise in both the



igure 24. A comparison of the mean (± S.E.) changes with time in the plasma lactoferrin concentration (/ug/l) and the white cell count (x 10^{9}/1) in 16 subjects with acute lobar pneumonia who survived and 5 who died.

lactoferrin concentrations and the white count. In contrast, the lactoferrin concentration fell steadily from its initial high levels in the non-surviving group, while the white count remained between 7.5 and 10.5 x 10^9/1. The only other index of the acute inflammatory response that was a predictor of a fatal outcome was the initial plasma iron concentration, which was significantly greater in the group that died ($p < 0.03$). Evaluation of the lactoferrin:leucocyte ratio in these 2 subgroups revealed that this ratio was strikingly elevated in the group that died (417 SD ± 211) whereas it was only moderately elevated in the group that survived (80 SD + 46). In addition, the ratio returned to normal in the survivors within 2 days, while it decreased very slowly in the group that succumbed.

12.3.2 Relation Between Plasma Lactoferrin, C-Reactive Protein, Ferritin, Iron and Iron-Binding Capacity

12.3.2.1 Acute lobar pneumonia

The mean plasma lactoferrin was initially about 3 times normal and while it declined gradually over the next 15 days, it was still above normal at the end of the study (Figure 25). Changes in plasma C-reactive protein and ferritin were very similar to each other. Both were approximately 10 times normal at the onset of the illness and altered very little during the period of observation. There was a slow rise in the plasma iron and in the total iron-binding capacity over the period of the study, which was well demonstrated by comparing the first two observations with the last two. The mean plasma iron at the

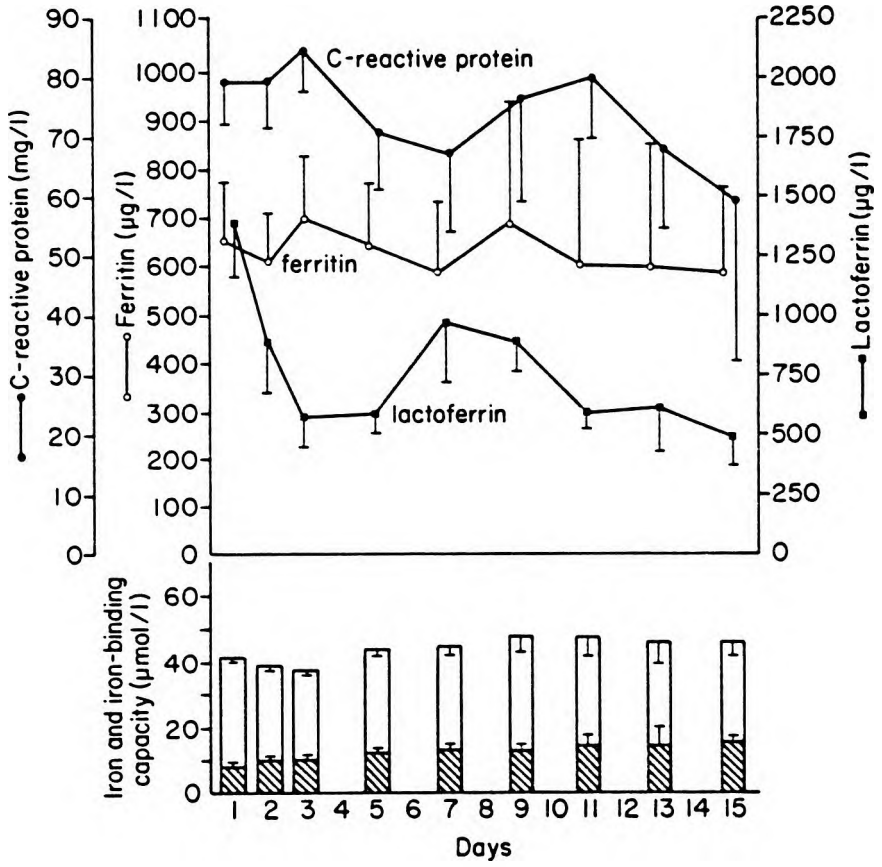


Figure 25. Sequential changes during treatment in the mean (± S.E.) plasma concentrations of lactoferrin, ferritin, C-reactive protein, iron (hatched columns) and iron-binding capacity (total columns) in 21 patients with acute lobar pneumonia.

onset was 9.0 /umol/l, the total iron-binding capacity 39.4 /umol/l and the percent saturation 14. In contrast, comparable figures at the end of the study were 14.1 /umol/l, 44.9 /umol/l and 31 percent respectively.

12.3.2.2 Active pulmonary tuberculosis

The patients with tuberculosis were followed weekly for 11 weeks. During this period there was a steady and progressive drop in each of the measurements (Figure 26). The plasma lactoferrin was initially about 5 times normal and by the end of the study was within the normal range. At the onset, plasma ferritin and C-reactive protein were approximately 8 to 10 times normal, while final values were within or just above the normal range. Changes in plasma iron and total iron-binding capacity were unremarkable, with a modest but sustained decrease in each. This pattern was apparent when mean values over the first two weeks were compared with the last two observations. At the onset, the plasma iron was 13.3 /umol/l, the total iron-binding capacity 39.9 /umol/l, and the saturation 32 percent, while final values were 11.3 /umol/l, 43.3 /umol/l and 26 percent respectively.

12.3.2.3 Rheumatoid arthritis

Plasma lactoferrin concentrations were only mildly raised and did not alter throughout the 20 weeks of observation (Figure 27). On the other hand, C-reactive protein concentrations were about 8 times normal prior to the commencement of gold therapy.

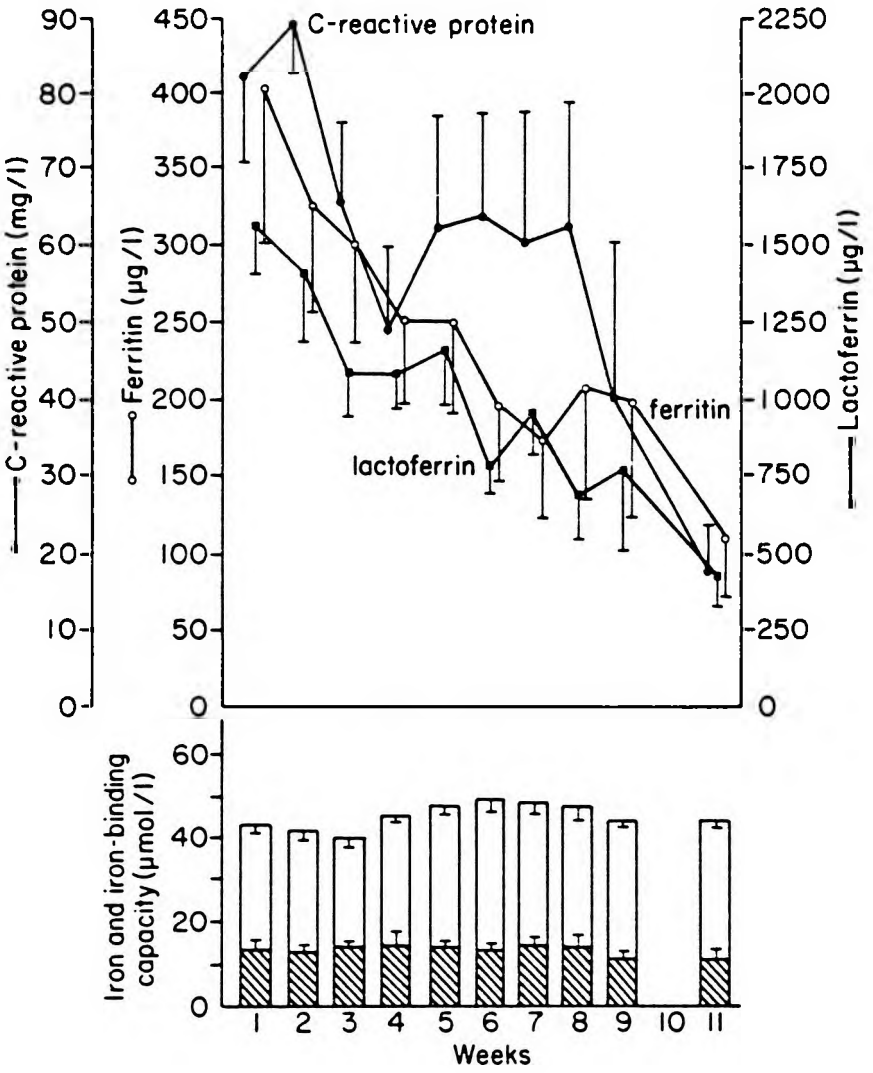


Figure 26. Sequential changes during treatment in the mean (± S.E.) plasma concentrations of plasma lactoferrin, ferritin, C-reactive protein, iron (hatched columns) and iron-binding capacity (total columns) in 7 patients with active pulmonary tuberculosis.

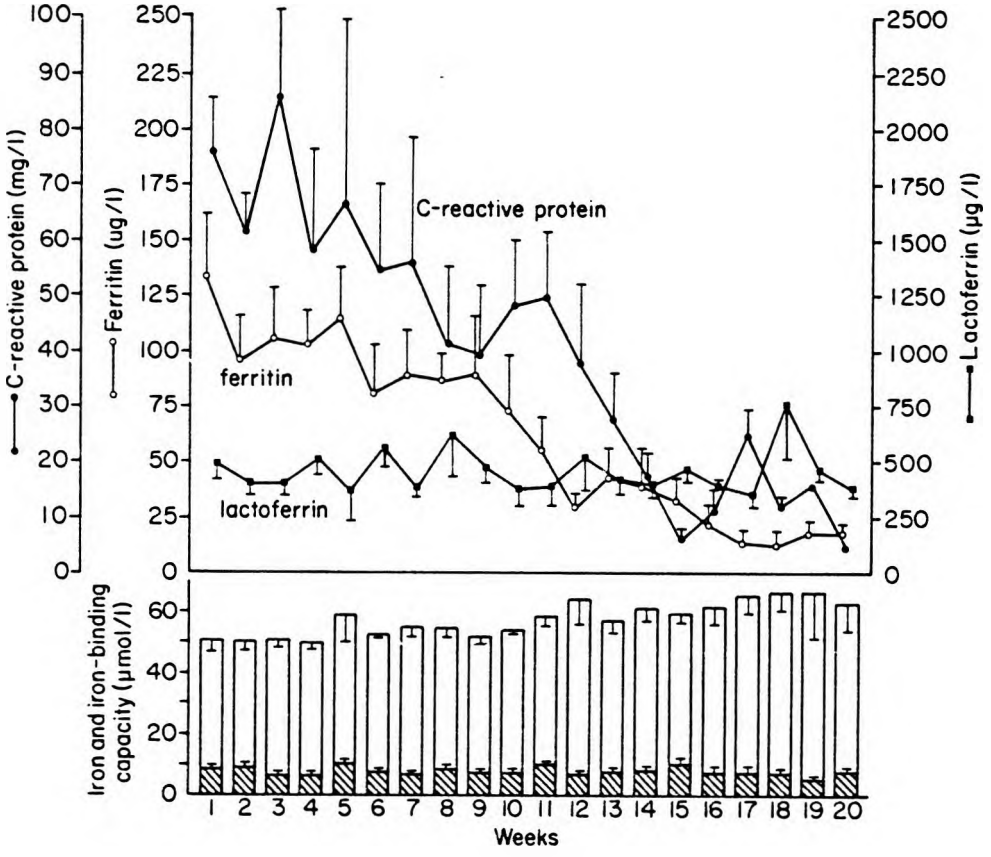


Figure 27. Sequential changes during treatment with gold in the mean (± S.E.) plasma concentration of lactoferrin, ferritin, C-reactive protein iron (hatched columns) and iron-binding capacity (total columns) in 6 patients with active rheumatoid arthritis.

Thereafter they declined steadily, so that by the end of the study they were within normal limits. A similar pattern of decline was noted insofar as plasma ferritin concentrations were concerned. Although initial concentrations were only within the high normal range they dropped steadily and at the end of the observation period the values were compatible with iron deficiency. The presence of concomitant iron deficiency was also suggested by the changes in the plasma iron and iron-binding capacity. The mean plasma iron concentration during the first month was 7.5 /umol/l, the total iron-binding capacity was 50.1 /umol/l and the percentage saturation 15.

During the period of treatment there was a steady rise in the total iron-binding capacity so that it had risen to 64.4 /umol/l by the last month. The mean plasma iron was little changed (6.6 /umol/l) but the percentage saturation had fallen to 10. The fact that mean haemoglobin concentration at the start of the study was 11.9 (± 0.73) g/dl and remained constant throughout the study to end at 11.5 (± 0.91) g/dl indicates that the fall in ferritin concentrations was not simply due to a deviation of storage iron into haemoglobin.

12.3.2.4 Neutropenic sepsis

Plasma concentrations of C-reactive protein and ferritin were approximately 10 times normal in patients with bone marrow suppression and neutropenic sepsis and remained at these levels throughout the period of infection (Figure 28). With the subsidence of the infection the C-reactive protein concentration

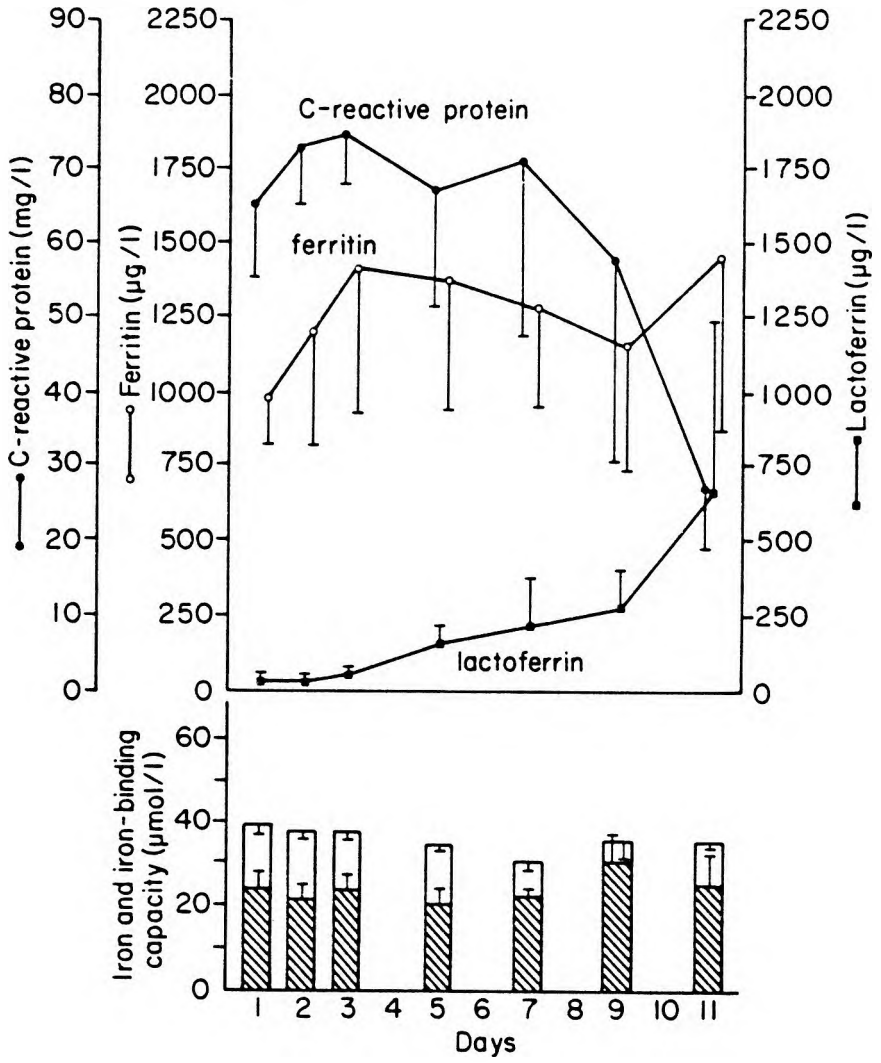


Figure 28. Sequential changes during treatment in mean (± S.E.) plasma concentrations of lactoferrin, ferritin, C-reactive protein, iron (hatched columns) and iron-binding capacity (total columns) in 9 patients with neutropenic sepsis.

dropped sharply but the plasma ferritin was still markedly raised at the end of the period of observation. Lactoferrin concentrations were very low initially and only started rising with recovery of the white cell count (Figure 22). The percentage saturation of transferrin was raised throughout the study period. This was due more to a reduction in the total iron-binding capacity than to a rise in the plasma iron concentration. Comparison of the first two and last two observations were as follows:- plasma iron 23.3 and 29.6 /umol/l, total iron-binding capacity 39.4 and 37.4 /umol/l and percentage saturation 60 and 79 percent respectively.

An attempt was made to find out whether the lack of an overall hypoferraemic response in the patients with neutropenic sepsis was related to the low lactoferrin concentrations. Amongst the eleven infective episodes in 10 patients there were 5 in which the saturation was never below 80 percent and 4 in which it fell to below 30 percent during the period of infection. The mean lactoferrin concentrations in the two groups were both very reduced, being 38 and 73 /ug/dl respectively.

Further evidence suggesting that the plasma iron concentration was not dependent on the lactoferrin concentration was obtained by a longitudinal study in which repeated measurements were made in a patient who had received ablative chemotherapy prior to a bone marrow transplantation. During the period of severe marrow hypoplasia there were two episodes of infection, characterised in both by a sharp rise in the plasma concentrations of C-reactive protein (Figure 29). The plasma

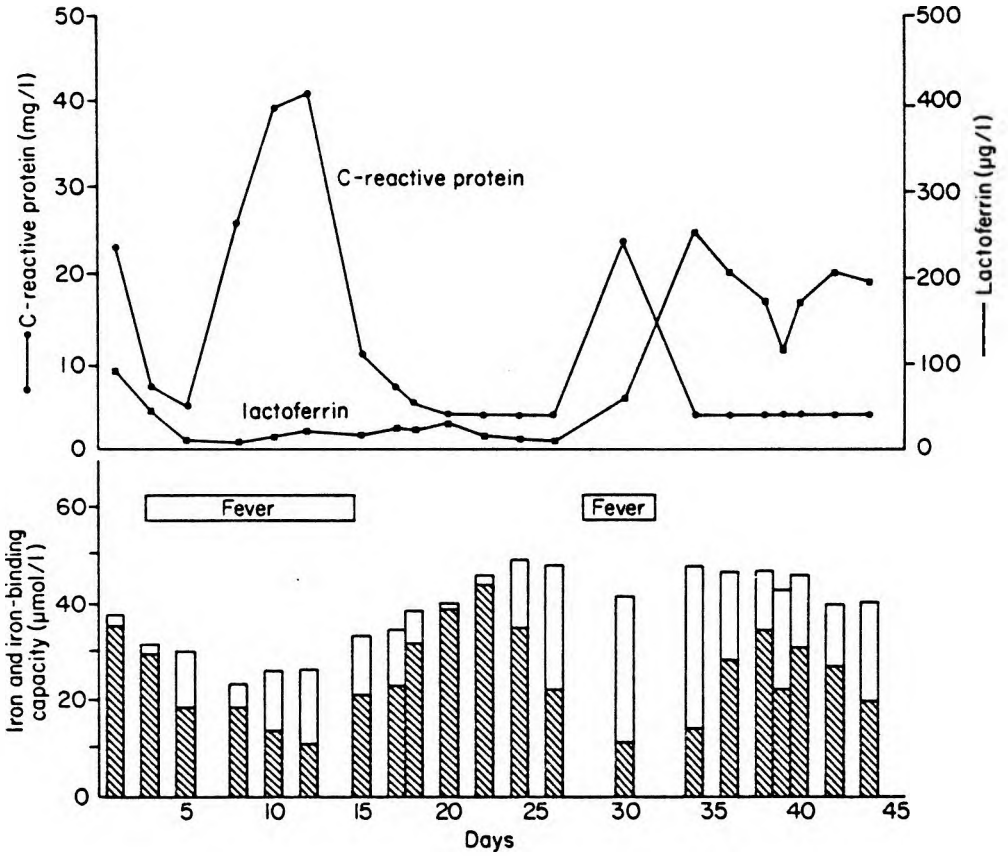


Figure 29. Sequential changes in plasma lactoferrin, C-reactive protein, iron (hatched columns) and iron-binding capacity (total columns) in a patient with refractory leukaemia who was treated with chemotherapy followed by a bone marrow transplantation from an HLA-compatible sibling.

iron and percentage saturation dropped during both episodes, despite the fact that lactoferrin concentrations were sharply reduced over this period. (The fact that the total iron-binding capacity, which reflects the concentrations of plasma transferrin, was initially so low, presumably reflected the generalised depression in protein synthesis induced by the intense chemotherapy).

12.4 DISCUSSION

The present study was done in an attempt to gain further insight into the possible interrelationships between various components of the non-immune inflammatory response. Included in the chain of events triggered by interleukin-1 is the release of lactoferrin from mobilised neutrophils. There is a rise in the plasma concentration of C-reactive protein, which is derived from the liver (Kushner and Feldman, 1978), and a raised plasma ferritin has also been suggested as a part of the response (Konijn and Hershko, 1977). In addition, there is a drop in the plasma iron and a similar but less marked one in its protein carrier, transferrin (Kampschmidt and Upchurch, 1969; Birgegard et al. 1978).

It was realised at the outset that interpretation of the data might be difficult, since the changes in all the components that were being measured were being directly or indirectly set off by a common stimulus and, as a result, spurious correlations between them might be found. In addition, it was possible that

certain of the measurements were being affected by factors unrelated to the inflammatory response. For example, patients with rheumatoid arthritis are also often iron deficient as a result of therapy with non-steroidal anti-inflammatory agents, while suppression of erythroid marrow activity associated with chemotherapy leads to a diversion of iron into storage depots (Finch et al. 1970). In both instances plasma iron and ferritin concentrations would be affected and these changes might complicate the interpretation of the findings during a period of inflammation. Another possible constraint on the accurate interpretation of the findings was the difference in turnover rates of the various components being measured. Their half times vary from 1-2 hours for plasma iron (Bothwell et al. 1979) to about 30 hours for ferritin (Worwood et al, 1982). Values for C-reactive protein (4 to 9 hours) (Gewurz et al, 1982; Pepys and Baltz, 1983) and lactoferrin (about 2 to 16 hours) (Chapter 8) fall in the intermediate range. However, even with these reservations, it was possible from the findings to reach a number of conclusions relating to the non-immune inflammatory response.

The present results confirmed the close relationship between plasma lactoferrin concentrations and the neutrophil count (Hansen et al. 1975; Lash et al. 1983; Spitznagel et al.1974). Of particular interest was the lactoferrin:leucocyte ratio, since a raised ratio implies that extra lactoferrin is being produced, either from the degranulation of circulating neutrophils or from marginated or extravascular neutrophils or from a combination of these sources. The elevated ratio in acute lobar pneumonia

confirms the previous finding (Hansen et al. 1976) that high plasma concentrations in such a situation are associated with a decreased intracellular lactoferrin content. The significant difference between the ratio in surviving and non-surviving pneumonia subjects (t 6.2591; $p < 0.0005$) is not only of prognostic significance but also may have pathogenetic significance. Previous work (Hansen et al. 1976) has shown that a markedly decreased intraneutrophilic lactoferrin concentration is associated with toxic granulation of neutrophils and that toxic granulation is associated with decreased bactericidal activity (McCall et al. 1971). The fact that a steady drop in lactoferrin concentrations occurred in non-surviving patients despite an unchanged white cell count suggests the presence of a functional neutrophil defect. The occurrence of such acquired abnormalities in lactoferrin synthesis and release have recently been described in two patients suffering from recurrent sepsis (Hanson et al. 1984). The group of patients with acute pneumonia showed an interesting biphasic reponse in both lactoferrin concentrations and white counts. An initial fall was followed at about 7 days by a secondary rise, possibly reflecting the proliferation and release of a new cohort of functional neutrophils. It is of note that this secondary peak of lactoferrin and white count was associated with a normal lactoferrin:leucocyte ratio. The lactoferrin:leucocyte ratio in subjects with pulmonary tuberculosis was significantly elevated at the start of the study and remained modestly yet significantly elevated throughout most of the study. However, the trend revealed a gradual return

towards normal. This is clearly different from the rapid settling of the ratio in acute pneumonia. The difference may reflect a more prolonged neutrophil activation stimulus and possibly significant extravascular neutrophil activity over a time scale of several weeks. The mildly raised lactoferrin concentrations in patients with rheumatoid arthritis remained at these levels during a period of many weeks, with a normal ratio despite marked clinical improvement on gold therapy. In passing, it should be noted that there is *in vitro* evidence that gold inhibits neutrophil degranulation (Wolach et al. 1982; Coates et al. 1983). However, the present findings together with other *in vivo* observations (Wolach et al. 1982) suggest that the effect is not a clinically important one. In the case of marrow hypoplasia induced by chemotherapy the plasma lactoferrin concentration paralleled leucocyte recovery, with a normal ratio except during the short period when the plasma lactoferrin concentration was increasing rapidly. This occurred in the face of sepsis and may indicate active degranulation or repopulation of the marginated pool.

Plasma C-reactive protein concentrations paralleled plasma ferritin concentrations in both acute pneumonia and tuberculosis. In acute pneumonia both remained elevated throughout the period of study, while they both settled over several weeks in tuberculosis. This time-scale is in keeping with previous findings (Elin et al. 1977; Birgegard et al. 1978). From this it would appear that ferritin was behaving as an acute phase protein. In the subjects with rheumatoid arthritis,

interpretation was more difficult, since concomitant iron deficiency was present. The rise in C-reactive protein concentrations suggested that the degree of inflammatory response was as great as in pneumonia and tuberculosis but the plasma ferritin concentrations at the height of inflammation were much lower. The explanation for this difference relates to iron stores. All the subjects with acute pneumonia or tuberculosis belonged to population groups in which iron stores are known to be normal or increased (Bothwell et al. 1979). On the other hand, by the end of the study the rheumatoid subjects were showing evidence of iron deficiency. It was therefore not surprising that the plasma ferritin did not reach high concentrations at any time in the rheumatoid arthritis subjects on gold therapy. At the same time, when followed over time it did follow the same pattern as the C-reactive protein. While ferritin therefore still behaved as an acute reactant, its response appeared to be dampened by the iron status of the group. This conclusion is in accordance with previous findings reported in rheumatoid arthritis (Hansen et al. 1983). The C-reactive protein changes in rheumatoid arthritis are similar to those of Amos and co-workers (1977) who found that concentrations of the protein reflected disease activity. With neutropenic sepsis both measurements were markedly raised. Indeed mean plasma ferritin concentrations of between 1000 and 1500 /ug/l suggested that other factors, such as deviation of iron into stores as a result of bone marrow depression, might be contributing to their marked rise. With recovery, there was a sharp drop in C-reactive protein concentrations but not in the

plasma ferritin. This may have been due to the longer half life of plasma ferritin or to the fact that erythropoiesis was still depressed.

Changes in plasma iron and iron-binding capacity followed similar patterns in patients with pneumonia and tuberculosis. Both the plasma iron and the total iron-binding capacity were reduced initially with the major change being in the plasma iron concentration. As a result the percentage saturation was reduced. The lowered plasma iron concentration has been ascribed to decreased release of iron from reticuloendothelial cells (Cartwright and Lee, 1971; Kampschmidt and Upchurch, 1969), while the decreased total iron-binding capacity reflects increased catabolism of transferrin (Aisen, 1984). In rheumatoid arthritis, there was a steady rise in the total iron-binding capacity with resolution of the inflammation. This reflected an increase in transferrin concentrations from the reduced levels associated with inflammation to the raised ones associated with iron deficiency. Interpretation of the plasma iron and total iron-binding capacity measurements in neutropenic sepsis were complicated by 2 factors - a relative rise in plasma iron as a probable result of depressed erythropoiesis, and a drop in transferrin synthesis possibly as a consequence of chemotherapy. In consequence, the percentage saturation was raised throughout the period of observation.

