THE EFFECTS OF HYPOFERREMIA ON A MURINE LYMPHOMA

AND

A COMPARISON WITH *NEISSERIA MENINGITIDIS*

by

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ABSTRACT

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Both neoplastic and *Neisseria meningitidis* cells can obtain iron from transferrin *in vivo*. Previous findings (Holbein, 1980) showed that the murine hypoferremic response inhibits *Neisseria meningitidis* infection by iron deprivation. The present studies demonstrated that hypoferremia does not limit Fe acquisition by or growth of murine lymphoma cells. Transferrin binding sites on lymphoma cells and *N. meningitidis* were enumerated and the transferrin binding affinities were determined. Iron deprivation increased the number of transferrin binding sites on *N. meningitidis* cells. *N. subflava* and *Escherichia coli* were unable to bind transferrin. Competition binding studies demonstrated that lymphoma cells bound human transferrin over murine and bovine transferrin, conalbumin, and lactoferrin and suggested that neisserial transferrin binding sites had lower specificity. Further competition studies indicated that lymphoma transferrin binding sites had higher affinity for ferri-transferrin than for apo-transferrin while neisserial sites bound these two ligands to the same extent. This can explain why hypoferremia inhibits iron acquisition by *N. meningitidis* but not by lymphoma cells.

RESUME

Ph.D. Margaret Caldwell Microbiologie et Immunologie

EFFETS DE LA REPONSE HYPOFERREMIQUE SUR LES CELLULES LYMPHOIDES DE SOURIS ET UNE COMPARAISON AVEC *NEISSERIA MENINGITIDIS*

Les cellules tumorales et *Neisseria meningitidis* peuvent obtenir du fer de la transferrine *in vivo*. Les découvertes antérieures (Holbein, 1980) démontraient que la réponse hypoferrémique chez les souris freinait l'infection de *Neisseria meningitidis* en limitant l'accessibilité au fer. Cette étude démontrait que la réponse hypoferrémique ne restreint ni l'acquisition du fer ni la croissance des cellules lymphoides de souris. Le nombre de récepteurs spécifiques à la transferrine sur la surface des cellules lymphoïdes et sur *N. meningitidis* a été mesuré et les K_d's pour l'association de la transferrine avec ses récepteurs ont été déterminés. La carence en fer augmentait le nombre des récepteurs spécifiques à la transferrine des cellules *N. meningitidis*. Aucun site d'attachement à la transferrine ne fut trouvé chez *N. subflava* et *Escherichia coli*. Les études competitives d'attachement démontraient que les cellules lymphoides attachaient mieux à la transferrine humaine qu'à la transferrine bovine ou de souris ou à la lactoferrine ou à la conalbumine. Les résultats suggéraient aussi que les récepteurs spécifiques à la transferrine de *N. meningitidis* avaient une specificité plus basse que les récepteurs des cellules tumorales. Les récepteurs spécifiques à la transferrine des cellules

lymphoides avaient une affinité plus forte pour la ferritransferrine que pour l'apotransferrine alors que les récepteurs de *N. meningitidis* ne pouvaient différencier ces deux ligands. Ce résultat peut expliquer pourquoi la réponse hypoferrémique inhibe l'acquisition du fer chez *N. meningitidis* mais non chez les cellules lymphoides.

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CLAIM OF CONTRIBUTION TO KNOWLEDGE

1. It was demonstrated using the EL4 murine lymphoma, a tumor of C57 black mice, that the hypoferremic response was not beneficial in inhibiting tumor cell proliferation or iron acquisition.

2. The EL4-1 lymphoma cell subline which could grow *in vitro* without serum in synthetic tissue culture media and human transferrin was established.

3. A model of hypoferremia induced by turpentine inflammation was established in C57 black mice.

4. It was demonstrated that the EL4-1 cells could utilize the following iron sources for growth: human, bovine and murine transferrins, hemoglobin, ferritin and inorganic iron. Lactoferrin and conalbumin could not be used. The number of surface transferrin binding sites for these cells was determined as was the transferrin-transferrin receptor dissociation constant (K_d). It was found that these murine cells bind human transferrin preferentially over murine transferrin and the transferrin receptors of these cells have very low affinity for bovine transferrin, lactoferrin or conalbumin.