It has been suggested that lactoferrin may cause the drop in plasma iron and the rise in plasma ferritin by removing iron from transferrin and delivering it to macrophages where it is

incorporated into ferritin (Van Snick et al. 1974; 1975). The major evidence in favour of the hypothesis is the lack of a hypoferraemic response in neutropenic animals (Kampschmidt and Upchurch, 1969). In the current study some evidence that might support such an hypothesis was obtained - in pneumonia and tuberculosis the plasma lactoferrin concentrations were high and there was hypoferraemia, while the low concentrations of plasma lactoferrin in neutropenic sepsis were not associated with hypoferraemia. However, such an interpretation ignores the effects on iron kinetics of chemotherapy induced marrow suppression. Marrow suppression results in defective iron utilisation, with an associated high plasma iron concentration and percentage saturation (Finch et al. 1970). In fact, the results in the present study suggest that lactoferrin does not play any role in the hypoferraemic, hyperferritinaemic response to inflammation. This conclusion is based on the fact that plasma lactoferrin and ferritin concentrations did not change in parallel with each other in patients with rheumatoid arthritis receiving gold therapy. Secondly, there were several patients with neutropenic sepsis and low lactoferrin concentrations in whom the plasma iron concentrations were lowered. The lack of a similar response in the other patients can be ascribed to the fact that the plasma iron concentration was also being affected by its slow rate of removal as a result of reduced erythroid marrow activity. Indeed, erythrokinetic data show that inflammation is not associated with increased clearance of iron from plasma, but rather with diminished return of iron from

effete red cells by macrophages to the plasma (Finch et al. 1970; Letendre and Holbein, 1983; 1984). Quantitative considerations also tend to preclude a role for lactoferrin in hypoferraemia (Taylor et al. 1987). On the basis of the present findings raised neutrophil and plasma lactoferrin concentrations can be seen as one component of the acute inflammatory response, while increased plasma C-reactive protein and ferritin together with hypoferraemia can be seen as another, with no evidence that they are interdependent on each other. Furthermore, iron status appears to affect markedly the degree of hyperferritinaemia.

One final point merits discussion. The present findings illustrate several of the mechanisms that make patients receiving cytostatic chemotherapy so vulnerable to infections. Not only is host defence lowered by neutropenia and the associated low plasma lactoferrin concentrations but there is also a high percentage saturation of transferrin. The increased availability of transferrin bound iron has been shown to promote the growth of invading organisms (Weinberg, 1984). Finally, the synthesis of C-reactive protein is probably depressed by chemotherapy so that the concentrations of this protein, which plays an important role in host defence (Pepys and Baltz, 1983), are significantly lower than is normally the case in infection (Harris et al, 1984).

12.5 SUMMARY

The interrelationships between various components of the non-immune inflammatory response (white cell count, plasma

lactoferrin, C-reactive protein, ferritin, iron and iron-binding capacity), were studied serially in a variety of inflammatory conditions including acute lobar pneumonia, active pulmonary tuberculosis, rheumatoid arthritis on gold therapy, and sepsis in the face of marrow hypoplasia induced by chemotherapy.

Lactoferrin concentrations paralleled the white count in all groups. These were highest in pneumonia and tuberculosis, mildly elevated in rheumatoid arthritis and markedly decreased in neutropenic sepsis. Very high initial lactoferrin concentrations were associated with a poor prognosis in acute pneumonia.

The patterns of change in C-reactive protein and ferritin were similar to each other. Both remained elevated through the period of study in acute pneumonia and neutropenic sepsis, while they gradually normalised over weeks in subjects with tuberculosis or rheumatoid arthritis on therapy. Iron deficiency in the rheumatoid subjects appeared to limit the hyperferritinaemic response. In pneumonia and tuberculosis moderate hypoferraemia and reduced iron-binding capacity were evident. In contrast, a raised percentage saturation was present in neutropenic sepsis, probably related to erythroid marrow suppression.

Comparisons between ferritin, lactoferrin and C-reactive protein in the various groups supported the concept that ferritin behaves in part as an acute phase reactant and that hypoferraemia in inflammation is due to deviation of iron into ferritin stores. The suggestion that lactoferrin is responsible for the hypoferraemia and hyperferritinaemia was not supported by the

present data.

SECTION 3

Chapter Thirteen

IRON-TRANSFERRIN-CELL INTERACTIONS: INFECTION, INFLAMMATION AND NEOPLASIA

13.1 INTRODUCTION

The protein transferrin (molecular weight 80 000 D) plays a pivotal role in iron homeostasis. It circulates in plasma and interstitial fluid in four forms depending upon its degree of saturation with iron. The transferrin molecule has two iron binding sites and thus may exist as the diferric-, monoferric a-, monoferric b- or apotransferrin form. The diferric, and to a lesser extent monoferric forms, are involved primarily in the delivery of iron to growing and dividing cells (Morgan, 1981). The apotransferrin molecule is concerned primarily with the acquisition of iron from the cells concerned with iron absorption (gastrointestinal cells), iron storage (reticuloendothelial cells and hepatocytes), iron release from degraded iron containing proteins (reticuloendothelial cells and hepatocytes) and iron transfer (placental cells) (Morgan, 1981). There is a considerable body of information on the mechanism of iron delivery to cells by diferric transferrin. In this chapter the evidence will be reviewed and later in the thesis some data will be presented in relation to neoplastic cells, macrophages and inflammation. Virtually nothing is known of how iron is released from cells to apotransferrin. Some speculative literature on this subject will be reviewed in this chapter and later in the thesis my own experimental findings in relation to this topic will be presented. The regulation of internal and external iron exchange will also be briefly discussed.

13.2 IRON DELIVERY TO CELLS BY TRANSFERRIN

An orderly sequential process exists in which iron carrying transferrin binds to specific receptor sites on the cell membrane, enters the cell by a process of receptor mediated endocytosis, releases the iron intracellularly and is then recycled to the cell surface as apotransferrin. This released apotransferrin is then able to procure 2 further iron molecules (Morgan, 1981). Most of our current understanding of this process has been derived from studies involving immature red cells but most of the data also apply to non-erythroid cells.

13.2.1 The Transferrin Receptor

Diferric transferrin exhibits specific, reversible and saturable binding to reticulocytes (van Bockxmeer et al. 1975; 1978; van Bockxmeer and Morgan, 1977; 1979). Each reticulocyte carries approximately 80 000 transferrin-binding sites with an equilibrium association constant of roughly 10^7 l/mole. With maturation of red cell precursors there is a progressive disappearance of functional receptors (Pan and Johnstone, 1984). Proteolytic digestion of reticulocytes reduces transferrin binding (Hemmaplardh and Morgan, 1976). Binding of transferrin is calcium dependent (Hemmaplardh and Morgan, 1977) and maximal at pH 7.8 (van Bockxmeer et al. 1978). The binding of apotransferrin to reticulocytes has been demonstrated to be significantly less avid, with an equilibrium association constant of 10^5 l/mole (Morgan, 1981). The development of monoclonal

antibodies with specificity for the transferrin receptor has allowed for the immunological demonstration of transferrin receptors on cell surfaces (Schneider et al. 1982). Each receptor monomer can bind 1 molecule of transferrin (May and Cuatrecasas, 1985), which in turn carries two molecules of iron. The binding process is therefore very efficient in terms of iron economy.

The receptor has been fairly completely characterised. It is a dimer, with each subunit having a molecular weight of ± 90 000 D (Seligman, 1983; Enns and Sussman, 1981). It is a glycoprotein containing about 5% carbohydrate by weight (Seligman et al.1979). The dimers are linked by a single disulphide bond (Huebers and Finch, 1984). The receptor gene has been cloned and its sequence established (McClelland et al. 1984; Schneider et al. 1984). Each receptor dimer has a large carboxy terminal extracellular domain and a smaller amino terminal domain which is intracellular. Such an extracellular carboxy terminal domain is biologically unusual for transmembrane proteins (May and Cuatrecasas, 1985). It does, however, share this characteristic with the hepatic asialoglycoprotein receptor (Drickamer et al. 1984). This latter observation is of interest because of this protein's suggested role in hepatic iron metabolism (see Chapter 18, pages 315-352).

13.2.2 Diferric Transferrin Internalisation

Having bound to specific transferrin receptors on the cell surface the diferric transferrin, together with its receptor, is taken into the cell by a process of receptor mediated endocytosis (Morgan, 1981; May and Cuatrecasas, 1985; Wileman et al. 1985; Bomford and Munro, 1985; Stahl and Schwartz, 1986). This process

is a commonly employed biological strategy for the cellular internalisation of various substances and particles. The original description of receptor mediated endocytosis related to cholesterol, its low density lipoprotein ligands and the specific receptor for the complex of the two (Anderson, Brown and Goldstein, 1977). Other substances which undergo receptor mediated endocytosis include cell nutrients, such as Vitamin B_{12} attached to transcobalamin, growth factors such as epidermal growth factor and platelet derived growth factor, hormones such as insulin and gonadotrophins, toxins such as diphtheria and pseudomonas exotoxins and viruses such as influenza (Stahl and Schwartz, 1986). In addition, mannosylated proteins and asialoglycoproteins are cleared from the plasma by receptor mediated endocytosis. Receptors for these various molecules are supported in the cell membrane by a protein called clathrin to form clathrin coated pits. Dissociated clathrin exists as a trimer containing 3 polypeptides of molecular weight 180 000 in association with 3 clathrin light chains (Kirchhausen et al. 1983). On electron microscopy the trimers have a characteristic triskelion (three-legged) appearance. Morphological evidence suggests that a number of ligands may enter via the same coated pit (Carpentier et al. 1982). These clathrin coated pits then bind intracellularly and lose their clathrin by an energy dependent process to form a prelysosomal endosome termed a receptorsome (May and Cuatrecasas, 1985). As yet the mechanisms that cause receptor molecules to cluster over coated pits and that lead to invagination and clathrin uncoating are unclear

(Stahl and Schwartz, 1986). It was to be hoped that with receptor cloning and sequencing, a common factor might have been identified in different receptors which could act as a signal for receptor mediated endocytosis. In fact, four such receptors have now been cloned and sequenced. They include the receptors for epidermal growth factor (Ullrich et al. 1984), transferrin, low density lipoprotein (Russell et al. 1984) and asialoglycoproteins. However, no features common to all of them have been identified (May and Cuatrecasas, 1985).

Transferrin binding to a receptor is not the only stimulus which induces internalisation of the transferrin receptor. It is also stimulated by the presence of monoclonal antibodies to the transferrin receptor (McArdle and Morgan, 1984). In addition, incubation of cells with phorbol esters which are structurally unrelated to transferrin induces internalisation of the transferrin receptor, even in the absence of ligand (May et al. 1984; 1985). These esters are thought to act by stimulating the production of calcium-dependent protein kinase C, which leads to phosphorylation of the transferrin receptor. It is, however not clear whether phosphorylation of the receptor is the mechanism responsible for stimulating endocytosis. Receptor mediated endocytosis of transferrin iron is reduced by inhibitors of protein kinases, such as phenothiazines and tricyclic antidepressants (Hebbert and Morgan, 1985; Hunt and Marshall-Carlson, 1986). These agents, which also exhibit anti-calmodulin activity, are, however, not specific, (Hebbert and Morgan, 1985). There are also data to show that the immunopurified transferrin receptor may possess intrinsic protein kinase activity (Johnstone

et al. 1984) but most studies that have evaluated the effect of transferrin binding to receptor on receptor phosphorylation, have failed to demonstrate such activity (May and Cuatrecasas 1985). In addition, recent work by May and co-workers (1985) has demonstrated that cytoskeleton inhibitors inhibit phorbol ester induced transferrin receptor internalisation without simultaneously inhibiting receptor phosphorylation. All this evidence suggests that there is a complex interrelationship between receptors, phosphorylation enzymes, calcium, calmodulin and cytoskeletal elements.

Once invagination and formation of the receptorsome has occurred, the vesicle is transported by saltatory motion within the cell cytoplasm along microtubule and microfilament tracts (May and Cuatrecasas, 1985). Earlier data, obtained in reticulocytes, demonstrated very clearly that receptor mediated endocytosis of transferrin could be blocked by microtubular and microfilament inhibitors such as the vinca alkaloids and colchicine (Hemmaplardh et al. 1974). Thus, receptor internalisation involves interactions between membranes, clathrin, the cytoskeleton, microtubules, microfilaments and enzymes regulating phosphorylation of these structures. In addition, calcium and calmodulin appear to play a role.

13.2.3 Release of Iron from Transferrin in the Endocytic Vesicle

During the first 2 minutes after endocytosis, ligands still bound to their receptors leave the clathrin coated vesicles and become small peripheral vesicles and tubules. In the following 8-

10 minutes the intraluminal area of the vesicles becomes acidified by an active protonation which is energy dependent (Wileman et al. 1985). During this process the endocytic vesicles become larger and gain arm-like extensions. The acidified vesicle is intrinsic to the dissociation of ligand from its receptor and for this reason is referred to as the compartment of uncoupling of receptor and ligand (CURL) (Goldstein et al. 1979). In the acidic endocytic vesicle the ligand dissociates from its receptor and in this way is released for further cellular metabolism. Transferrin, however, is a little different. In the acidic environment of CURL, the iron dissociates from its carrier diferric transferrin to leave an iron free apotransferrin. The apotransferrin, which has a low receptor affinity at physiological pH, acquires very significantly increased affinity for the receptor in the acidic milieu of the endocytic vesicle. This has been demonstrated in hepatoma cells (Dautry-Varsat et al. 1983), in reticulocytes (Morgan, 1983) and in erythroleukaemia cells (Klausner et al. 1983). The endocytic vesicle is returned to the cell surface and here fuses with the cell membrane. In the process, the vesicle contents are exposed to the physiological pH. At this pH the apotransferrin dissociates from its receptor and thus becomes available again for further iron transport. The variable pH dependent affinity of differic- and apotransferrin for the transferrin receptor seems to be fundamental to transferrin recycling through the cell. Other receptors, such as those for epidermal growth factor, are not recycled and undergo digestion in the endocytic vesicle (Wileman et al. 1985).

There are a number of ways in which the endocytic cycle of transferrin can be disturbed. Weak bases (eg. chloroquine, ammonium chloride and methylamine) diffuse across membranes into cells in their uncharged, lipid soluble forms. They accumulate in endocytic vesicles and by becoming protonated, buffer the endocytic vesicle against acidification (Wileman et al. 1985). These weak bases have been demonstrated to inhibit iron delivery to cells, almost certainly by preventing endosomal acidification (Harding and Stahl, 1983; Paterson et al. 1984). In addition, inhibitors of energy metabolism have also been demonstrated to limit endosomal acidification and hence iron delivery to cells (Kailis and Morgan, 1974). Such a metabolic inhibitor is 2,4 dinitro-phenol (2,4 DNP). These metabolic inhibitors seem to affect iron delivery more than diferric transferrin internalisation, which is surprising, since the stripping of clathrin from the endocytosed vesicle appears to be an energy dependent process. Proton ionophores such as monensin dissipate pH gradients between cell compartments and, as a result, inhibit iron delivery (Stein et al. 1984). Their action is, however, a little more complex, since they also alter cellular ionic gradients (Wileman et al. 1985) and thereby inhibit transferrin recycling (Stein et al. 1984). Thus, the iron delivery to the cell after initial endocytosis appears to relate to energy dependent acidification of the endocytic vesicle and possibly also to other ionic fluxes across the endocytic vesicle membrane.

13.2.4 Biological Significance of Transferrin Receptors

As will be outlined in subsequent sections, virtually every human cell-type that has been evaluated has been demonstrated to have transferrin receptors. The question therefore arises as to their functional significance.

All living systems require iron for growth and survival (Aisen and Listowski, 1980). Iron is involved in several key areas of cellular metabolism, including electron transfer and energy generation by oxidative cytochromes, activation of molecular oxygen, nitrogen and hydrogen, and the generation of free radicals (May and Cuatrecasas, 1985). In addition, some work has suggested that iron is essential in the generation of the nucleotides necessary for DNA synthesis (Hoffbrand et al. 1976). Evidence in favour of iron's involvement in cell proliferation includes the finding of abnormal morphology in rapidly proliferating cells of the gastro-intestinal tract in iron deficiency (Ikkala and Siurala, 1964), and growth retardation in iron-deficient infants (Oski, 1979).

There are several pieces of evidence to suggest that the transferrin-iron-cell interaction is of importance in the regulation of cellular growth and proliferation. In this regard, there is some suggestion that such an effect may be independent of the iron content and that the transferrin-receptor interaction is the important component in growth regulation (May and Cuatrecasas, 1985). Transferrin has been demonstrated to be an essential requirement for cultured cells grown in serum-free conditions (Barnes and Sato, 1970). If cultured cells are

deprived of transferrin or if the transferrin receptor is blocked by monoclonal antibody specific for the binding site cellular proliferation is inhibited. This has been demonstrated with mitogen stimulated lymphocytes (Mendelsohn et al. 1983), mononuclear cells (Taetle et al. 1986), malignant leukaemic cells (Taetle et al. 1983; 1985; 1986), other tumour cells (Trowbridge and Lopez, 1982) and human erythroid burst forming units (Shannon et al. 1986). The inhibitory effect can be partially overcome by the addition of soluble iron. Mitogenic stimulation of lymphocytes induces sequential surface expression of receptors for both interleukin-2 (IL-2) and transferrin. This induction of IL-2 and transferrin receptors is essential for the initiation of proliferation of quiescent T-lymphocytes (Hamilton, 1982; Neckers and Cossman, 1983). The relationship between transferrin and cell growth may be even more complex. Activated T-cells, lymphoma cells and transformed melanoma cells have been shown to express or to produce a glycoprotein which is homologous to transferrin (Nishiya et al. 1980; Brown et al. 1981; 1982; Plowman et al. 1983) but which does not carry iron. In addition, a chicken B-cell lymphoma has been shown to contain an activated oncogene (ChBLYM-1) which has sequence homology with transferrin (Goubin et al. 1983) and which is capable of transforming fibroblasts on transfection (Cooper, 1982). The mechanisms by which transferrin might regulate growth are speculative, but may be related to an increased phosphorylation signal consequent upon the transferrin-receptor interaction (see page 237). Unfortunately any study of the importance of iron itself in the regulation of cell growth by transferrin-receptor interactions is bedevilled by difficulty in

preventing contamination of apotransferrin with iron.

There are data to show that the cell surface expression of transferrin receptor is not constant throughout the cell growth cycle. Fluorescent activated cell sorting techniques have demonstrated that the surface expression of immunoreactive transferrin receptors is maximal during the S, G_2 and M phases of the cell cycle (Seligman, 1983), which suggests that transferrin or iron or both are required after the G_1 phase of the cycle and that they are involved in the process of cell division. When monoclonal antibodies to the transferrin receptors are added to cell cultures, cell growth is arrested in the S (Trowbridge and Lopez, 1982) or the G_2 and M phases (Trowbridge et al. 1982). The phase at which arrest occurs appears to depend on the specific antibody.

It has been postulated that the transferrin receptor may play a part in host defence against neoplasia. The fact that there are so many transferrin receptors on rapidly proliferating and malignant cells initially raised the possibility that they might serve as a targetting mechanism for natural killer (NK) cells (Vodinelich et al. 1983; Baines et al. 1983). Evidence supporting this postulate included the inhibitory effect of diferric transferrin on NK cell killing, (Baines et al. 1983), the association between the extent of tumour cell expression of transferrin receptors and the degree of NK cell killing (Vodinelich et al. 1983) and, finally, the inhibition of NK cell killing by the addition of immunopurified transferrin receptors (Vodinelich et al. 1983). The finding that pre-incubation of NK

cells with transferrin enhanced NK cell activity, while antitransferrin antibody depressed it, suggested that the mechanism might involve occupied NK cell transferrin receptors interacting with non-occupied receptors on target cells (Baines et al. 1983). Another possible targetting mechanism that was considered was one which involved NK cells having a transferrin-like molecule on their surface. Suggestive evidence in favour of this hypothesis came from experiments which showed inhibition of NK cell activity by pretreatment with antitransferrin antibody and complement (Alarcon and Fresno, 1985). Doubt has, however, been recently cast on this postulated role of transferrin receptors in NK cell killing by the finding that up-regulation of receptor numbers on K562 cells by incubation with desferrioxamine and down-regulation by incubation with haemin had no effect on NK cell killing (Bridges and Smith, 1985). In addition, down-regulation of receptors on HeLa cells by incubation with iron saturated transferrin resulted in no change in NK activity. Furthermore infestation of HeLa cells with measles virus enhanced NK killing without affecting the numbers of receptors (Bridges and Smith, 1985). (It should be noted that HeLa cells are generally fairly resistant to NK cell killing (Bridges and Smith, 1985)).

Regulation of transferrin receptor expression is incompletely understood. As has been previously mentioned, proliferative stimuli and the phase of the cell cycle in some way regulate receptor numbers. This change is thought to be due to changes in the cellular distribution of receptors (Bomford and Munro, 1985). Limitation of the cellular iron supply by growth in

iron deficient media (Rudolph et al. 1985), or growth in the presence of chelating agents (Bottomley et al. 1985) results in increased receptor expression. This increase seems to be brought about by increased receptor synthesis (Bomford and Munro, 1985) and it has been suggested that the intracellular chelatable iron pool is the major regulator of transferrin receptor expression (Bridges and Cudkowicz, 1984; Bottomley et al. 1985). Haem has also been identified as a down regulator of transferrin receptor numbers in certain cells (Ward et al. 1984), while phorbol esters cause a redistribution of transferrin receptors between the surface and interior of cells. Recent work in mitogen stimulated and frankly neoplastic T cells has suggested that the increased expression of transferrin receptors, as a prerequisite for cell proliferation, is mediated, in part, by an increase in the intracellular concentration of calcium (Neckers et al. 1986). This can be blocked by the calcium channel blocking agent diltiazem (Neckers et al. 1986). Clearly, much has yet to be learned about the control of receptor expression.

13.2.5 Transferrin Receptor Activity in Various Cell Types

The data presented thus far on transferrin cell interactions are based predominantly on work with reticulocytes. From work on an array of other cells there would appear to be a striking homology of receptor function and behaviour between cell types. This section will highlight iron-transferrin-cell interactions in non-erythroid cells and particularly in hepatocytes and macrophages.

13.2.5.1 Transferrin receptor activity on hepatocytes

The liver contains 2 major cell types, namely reticuloendothelial (Kupffer) cells and parenchymal cells (hepatocytes). Hepatocytes take up transferrin iron, haem-haemopexin iron, haptoglobin-haemoglobin iron and ferritin iron (Hershko et al. 1972). Of these sources, plasma transferrin is thought to be the most significant, with some 10-20% of plasma iron turnover in rats being directed at the hepatocyte (Page et al. 1984). (In humans the figure is less than 5% (Bothwell and Finch, 1962)). A number of mechanisms are responsible for hepatocyte iron uptake from transferrin. Studies with isolated hepatocytes suggest the existence of specific diferric transferrin receptors analagous to reticulocytes (Grolich et al. 1977; Young and Aisen, 1980; 1981). However, other work has shown that some of the iron uptake by these cells is not inhibited by low temperatures (4^{o}C) or by proteolytic enzymes (Grolich et al. 1979), and is non-saturable at higher concentrations of transferrin (Cole and Glass, 1983; Page et al. 1984). These findings are incompatible with the concept that iron uptake is solely by receptor mediated endocytosis. It is now apparent that iron uptake from transferrin occurs via a combination of receptor mediated endocytosis, which is most effective at lower transferrin concentrations, and non-saturable transferrin binding which occurs mainly at higher concentrations; Fluid phase endocytosis also occurs but is very inefficient (Sibille et al. 1982; Page et al. 1984; Trinder et al. 1986). A large proportion of transferrin iron uptake by hepatocytes must involve energy-dependent processes, since it is inhibited by the energy

metabolism blocking agent cyanide (Thorstensen and Romslo, 1986).

Asialodifferic transferrin is an additional component to be considered in iron-transferrin-hepatocyte interactions. As discussed previously, the hepatocyte has an asialoprotein receptor which seems to be concerned with the removal of desialated proteins from the circulation. There is now evidence that desialated diferric transferrin may be taken into the hepatocyte either via the conventional diferric transferrin receptor or via an asialoprotein receptor (Young et al. 1983; Dekker et al. 1985). Desialated transferrin is processed normally by reticulocytes (Dekker et al. 1984) but whether this is also true for the hepatocyte is not quite clear. It has been claimed that hepatocytes and Kupffer cells do not have conventional transferrin receptors and that previous reports of such receptors were artefacts of impure cell populations (Soda and Tavassoli, 1984). In fact, it has also been claimed that only endothelial cells possess conventional transferrin receptors in the liver and that these cells take up diferric transferin (Kishimoto and Tavassoli, 1985). It has been postulated that diferric transferrin becomes desialated during its passage transendothelially and that the desialated transferrin then gains access to the hepatocyte via the asialoprotein receptor (Tavassoli et al. 1986). Such a pathway would not be incompatible with the observation that asialotransferrin is taken up by an asialoprotein receptor on the hepatocyte, with its iron being released intracellularly and the transferrin catabolised (Young et al. 1983). However, it is clearly incompatible with the

finding that desialated transferrin only donates iron to the hepatocyte if it is following the conventional transferrin receptor pathway (Dekker et al. 1985). When it is taken up by the asialoprotein receptor the transferrin is recycled without unloading its iron (Dekker et al. 1985). To confuse the issue further, the proponents of the asialoreceptor model have recently reported that the apparent absence of diferric transferrin receptors on hepatocytes was, in fact, an artefact of cell preparation with collagenase (Kishimoto and Tavassoli, 1986). Other workers have however demonstrated that very pure populations of hepatocytes, prepared from rat livers by collagenase perfusion, do express very significant numbers of transferrin receptors (Vogel et al. 1987).

From the previous discussion it is apparent that the iron-transferrin-hepatocyte interaction is complex and incompletely understood, with the relative importance of the diferric transferrin receptor to hepatocyte iron uptake yet to be defined. In common with other data on transferrin's cell proliferative role, it has been noted that partial hepatectomy results in significant increases in transferrin receptor numbers (Hirose-Kumagai et al. 1984; Tei et al. 1984). In addition, a clear correlation has been demonstrated between the disappearance rate of transferrin and the growth rate of hepatomas in rats (Aulbert et al. 1980). The handling of diferric transferrin and desialated transferrin by hepatoma cells will be explored further in this thesis (Chapter 18 pages 315-338).

13.2.5.2 Transferrin receptor activity on macrophages

After leaving the bone marrow, mononuclear phagocytes travel

through the blood as monocytes before reaching those target tissues where they constitute the resident macrophage population (Adams and Hamilton, 1984). The resident tissue macrophage is thought to represent a quiescent cell, and as such, does not respond to lymphokine or endotoxin by becoming cytolytic (Adams and Hamilton, 1984). Various stages of macrophage activation against tumour cells have been described (Adams and Hamilton, 1984). A responsive macrophage becomes cytolytic when exposed to endotoxin after prior lymphokine stimulation. In contrast, a primed macrophage becomes cytolytic after the exhibition of endotoxin only, while an activated macrophage is fully cytolytic. Not only are macrophages heterogeneous in terms of their cytolytic response to activators, but they also differ in their expression of transferrin receptors. Transferrin receptor expression is decreased or absent on human monocytes and on resident and activated macrophages, while it is enhanced on responsive and cultured macrophages (Andreesen et al. 1983; 1984; Parmley et al. 1983; Adams and Hamilton, 1984; Hamilton et al. 1984; Weiel et al. 1984; Yeh et al, 1984). These findings are compatible with previous observations of progressive uptake of transferrin iron by cultured macrophages (MacSween et al. 1969, Summers and Jacobs, 1976; Wyllie, 1977; Sizemore and Bassett, 1984). Although it has been assumed that the iron uptake in this setting is by receptor-mediated endocytosis, there is in fact little direct evidence for this. In addition, the reason for the existence of such a pathway in these cells is unclear, since reticuloendothelial cells acquire large amounts of iron from the

haemoglobin of old and damaged red cells. Whether these receptors may be playing more of a role in regulating the differentiation of the resident macrophage is unclear. The iron-transferrin-macrophage interactions will be explored further in this thesis (Chapters 15, 16). The extent to which transferrin receptors on the macrophage may be modulated in inflammation will also be discussed (Chapter 17).

13.2.5.3 Transferrin receptor activity on other cell types

Transferrin receptor function has been evaluated in a large number of different cell types. Significant numbers of receptors have been demonstrated on placental cells (Brown et al. 1982), mitogen stimulated lymphocytes (Larrick and Cresswell, 1979; Galbraith et al. 1980; Hammarstrom et al. 1982; Bomford et al. 1983; 1986), cultured fibroblasts (Octave et al. 1981; Ward et al. 1982), K562 erythroleukaemia cell lines (Enns et al. 1983), testicular cell lines (Perez-Infante and Mather, 1982), HeLa cells (Ward et al. 1982), malignant and non-malignant breast tissue (Vandewalle et al. 1985; Walker and Day, 1986) and Friend erythroleukaemic cells (Glass et al. 1978). Of considerable interest is the finding that receptors from both normal and malignant human cells appear to be identical both electrophoretically and by peptide mapping (Stein and Sussman, 1983).