5. High affinity binding sites for human transferrin on *Neisseria meningitidis* cells were enumerated by Scatchard analysis and the dissociation constant (K_d) was also determined.

6. Iron deprivation of *Neisseria meningitidis* causes enhanced binding of human transferrin to the cells. No difference was observed in the K_d's between iron-sufficient and iron-deprived cells.

7. Unlike *Neisseria meningitidis*, the non-pathogens *Neisseria subflava* and *Escherichia coli* do not possess surface cellular binding sites for human transferrin.

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1. Introduction

All procaryotic cells except certain lactic acid bacteria and all normal and malignant eucaryotic cells examined require iron (Fe) as a nutrient for growth. Therefore in order for a pathogen to establish an infection or for tumor cells to proliferate sufficient Fe must be obtained from the host. The primary Fe source *in vivo* is the serum Fe transport protein transferrin (Tf). During inflammation, infection or neoplastic disease the amount of Fe carried by this protein decreases. This process, known as the hypoferremic response, has been shown repeatedly to be effective in controlling infection by limiting Fe availability to infecting microorganisms. A series of studies by Holbein (1980, 1981) and Letendre and Holbein (1983, 1984) demonstrated that hypoferremia is important in controlling *Neisseria meningitidis* infection in mice. This bacterial pathogen is unusual as it can obtain host Fe by binding Tf directly. Weinberg, in a series of reviews, (1981, 1983, 1984, 1986) has pointed out that since proliferating tumor cells require a continuous supply of Fe (which nondividing cells do not) and since they also obtain Fe chiefly or solely by binding Tf directly, the hypoferremic response might well inhibit tumor cell growth by Fe deprivation. The primary purposes of this work were to use a murine model of neoplastic disease to investigate whether the hypoferremic response could effectively deprive tumor cells of Fe and limit their growth and to compare the characteristics of procaryotic and eucaryotic Tf binding. Subsequently, several other facets of tumor cell and neisserial Fe acquisition were examined.

As background for this work, neoplastic and meningococcal disease as

well as certain aspects of Fe metabolism will be reviewed. These aspects will include a description of vertebrate Fe metabolism, the proteins involved in Fe metabolism and a discussion of the changes which occur in this cycle during infection and neoplasia. The possible role of Fe in contributing to neoplastic disease will be mentioned and microbial Fe acquisition will be discussed. Finally, the mechanism of tumor cell Fe acquisition will be reviewed in terms of the information currently available concerning the eucaryotic Tf receptor, its cellular expression and the endocytic Fe acquisition cycle.

2. LITERATURE REVIEW

2.1 Neoplasia

The word neoplasm means new growth and cancer is a general term used for all malignant tumors. A malignant neoplasm is an abnormal mass of tissue whose growth rate often exceeds that of normal tissue. Malignant neoplastic cells do not respond to normal host signals to regulate or halt division (Maugh and Marx, 1975). Neoplastic cells may arise in any tissue containing cells capable of division but occur most frequently in tissues typified by active cell loss and replacement and in tissues exposed to environmental carcinogens such as the skin, the respiratory tract and alimentary tract. Cancer cells are more variable in size and shape than normal cells and the nucleus is generally larger and often irregular There is often an increase in the size and number of nucleoli and there is generally a large proportion of the cells undergoing mitosis at any one time. Neoplastic cells are also generally less well-differentiated than the original normal tissue (Ruddon, 1981).

The hallmarks of neoplastic disease are invasion and metastasis. Malignant cells invade and destroy the surrounding normal tissue disrupting tissue function. These cells further metastasize via blood vessels and lymphatic channels to distant organs. These properties of malignant tumors have devastating effects on the host including: bleeding or hemorrhage, infection, fever, anorexia, cachexia or body-wasting, anemia and pain due to tissue destruction, pressure or obstruction of organ function (Ruddon,

1981).

Generally, tumors *in vivo* grow slowly initially when they are avascular but subsequent vascularization may occur due to production of tumor angiogenesis factor. Once vascularization occurs, tumors have access to more nutrients and oxygen and therefore grow more quickly.

Transformed cells *in vitro* have many cytological characteristics similar to those described above for cancer cells *in vivo*. Normal diploid mammalian cells have a finite life span *in vitro* while malignant cell cultures are able to live indefinitely. Neoplastic cells *in vitro* grow to a higher cell density than non-transformed cells; they no longer demonstrate contact inhibition or anchorage dependence and can grow in suspension or soft agar. Transformed cells also demonstrate loss of restriction point control. Certain tumor cells can synthesize some of their own growth factors and therefore may require a lower serum concentration. Many cellular properties might be changed including: surface glycoproteins and glycolipids; tumor-associated antigens may be present and the cell cytoskeleton, microtubules and microfilaments, may be altered. Changes in cell enzyme patterns, cyclic nucleotide levels and in amino acid and sugar transport may be evident. Finally, malignant cells demonstrate tumorigenicity when injected into experimental animals (Ruddon, 1981).

2.2 *Neisseria meningitidis*

Neisseria meningitidis, the causative agent of meningococcal meningitis, is a gram-negative, non-motile diplococcus. The individual cocci are small (0.8 X 0.6 um) and are kidney-shaped. This bacterium is obligately aerobic but readily degrades glucose and maltose producing acetic acid. It is TMPD (tetramethyl-p-phenylenediamine) or terminal oxidase, superoxide dismutase and catalase positive The meningococci are divided into 10 serogroups on the basis of polysaccharide capsular antigens: A, B, C1+, C1-, D, W-135, X, Y, Z and Z'. This organism is further classified on the basis of outer membrane protein and lipopolysaccharide antigens (Gotschlich, 1980).

N. meningitidis causes 10-30% of cases of adult meningitis and 30-40% of cases of meningitis in children under age 15. The human nasopharynx is the natural reservoir of this pathogen and 5-30% of the population normally are asymptomatic carriers. In the susceptible host, the organism may cause pharyngitis and from the nasopharynx may invade the blood stream to cause meningococcemia. Meningococci may spread to the skin, eyes, joints, heart, adrenals and meninges in which case the leptomeninges, pia and arachnoid matter become inflamed Most patients develop a petechial rash. In 10-20% of cases fulminant meningococcemia occurs and shock and vascular collapse may ensue; death may occur due to cardiac or respiratory failure.

In a series of studies by Archibald and DeVoe (1978, 1979, 1980) the Fe requirements for the growth of *N. meningitidis* were determined and the ability of the organism to grow on many different Fe-containing compounds was examined. It was found that *N. meningitidis* SD1C in continuous culture

required 11-17 ug Fe per g (dry weight) for slow cell division and maintenance of viability (Archibald and DeVoe, 1978). *N. meningitidis*, unlike most bacteria, was not found to produce siderophores (Archibald and DeVoe, 1979). *N. meningitidis*, however, demonstrated the rare procaryotic ability to obtain Fe from Tf when Fe deprived Contact between the organism and the Tf protein was necessary proving that the meningococcus obtains Fe by binding Tf directly Yancey and Finkelstein (1981) reported that hydroxamate siderophores had been isolated from concentrated supernatants of both *N meningitidis* and *N. gonorrhoeae* cultures. These siderophores were stimulatory in the *Arthrobacter flavescens* JG-9 bioassay for hydroxamic acid siderophores. West and Sparling (1985), however, found that the uninoculated culture media which included Proteose Peptone contained small amounts of siderophore-like activity and no increased amount of siderophore activity above that found in uninoculated media could be found in supernatants from *N gonorrhoeae* cultures. West and Sparling further pointed out that the very small amount of siderophore found in supernatants by Yancey and Finkelstein would be unlikely to be sufficient to support the growth of the gonococcus. Thus the results of Archibald and Devoe, West and Sparling and also of Simonson *et al.* (1982) indicate that the pathogenic neisseriae do not produce siderophores *in vitro*. The possibility that these organisms may produce siderophores *in vivo* cannot be ruled out but this would be difficult to test.

N. meningitidis can also use Fe from $FeCl_3$, ferric citrate, hog gastric mucin, hemoglobin and myoglobin but cannot readily use Fe from conalbumin, ferritin, cytochrome c or certain microbial siderophores including desferrioxamine, ferrichrome or enterochelin (Archibald and DeVoe, 1979;

1980). Mickelsen and Sparling (1981) reported that all *N. meningitidis* and *N. gonorrhoeae* strains examined could utilize Fe from 25% Fe-saturated Tf but most commensal strains were inhibited by this protein. In a subsequent study, Mickelsen *et al.* (1982) examined 107 *Neisseria* strains to determine which ones could use lactoferrin (Lf) Fe for growth. All meningococcal strains examined, 53% of gonococcal strains and 24% of commensal strains (*N. lactamica, N sicca, N. flava* etc.) could utilize Fe from this compound.