13.3 HOW DOES IRON GET OUT OF CELLS?

To maintain iron economy there must be a balance between the amounts of iron delivered to and released from cells each day.

Transferrin iron is principally directed to red cell precursors in the bone marrow. The major source of iron returning to the plasma is the reticuloendothelial system where senescent red cells are broken down (Morgan, 1981). The liver and gastrointestinal mucosal cells also contribute in a small but significant way to the plasma iron content (Morgan, 1981). The mechanism by which iron is released from cells is unknown. Whether iron is released to apotransferrin as an active process or whether apotransferrin picks up the iron after it has been released is not clear. Clinical observations suggest that there must be an active mechanism regulating the process, since iron is released from reticuloendothelial cells and from gastrointestinal cells in proportion to the body needs. However, in the anaemia of chronic disorders iron is held up in reticuloendothelial cells and in gut mucosal cells in the face of a fall in the plasma iron and transferrin saturation. Iron is thus being retained in these cells despite a significant concentration gradient (Roeser, 1980; Lee, 1983). In contrast, there is relatively little iron in the gut mucosal cell and reticuloendothelial cell relative to the striking overload of parenchymal cells and plasma transferrin in subjects with idiopathic haemochromatosis (Brink et al. 1976). This implies that iron is being released from these cells against a significant concentration gradient. These various observations on disturbances of cellular iron handling suggest that iron release or retention must be an active process. Previous work has shown that the release of labelled ferritin from rat peritoneal macrophages is greater in the presence of iron-free fetal calf

serum than it is with iron-saturated serum (Fedorko, 1974). Similar results have been obtained with hepatic cells, isolated perfused rat livers (Baker et al. 1975) and rat hepatocyte suspension studies (Baker et al. 1977). However, specific apotransferrin-hepatocyte interaction did not seem to be essential, since iron chelators such as desferrioxamine were equally effective in promoting iron release. In 1982 it was claimed that specific apotransferrin receptors had been noted on cultured rat peritoneal macrophages. This specific binding was not influenced by diferric transferrin but was displaceable by cold apotransferrin (Nishisato and Aisen, 1982). If such apotransferrin receptors do exist on reticuloendothelial cells, their presence would certainly help to explain the mechanisms involved in iron release. Such a finding would also be compatible with the observation that the intravenous infusion into experimental animals of transferrin raises the plasma iron concentration (Morgan, 1981). The same group of workers who initially identified apotransferrin receptors on reticuloendothelial cells, subsequently reported that incubation of iron loaded macrophages in media containing apotransferrin resulted in the progressive saturation of the apotransferrin with iron (Saito et al. 1986). Iron release was also enhanced by the addition of apotransferrin but its presence was not essential. It was, however, noteworthy that the macrophages also released considerable amounts of iron as ferritin which raises the question as to whether leakage was occurring from the cells as a result of decreased viability. Questions relative to the possible existence of apotransferrin receptors on reticuloendothelial

cells are addressed experimentally later in this thesis (Chapter 16 page 293).

13.4 TRANSFERRIN AND INTESTINAL IRON ABSORPTION

While the quantitative aspects of iron absorption have been well defined, the manner and form(s) in which iron is transferred from the gastrointestinal lumen to mucosal cells are still unclear. On the one hand, it has been suggested that inorganic iron is taken up by diffusion with a concentration gradient, while there is also evidence that transferrin is involved in the process of inorganic iron absorption. A major factor limiting the absorbability of inorganic iron is its tendency to be converted to insoluble ferric hydroxide at high pH's unless ligands such as ascorbic acid are present (Bothwell et al. 1979). In this context, it has been claimed that small intestinal epithelium contains an isotransferrin which is secreted into the gastrointestinal lumen in much the same way as bacteria secrete siderophores (Huebers et al. 1971; 1983; Pollack and Lasky, 1976). The intestinal transferrin picks up inorganic iron in the lumen and the iron-transferrin complex is then taken up by the intestinal epithelium via a process of receptor mediated endocytosis. Evidence in support of this hypothesis includes the finding of a high molecular weight elutable factor in the lumina of iron deficient rats which promotes iron absorption (Huebers et al. 1971). Mucosal transferrin has been found in the lumen, on the mucosal surface and inside the mucosal cells of the duodenum

and jejunum (El-Shobaki et al. 1977; Huebers et al. 1976). The concentration of intestinal transferrin has been claimed to increase in rats when iron deficiency is present (Halliday et al. 1976). In addition, it has been shown that the iron-transferrin complex is stable at neutral pH, that transferrin is resistant to digestive breakdown, and that the concentration of biliary transferrin increases in iron deficient rats (Huebers et al. 1983). A significant correlation has also been demonstrated between iron absorption and the transferrin:ferritin ratio in isolated mucosal cells (Savin and Cook, 1980). Despite these seemingly plausible arguments, there are a number of problems in accepting the mucosal transferrin model as being of relevance to human iron absorption.

It has not been possible to demonstrate that the mucosal cells actually produce an isotransferrin. Indeed, recent molecular biological work has been unable to demonstrate any transferrin mRNA in gastrointestinal mucosal cells (Idzerda et al. 1986). This does not completely negate the mucosal transferrin model, since the bile may be the source of intestinal transferrin. An additional problem has been raised by a recent study in which it was not possible to demonstrate transferrin receptors on the luminal surface of mucosal cells (Parmley et al. 1985) and on brush border (Banerjee et al. 1986) using immunocytochemical tests. Because of these uncertainties, our own laboratory recently did a study in which the possible relevance of transferrin to iron absorption was investigated in human subjects. The absorption of iron bound to transferrin was evaluated in 7 subjects with pernicious anaemia and acid fast

achlorhydria. These particular subjects were chosen because the presence of achlorhydria would be expected to prevent any acid induced dissociation of iron from transferrin in the lumen of the stomach. All subjects were shown to be able to absorb inorganic iron (the geometric mean absorption of ferrous ascorbate was 18.9% (11.9-30%)). The diferric transferrin complex gave a geometric mean iron absorption of 1.4% (0.4-5.1%), whilst ferric chloride alone gave a geometric mean iron absorption of 1.9% (1.0-3.4%) (Bezwoda et al. 1986).

The inability to demonstrate mucosal transferrin synthesis, mucosal transferrin receptors or an enhancing effect of transferrin on iron absorption in achlorhydric subjects, mitigate strongly against the hypothesis that mucosal transferrin plays a key role in iron absorption. It could be argued that since there are minor biochemical differences between mucosal and serum transferrin (Huebers et al. 1976), experimental findings obtained with serum transferrin may not be valid. However, recent data indicate that the treatment of serum transferrin with bile results in an acid shift in its isofocussing behaviour and after such treatment it co-migrates with intestinal transferrin (Idzerda et al. 1986).

13.5 THE REGULATION OF INTERNAL AND EXTERNAL IRON EXCHANGE

There exists good experimental evidence that there is an inverse relationship between iron absorption and iron stores and a direct relationship between iron absorption and erythropoietic activity (Bothwell et al. 1958). With depletion of iron stores, there is a reciprocal rise in iron absorption, while any sudden change in erythroid activity is associated with a corresponding change in iron absorption. In clinical situations the storage iron content is the single most important determinant of iron absorption (Bezwoda et al. 1979; Baynes et al. 1987). Erythropoietic activity also exerts an effect especially in conditions such as thalassaemia major, where iron absorption is markedly increased (Bothwell et al. 1979). How this coupling of iron absorption to the iron storage status and the erythropoietic iron need is accomplished is unknown. The most plausible of current hypotheses identifies the iron content of individual tissues as an important determinant of iron absorption (Bannerman et al. 1962; Crosby 1963; Cavill et al. 1975). According to this hypothesis individual tissues donate iron to plasma transferrin in proportion to (a) the size of the iron pools within them, and (b) the number of empty transferrin iron binding sites in circulation. The number of empty sites depends, in part at least, on the rate of iron delivery to the erythroid marrow, which, in turn, depends on the degree of erythropoietic activity. Thus, iron absorption would be expected to rise both in iron deficiency, a condition in which tissue pools of iron are small,

and in states of increased erythropoiesis, where the requirements of the major receptor tissue are increased.

Evidence in favour of the empty site hypothesis was produced by Taylor and Gattenby (1966) when they showed that the percentage saturation of transferrin influenced iron absorption. In addition, Hallberg and Solvell (1960) reported a fall in iron absorption when the saturation of transferrin was raised by the infusion of iron. In contrast, intravenous apotransferrin appeared to enhance iron absorption. These results were in agreement with the finding that iron absorption in rats was enhanced when the total iron-binding capacity was increased (Hyde, 1957). These various results were, however, not confirmed in other studies. Raising the transferrin saturation was not found to influence iron absorption in rats, dogs or humans (Wheby and Jones, 1963; Pollack et al. 1963; Wheby and Umpiere, 1964; Fronstein et al. 1967), while elevation of the unsaturated iron-binding capacity failed to enhance iron absorption in rats (Schade et al. 1969; Levine et al. 1972). The conclusion that apotransferrin is not essential for iron release from the mucosal cell was further suggested by the high rates of iron absorption noted in subjects with atransferrinaemia (Goya et al. 1972).

In recent experiments, Finch and co-workers (1982) studied the relationship of internal and external iron exchange in a rat model in which the size of the erythroid receptor pool could be rapidly increased by the infusion of varying numbers of reticulocytes. Sudden increases in the erythroid receptor mass were accompanied by an increase in the plasma iron turnover and an increase in gastrointestinal iron absorption. These changes

occurred despite the fact that there was no detectable change in the plasma iron concentration. The possibility that a subtle increase in the unsaturated iron-binding capacity might have been responsible for the increased absorption was excluded by the finding that there was no increase in iron absorption after the injection of large amounts of apotransferrin. It was concluded that iron turnover through the plasma is primarily determined by the number of tissue receptors for transferrin iron and that the amount of iron supplied by each donor tissue, including the intestinal mucosa, is dependent on the output from the other donor sites (Huebers et al. 1982). These workers speculated that the donation of iron by transferrin immediately facilitates its ability to procure more iron. This "activated transferrin" would then be responsible for an increased rate of iron acceptance from the donor sites. This "activated transferrin hypothesis" was experimentally evaluated in this laboratory in an *in vitro* model and no confirmatory evidence could be found for it (Aron et al. 1985).

13.6 SUMMARY

Receptor-mediated endocytosis of diferric transferrin has been briefly reviewed in this chapter. Its biological significance in terms of iron delivery, regulation of proliferation and differentiation and host defence has been outlined and the ways in which receptor mediated endocytosis of diferric transferrin can be inhibited have been described.

Specific aspects of diferric transferrin-hepatocyte, diferric transferrin-reticuloendothelial cell and apotransferrin-reticuloendothelial cell interactions have been reviewed. The possible role of the hepatocyte asialoprotein receptor in the uptake of iron has been mentioned. Finally, some aspects of the mechanisms of inorganic iron absorption and the regulation of internal and external iron exchange have been reviewed.

Chapter Fourteen

METHODS EMPLOYED IN THE STUDY OF TRANSFERRIN-IRON-CELL INTERACTION

14.1 INTRODUCTION

In this section of the thesis, various studies are described in which the nature of the interaction of transferrin with certain cells, which have been grown in tissue culture, has been explored. Transferrin was studied in both its apo- and diferric-forms. The cells studied have specific relevance to inflammation and neoplasia. The reticuloendothelial cell, which has a central role in regulating the inflammatory response, was studied by setting up human monocytes in short term tissue cultures. The two other cell types which were studied were both human malignant cells in long term tissue culture. The one was a malignant hepatoma cell line (PLC/PRF 5) and the other was a line developed in this laboratory from a human ovarian malignancy (UWB-17).

14.2 CELL CULTURE TECHNIQUES

14.2.1 Cultured Human Blood Monocytes

Peripheral blood mononuclear cells were prepared from whole blood by centrifugation over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) by the method of Boyum (1968). The mononuclear cells were then added to 35mm Petri dishes in aliquots, each of which contained 1×10^6 monocytes. Two ml of Basal Medium Eagle S.S. (BMESS) (Highveld Biological, Johannesburg, South Africa) containing 10% autologous serum and antibiotics was added to each dish. The cells were cultured in this medium at 37°C in a 5% CO_2 incubator (Flow Laboratories, Irvine, Scotland) for 7 days.

Transferrin binding and iron uptake studies were performed on day 7. At this time 100% of the adherent cells excluded trypan blue, more than 98% stained positively with non-specific esterase (Yam et al. 1971) and more than 98% phagocytized opsonised yeast particles (Miller et al. 1969). It was not possible to quantitate accurately the number of cultured monocytes in each Petri dish. However reasonable uniformity between Petri dishes was ensured by adding the same plating density of mononuclear cells to each dish in individual experiments. Morphologically, the cultured macrophages were heterogenous in terms of size, shape and nuclear ploidy. An example of the culture density achieved is shown in Figure 30. In this example the Petri dish had had the medium removed, been air dried, fixed in methanol and stained with Giemsa stain. One of the cultured blood monocytes is shown in Figure 31. The phagocytic capacity of the cultured monocyte is demonstrated in Figure 32 in which hyperphagocytosis of opsonised yeasts and red blood cells is evident.

14.2.2 Cultured Hepatoma Cells (PLC/PRF 5 line)

These cells were initially grown up in tissue culture flasks. They grew in adherent fashion on the bottom of the flasks. The tissue culture medium employed was Dulbecco's modified Eagle medium with glutamine but without sodium bicarbonate (Flow Laboratories, Irvine, Scotland). This had 20mM Hepes and antibiotics added to it. The medium was further enriched by the addition of 5% heat inactivated foetal calf serum (Highveld Biological, Johannesburg, South Africa). Cells were

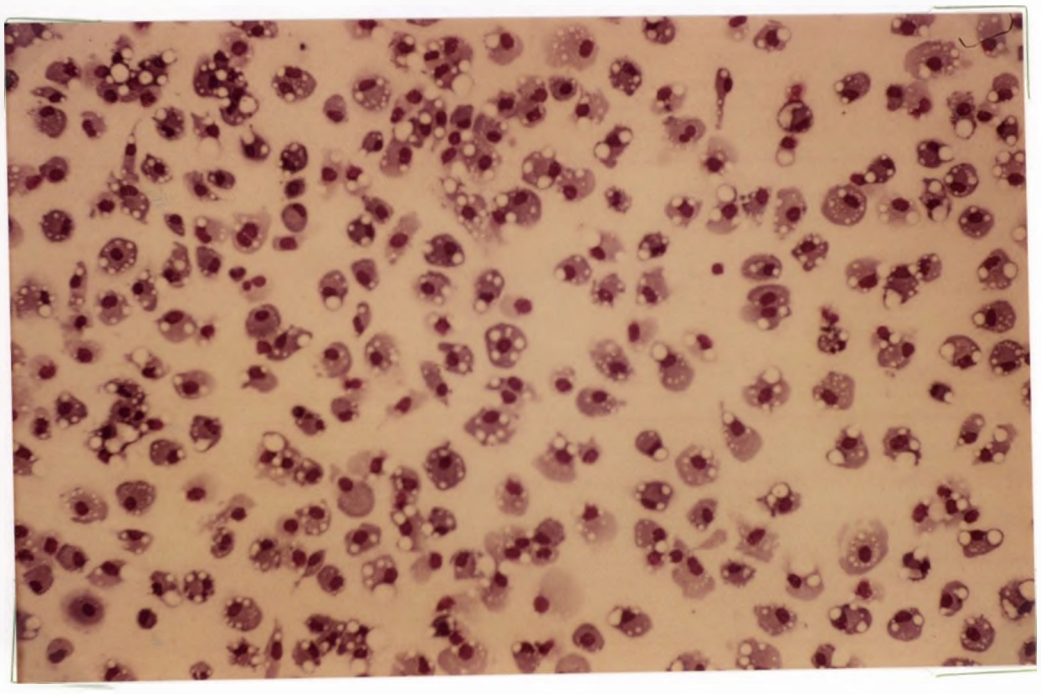


Figure 30. An example of the culture density obtained with cultured human blood monocytes.

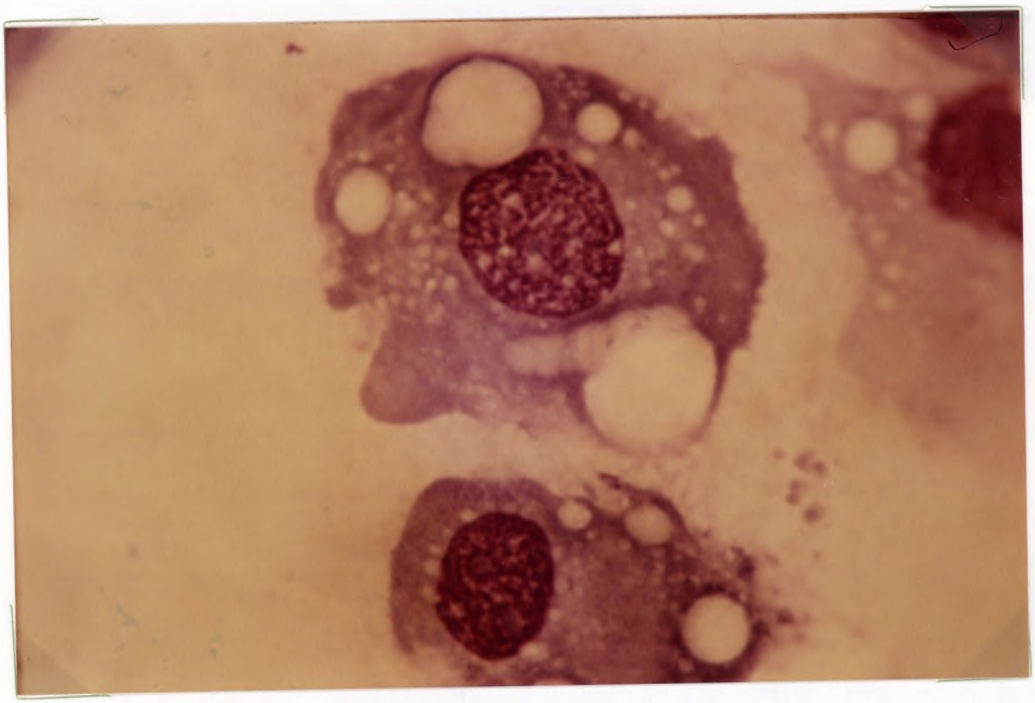


Figure 31. Magnified view of a cultured blood monocyte.

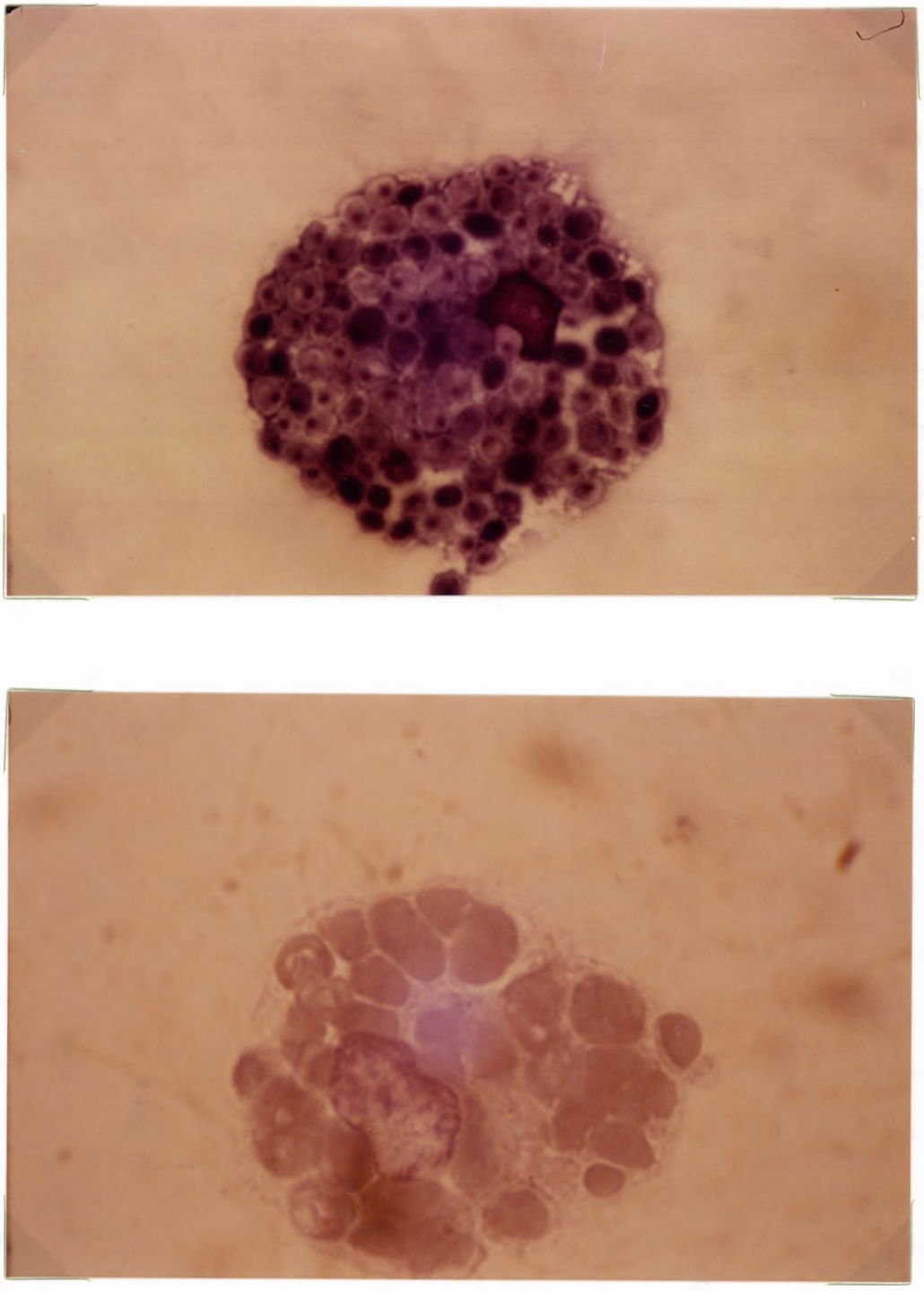


Figure 32. Demonstration of phagocytic capacity of cultured blood monocytes. The ingestion of opsonised yeast particles and red blood cells by cultured blood monocytes is demonstrated.

grown in flasks containing 20ml of medium in a 5% CO_2 incubator. When cells reached confluence they were either split into three to four flasks after trypsinisation (see 14.2.4) or plated into Petri dishes at a plating density of 1×10^5 cells per dish. They were then grown up in the Petri dishes to a time when they were nearly confluent. The relevant binding studies were then performed. The numbers of cells employed in binding studies were quantitated as described in 14.2.4. The hepatoma cells were a gift from Professor J Alexander who had established the line.

14.2.3 Cultured Ovarian Carcinoma Cells (UWB-17)

The ovarian cell carcinoma line was established in this laboratory by Ms T Golombick from malignant ovarian ascites. The characterisation of the line is currently being prepared for publication. These cells, which also grew in an adherent fashion, were cultured in an enriched medium 199-SS (Highveld Biological, Johannesburg, South Africa). The medium was then further enriched with 10% foetal calf serum and antibiotics were also added. Cells were grown in a 5% CO_2 incubator in flasks containing 20ml of medium. The cells were handled in similar fashion to the hepatoma cells described above. However, they took almost 7 days to reach near confluence once plated into Petri dishes.

14.2.4 Splitting, Plating and Counting Cultured Hepatoma and Ovarian Cells

Cells were trypsinised in a sterile solution of 0.1% trypsin, 0.05% EDTA, 0.05% glucose in magnesium and calcium free

phosphate buffered saline. To do this, the medium was decanted from flasks, the flasks were then rinsed with the 0.1% trypsin mixture (5ml) and thereafter they were incubated for 3-5 minutes in another 5ml of the 0.1% trypsin mixture. Incubation resulted in the cells lifting off the culture surface. These cells were then decanted into a sterile Falcon tube (15ml). The flask was then rinsed with 10ml of respective medium containing 10% foetal calf serum. This serum inactivates trypsin activity. Cells were then sedimented by centrifugation at 1000 rpm for 3 minutes. The supernatant was discarded and the cells resuspended in complete medium. They were then either split into 3-4 flasks or aliquoted into Petri dishes at a density of 1×10^5 per dish. To quantitate the number of cells present in binding studies five representative Petri dishes were chosen and trypsinised as outlined above, except that only 1 ml of 0.1% trypsin solution was employed. After washing, the cells were resuspended in a known volume of medium and representative aliquots were counted by means of a haemocytometer.

14.3 PREPARATION OF APO- AND DIFERRIC TRANSFERRIN

14.3.1 Preparation of ^{59}Fe Diferric Transferrin and Doubly Labelled ^{59}Fe, ^{125}I Diferric Transferrin

Human transferrin was obtained from Sigma (St. Louis, Missouri, USA), while ^{59}Fe as $FeCl_3$ and ^{125}I as Bolton Hunter reagent were purchased from Amersham (Amersham, Buckinghamshire, United Kingdom). The transferrin was labelled with ^{59}Fe as

diferric transferrin by dissolving apotransferrin in normal saline. The pH was adjusted to 7.6 by the addition of 40 /ul 0.2M Tris-Hcl buffer and 20 /ul 1M bicarbonate solution per mg transferrin. Sufficient $^{59}FeCl_3$ to saturate the apotransferrin was then added and the final pH was adjusted to 7.4. Excess iron was removed by passage through a Dowex 1 x 8 column. The diferric nature of the transferrin was confirmed both spectrophotometrically and by 6M urea polyacrylamide gel electrophoresis using the method of Makey and Seal (1976). The ^{59}Fe labelled diferric transferrin was iodinated with ^{125}I by the method of Bolton and Hunter (1973). The end concentration of diferric transferrin was determined by radial immuno-diffusion.

14.3.2 Preparation of ^{125}I Apotransferrin

Apotransferrin was produced by taking doubly labelled diferric transferrin and subjecting it to dialysis for two periods of 12 hours against two changes of a 0.1 M acetate buffer at pH 4.5. The buffer contained 0.5 mM desferrioxamine (Ciba-Geigy, Basle, Switzerland). The sample was then dialysed against three changes of acetate buffer (pH 4.5) to remove the desferrioxamine. The purity of both the iodinated apotransferrin and the doubly labelled diferric transferrin was confirmed by electrophoresis on 6M urea polyacrylamide gels. In order to minimise the effects of contaminating iron, all buffers were prepared with water which had been distilled, deionised, extracted with 200-400 mesh Chelex resin (Biorad, Richmond, California, USA) and extracted with Dithizone (BDH, Poole, United

Kingdom).

14.3.3 Demonstration of Protein Purity

The labelled diferric transferrin and apotransferrin were checked for electrophoretic purity on 6M urea polyacrylamide gels after the method of Makey and Seal (1976). By this method transferrin can be resolved into four species depending on the degree of iron saturation of the protein. Thus, transferrin can exist as apotransferrin, monoferric-a transferrin, monoferric-b transferrin and diferric transferrin. This separation into four distinct species is demonstrated on a Coomassie blue stained 6M urea polyacrylamide gel in Figure 33.

14.4 BINDING STUDIES

14.4.1 Diferric Transferrin Binding Studies to Cultured Monocytes

The culture medium and any non-adherent cells were removed from the Petri dishes on day 7. The Petri dishes were then washed three times with phosphate buffered saline. One ml RPMI 1640 (Flow Laboratories, Irvine, Scotland) was then added to each Petri dish. The RPMI contained 1% bovine serum albumin. The dishes were then incubated for 30 minutes. In studies employing potential pharmacological inhibitors, the inhibitor was next added in the required concentration and the incubation was continued for a further 30 minutes unless otherwise stated. Where potential inhibitors were diluted in dimethyl sulphoxide (DMSO),

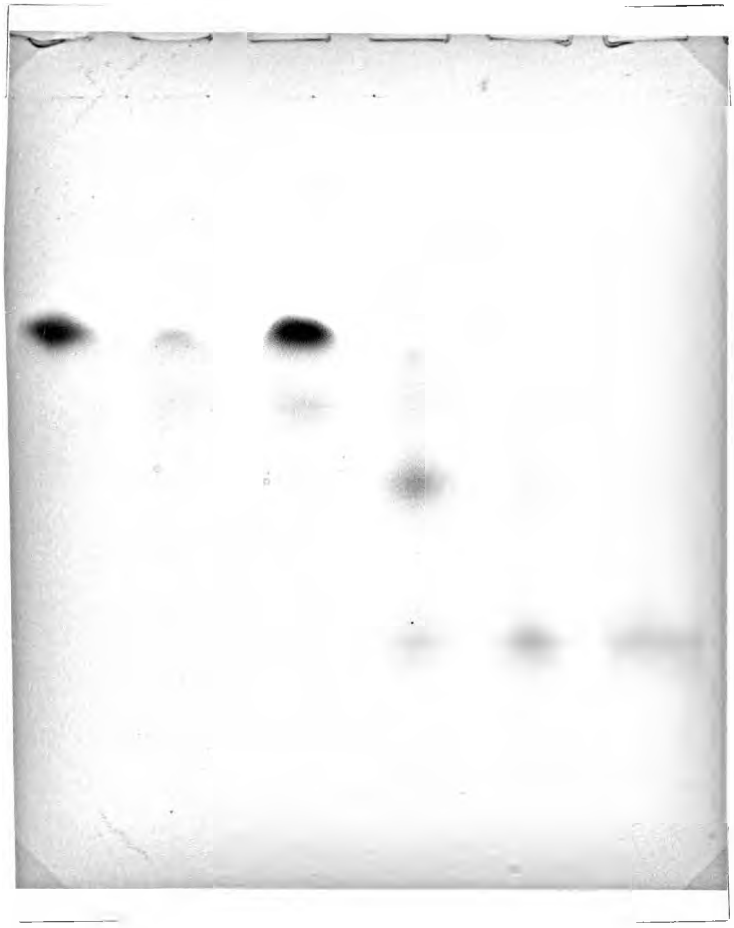


Figure 33. 6M Urea polyacrylamide gel electrophoresis showing resolution of transferrin from above downwards into apo-, monoferric-a, monoferric-b and diferric species (Coomassie blue stain).

control dishes contained RPMI with DMSO in the same concentration. On completion of the pre-incubation, doubly labelled diferric transferrin was added to each Petri dish in both the dose response and time response studies. Hamilton syringes were utilised for accurate dispensing. Control Petri dishes containing no cells were included for each dose or time component. Petri dishes were also set up containing a 100 fold excess of cold diferric transferrin. The Petri dishes were then incubated in a 5% CO_2 incubator at 37°C. In some experiments a temperature of 4°C was employed. The supernatants were removed for counting on completion of the incubation. The adherent cells were then washed three times with ice cold phosphate buffered saline. The washing fluid was also collected for counting. One ml of a solution containing 0.1M NaOH and 0.1% "Tween" 80 (BDH Chemicals, Poole, England) was added to each Petri dish and they were then incubated at room temperature with agitation for 10 minutes. The cultured monocytes, which were now completely lysed, were removed to test tubes and the Petri dishes were then each washed once more with 1ml of the detergent solution. This was also collected. The resultant cellular lysate was counted for radioactivity in a Packard Autogamma counter (Model 5650, Packard Instruments, Downers Grove, Illinois, USA). Appropriate corrections for cross counting were made in the experiments with doubly labelled diferric transferrin. Specific binding was calculated as the total binding less the counts obtained from control Petri dishes without cells and less the counts obtained from Petri dishes which had also had a 100 fold excess of cold

diferric transferrin added. The dose dependent binding curve was obtained by incubation for 180 minutes and was analysed after the method of Scatchard (1949).

In the chase experiments, Petri dishes of cells were incubated with doubly labelled diferric transferrin for 2 hours. After removal of the supernatants, the cells were washed 3 times with phosphate buffered saline and 1ml of RPMI containing unlabelled diferric transferrin was then added. Time related changes in cellular transferrin and iron were then measured. At each point in each study the mean value was calculated from four separate Petri dishes.

14.4.2 Apo- and Diferric Transferrin Binding to Cultured Monocytes at pH 7.4 and pH 6.0

The culture medium and any non-adherent cells were removed from the Petri dishes on day 7. The Petri dishes were then washed three times with RPMI 1640 (Flow Laboratories, Irvine, Scotland). One ml of transferrin-free RPMI containing 1% defatted bovine serum albumin (Sigma, St. Louis, Missouri, USA) with a pH adjusted to either 6.0 or 7.4 was then added to each Petri dish and they were incubated for 30 min. At both pHs the macrophages remained firmly adherent to the Petri dish. Desferrioxamine (10/uM) was also included in the incubation medium in the studies in which apotransferrin was present. All media and buffers were prepared from deionised, distilled water which had been extracted as previously described with Chelex and Dithizone. Incremental doses of either doubly labelled diferric transferrin or radio-

iodinated apotransferrin were then added to the Petri dishes at time 0. Incubation, washing, lysing, counting and calculating procedures were as described in the previous section. To check on the degree of contamination of apotransferrin occurring during the incubation, supernatant controls were obtained from the Petri dishes, concentrated and applied to similar 6M urea gels. On completion of the electrophoresis, individual lanes were sliced into 3mm strips and counted for radioactivity.

14.4.3 Diferric Transferrin Binding Studies to Cultured Hepatoma Cells and Ovarian Cancer Cells

The binding study procedures were the same as those outlined for cultured blood monocytes, but with a few modifications. In the case of hepatoma cells the binding was conducted in transferrin and foetal calf serum-free Dulbecco's modified Eagle medium in the presence of 1% bovine serum albumin. In the case of the ovarian cancer cells, the binding was conducted in transferrin free RPMI in the presence of 2% bovine serum albumin. The 2% albumin was necessary to limit nonspecific binding of diferric transferrin.

14.4.4 Immunofluorescent Demonstration of Transferrin Receptors on Cultured Monocytes

A chamber slide containing 1 ml medium and $\pm 10^6$ cultured blood monocytes was incubated for 30 min at $4^{o}C$ after the addition of 20/ul of a saturating concentration of OKT9 monoclonal antibody (Ortho-mune, Ortho Diagnostic Systems,

Rarlton, New Jersey, USA). The cells were washed and labelled with 5/ul of fluorescein isothiocyanate-labelled $F(ab)_2$ fragments of goat anti-mouse immunoglobulin. The slides were then examined under a fluorescent microscope. Appropriate negative controls were employed.

14.4.5 Reagents and Chemotherapeutic Agents used in Inhibition Studies

Various reagents and chemotherapeutic agents were used in transferrin binding studies to delineate patterns of inhibition. The chemical reagents which were used were of analytical quality and included ammonium chloride (Protea Laboratory Services, Johannesburg, South Africa) and 2,4-dinitrophenol (BDH, Poole, England). The chemotherapeutic agents vindesine and vincristine were obtained from Eli Lily (Isando, South Africa). Other pharmacological agents included verapamil (Knoll, Johannesburg, South Africa), chlorpromazine (May Baker, Johannesburg, South Africa), imipramine (Ciba-Geigy, Isando, South Africa), cytochalasin B, colchicine and dimethyl sulphoxide (Sigma, St. Louis, Missouri, USA).

14.5 ESTABLISHING THE FATE OF THE ENDOCYTOSED IRON

Cultured monocytes were incubated with diferric transferrin labelled with ^{59}Fe for various times. At the end of each time interval the supernatants were removed and the macrophages washed 3 times with phosphate buffered saline. Thereafter the cells were

removed in 1 ml of either phosphate buffered saline or column equilibration buffer using a rubber policeman and the cells were then disrupted by 3 x 30 second bursts of sonication followed by ultracentrifugation at 100 000 x g for 20 minutes. The resultant supernatants (representing cytosolic material) were either electrophoresed on a 5% sodium dodecyl sulphate (SDS) polyacrylamide gel with ferritin and transferrin markers, or were applied to an AcA 44 ultrogel (LKB, Bromma, Sweden) column which had been calibrated with transferrin and ferritin. The pellet resulting after centrifugation was thought to represent membrane material. The column procedures and buffers were those described by Pollack and co-workers (1985). Cultured hepatoma cells and ovarian cancer cells were prepared in similar fashion after prior loading with transferrin iron after a prolonged period of incubation.

Chapter Fifteen

TRANSFERRIN RECEPTORS AND TRANSFERRIN IRON UPTAKE BY CULTURED HUMAN BLOOD MONOCYTES

15.1 INTRODUCTION

As has been discussed in Chapter 13.2.5.2 the reticulo-endothelial cells of the body are heterogeneous in terms of their cytolytic responses to lymphokines and other activators and in terms of their expression of transferrin receptors (Adams and Hamilton, 1984). Expression of transferrin receptors is reduced or absent on human monocytes and on resident and some activated macrophages, while it is enhanced on macrophages in tissue culture and responsive macrophages (a macrophage that is cytolytic after exposure to endotoxin and pre-treatment with lymphokines) (Andreesen et al. 1983; 1984; Parmley et al. 1983; Adams and Hamilton, 1984; Hamilton et al. 1984; Weiel et al. 1984; Yeh et al. 1984). As discussed in Chapter 13.2.5.2 various workers have reported transferrin-iron uptake by cultured blood monocytes (MacSween and MacDonald, 1969; Summers and Jacobs, 1976; Wyllie, 1977; Sizemore and Basset, 1984). This transferrin iron uptake has been inferred rather than demonstrated to involve a process of receptor mediated endocytosis. The current study was done to obtain further insight into the interaction between diferric transferrin and transferrin receptors on cultured human blood monocytes with special attention being paid to the effects of a number of metabolic inhibitors of receptor mediated endocytosis.

15.2 RESULTS

The cultured human blood monocytes used in the various studies were shown to possess transferrin receptors using the OKT9 antibody, with virtually all the cells exhibiting dense fluorescence on their surfaces. An example of this is shown in Figure 34.

Several experiments demonstrated a dose dependent, saturable uptake of transferrin, with a Kd of 3.6×10^{-8}M (Figure 35). Allowing for a 50-90% loss of monocytes in culture (Johnson et al. 1977) the calculated number of receptors varied between 1.25 - 2.5×10^{5} per cell.

Fifty pmol doubly labelled diferric transferrin were added to each Petri dish in order to assess the time dependent uptake of transferrin and of transferrin iron at 37°C and at 4°C (Figure 36). The molar ratio of iron to transferrin uptake was also followed as a function of time and temperature. At 37°C there was a steady accumulation of iron in cultured monocytes with time and this was associated with a similar rise in the molar ratio of iron to transferrin. Some transferrin binding was also noted at 4°C but uptake and internalisation of iron was only noted at 37°C. At 4°C the cellular iron to transferrin ratio remained at 2 throughout the experiment.

The internalisation of iron was further shown in a chase experiment (Figure 37). Doubly labelled diferric transferrin (50 pmol) was added to Petri dishes containing cultured monocytes and after 2 hours' incubation the supernatants were removed, the

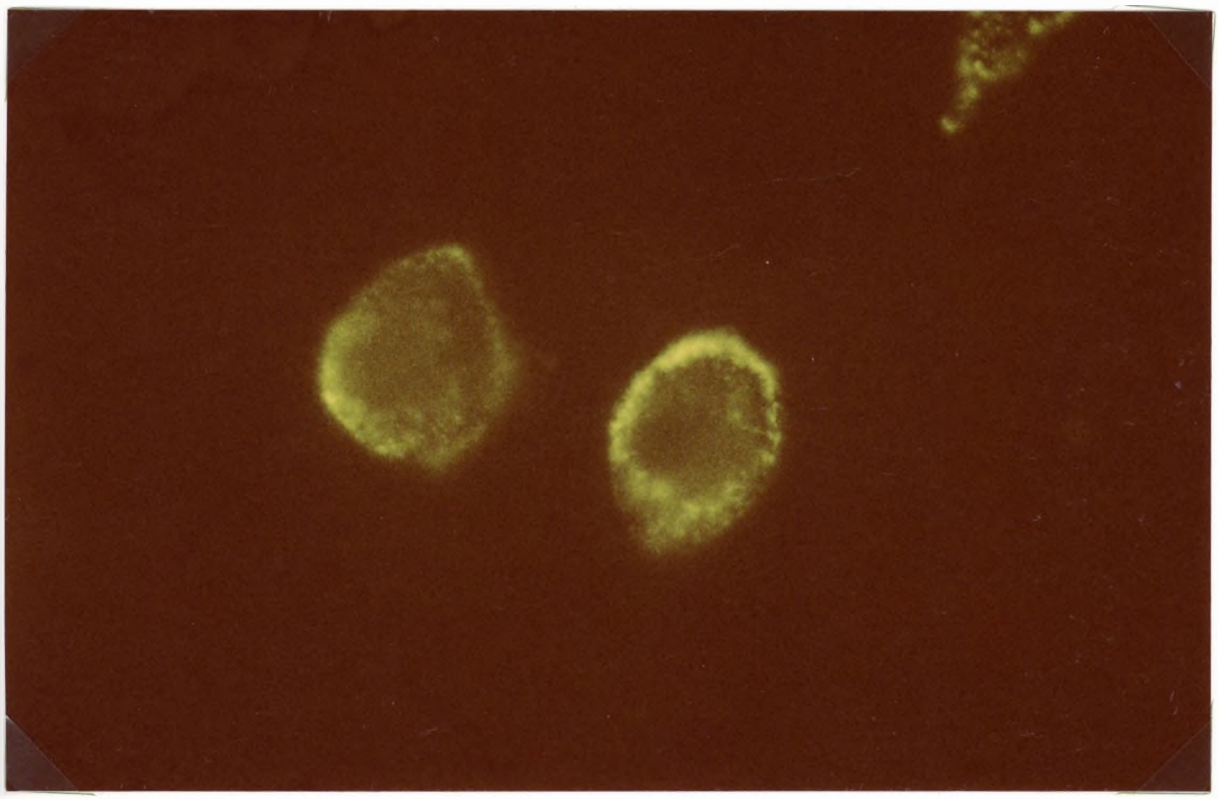


Figure 34. Immunofluorescent pattern obtained when using an indirect fluorescent technique to demonstrate immunoreactive transferrin receptors on the surface of cultured blood monocytes.

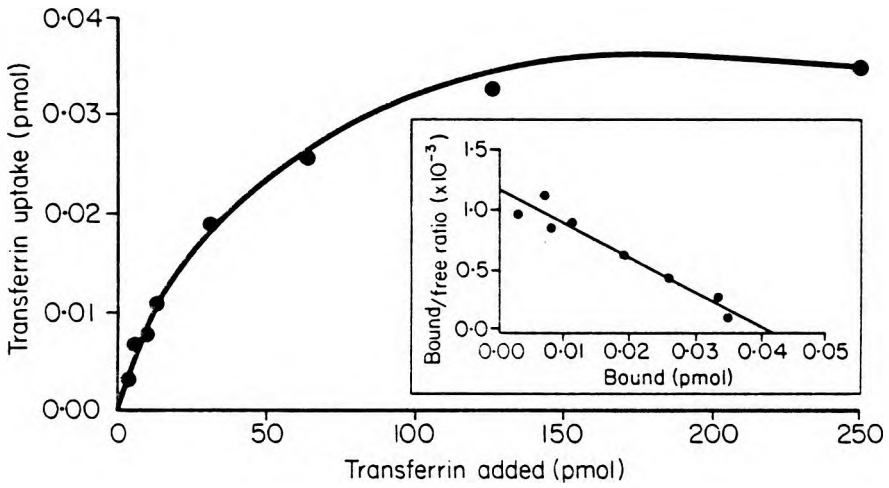


Figure 35. The dose dependent uptake of diferric transferrin by cultured blood monocytes. The inset Scatchard analysis ($r = -0.97$) shows a Kd of 3.6×10^{-8} M. The calculated number of receptors per cultured monocyte was approximately $1.25\text{-}2.5 \times 10^{5}$.

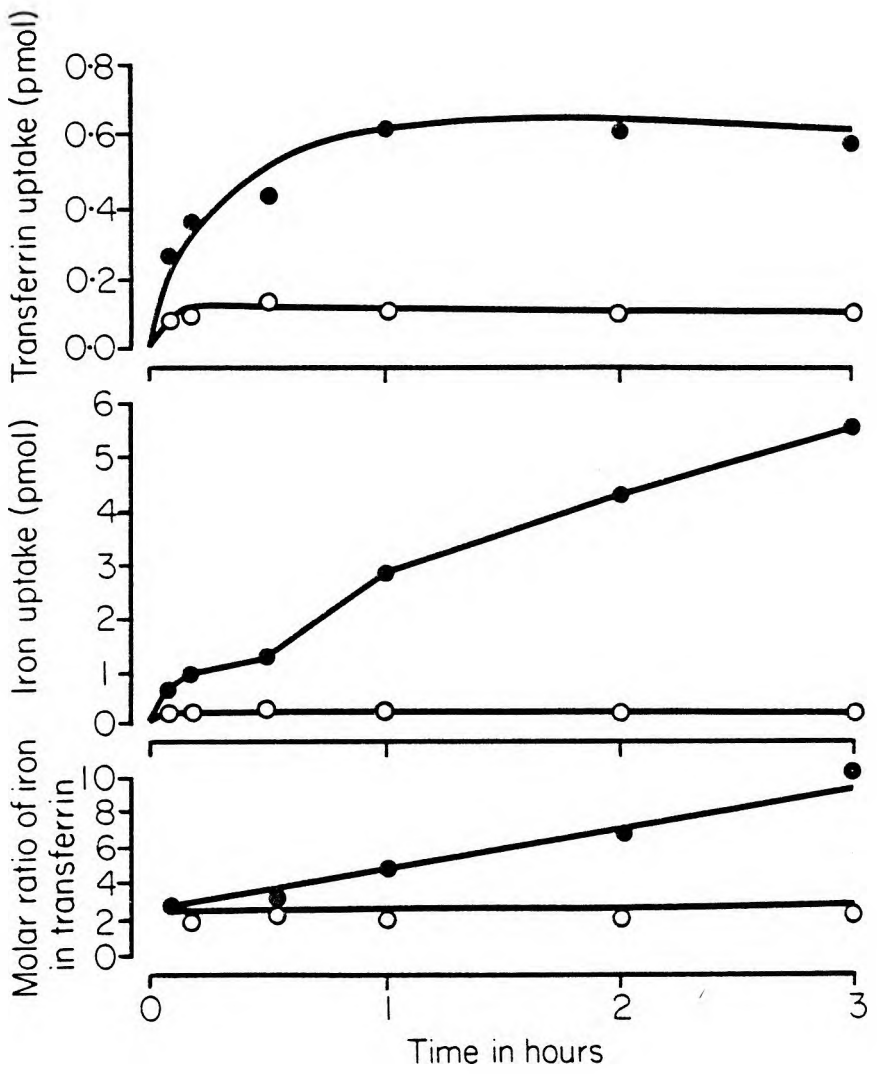


Figure 36. The time dependent uptake of transferrin and of iron by cultured blood monocytes at 37°C (●) and 4°C (○).

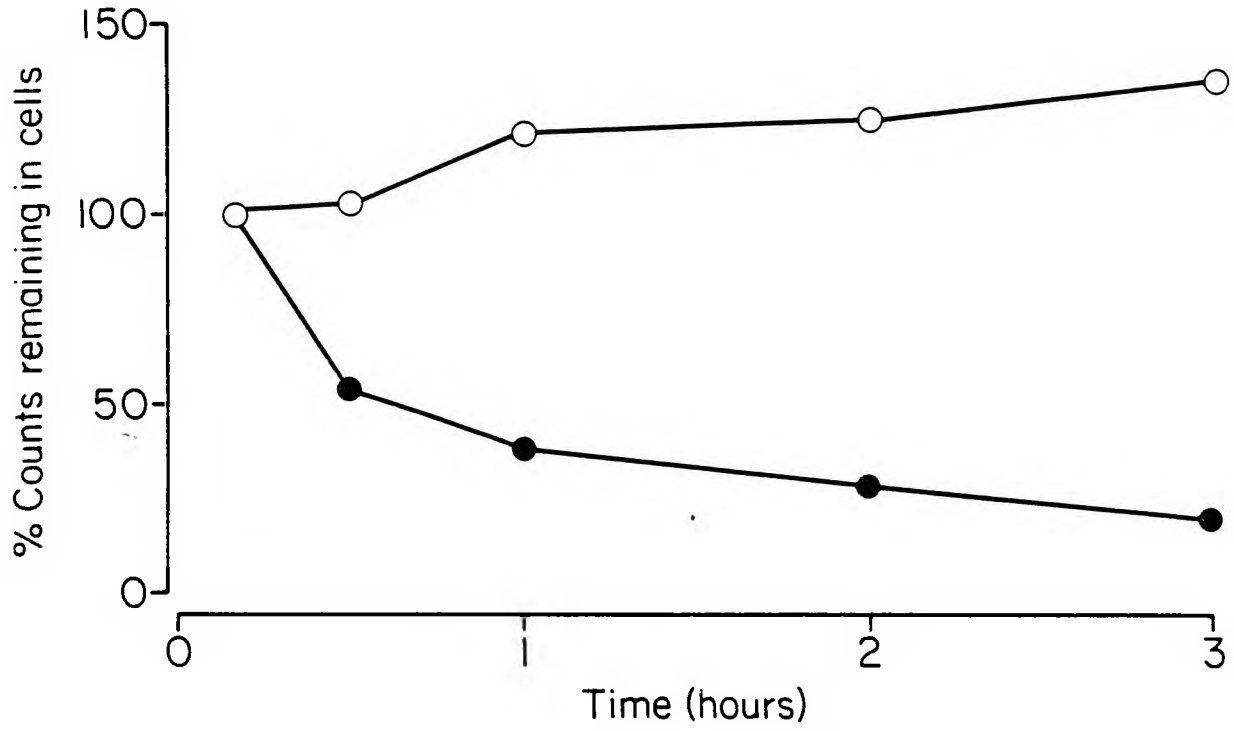


Figure 37. The results of a chase experiment in which monocytes were cultured for 3 hours in a medium containing 100 pmol/ml cold diferric transferrin after having been previously incubated in a medium containing 50 pmol/ml of diferric transferrin doubly labelled with ^{125}I and ^{59}Fe. The transferrin (●) and iron (O) counts are expressed as percentages of the values at 10 minutes.

cells washed and equivalent volumes of medium containing 100 pmol/ml of cold diferric transferrin were then added to each dish. There was a steady fall in ^{125}I activity in the cells over the following 3 hours, while ^{59}Fe activity remained relatively constant.

The effects of an inhibitor of endosomal acidification (20 mM ammonium chloride) on the uptake of transferrin and iron by cultured monocytes were studied over a period of 3 hours (Figure 38). Transferrin uptake was moderately suppressed, while iron uptake was almost totally abolished. In consequence, there was no rise in the molar ratio of iron to transferrin over the period of study. The effects of different concentrations of 2,4-dinitrophenol, which is another inhibitor of endosomal acidification, were studied in another experiment (Table 17). There was a dose dependent reduction in transferrin and iron uptake, with the effect being far greater on iron uptake. As a result, there was a marked drop in the molar ratio of iron to transferrin.

The effects of two microtubular poisons (colchicine and vindesine) and of one microfilament poison (cytochalasin B) on transferrin and iron uptake by cultured monocytes were studied in 3 further experiments (Table 17). Each was associated with a dose dependent decrease in transferrin and iron uptake. The relative depression of transferrin and iron uptake was similar except in the case of vindesine where the uptake of iron was somewhat more affected than was that of transferrin.

The calcium antagonist, verapamil, was tested in a final

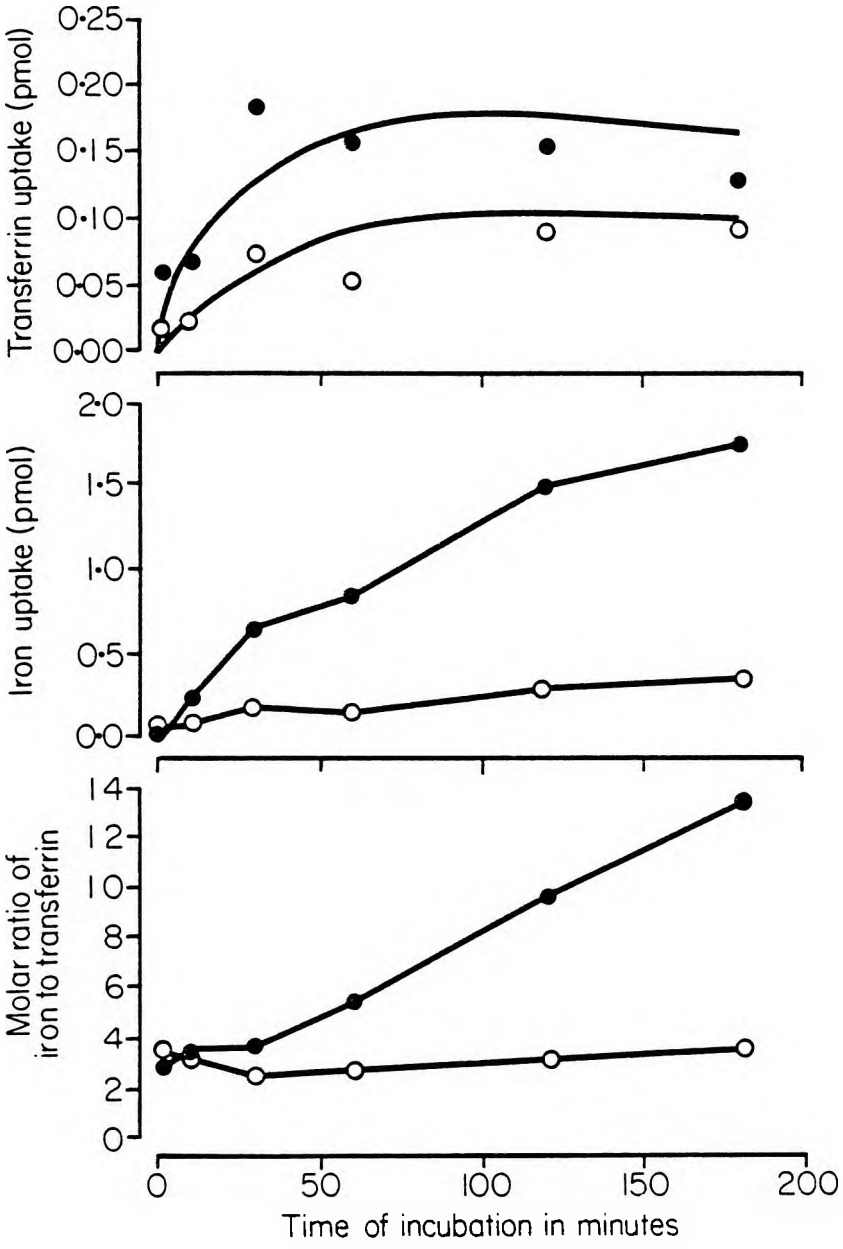


Figure 38. The effects of 20 mM NH_4Cl on the uptake of transferrin and of iron by cultured blood monocytes. Results are shown in the absence (●) and presence (○) of NH_4Cl.

Table 17. The effects of various chemical agents on transferrin and iron uptake by cultured monocytes

Chemical	Mechanism of inhibition	Dose	% Basal transferrin uptake	% Basal iron uptake	% Basal molar ratio of iron to transferrin
2.4 Dinitro-phenol	Inhibitor of glycolysis and ATP production	0	100	100	100
		0.10 mM	83	34	41
		0.25 mM	91	8	9
		0.50 mM	57	7	12
		0.75 mM	44	2	5
		1.00 mM	1	0	0
Colchicine	Microtubular inhibitor	0	100	100	100
		2 /ug/ml	64	45	71
		10 /ug/ml	59	45	76
		50 /ug/ml	53	40	74
		100 /ug/ml	47	37	78
		500 /ug/ml	15	14	95
Vindesine	Microtubular inhibitor	0	100	100	100
		2 /ug/ml	53	38	71
		10 /ug/ml	38	26	70
		50 /ug/ml	22	15	67
		100 /ug/ml	16	5	34
		500 /ug/ml	0	2	-
Cytochalasin B	Microfilament inhibitor	0	100	100	100
		0.25 /ug/ml	50	52	103
		0.50 /ug/ml	64	54	83
		1.00 /ug/ml	55	55	99
		2.00 /ug/ml	38	42	110
Verapamil	Calcium ion antagonist	0	100	100	100
		0.25 /ug/ml	83	93	111
		0.50 /ug/ml	77	86	112
		1.00 /ug/ml	74	77	104
		2.00 /ug/ml	72	79	110

dose related experiment (Table 17). It caused a modest depression in transferrin and iron uptake when present in concentrations of 0.25 to 2.0 /ug/ml.

The time dependent effects of chlorpromazine (112 /umol/l) and imipramine (102 /umol/l), which are calmodulin antagonists (Hebbert and Morgan, 1985), were studied in further experiments on cultured monocytes. The agents were diluted in dimethyl sulphoxide, which was also added in similar amounts to the control dishes. Chlorpromazine caused a 50% reduction in transferrin uptake and a 60% reduction in iron uptake at times up to 3 hours. In consequence, there was a 20% reduction in the molar ratio of iron to transferrin. Imipramine caused a 30% reduction in both transferrin and iron uptake, with the molar ratio of iron to transferrin remaining constant.

The fate of the endocytosed iron was studied in 2 experiments. In the first, 250 pmol diferric transferrin labelled with ^{59}Fe were added to Petri dishes containing cultured monocytes. The cellular uptake of ^{59}Fe was measured at 4, 24 and 48 hours. After centrifugation the 48 hr pellet (membrane fraction) contained 9% of the cell associated iron. The cytosolic cellular lysates were then applied to AcA 44 ultrogel columns and the elution profiles were compared with those obtained with ^{59}Fe labelled diferric transferrin and ^{59}Fe labelled ferritin. There was a linear increase in ^{59}Fe uptake over the period of observation. Over 70% of the counts applied to the columns were recovered at each time. At 4 hours 53% was identified as ferritin and 12% as transferrin bound. At the 2 later times more

than 90% was in ferritin and between 2 and 3% in the transferrin peak (Figure 39). No low molecular weight pool could be identified. The presence of labelled ferritin was further confirmed by electrophoresis of cellular lysates on 6% SDS polyacrylamide gel which was equilibrated for transferrin and ferritin. The major radioactive peak corresponded exactly to the position of the ferritin marker (Figure 40).

15.3 DISCUSSION

The present results clearly confirm the existence of diferric transferrin receptors on the surface of cultured human blood monocytes and demonstrate that the interaction between transferrin iron and cultured monocyte receptors is very similar to that reported for erythroid precursors and other cells in relation to iron uptake, intracellular release of iron and transferrin recycling (Morgan, 1981). Transferrin uptake was found to be dose dependent and saturable, with a calculated Kd of 3.6×10^{-8}M. At 37^{o}C there was a steady build-up of iron in cultured monocytes, with a steady rise in the molar ratio of iron to transferrin. At 4^{o}C there was a marked inhibition of transferrin uptake and a total loss of iron uptake. The results of chase experiments were compatible with the presence of an endocytic pathway. Cold diferric transferrin displaced the labelled transferrin but not the iron. Inhibitors of endosomal acidification, ammonium chloride and 2,4 dinitrophenol, were shown to be powerful inhibitors of iron unloading from

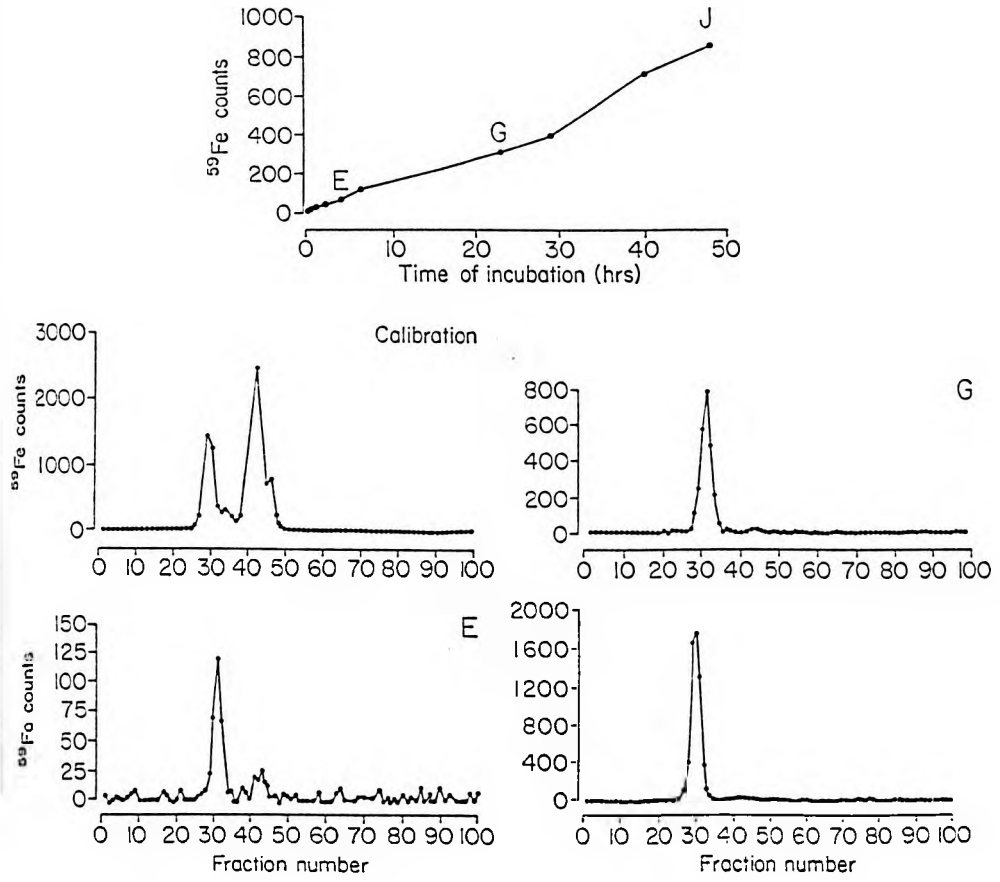
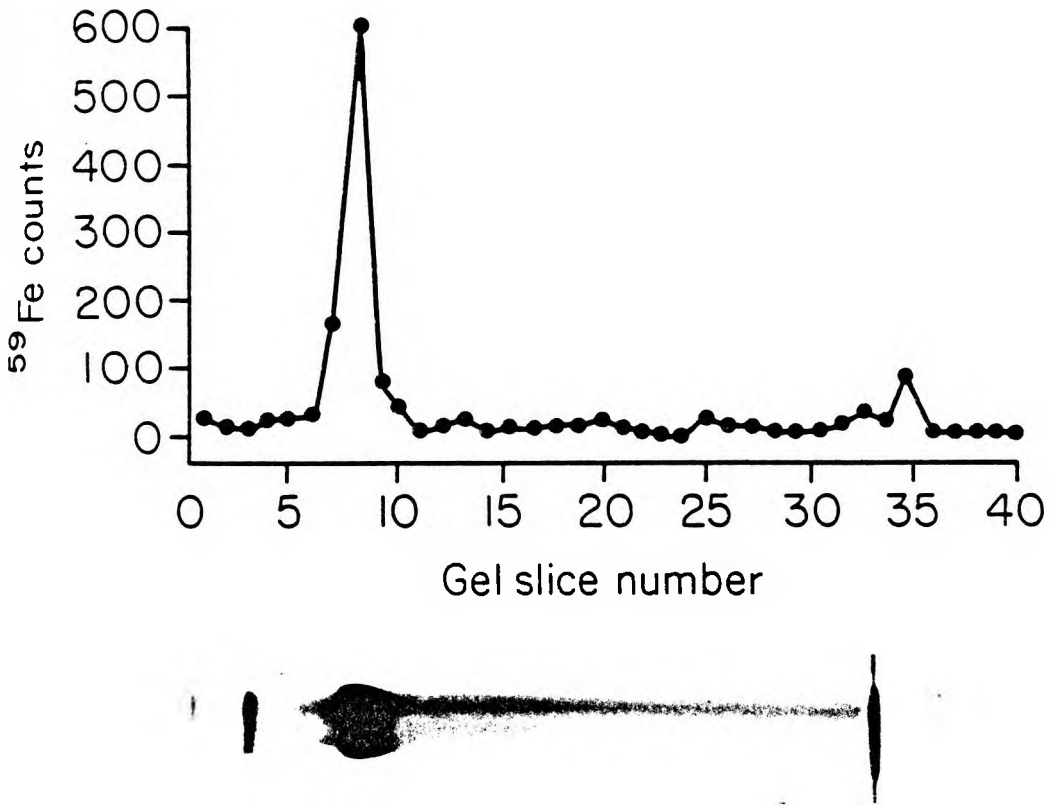


Fig. 39. Progressive uptake of iron from transferrin by cultured blood monocytes. It can be noted from the AcA 44 (ultrogel) separation that the iron accumulates progessively in ferritin. E, G and J represent durations of incubation. Calibration shows the ferritin (first) and transferrin (second) peaks.



ure 40. Results of SDS polyacrylamide gel electrophoresis on the cellular lysate obtained from blood monocytes which had been incubated for 48 hours in a medium containing ^{59}Fe diferric transferrin. The radioactive counts are shown in the upper frame and a ferritin marker in the lower one.

cell associated diferric transferrin (Kailis and Morgan, 1974; Harding et al. 1983a; 1983b; Wileman et al. 1985). The microtubular and microfilament inhibitors, colchicine, vindesine and cytochalasin B, inhibited transferrin and iron uptake to the same degree, which, in general, is similar to what Hemmaplardh and Morgan (1974) found using rabbit reticulocytes. The only difference related to cytochalasin B, the microfilament inhibitor, which was not shown to inhibit uptake in reticulocyte experiments (Hemmaplardh and Morgan, 1974). A further important factor in the regulation of transferrin receptor mediated endocytosis has recently been identified. It has been shown that the phosphorylation enzyme protein kinase C, which requires calcium and probably calmodulin as co-factors, is involved in the interaction between the cytoskeleton and the cell membrane (Hebbert and Morgan, 1985; May et al. 1985; Hunt and Marshall-Carlson, 1986). In this regard, it was of interest that the calcium ion antagonist, verapamil, and the calmodulin inhibitors, chlorpromazine and imipramine, caused some inhibition of both transferrin and iron uptakes. They did not, however, seem to affect iron unloading, since the molar ratio of iron to transferrin was not altered. Recent work has indicated that calcium channel blockers, by reducing intracellular calcium, inhibit transferrin receptor synthesis in mitogen stimulated and neoplastic lymphocytes (Neckers et al. 1986). The time course of the current study suggests, however, that the current findings were independent of this inhibition.

The results of the present study have demonstrated receptor

mediated uptake of transferrin iron by cultured monocytes and suggest an endocytic pathway very similar to that noted in erythroid precursors. The reason why such a pathway should be present in reticuloendothelial cells is, however, not clear, since they acquire large amounts of iron from the haemoglobin of broken down red cells. In this context, the interaction between transferrin iron and macrophages may be important in a wider context, which includes cellular differentiation (Andreesen et al. 1983; 1984; May et al. 1985) and cellular proliferation Galbraith et al. 1980).

15.4 SUMMARY

Transferrin receptors have been previously found on human macrophages and it has also been shown that transferrin iron is taken up by these cells. It has therefore been inferred that the uptake is receptor mediated and involves an endocytic pathway. The subject was addressed directly in the present study in which the transferrin-iron-receptor interaction was characterised in cultured human blood monocytes. Specific, saturable diferric transferrin binding was demonstrated, with a Kd of 3.6×10^{-8}M and a calculated receptor density of $1.25 - 2.5 \times 10^{5}$ receptors per cell. Incubation at 4^{o}C markedly reduced transferrin binding and completely inhibited iron uptake. Chase experiments confirmed progressive cellular loading of iron, with concomitant loss of transferrin. Inhibitors of endocytic vesicle acidification (ammonium chloride and 2.4-dinitrophenol) inhibited iron

unloading from cell associated diferric transferrin, while microtubular inhibitors (colchicine and vindesine) and a microfilament inhibitor (cytochalasin B) reduced diferric transferrin uptake but had little effect on the iron unloading pathway. A similar effect was noted with a calcium ion antagonist (verapamil) and with 2 calmodulin antagonists (chlorpromazine and imipramine). These latter findings suggest the importance of cytoskeleton-membrane interactions via a calcium, calmodulin and protein kinase C mediated system. Internalised iron accumulated progressively as ferritin within the cultured monocytes. Most of the internalised iron was demonstrated to be present intracellularly in ferritin.

Chapter Sixteen

APOTRANSFERRIN RECEPTORS AND THE DELIVERY OF IRON FROM CULTURED HUMAN BLOOD MONOCYTES

16.1 INTRODUCTION

Most cells obtain their iron requirements from the iron transport protein, transferrin, by a process of receptor mediated endocytosis (Morgan, 1981) and the iron is then retained and used in a variety of metabolic processes. In contrast, there are specialised transport cells which subserve a different function in that they procure iron in a variety of ways, process it and then deliver it to the transferrin of plasma. The mucosal cell obtains its iron from the lumen of the gut, the placental cell from transferrin and the reticuloendothelial cell from effete red cells. However, the mechanisms by which these cells then release iron into the circulation is not known. Elucidating the factors involved could well be of importance in understanding a number of disorders of iron transport. For example, the anaemia of chronic disorders is associated with a decreased plasma iron concentration in the face of normal or increased reticuloendothelial iron (Lee, 1983), whilst the intracellular iron content of both reticuloendothelial and mucosal cells is relatively reduced in the inherited disorder, idiopathic haemochromatosis, while circulating transferrin is saturated with iron (Brink et al. 1976; Bothwell et al. 1983). Both these findings suggest alterations in the release mechanisms of iron from transport cells. It is against this background that recent observations on rat peritoneal macrophages are of particular interest. Large numbers of apotransferrin receptors have been identified on these cells (Nishisato and Aisen, 1982; Saito et

al. 1986) and they have been shown to be distinct from diferric transferrin receptors in that they are not displaceable by diferric transferrin (Nishisato and Aisen, 1982). However, the importance of these apotransferrin receptors in iron delivery to the plasma is not clear (Saito et al. 1986). The present study was done to find out whether they are present on human reticuloendothelial cells and to evaluate their possible role in internal iron exchange.

16.2 RESULTS

The relative purity of the labelled diferric transferrin and the apotransferrin was assessed electrophoretically on a 6M polyacrylamide gel (Figure 41). The diferric transferrin was relatively pure, with only a small amount of the monoferric species. Counts from ^{59}Fe and ^{125}I were closely associated. The apotransferrin, which had been obtained by acid dialysis against desferrioxamine, was pure.

The binding of diferric transferrin and apotransferrin to macrophages cultured from blood monocytes was studied at pH 7.4 (Figures 42 and 43) and at pH 6.0 (Figures 44 and 45). Specific binding of diferric transferrin occurred at ph 7.4 (Figure 42), with a Kd of 1.3×10^{-8}M (Figure 43). In contrast, binding of apotransferrin was minimal (Figure 42) and could not be analysed by Scatchard analysis. It was noted that excess unlabelled apotransferrin displaced diferric binding but to an appreciably lesser degree than an excess of diferric transferrin. The binding

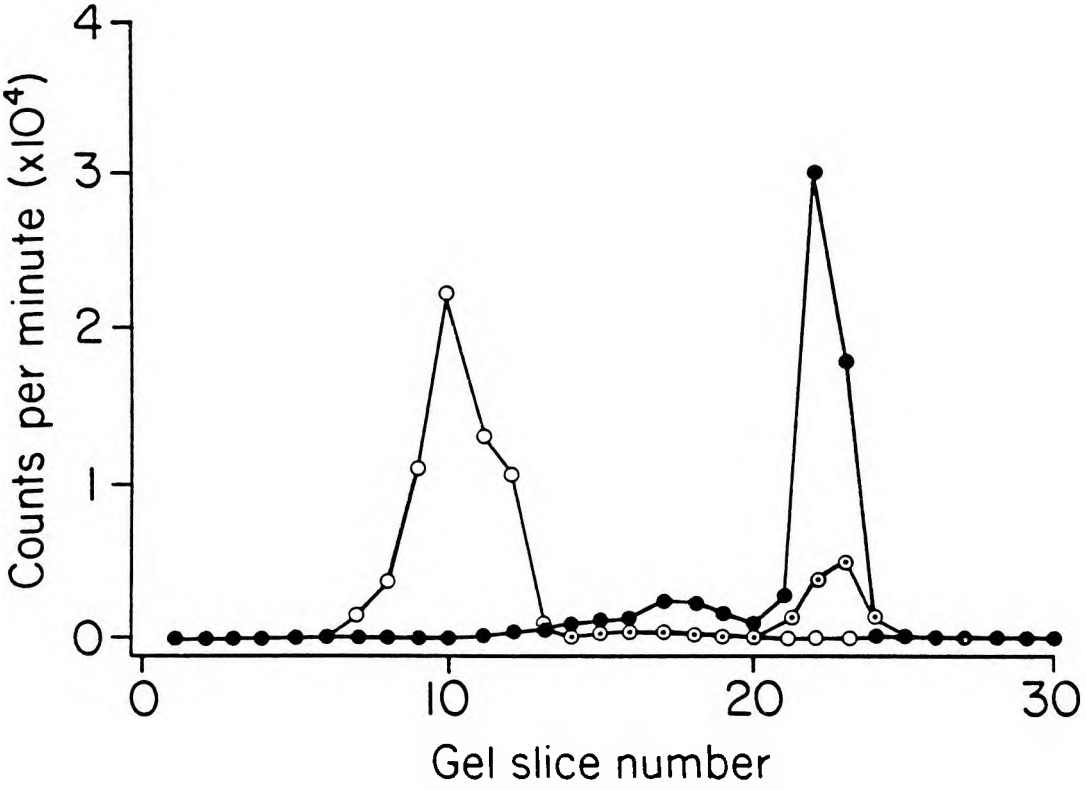


Figure 41. Radioactive counts in sliced lanes removed from a 6M urea polyacrylamide gel. Diferric transferrin (^{125}I counts (●) and ^{59}Fe counts (⊙)) is clearly separated from apotransferrin (^{125}I counts (○)).

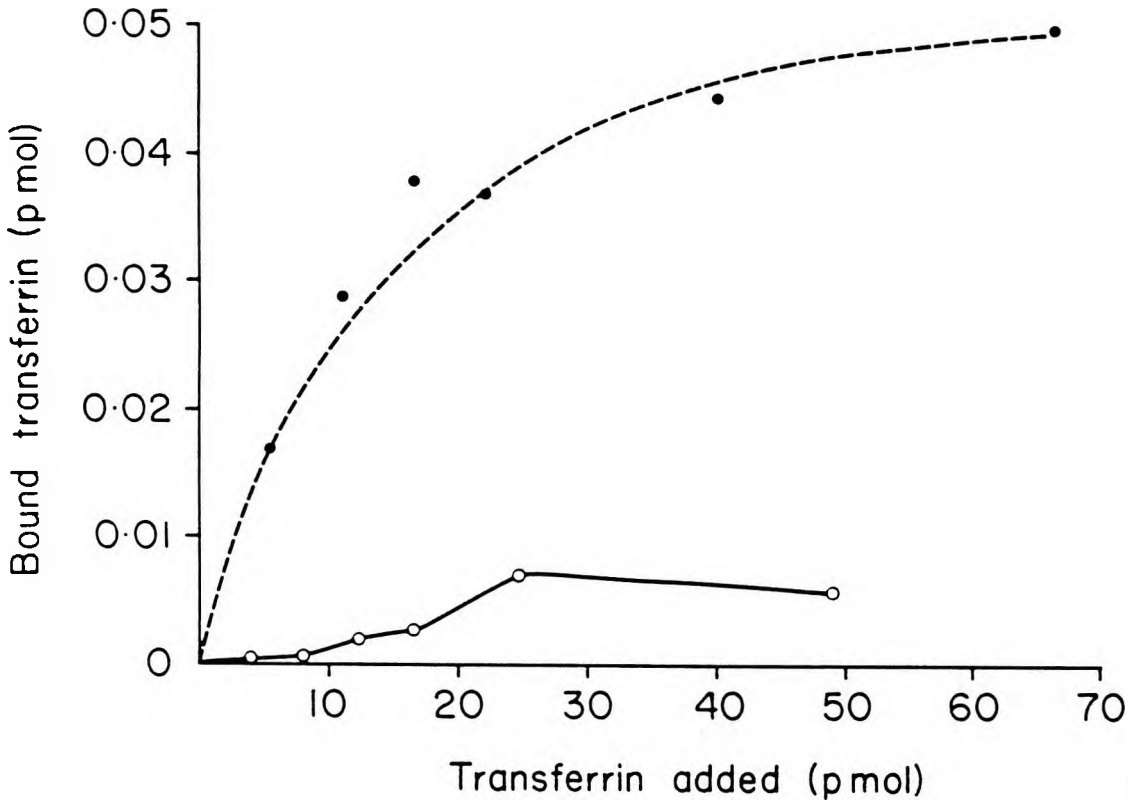
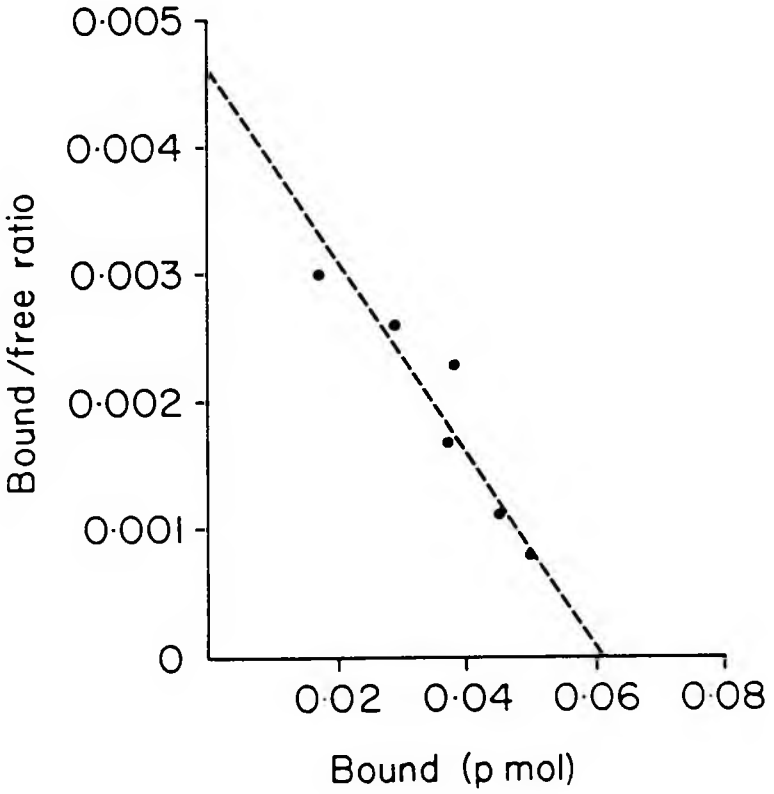


Figure 42. Binding of apotransferrin (o—o) and diferric transferrin (●--●) to day 7 human macrophages at pH 7.4.



re 43. Scatchard analysis of the binding of diferric transferrin to 7 day human macrophages at pH 7.4 (Kd 1.3 x 10^{-8}M).

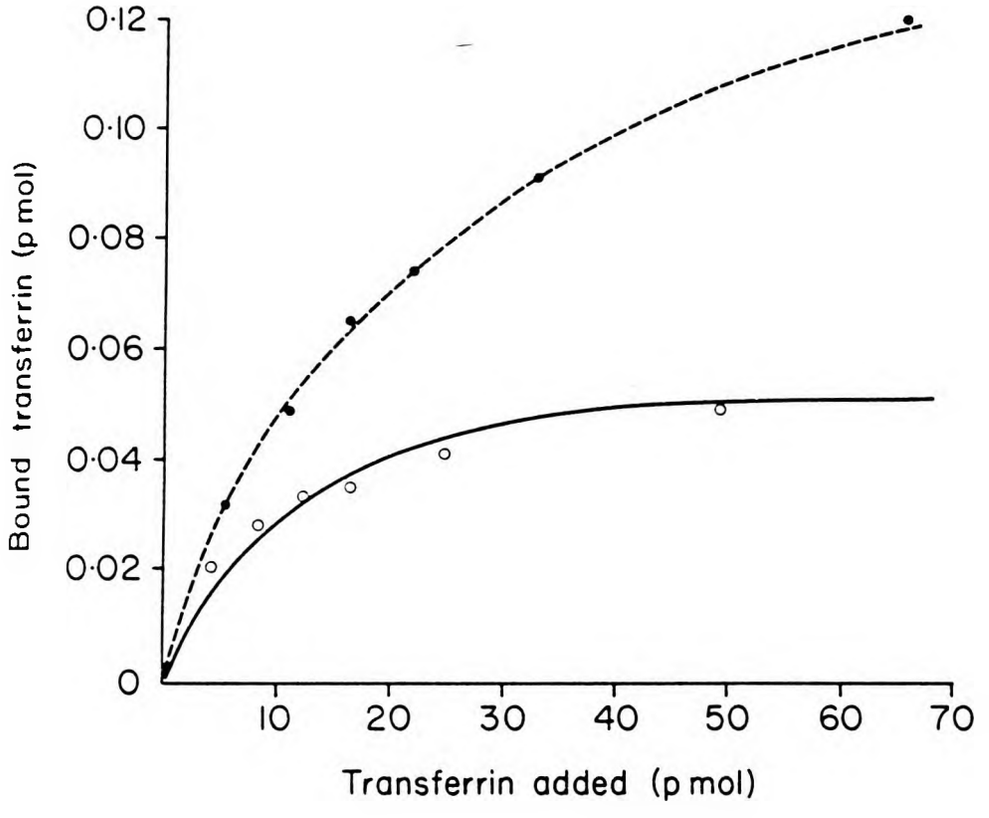
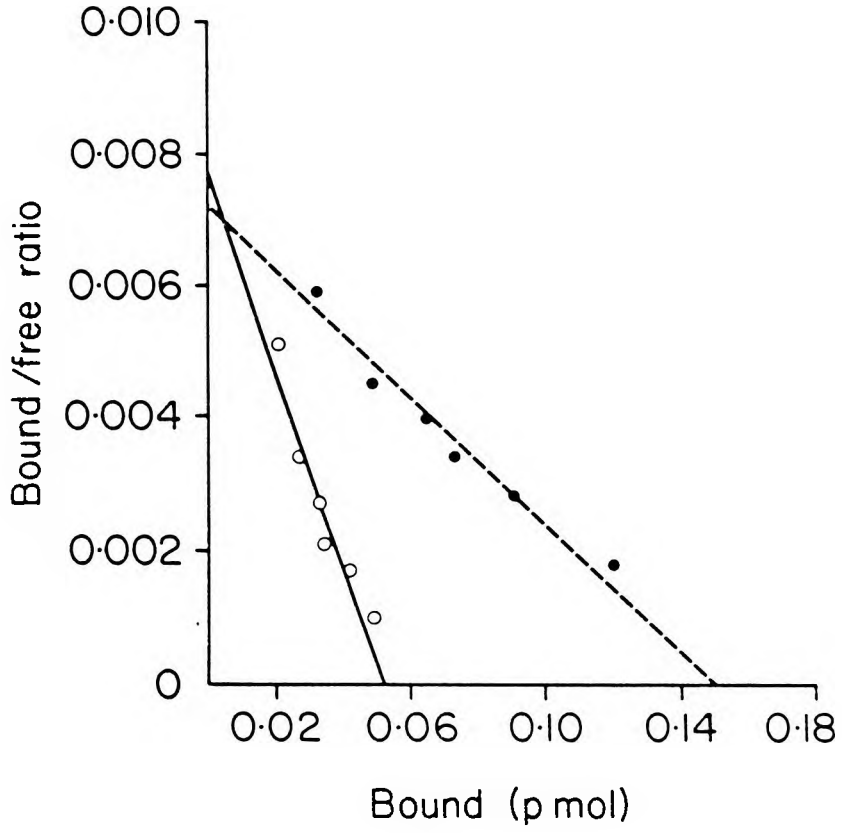


Figure 44. Binding of apotransferrin (o——o) and diferric transferrin (●--●) to 7 day human macrophages at pH 6.0.



ure 45. Scatchard analysis of the binding of apotransferrin (o—o) and diferric transferrin (●--●) to 7 day human macrophages at pH 6.0. The Kd for apotransferrin is 6.8 x 10^{-9}M and that for diferric transferrin is 2.1 x 10^{-8}M.

of diferric transferrin at pH 6.0 was similar to that at 7.4 (Figure 44), with a Kd of 2.1 x 10^{-8}M (Figure 45). Specific binding of apotransferrin was also noted at this pH (Figure 44). The X intercept (Figure 45) suggested that there were fewer apotransferrin receptors than diferric transferrin receptors at pH 6.0, while the Kd of 6.8 x 10^{-9}M indicated that they had a somewhat higher affinity. It was also noted that apotransferrin binding was equally displaceable by excess apotransferrin or diferric transferrin.

The fact that the cells being studied were adherent made quantitation of their numbers, and hence of receptor numbers, difficult. However, it was possible to make rough calculations from the initial plating density of monocytes (1 x 10^6) and from the previous observation that between 50 and 90% are lost during a 7 day period of macrophage differentiation (Johnson et al. 1977). On this basis, the final macrophage count would be 1 to 5 x 10^5 macrophages and the diferric transferrin receptors at pH 7.4 would be between 6 x 10^4 and 3 x 10^5 per cell. These figures may represent underestimates, since only a proportion of the receptors on adherent cells are available to potential ligands. Calculated figures for the numbers of diferric and apotransferrin receptors at pH 6.0 were 2 to 9 x 10^5 and between 6 x 10^4 and 3 x 10^5 respectively.

In a final experiment, an attempt was made to find out the degree to which iron contamination of the apotransferrin by the incubation medium, reagents and Petri dishes might be influencing the binding studies. To this end, supernatants from the control

Petri dishes (ie. the ones not containing cells) were removed at the end of the binding studies, concentrated and then electrophoresed on 6M urea polyacrylamide gels. Variable amounts of diferric- and monoferric transferrin were consistently found in the pH 7.4 medium (RPMI, defatted BSA and 10mM desferrioxamine) but no such contamination was noted at pH 6.0 (Figure 46).

16.3 DISCUSSION

The reticuloendothelial system occupies a unique role in iron transport, since it is responsible for supplying all the iron requirements of the erythroid marrow. It, in turn, obtains its own iron supply from the haemoglobin of effete red cells. The haemoglobin is catabolised and most of the released iron is then rapidly returned to the plasma. Immunologic studies have revealed that macrophages also possess diferric transferrin receptors (Chapter 15, page 279) but why they should require this extra pathway for iron acquisition is not clear. Perhaps it has a wider relevance in relation to cellular proliferation (Galbraith et al. 1980) and differentiation (Andreesen et al. 1983; 1984; May et al. 1985). The functional importance of diferric transferrin receptors was confirmed in the present study which was done on cultured human blood monocytes. Diferric transferrin was shown to bind at pH 7.4 with a Kd of 1.3×10^{-8} and the number of receptors was calculated to be between 6×10^{4} and 3×10^{5} per cell. This is comparable with what has been found on immature

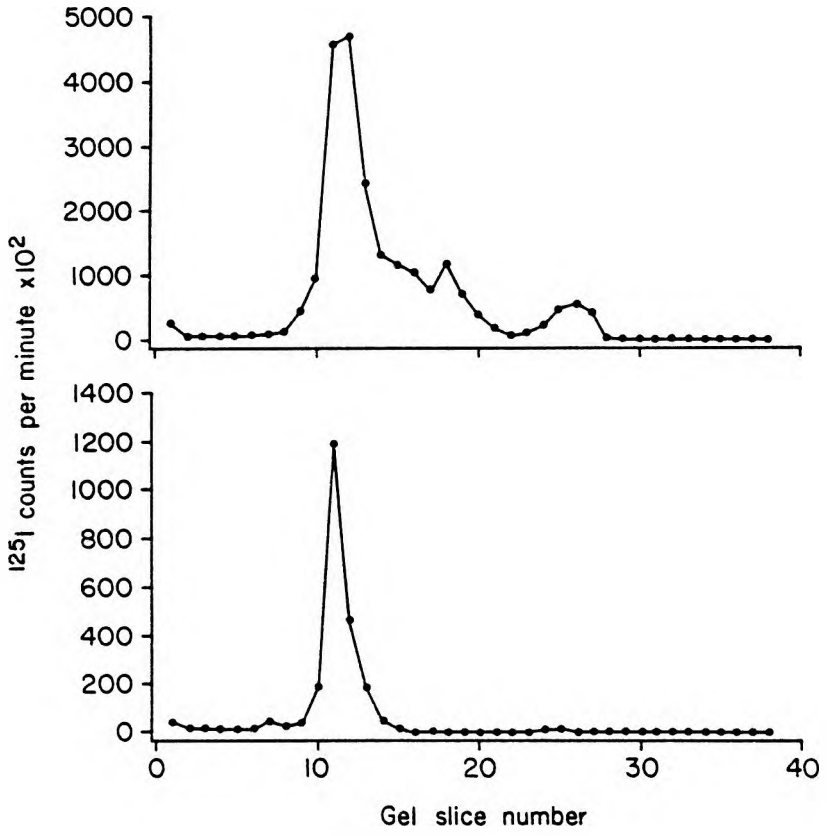


Figure 46. An example of control supernatants electrophoresed on 6M urea polyacrylamide gel. In addition to the major peak of apotransferrin, small amounts of mono- and diferric transferrin were consistently present (upper frame). At pH 6.0 there was no significant contamination with iron and only apotransferrin was present (lower frame).

erythroid cells and Friend leukaemia cells (Glass et al. 1978; Iacopetta et al. 1982), on mitogen stimulated lymphocytes (Hammerstrom et al,. 1982; Bomford et al. 1983), on hepatocytes (Young and Aisen, 1980), on human fibroblasts (Ward et al. 1982) and on HeLa cells (Ward et al. 1982).

The major question addressed in the present study related to the possible role that apotransferrin receptors might play in the delivery of iron from reticuloendothelial cells to the transferrin of plasma. No good evidence could be obtained for the presence of apotransferrin receptors at the physiological pH of 7.4 and it seemed likely that the small amount of apotransferrin binding that did occur resulted from iron contamination. Indeed, it is possible that the discrepancy between the present results and the previous finding of apotransferrin receptors on rat peritoneal macrophages (Nishisato and Aisen, 1982; Saito et al. 1986) may be due, in part at least, to minor degrees of iron contamination. On the current evidence it would seem unlikely that apotransferrin receptors are required in the normal transport of iron from reticuloendothelial cell to the plasma. In this context, it should be noted that reticuloendothelial cells deliver as much iron daily into the plasma as is removed by erythroid precursors. Were apotransferrin receptors to be of physiological significance, it might be anticipated that they would be present on reticuloendothelial cells in much greater numbers than diferric transferrin receptors. This is due to the fact that iron delivered by transferrin to such cells represents only a small fraction of reticuloendothelial turnover; the major

source is iron released from catabolised red cells.

While apotransferrin receptors were not found at pH 7.4, specific apotransferrin binding was demonstrable at pH 6.0. This pattern of apotransferrin binding is not confined to reticuloendothelial cells but has previously been demonstrated using reticulocytes (Morgan, 1983; Harding and Stahl, 1983), hepatoma cell lines (Dautry-Varsat et al. 1983) and K562 cells (Klausner et al. 1983). On this basis, a model has been proposed for the delivery of transferrin iron to cells (Wileman et al. 1985). Diferric transferrin is bound to surface receptors which are then internalised. Acidification of the endocytic vesicle leads to the release of the iron but the affinity of the apotransferrin for its receptor remains high at the acidic pH within the vesicle. As a result, it does not dissociate and thus escapes digestion. When the apotransferrin receptor complex reaches the surface of the cell it is exposed to the physiological pH and dissociation occurs. In this way the receptor is made available again for further transferrin iron uptake.

Calculated figures for the number of diferric and apotransferrin receptors at pH 6.0 were 2×10^5 and between 6×10^4 and 3×10^5 respectively. The values for apotransferrin receptors are similar to those documented on hepatoma cell lines at acid pH (Dautry-Varsat et al. 1983). The reason why there appeared to be more diferric than apotransferrin receptors was not clear but raises the possibility of other mechanisms for the binding of diferric transferrin to reticuloendothelial cells.

16.4 SUMMARY

A study was done to find out whether apotransferrin receptors are involved in the release of iron from reticuloendothelial cells. To this end, human macrophages which had been obtain by culturing blood monocytes for 7 days were incubated with either diferric or apotransferrin at the physiological pH of 7.4 or at an acidic pH (6.0). While specific diferric transferrin receptors (Kd 2.1 x 10^{-8}M) were demonstrated at pH 7.4, no apotransferrin receptors were found. In contrast, both diferric receptors (Kd 2.1 x 10^{-8}M) and apotransferrin receptors (Kd 6.8 x 10^{-9}M) were found at pH 6.0. The finding of specific apotransferrin binding at acidic pH fits in with the current understanding of iron uptake by cells, in which the iron-transferrin complex is endocytosed and the iron is released at acidic pH. The present results suggest that the apotransferrin remains attached to its receptor in the endocytosed vesicle at this acidic pH, but that it becomes detached at the cell surface where the pH is neutral. No evidence was found to indicate that iron is transported out of macrophages via apotransferrin receptors at the physiological pH.

Chapter Seventeen

ROLE OF A SERUM FACTOR IN ENHANCING TRANSFERRIN IRON UPTAKE BY HUMAN MACROPHAGES IN INFLAMMATION

17.1 INTRODUCTION

Cultured animal and human macrophages take up transferrin iron (MacDonald et al. 1969; MacSween and Macdonald, 1969; Summers and Jacobs, 1976; Wyllie, 1977; Sizemore and Basset, 1984) and there is evidence that this occurs by a process of receptor mediated endocytosis similar to that occurring with erythroid and a number of other cellular types (Chapter 15). There is also experimental *in vitro* evidence in animals that transferrin iron uptake by macrophages is enhanced in the presence of inflammation (Macdonald et al. 1969; Birgegard and Caro, 1984). In the present study, *in vitro* experiments were carried out on cultured human macrophages in order to obtain further insight into the increased transferrin iron uptake that has been noted to occur in inflammatory states.

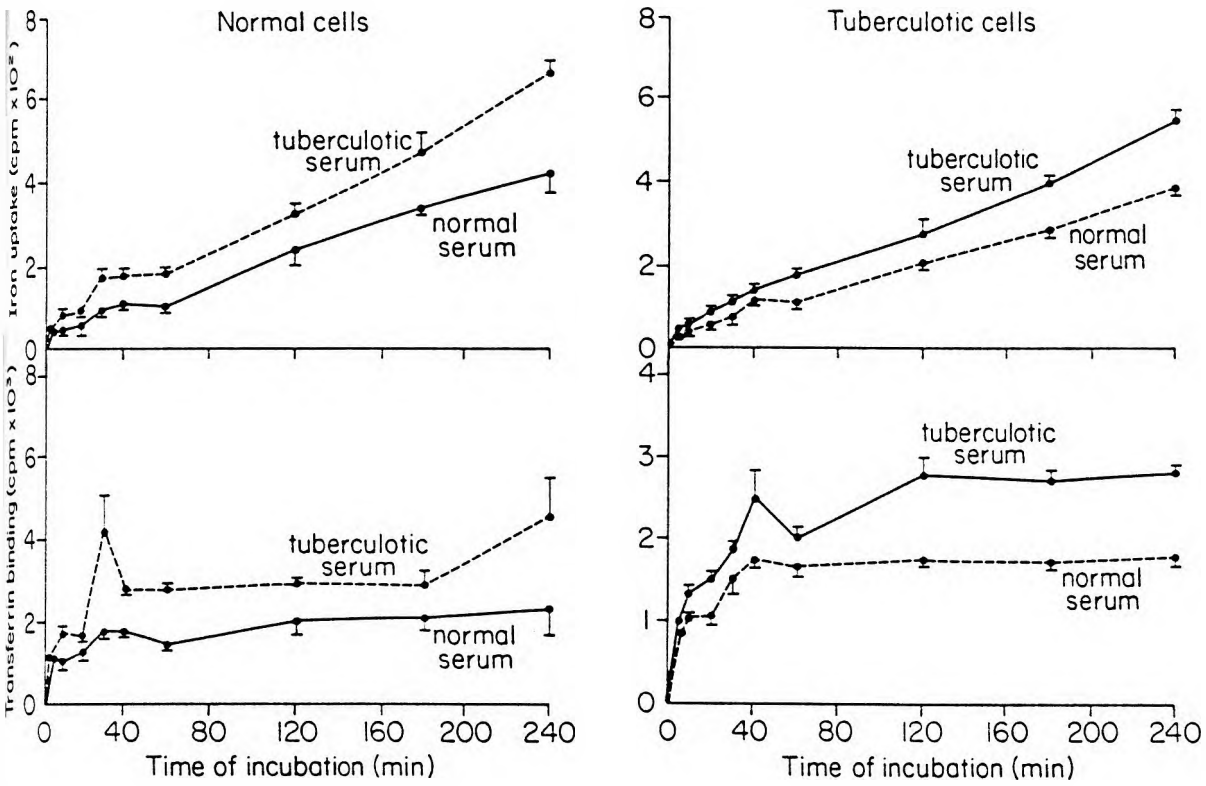
17.2 MATERIALS AND METHODS

Blood monocytes were collected from 3 patients with inflammatory disorders and from 3 normal subjects. The former group included one with active sero-positive rheumatoid arthritis and a marked systemic disorder and 2 others with untreated and active pulmonary tuberculosis. Monocytes from each of the patients with inflammation were cultured in parallel with monocytes from an ABO matched normal subject. After 4 days of culture in 10% autologous serum, each subject's macrophages were separated into 2 equal numbers of Petri dishes. The medium was

removed and the cells were washed 3 times with iron free RPMI (Flow Laboratories, Irvine, Scotland). After the third wash, half of each subject's Petri dishes were refilled with culture medium containing 10% autologous serum, while the other half were filled with culture medium containing 10% serum from an ABO matched normal control. Macrophages from normal subjects were processed in the same way. All cells were then grown for a further 3 days and were finally studied on day 7. Culture techniques, preparation of proteins, binding studies and counting methods were as described in Chapter 14.

17.3 RESULTS

The time dependent uptake of transferrin and of iron by normal cultured macrophages was increased at each time interval by 10% inflammatory serum in all 3 experiments. When figures for the uptake of transferrin in normal serum were all corrected to 100% the corresponding results with inflammatory serum were 167.3% (±41.3), 148.0% (±37.6) and 117.6% (±12.2) in 3 studies, while the corresponding figures for iron uptake were 160.4% (±27.1), 146.1% (±26.1) and 121.3% (±20.8). In contrast, 10% normal serum inhibited transferrin and iron uptake by inflammatory macrophages when compared with inflammatory serum. The transferrin uptake figures were 70.9% (±7.8), 88.9% (±6.3), and 61.0% (±8.7) and those for iron uptake were 73.1% (±12.8), 83.1% (±5.9) and 62.3% (±9.1) respectively. The changes with time in one of the experiments are shown in Figure 47.



ure 47. Time dependent mean (± SEM) uptake of transferrin and of iron by cultured macrophages from a normal subject and from a patient with active pulmonary tuberculosis in the presence of normal and tuberculotic serum.

When similar studies were performed with inflammatory macrophages, but using autologous serum incubated for 30 minutes at 70°C, inhibition of transferrin and iron uptake relative to non-heat inactivated serum was observed (3 subjects).

17.4 DISCUSSION

The current results show that a serum factor present in inflammation enhances transferrin and iron uptake by cultured human macrophages irrespective of whether the cells originate from normal subjects or from patients with inflammatory states. In view of the multiple roles that Interleukin-1 (IL-1) plays in regulating the inflammatory response (Dinarello, 1984a), it would seem to be a likely candidate for causing these changes. The fact that this factor appeared to be inactivated by heating to 70°C for 30 minutes was in keeping with it being IL-1 (Dinarello, 1984a). The effect could either be a direct one on transferrin receptor expression or could be indirectly mediated. In this latter context, there is evidence of increased ferritin synthesis in inflammation (Konijn and Hershko, 1977) and this may well be induced by Interleukin-1, which is known to be released in inflammatory states (Chensue et al. 1986). Enhanced ferritin synthesis would, in its turn, remove iron from the intracellular chelatable pool and thereby enhance transferrin receptor expression (Bridges and Cudkowicz, 1984; Bottomley et al. 1985).

It has been suggested that increased transferrin receptor expression on macrophages and lymphocytes may contribute to the

hypoferraemia of inflammation (Birgegard and Caro, 1984). However, kinetic data have shown that the transferrin iron pathway to erythroid precursors is normal in inflammatory states (Finch et al. 1970; Letendre and Holbein, 1983) and that the hypoferraemia results from the impaired release of iron from reticuloendothelial cells (Letendre and Holbein, 1984). An alternative more likely explanation of increased transferrin receptor expression in inflammation relates to the effects of transferrin on cellular proliferation and differentiation. For example, activated lymphocytes express transferrin receptors as an essential component of proliferation (Hamilton, 1982; Neckers and Cossman, 1983). Insofar as macrophages are concerned, transferrin receptor expression is decreased or absent on human blood monocytes but is enhanced on cultured macrophages (Adams and Hamilton, 1984). These findings suggest that transferrin-receptor interaction may be of importance in the macrophage differentiation which occurs as part of the inflammatory response and that it may be regulated by a serum factor.

17.5 SUMMARY

Monocytes from 3 normal subjects and from 2 patients with active tuberculosis and 1 with rheumatoid arthritis were cultured in 10% autologous serum for 4 days. Paired experiments were then done in which ABO matched normal and inflammatory macrophages were cultured for a further 3 days in either 10% normal or 10% inflammatory serum. Transferrin and iron uptake were then

measured for periods varying between 5 and 240 minutes. The uptake of transferrin and iron by normal cells was uniformly increased by mean figures of 44.3% and 42.6% respectively in the presence of inflammatory serum. In contrast, normal serum decreased the uptake of transferrin and iron by inflammatory macrophages, with mean reductions of 26.4% and 27.2% respectively. The reason for the serum induced increase in transferrin receptor expression in inflammation is not clear but may relate to macrophage differentiation. Heat inactivation of this serum factor in inflammation by heating to 70^{o}C for 30 minutes is in keeping with this factor being IL-1.

Chapter Eighteen

TRANSFERRIN-IRON-CELL INTERACTIONS : HEPATOMA CELLS (PLC/PRF 5 LINE)

18.1 INTRODUCTION

Hepatic iron uptake from diferric transferrin is a fairly complex phenomenon which involves several distinct processes. One is receptor mediated endocytosis analagous to that found in erythroid and other non-erythroid cells (Young and Aisen, 1980; Cole and Glass, 1983; Page et al. 1984; Baker et al. 1985; Trinder et al. 1986). This receptor pathway is probably the most significant pathway at relatively low concentrations of transferrin (Cole and Glass, 1983; Page et al. 1984) and is mediated by low capacity high affinity receptors (Ka 2 x 10^6 l/mol; receptor number 8 x 10^5 per hepatocyte (Trinder et al. 1986). At higher concentrations, which are more biologically relevant, the transferrin binding is non-saturable (Page et al. 1984). Part of the explanation for this phenomenon appears to be the existence of high capacity low affinity transferrin receptors (Ka 2.6 x 10^5 l/mol; receptor number 4.2 x 10^6 per hepatocyte). Both these receptor populations are capable of recycling transferrin and unloading iron intracellularly. The high affinity receptor appears to cycle transferrin more rapidly (9 minutes) as compared to the low affinity receptor (25 minutes). A third component of transferrin-iron uptake by hepatocytes is that of non-specific fluid phase endocytosis. Although it accounts for as much as 50% of transferrin uptake after 15 minutes at higher transferrin concentrations, it is relatively inefficient in terms of iron delivery and accounts for only 2% of iron uptake.

To make the picture more complex, a fourth transferrin iron

uptake pathway has recently been described. It involves the interaction of desialated transferrin (particularly the triantenary form) with non-specific asialoprotein receptors on hepatocyte surfaces (Young et al. 1983; Dekker et al. 1985; Regoeczi and Koj, 1985; Tavassoli et al. 1986). These receptors have specificity for the galactosyl residues which are exposed when sialic acid is stripped from glycoproteins. To understand the possible significance of these receptors it is necessary to discuss briefly some technical problems related to the culture of hepatocytes. In 1984 it was claimed that hepatocytes do not, in fact, exhibit normal transferrin receptors and that those which had been previously reported were an artefact of impure hepatocyte preparations, with significant contamination by endothelial cells and Kupffer cells (Soda and Tavassoli, 1984). In their preparation of pure hepatocytes, these workers used collagenase and they later showed that it was stripping receptors off hepatocyte membranes. While these transferrin receptors recovered if the cells were left in culture prior to study (Kishimoto and Tavassoli, 1986), they had a relatively low affinity (Kd 0.7 x 10^{-7}M) and were relatively few in number (4.4 x 10^{4} per hepatocyte). In contrast, hepatic endothelium was found to have similar affinity for transferrin (Kd 1.9 x 10^{-7}), but a manyfold greater number of receptors (1.8 x 10^{6}) (Kishimoto and Tavassoli, 1986). In addition, hepatic endothelium was shown to possess the ability to transport endocytosed transferrin from the vascular lumen to the hepatocyte side of the endothelium. In the process the transferrin was desialated (Tavassoli et al. 1986). On the basis of these findings it was suggested that a fourth

pathway might be important in the hepatocyte uptake of transferrin iron. It was postulated that this pathway involved the transendothelial desialation of transferrin with its subsequent uptake by the hepatic asialoprotein receptor (Tavassoli et al. 1986).

Other work has shown that desialated rat transferrin interacts with two types of receptors on rat hepatocytes (Young et al. 1983). One interaction occurs with the usual low capacity, high affinity transferrin receptors (31 000 receptors per hepatocyte; Ka 1.9 x 10^7 l/mol), while the other is with the high capacity lower affinity asialoglycoprotein receptors (110 000 receptors per hepatocyte; Ka 1.4 x 10^6 l/mol). Uptake of desialated transferrin by the high affinity transferrin receptor results in normal recycling of the transferrin (Young et al. 1983). The fate of endocytosed desialated transferrin which is taken up by the asialoprotein receptor is affected by the presence or absence of normal transferrin and by the concentration of desialated transferrin. When only desialated transferrin is present at low concentrations, there is recycling of the desialated transferrin, with release of iron in the normal way. When, however, the concentrations of desialated transferrin are higher, or normal transferrin is present, much of the desialated transferrin appears to be catabolised (Regoeczi and Koj, 1985). The suggested explanation for these observations invokes both the endocytosis of desialated transferrin via the asialoprotein receptor, and the endocytosis of either excess desialo- or normal transferrin via the normal transferrin

receptor in the same endocytic vesicle. It is also assumed that normal receptors can be endocytosed in the absence of ligand at low concentrations of either normal transferrin or desialated transferrin. In this explanation the desialated transferrin gives up its iron in the acidified endocytic vesicle and dissociates from the asialoglycoprotein receptor. If a free normal transferrin receptor is available the desialated apotransferrin molecule is recycled in the usual way. If, however, there are no free receptor sites (as might occur in situations where large amounts of desialated transferrin or normal transferrin are present) then the dissociated desialated transferrin molecule is degraded in the acid environment of the vesicle (Regoeczi and Koj, 1985).

After transferrin-iron is taken up by normal cultured hepatocytes roughly two thirds of the endocytosed iron accumulates in ferritin, irrespective of whether the uptake is mediated by high affinity low capacity receptors or the low affinity high capacity receptors (Trinder et al. 1986).

It was against this background that transferrin-iron-cell interactions were evaluated in a locally developed human hepatoma cell line (PLC/PRF 5). This was done in an attempt to gain insight into the pathways involved in a malignant hepatocyte. It was also hoped that it would be possible to assess the degree to which desialated transferrin was utilised by such a cell, since there is previous evidence that some human hepatoma cell lines do express asialo-glycoprotein receptors (Schwartz et al. 1981).

18.2 METHODS

The methods used for cell culture, splitting of cultures, plating out cells, cell binding studies, quantitating cells, radioactive counting and data analysis have been outlined in the methods chapter of Section 3 (Chapter 14). In addition, the preparation of labelled diferric transferrin has also been described in that chapter.

In this section the preparation of desialated diferric transferrin will be briefly described. Two ml of a 6mg per ml solution of apotransferrin was incubated for 18 hours with 80/ul of an insoluble neuraminidase (neuraminidase complexed with agarose) (Sigma, St Louis, Missouri, USA) at 37^{o}C (Morgan et al. 1967). The solution was agitated throughout the incubation. At the end of this period aliquots were subjected to polyacrylamide gel electrophoresis and isoelectric focussing in polyacrylamide gels in order to confirm the production of altered transferrin. The results of such an electrophoretic study are shown in Figure 48. The desialated transferrin thus obtained was doubly labelled with ^{125}I and ^{59}Fe as outlined in Chapter 14. The diferric nature of the desialated doubly labelled transferrin was confirmed on 6M urea polyacrylamide gel electrophoresis as outlined in Chapter 14.

18.3 RESULTS

Dose dependent studies with doubly labelled diferric

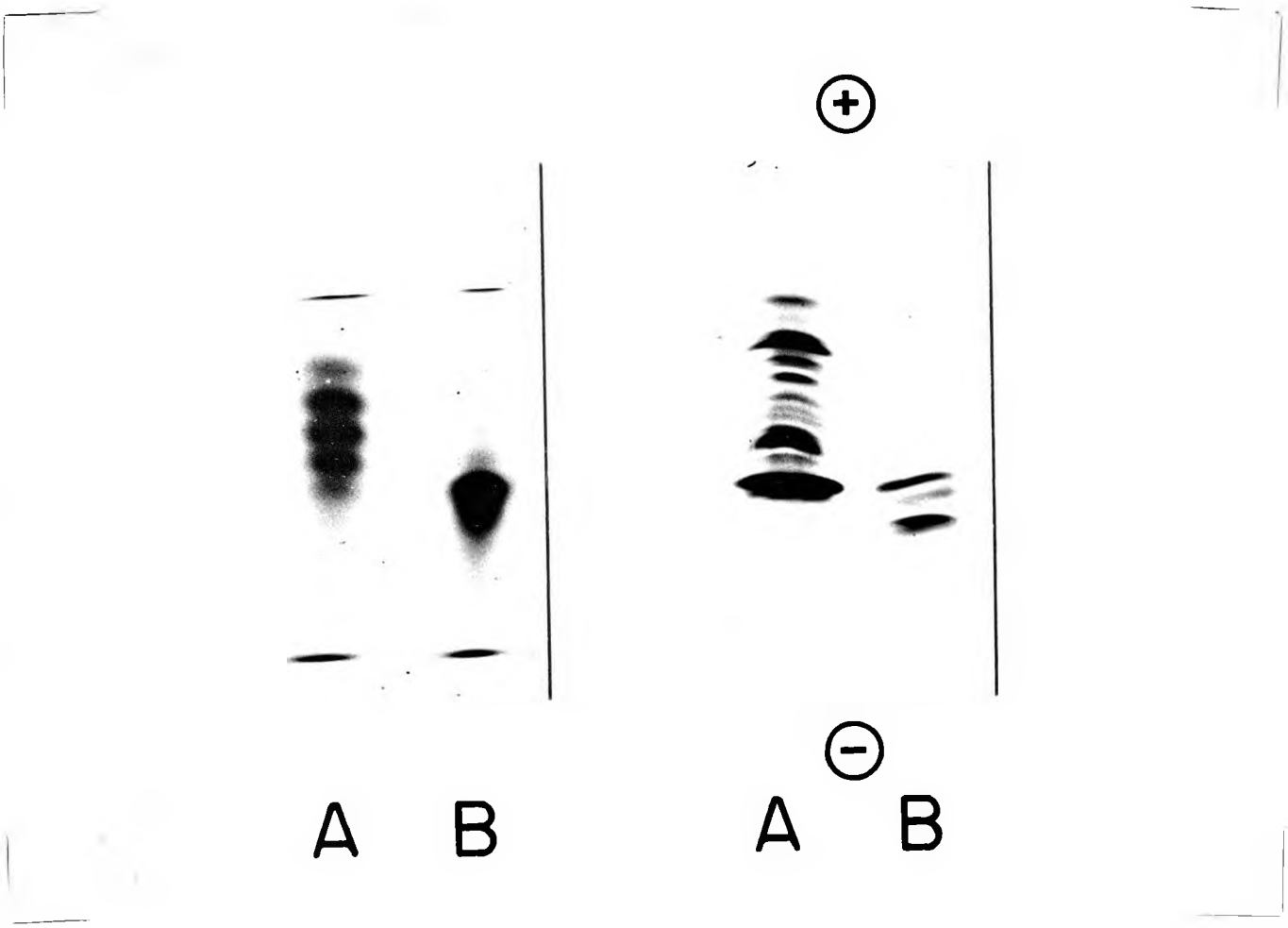


Figure 48. The two figures show the product of neuraminidase induced desialation of transferrin on polyacrylamide gel electrophoresis (left panel) and isoelectric focussing in polyacrylamide gels (right panel). B indicates normal transferrin and A indicates desialated transferrin in both the panels.

transferrin were conducted as outlined previously (Chapter 14), but incubation was continued for 4 hours. Results obtained by dose-dependent incubation in the low transferrin concentration range (up to 80pmol/ml) are summarised in Figure 49. Specific binding was arrived at by subtracting irreversible binding (in the presence of 100-fold excess of cold ligand) from total binding. It can be seen that specific binding appeared to be saturable over this concentration range. Scatchard analysis revealed 1.9×10^5 receptors per hepatoma cell with a Kd of 1.5×10^{-8}M. Dose dependent binding was then studied over a wider concentration range of diferric transferrin (0-600pmol/ml). A representative study is shown in Figure 50. It can be seen that specific binding was less saturable. Although a single line was fitted to the Scatchard analysis, 2 components were possibly present. Using a single line there was a slightly lower affinity (Kd 4.6×10^{-8}M) and a slightly increased receptor number (2.3×10^5).

Time dependent and temperature dependent uptake of transferrin and iron by human hepatoma cells are shown in Figure 51. In these experiments 40 pmol doubly labelled diferric transferrin was added to each Petri dish. Incubation was continued for 360 minutes. From this representative study it can be seen that with time there was a near linear uptake of iron at 37°C. The molar ratio of iron to transferrin showed a progressive rise with the passage of time, which indicated that there was recycling of the transferrin. Both transferrin and iron uptake were markedly reduced at 4°C with the result that the molar ratio

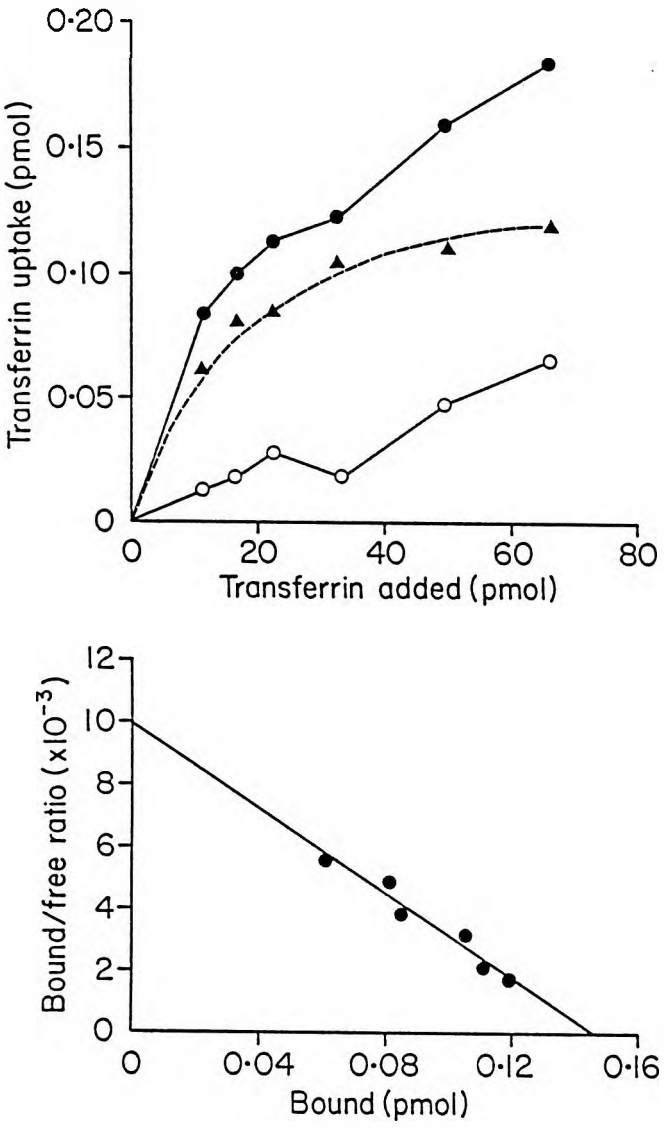


Figure 49. Dose dependent diferric transferrin binding over a low concentration range of 0-80pmol/ml. Total binding (●), irreversible binding in the presence of a 100-fold excess of cold ligand (o) and specific binding (▲) are shown. The Scatchard analysis revealed a receptor number per hepatocyte of 1.9×10^5 with a Kd of 1.5×10^{-8}M. Number of cells per Petri dish = 4.9×10^5.

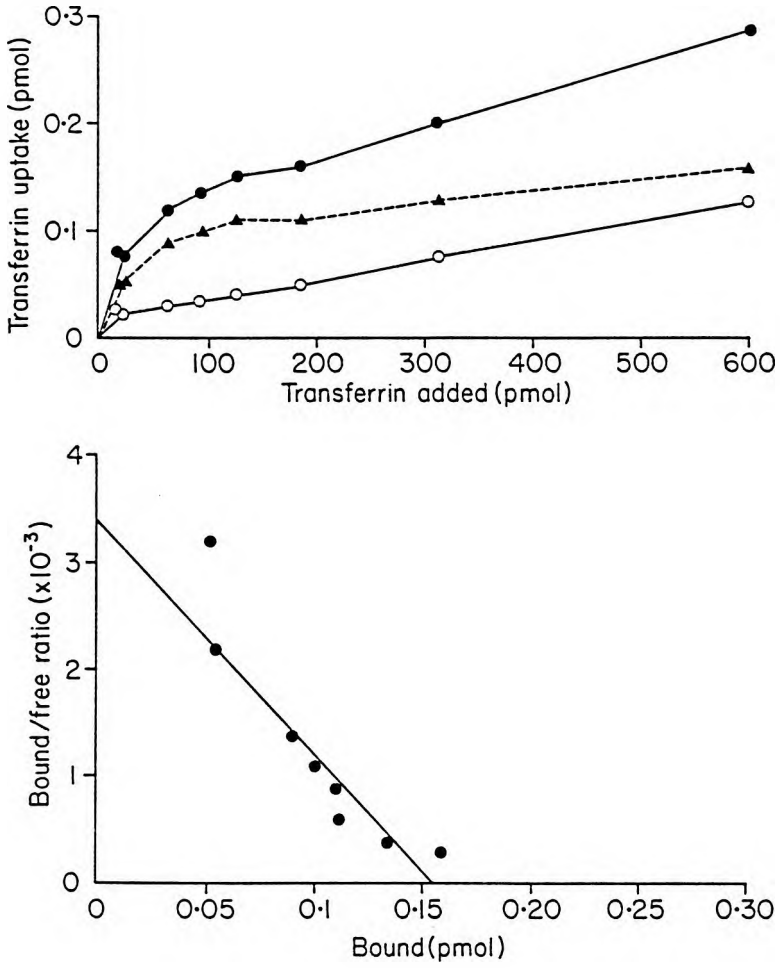


Figure 50. Dose dependent diferric transferrin binding over a higher concentration range of 0-600pmol/ml. Total binding (●), irreversible binding in the presence of a 100-fold excess of cold ligand (o) and specific binding (▲) are indicated. The Scatchard analysis revealed a receptor number per hepatocyte of 2.3×10^5 with a Kd of 4.6×10^{-8}M. Number of cells per Petri dish = 4.2×10^5.

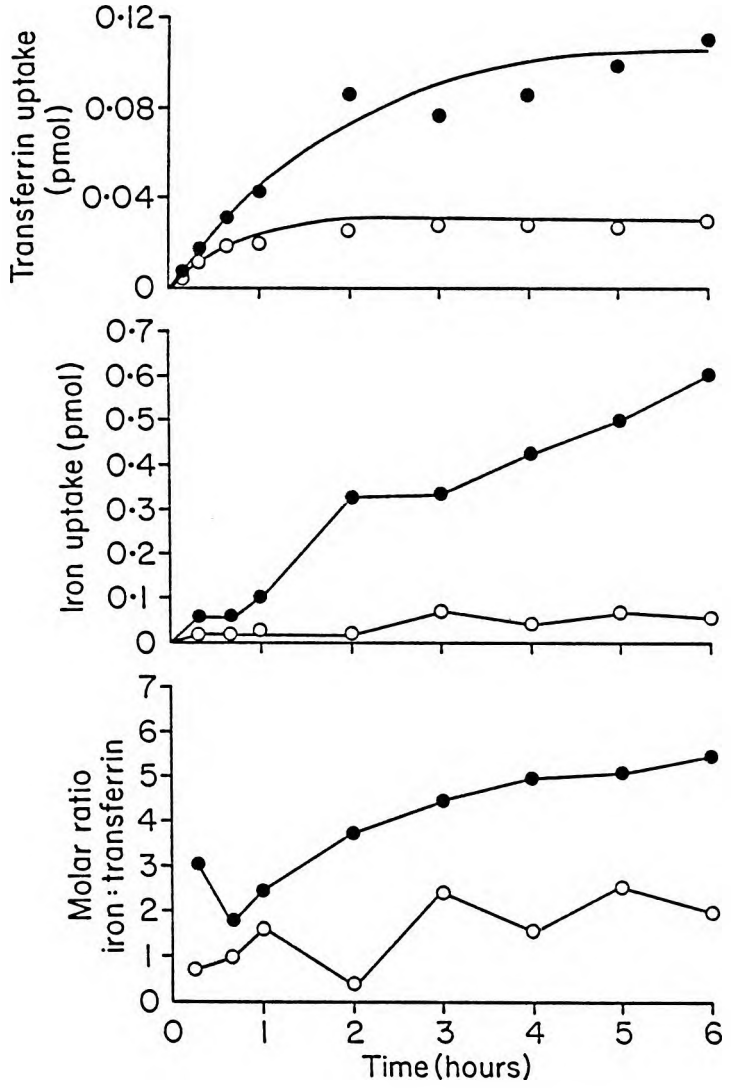


Figure 51. The time and temperature dependent binding of transferrin by human hepatoma cells is shown in the upper panel. The uptake of iron into the cells at 4°C (o) and 37°C (●) is shown in the middle panel and the molar ratio of cellular iron to transferrin can be seen in the lower panel.

of iron to transferrin remained close to 2. The fact that the iron was retained in the hepatoma cells whilst transferrin was returned to the extracellular environment at 37^{o}C was confirmed in conventional chase experiments. In these experiments 60 pmol of doubly labelled diferric transferrin was added to each Petri dish. After 3 hours of incubation the medium was removed, the adherent cells were washed twice in transferrin free media and 1ml of medium containing 60 pmol of cold diferric transferrin was replaced. Cell associated transferrin (^{125}I) and iron (^{59}Fe) were then followed in a time dependent fashion. An example of such a chase experiment is shown in Figure 52. It can be seen that while the iron which had been taken up by the hepatoma cells remained at a constant level, there was a fairly rapid decline in the transferrin. These findings are compatible with recycling of the transferrin molecule.

The effect of the weak base ammonium chloride on transferrin and iron uptake by hepatoma cells was next assessed. In these studies 20mM NH_4Cl was included in the medium in which the binding studies were conducted. Seventeen pmol of doubly labelled diferric transferrin were employed in these studies, which were conducted in a time dependent fashion. A representative example is shown in Figure 53. It can be clearly seen that ammonium chloride decreased transferrin binding but had an even greater effect on iron uptake. As a result, there was virtually no progressive increase of the molar ratio of iron to transferrin. The effect of the microtubular inhibitor, vincristine, on diferric transferrin uptake by hepatoma cells was measured in

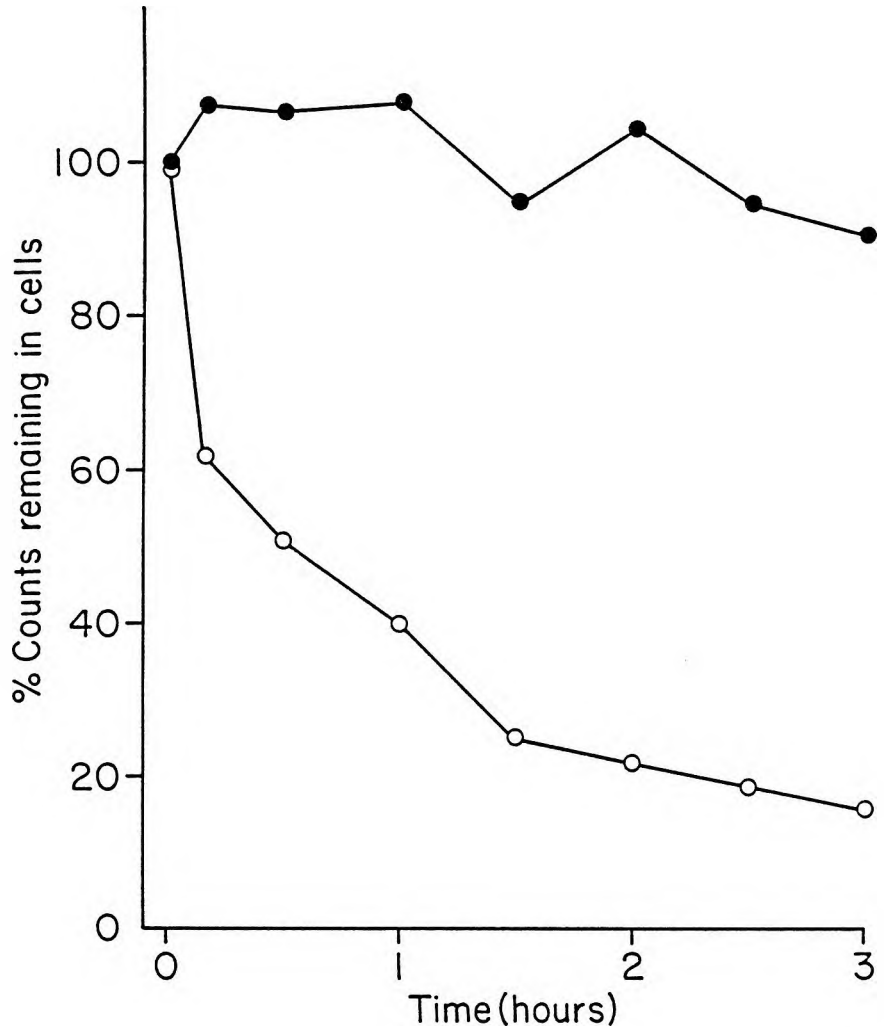


Figure 52. A chase experiment showing progressive loss of the transferrin in hepatoma cells while the labelled cellular iron content remained constant.

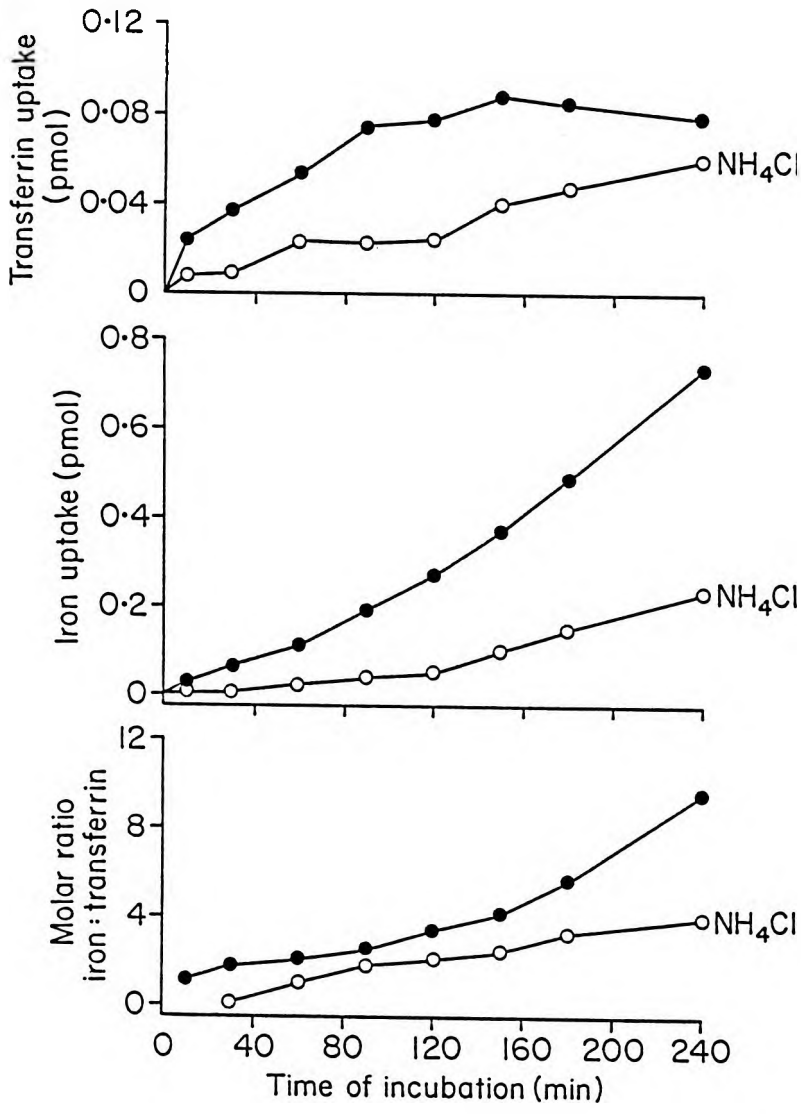


Figure 53. The effect of 20mM NH_4Cl on the time dependent uptake of doubly labelled diferric transferrin by human hepatoma cells. The top frame shows transferrin binding. The middle frame shows progressive iron uptake, while the bottom frame shows the molar ratio of iron to transferrin.

both dose and time dependent studies. An example of a dose dependent study is shown in Figure 54. It can be seen that there was a modest yet significant decline in both transferrin binding and iron uptake by hepatoma cells at relatively low concentrations of the vinca alkaloid. The dose response curve thereafter became very flat. Both transferrin binding and iron uptake were reduced proportionately so that the molar ratio of iron to transferrin was little affected. This was confirmed in time dependent studies in which vincristine (5/ug/ml) was included in each Petri dish. A modest time dependent reduction in both transferrin and iron uptake was evident. Since these reductions were proportional the molar ratio of iron to transferrin remained unaffected (Figure 55).

The binding of desialated transferrin to human PLC/PRF 5 hepatoma cells was evaluated in the next series of experiments. There was progressive, non-saturable, total binding of desialated diferric transferrin to these cells. When the incubation took place in the presence of a 100-fold excess of cold normal diferric transferrin, the binding of the labelled desialated transferrin was almost completely displaced. This finding suggests that the desialated transferrin binding to human hepatoma cells was occurring almost completely via normal transferrin receptors and not via asialo-glycoprotein receptors. The results are shown in Figure 56. Scatchard analysis revealed 9.3×10^5 receptors per cell with a Kd of 7×10^{-8}M. With time dependent uptake studies there was progressive uptake of iron and an increase in the molar ratio of iron to transferrin comparable

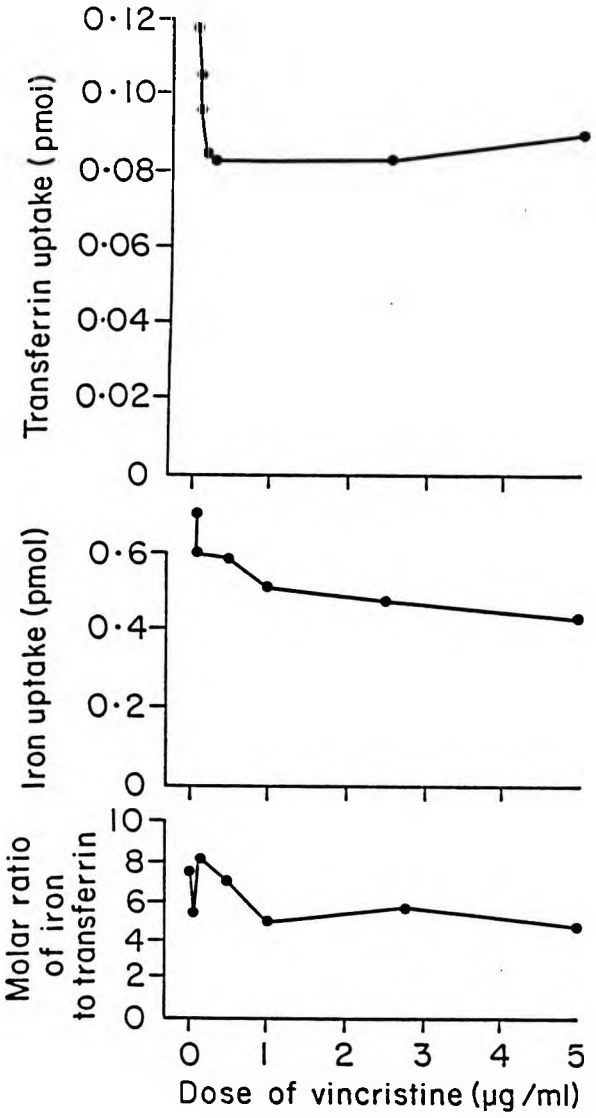


Figure 54. The dose dependent effect of the vinca alkaloid vincristine on transferrin uptake (upper panel) and iron uptake (middle panel). The molar ratio of iron to transferrin is shown in the bottom panel. In these studies 17pmol of doubly labelled diferric transferrin was added to each Petri dish after prior incubation in the presence of increasing concentrations of vincristine. Incubation was continued for 180 minutes.

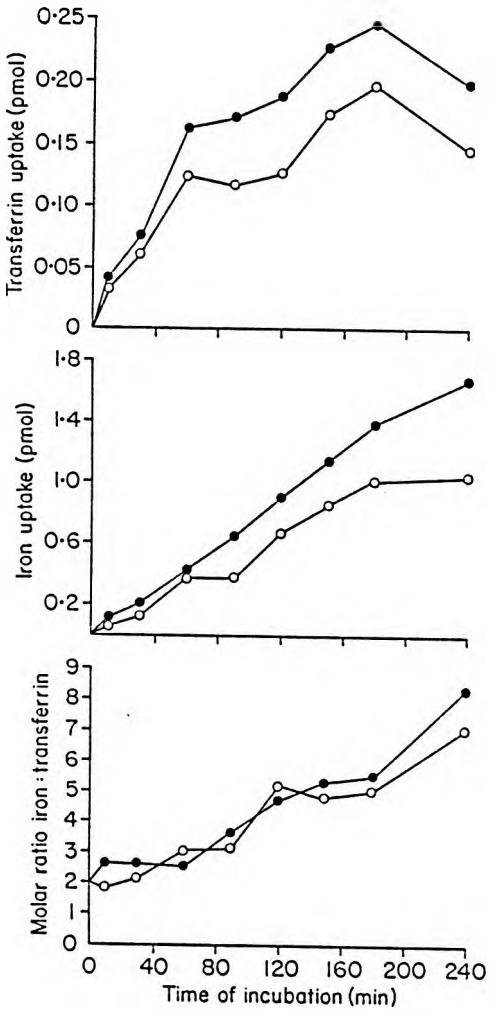


Figure 55. The effect of vincristine (5 ug/ml) on transferrin uptake (upper panel) and iron uptake (middle panel) by hepatoma cells. The molar ratio of iron to transferrin is shown in the bottom panel. Incubation was time dependent. Seventeen pmol doubly labelled diferric transferrin was added to each Petri dish. (Vincristine o; normal ●).

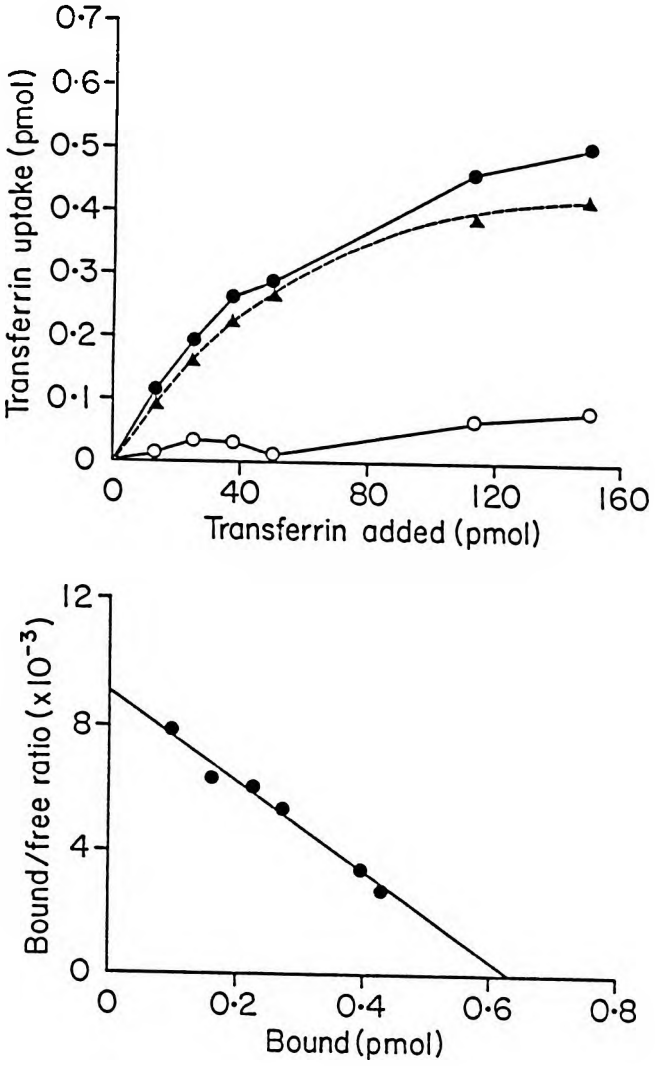


Figure 56. The dose dependent uptake of desialated diferric transferrin. Total diferric binding (●), non specific binding in the presence of a 100-fold excess of cold normal diferric transferrin (o) and "specific" binding of desialated transferrin (▲) are shown. Scatchard analysis reveals 9.3 x 10^5 receptors per hepatocellular carcinoma cell with a Kd of 7 x 10^{-8}M. Number of hepatoma cells per Petri dish = 4.2 x 10^5.

to that found with normal transferrin.

The fate of internalised diferric transferrin iron after a 48 hour incubation with ^{59}Fe labelled diferric transferrin was assessed in a final experiment. This was done by preparing cellular lysates and passing them through an AcA 44 ultrogel sizing column (see Chapter 14). The pellet obtained after centrifugation (membrane fraction) contained on average 24% of the ^{59}Fe counts. The remaining 76% was present in the cytosolic supernatant. After 48 hours only 33% of the iron in the cytosol was in ferritin, 31% was still bound to transferrin and 21% of the iron was present in a low molecular weight pool (Figure 57).

18.4 DISCUSSION

This study has demonstrated that at low concentrations of transferrin, cultured human hepatoma cells of the PLC/PRF 5 line have 1.9×10^5 transferrin receptors per cell. Since these studies were conducted at 37°C the transferrin uptake represents a composite picture of cell surface receptors and intracellular receptors in transit. Ciechanover and colleagues (1983), who studied the human hepatoma line Hep G 2, showed that each cell had 5×10^4 functional surface transferrin binding sites and 10×10^4 intracellular transferrin binding sites. This adds up to a total number of transferrin receptors of 1.5×10^5 which is in remarkably close agreement with the numbers found on the PLC/PRF 5 cell. The Kd as measured in the current study was 1.5×10^{-8}M. Over a wider concentration of transferrin there was a suggestion

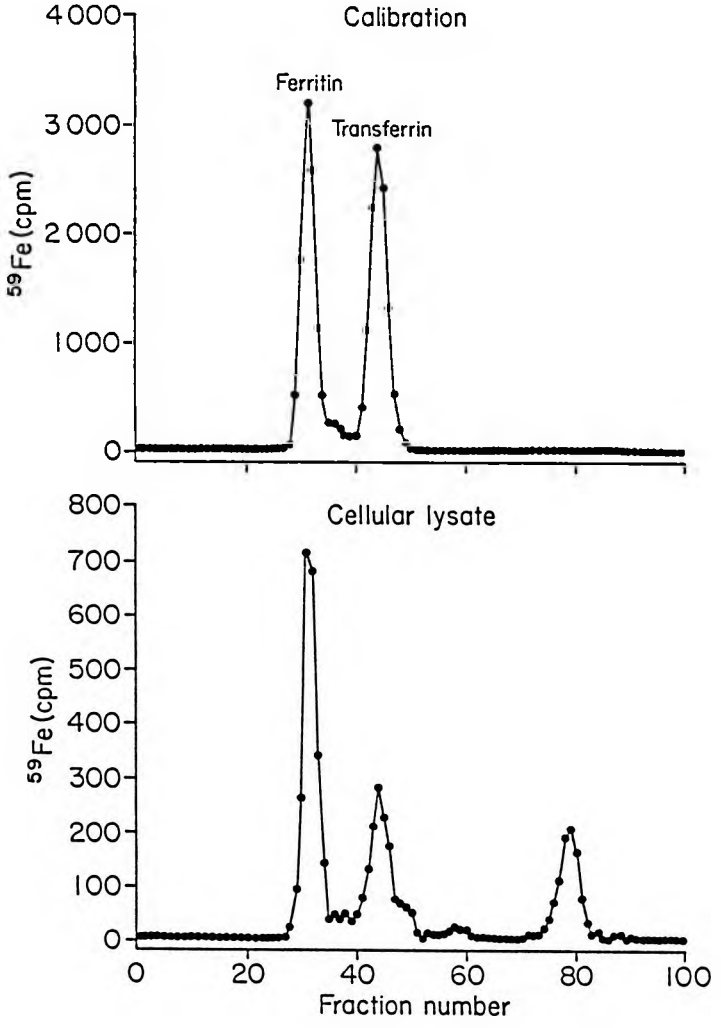


Figure 57. Results of the passage of the cytosolic component of cellular lysates through an AcA 44 ultrogel sizing column. The top frame shows the column calibrated with ferritin and transferrin whilst the bottom frame shows the analysis of cellular lysates. 33% of the iron appeared to be in ferritin, 31% in a transferrin bound fraction and 21% in a low molecular weight pool.

that the binding was non-saturable and that there were 2 receptor populations. This interpretation is in keeping with the results of other work on non-malignant hepatocytes. Low affinity high capacity receptors were demonstrated at higher concentrations of transferrin and fluid phase endocytosis possibly became relatively more important (Page et al. 1984; Trinder et al, 1986). However, the finding in the present study that the molar ratios of iron to transferrin remained relatively constant despite increasing doses of transferrin, suggests that only one pathway was playing the dominant role in iron delivery. An example of this is shown in Table 18, which presents iron and transferrin measurements for the second dose response experiment in this chapter (Figure 50). This, of course, assumes that different pathways of transferrin iron uptake have different rates of transferrin cycling (Trinder et al. 1986).

Time dependent studies showed that there was a progressive accumulation of iron in the hepatoma cells, with the molar ratio of iron to transferrin showing a progressive increase. This suggested that the pathway involved was unloading iron while recycling the transferrin. This was confirmed in the chase experiment. Using cold diferric transferrin there was a progressive release of transferrin from the hepatocellular carcinoma cells, whilst all the endocytosed iron was retained within them. Incubation at $4^{o}C$ completely inhibited iron and transferrin uptake by hepatoma cells. This finding is in keeping with the concept that the uptake of transferrin and iron is an active process.

TABLE 18 Transferrin binding, iron uptake and the molar ratio of iron to transferrin as a function of the incubating concentration of transferrin. Incubation was conducted for four hours. The number of cells per Petri dish was 4.2×10^5.

Incubating concentration of transferrin (pmol/ml)	Transferrin binding (pmol)	Iron uptake (pmol)	Molar ratio of iron to transferrin
16.25	0.051	0.362	7.1
24.38	0.053	0.284	5.3
63.38	0.089	0.603	6.8
94.25	0.100	0.711	7.1
125.13	0.110	0.771	7.0
186.88	0.111	0.814	7.3
312.00	0.133	0.896	7.4
625.63	0.159	1.374	8.6

Experiments employing the lysosomotropic agent NH_4Cl showed that it significantly inhibited iron and had a lesser inhibitory effect on transferrin uptake. Weak bases such as NH_4Cl are known to prevent acidification of endocytic vesicles (Chapter 13) and as such prevent iron unloading within the vesicle. To explain the effect of NH_4Cl on transferrin binding one must examine the endocytic cycle. NH_4Cl does not inhibit binding of transferrin to cell surface receptors, nor does it inhibit internalisation of the receptor-ligand complex (Klausner et al. 1983) but it does appear to slow the exocytosis of transferrin (Ciechanover et al. 1983).

Experiments utilising the microtubular inhibiting vinca alkaloid, vincristine, showed a very minor inhibition of both transferrin and iron uptake but no effect on the unloading of iron from transferrin. These findings are in keeping with the role of microtubules in the endocytotic pathway of transferrin iron uptake.

To evaluate the significance of the findings in relation to the uptake of desialated transferrin by PLC/PRF 5 hepatocellular carcinoma cells it is first necessary to review the results obtained by other workers. Recent work in whole rats has indicated that rat asialotransferrin is some 3 to 4 times more efficient in iron delivery than is normal transferrin (Rudolf et al. 1986). Insofar as the Hep G 2 human hepatocellular cancer cell is concerned, the numbers of asialoglycoprotein receptors have been found to be comparable to those of transferrin receptors (Ciechanover et al. 1983). Although the rate constant

for association of the asialoglycoprotein receptor was only half that of the transferrin receptor, that for internalisation was twice as great. It is against this background that the present result should be seen. It was found that most of the uptake of desialated transferrin by PLC/PRF 5 hepatocarcinoma cells could be inhibited by an excess of cold normal transferrin. This suggested that the desialated transferrin was being processed by the conventional transferrin receptor pathway rather than by an asialoprotein receptor pathway. The reason for these findings was elucidated very recently by Wu and Wu (1986), who showed that the PLC/PRF 5 cell line is different from the Hep G 2 one in that it lacks asialoglycoprotein receptors. The fact that it exhibits exuberant growth despite the absence of these receptors suggests that the pathway is quantitatively unimportant under neoplastic circumstances or that enhanced transferrin receptor expression as a consequence of the malignant process is able to offset any disadvantage consequent upon the lack of the asialoglycoprotein.

The studies in which the fate of internalised iron was evaluated indicated that only about a third of the cytosolic iron was in ferritin. Roughly another third was still bound to transferrin and about one fifth was found in a small molecular weight fraction. This pattern is very different to that observed with cultured human monocytes (Chapter 15), where the vast majority of the endocytosed iron was present in the iron storage protein ferritin. The reasons for this difference are not clear, but may relate to differences in cell metabolism, proliferation, growth and differentiation.

18.5 SUMMARY

This study has delineated, in part at least, the nature of transferrin-iron-cell interactions as they relate to the cultured PLC/PRF 5 human hepatocellular carcinoma cell. Specific transferrin receptors were identified on these cells. There were 1.9×10^5 receptors per PLC/PRF 5 hepatoma cell with a Kd of 1.5×10^{-8}M. At higher concentrations of transferrin, binding was not completely saturable. There was progressive near linear incorporation and concentration of iron in hepatoma cells when incubated with diferric transferrin. Transferrin and iron uptake were temperature dependent. Chase experiments confirmed that transferrin was being recycled. The lysosomotropic agent, NH_4Cl, appeared to inhibit partially iron unloading from transferrin. The microtubular inhibiting vinca alkaloid, vincristine, had a modest inhibitory effect on transferrin and iron uptake. Desialated transferrin appeared to be associated via transferrin receptors rather than via asialoprotein receptors. Approximately one third of the internalised cytosolic iron was found in ferritin, another one third still bound to transferrin, and a fifth was present in a small molecular weight compartment.

Chapter Nineteen

TRANSFERRIN-IRON-CELL INTERACTIONS: OVARIAN CARCINOMA CELLS (UWB-17 LINE)

19.1 INTRODUCTION

An ovarian carcinoma cell line (UWB-17) was developed in this laboratory from malignant ascitic fluid that had been removed from a subject with ovarian carcinoma. Characterisation and description of this line has been completed and is being prepared for publication. Transferrin receptor studies were performed on these cells as part of this characterisation. Methods employed were the same as those outlined in Chapter 14. The present chapter describes the results of the receptor studies.

19.2 RESULTS

Dose dependent diferric transferrin uptake in a low transferrin concentration range (0-180pmol/ml) gave a saturable binding curve. Specific binding was calculated by subtracting irreversible binding (in the presence of a 100-fold excess of diferric transferrin) from total transferrin binding. Non-specific transferrin binding was minimised by the addition of 2% bovine serum albumin to the incubation medium. A representative binding curve is shown in Figure 58. Scatchard analysis suggested that there were 4×10^5 receptors per cell with a Kd of 4.5×10^{-8}M. Temperature and time-dependent studies of transferrin and iron uptake by these cells revealed that there was progressive uptake at 37°C with a nett accumulation of iron. This resulted in the cellular iron to transferrin molar ratio increasing from 2 early

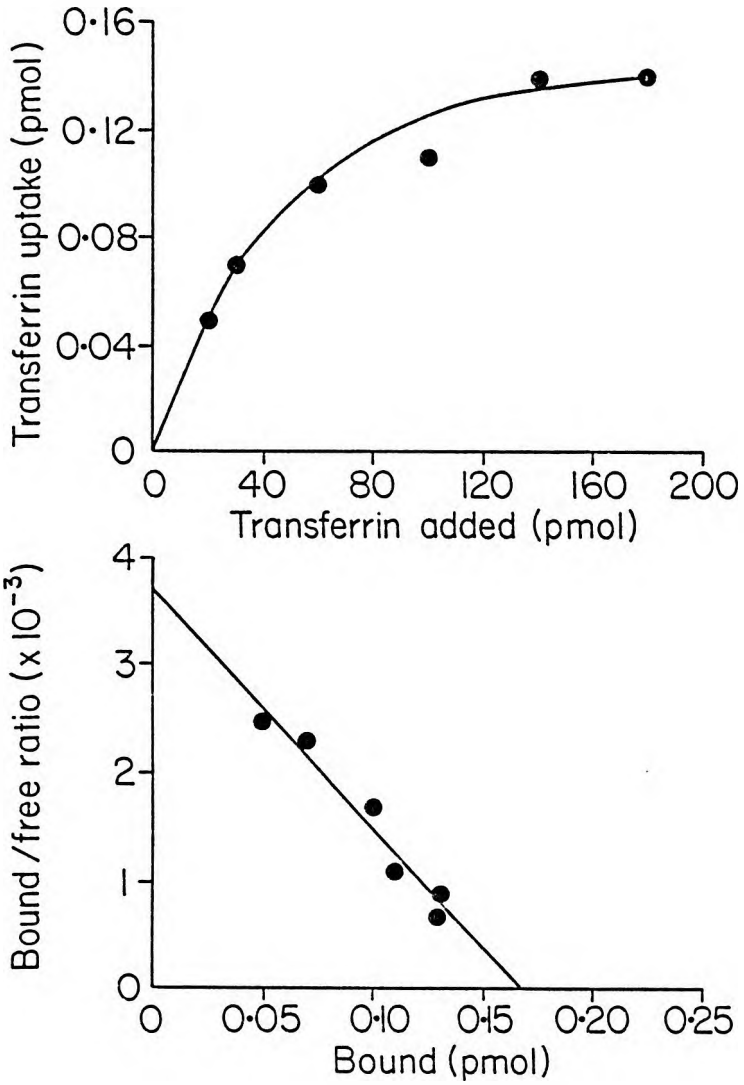
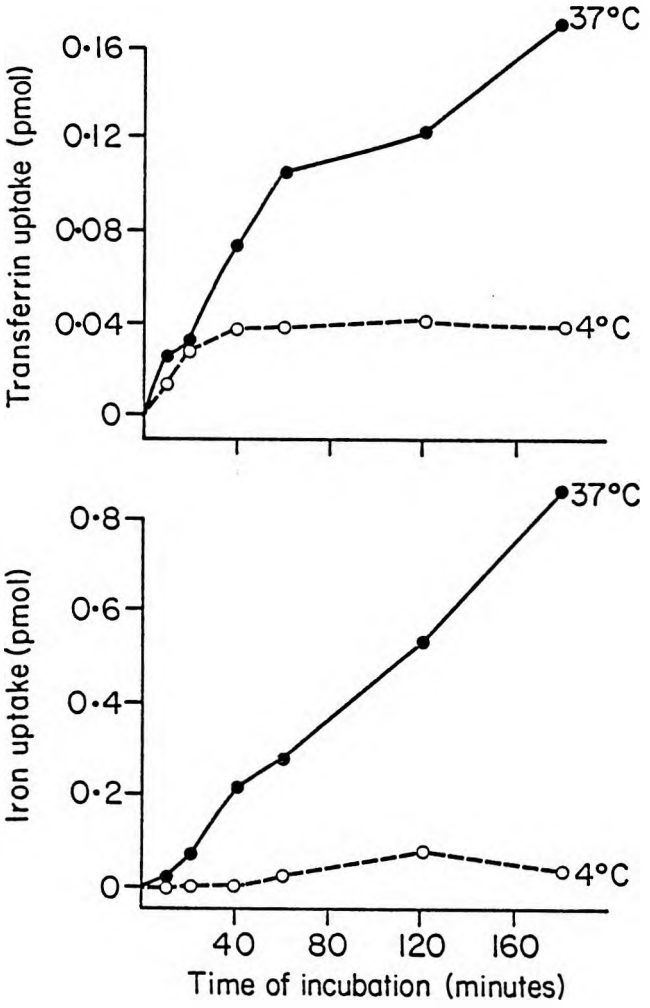


Figure 58. The dose dependent diferric transferrin uptake by cultured ovarian carcinoma cells (UWB-17) (upper panel) together with the Scatchard analysis (lower panel). The incubation time was 180 minutes. The mean number of cells per Petri dish was 2.5×10^5. From the Scatchard analysis the Kd was 4.5×10^{-8}M and the number of receptors per cell was 4×10^5.

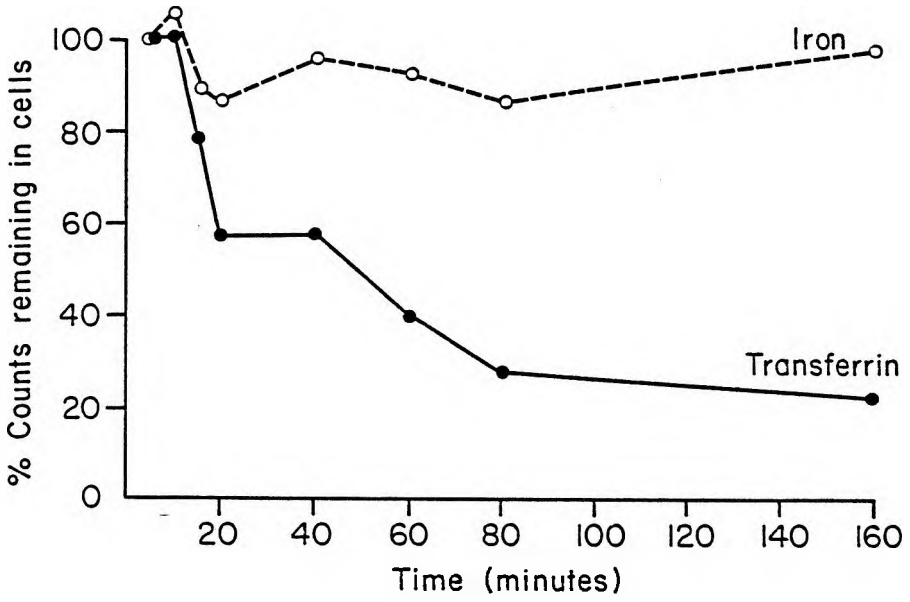
in the incubation to ± 6 after 180 minutes of incubation. At 4°C transferrin binding was reduced to a lower level than at 37°C by some 75% after 180 minutes of incubation. There was no progressive uptake of iron at 4°C. Thus, the cellular molar ratio of iron to transferrin at 4°C did not exceed 2 at any stage. A representative example is shown in Figure 59.

The recycling of transferrin and the intracellular unloading of iron were demonstrated by chase experiments. In these experiments Petri dishes were preincubated in doubly labelled diferric transferrin for 3 hours as described previously. Thereafter, the medium containing labelled diferric transferrin was removed, the cells were washed and medium containing cold diferric transferrin replaced. The cellular contents of ^{125}I and ^{59}Fe were then followed sequentially. A representative example is shown in Figure 60. It can be seen that the intracellular iron remained constant while the transferrin was cleared from the cell.

The effects of an inhibitor of endocytic vesicle acidification (NH_4Cl) and a microtubular inhibitor (vincristine) on diferric transferrin handling were evaluated in the next series of experiments. Dose dependent studies up to a vincristine concentration of 5 ug/ml showed no inhibition of transferrin or iron uptake. As in previous studies on human macrophages (Chapter 15) and hepatoma cells (Chapter 18), NH_4Cl inhibited iron unloading from transferrin in the ovarian carcinoma cells. Only one small difference was noted. In the presence of NH_4Cl there was increased uptake of transferrin by the ovarian carcinoma



igure 59. Temperature and time dependent transferrin and iron uptake by cultured ovarian carcinoma cells (UWB-17). 35pmol diferric transferrin was added to each Petri dish.



ure 60. Chase experiment in which cells were preloaded with doubly labelled diferric transferrin and then followed over a 160 min period of incubation in the presence of cold diferric transferrin. Counts were expressed as a percentage of those intracellularly at 5 minutes.

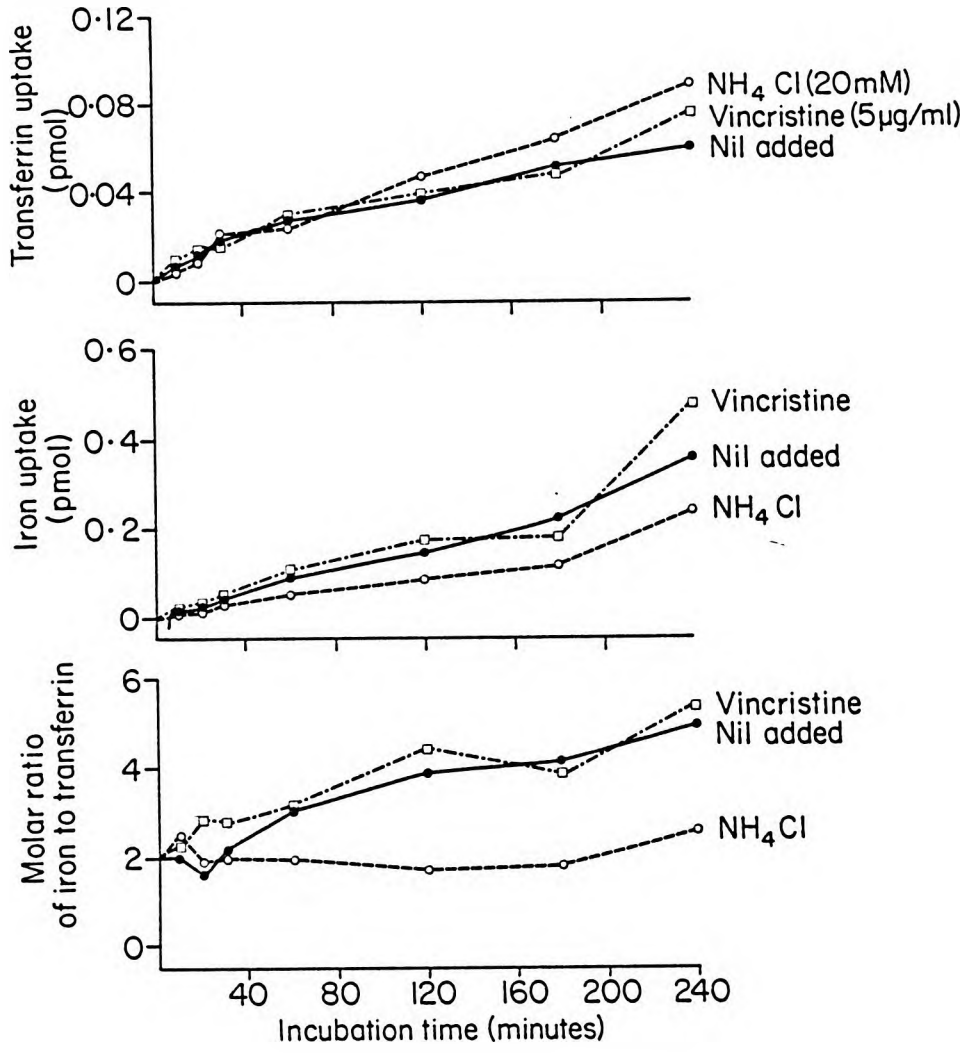
cells. In contrast, a minor reduction in transferrin uptake was noted with hepatoma cells and macrophages. A representative study showing the effects of 20mM NH_4Cl and 5/ug/ml vincristine on the time dependent uptake of transferrin and iron by cultured ovarian cancer cells is shown in Figure 61. In addition, their effects on the cellular molar ratio of iron to transferrin are indicated.

To determine the fate of the internalised iron, cellular lysates were prepared as described in Chapter 14 and passed through an AcA 44 ultrogel column as previously outlined. When this was done the vast majority (81%) of the radioactive iron counts in the cytosolic supernatant were present in ferritin by 48 hours of incubation. A representative example of ovarian cancer cells incubated in the presence of ^{59}Fe diferric transferrin for 48 hours is indicated in Figure 62.

In a final experiment the UWB-17 cell line was compared to another ovarian carcinoma cell line (OVCAR-3) which was obtained from the American Tissue Culture Collection and developed at the National Institutes of Health. The two lines were very similar in terms of transferrin receptors. When OVCAR-3 cells were studied by methods similar to those employed for the UWB-17, there appeared to be 6×10^5 transferrin receptors per cancer cell with a Kd of 1×10^{-8}M. Time dependent and chase experiments were also very similar.

19.3 DISCUSSION

The aim of the current study was to characterise



61. The effect of 20mM NH_4Cl and 5/ug/ml vincristine on the time dependent uptake of transferrin and iron by cultured ovarian cancer cells and the cellular molar ratio of iron to transferrin.

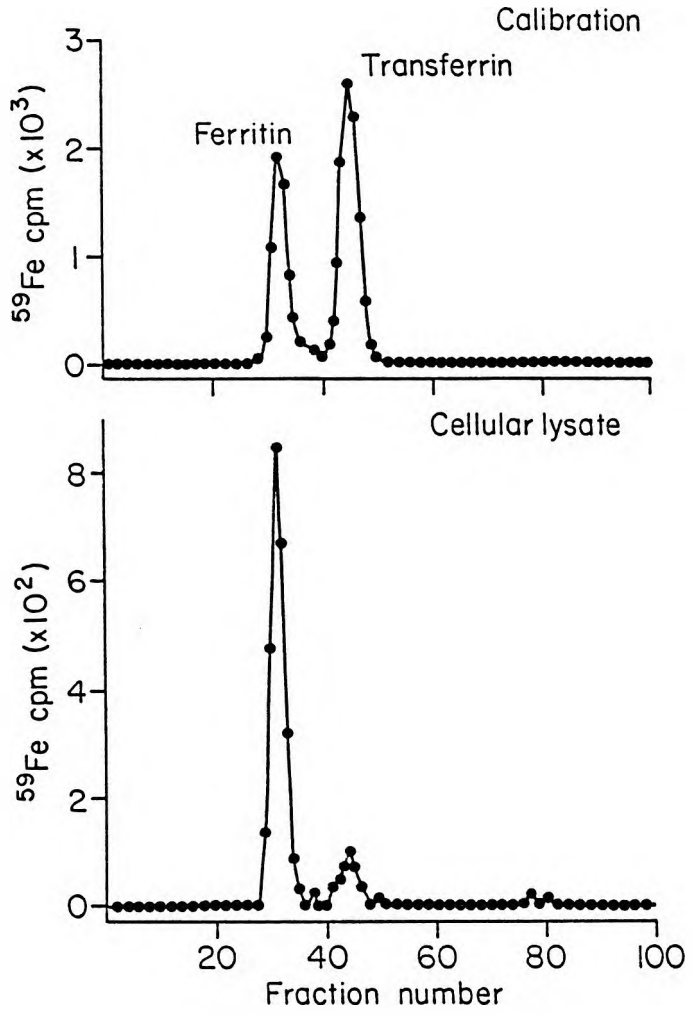


Figure 62. Results of AcA 44 ultrogel separation of ^{59}Fe in cytosol supernatants from cellular lysates. The top frame shows column calibration with labelled ferritin (first peak) and labelled transferrin (second peak). The bottom frame shows the separation of the cellular lysate obtained after 48 hours' incubation in a medium containing ^{59}Fe diferric transferrin. The largest peak is present in the ferritin region. The next large peak is in the transferrin region. A small number of counts are present in the low molecular weight region.

transferrin-iron interaction with a locally developed ovarian carcinoma cell line. As with the case of cultured blood monocytes and cultured human hepatoma cells, large numbers of receptors for diferric transferrin were found to be present. Transferrin and iron uptake appeared to be active processes, which were inhibited at 4°C. Chase experiments indicated that transferrin was recycled while iron was unloaded intracellularly. The inhibition of iron unloading by NH_4Cl was compatible with endocytic vesicle acidification playing a major role in the process. It was interesting to note the lack of an inhibitory effect of the microtubular inhibitor, vincristine, on transferrin-iron uptake. A possible explanation for this observation is the fact that this ovarian cancer line exhibits a multi-drug resistant phenotype (data not shown). The column separation of cellular lysates indicated that most of the internalised iron was detectable in ferritin. Comparison with the NIH cell line OVCAR-3 showed a striking similarity in terms of transferrin-iron interactions.

The possible biological significance of transferrin receptors was reviewed in Chapter 13. To summarise briefly, receptor mediated endocytosis of diferric transferrin appears to be fundamental to cell growth and division. Transferrin receptor number varies through the cell cycle. There is some evidence that the transferrin receptor endocytosis is important for cell growth, possibly independent of diferric transferrin uptake. Transferrin receptors may play a role in the targeting of natural killer cells. They may also play a role in cellular differentiation. Whatever ancilliary roles they may have, they

are essential for malignant cell proliferation. These observations lead to the intriguing possibility that these receptors, present in large numbers on malignant cells, might be utilised for therapeutic purposes. One immediate objection, however, relates to the presence of large numbers of similar receptors on a vast array of normal cells. Thus it seems that their ubiquitous nature will always mitigate against selective therapeutic manipulations. This lack of selectivity is underlined by the marked similarities between transferrin receptors on normal and malignant cells (Young and Bomford, 1984). However, there are other reasons why this therapeutic option merits further evaluation. Where neoplasms are confined to a body space (eg. ovarian carcinoma in the peritoneum) or are accessible to regional perfusion, systemic toxicity might be limited and therapy utilising the transferrin receptor might thus be feasible. Since transferrin receptors are present in large numbers on malignant cells, and since they are not subject to antigenic and functional change, they provide ideal targets for transferrin or anti-transferrin receptor monoclonal antibodies linked to either chemotherapeutic agents or toxins. In this regard, promising <u>in vitro</u> data indicate that ovarian carcinoma cell proliferation is markedly inhibited by anti-transferrin receptor antibody linked to pseudomonas exotoxin (Pirker et al. 1985). Other <u>in vitro</u> studies have obtained similar results with human transferrin conjugated to adriamycin (Yeh and Faulk, 1984). In this connection there is recent work showing that adriamycin is a weak base which becomes concentrated in endocytic vesicles

and here, at acid pH, acts as a powerful iron chelator. In this way iron delivery to the cell is limited (Demant and Norskov-Lauritsen, 1986). Clearly further work will be required in order to explore these therapeutic possibilities.

19.4 SUMMARY

In this chapter a cell line developed in this laboratory from malignant ovarian carcinoma ascitic fluid was evaluated in terms of its interaction with diferric transferrin. The cells exhibited saturable, specific and high affinity binding for transferrin. Each cancer cell appeared to have 4×10^5 diferric transferrin receptors per cell, with a Kd of 4.5×10^{-8}M. Transferrin binding and progressive iron uptake was demonstrated to be temperature dependent. Chase experiments showed that the transferrin was being recycled while iron was progressively unloaded intracellularly. The weak base NH_4Cl was found to inhibit intracellular iron unloading from transferrin. An interesting finding was the failure of the microtubular inhibitor, vincristine, to limit transferrin and iron uptake. This was possibly explained on the basis of the cell's multi-drug resistant phenotype. Comparison with an established NIH ovarian carcinoma line OVCAR-3 indicated marked similarities in terms of diferric transferrin uptake, with each cell having 6×10^5 receptors and a Kd of 1×10^{-8}M. When the fate of the internalised iron was evaluated, it appeared that most of it was present in ferritin. Finally, some speculations are offered as to how these

receptors might be put to therapeutic advantage.

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