Simonson *et al.* (1982) found that both Fe-deprived and Fe-sufficient meningococci could bind Tf but only the Fe-deprived cells could take up Fe from this protein; maximal uptake occurred after 4 h of Fe deprivation. Binding of Tf was energy-independent but Fe uptake required a functional electron transport chain as azide, cyanide and antimycin A inhibited uptake. If cells were exposed to trypsin or 60° C temperatures for 5 min Fe uptake was abolished Apo-Tf was bound to the same extent as 30% Fe-saturated Tf suggesting that the bacterium recognizes the protein not the Fe moiety of the molecule The ability of *N. meningitidis* to obtain Fe from Tf may represent an important virulence factor for this organism as the non-pathogenic *N. flava* and *N. sicca* could not obtain Fe from Tf even when Fe-starved.

Brener *et al.* (1981) found that if *N. meningitidis* was grown at low pH (6.6) under Fe-limited conditions the virulence of the organism for mice was enhanced 1200-fold. Cells grown at pH 6 6 or under Fe-deprived conditions at pH 7.2 demonstrated enhanced synthesis of an outer membrane protein of 69,000 daltons. Masson and Holbein (1985) demonstrated that when *N. meningitidis* was grown in Fe-limited media at low pH the virulence

of the organism for mice, the amount of capsular polysaccharide and cell surface hydrophilicity were all increased. Mietzner *et al.* (1986) examined many *Neisseria* strains for the presence of a 37,000 dalton Fe-regulated outer membrane protein which was first observed in *N. gonorrhoeae* (Mietzner *et al.*, 1984). This protein was found in 40 strains of *N. gonorrhoeae*, *N. meningitidis*, *N. lactamica* and *N. cinerea* while 17 strains from other species of *Neisseria* and *Branhamella* did not demonstrate the presence of this Fe-regulated protein except for one strain of *N subflava*. This antigen therefore appeared to be conserved among pathogenic neisseriae. It was also reported that sera from patients recovering from gonococcal infections had antibodies to this 37,000 dalton protein. As the authors point out, this latter finding suggests that this protein is expressed and antigenic *in vivo*. This finding coupled with the fact that the protein appears among pathogenic neisseriae suggests that the protein may have an essential pathogenic function *in vivo*. However, the precise role of this protein in Fe acquisition and pathogenicity remains unknown at this time.

In a recent study Black *et al* (1986) wanted to determine whether Fe-repressible outer membrane proteins in *N. meningitidis* were expressed and immunogenic during infection. They examined purified membranes from four disease isolates by Western blotting with patient sera. The patients' convalescent sera contained IgG and IgM antibodies to a 70,000 dalton Fe-regulated protein and IgG antibody to a 94,000 dalton protein in the outer membrane of the *N. meningitidis* culture isolated from the corresponding patient. Neither acute phase nor pooled normal sera contained antibodies to these proteins. Antigenic cross-reactivity was apparent as certain patient antibodies would react with Fe-regulated membrane

antigens from isolates of other patients. These authors further described an interesting meningococcal mutant which did not possess the 70,000 dalton Fe-regulated protein and was unable to use Tf or Lf for growth or to take up appreciable ^{55}Fe from Tf or Lf. Unfortunately, this mutant has proved transformation incompetent and genetic analysis of the system has not progressed (D Dyer, personal communication). The initial findings, however, suggest that this protein has a central role in Fe acquisition from Tf or Lf and may be an important virulence factor particularly as the ability to deferrate Tf directly has an obvious advantage in the meningococcal invasion of the blood and cerebrospinal fluid, and because the host Fe status clearly affects the course and outcome of experimental murine meningococcemia (Holbein, 1980, 1981).

2.3 Importance of Fe in biological systems

Certain atomic properties of Fe, primarily its unpaired electrons and two valences make it the most versatile transition metal and thus an important component of many biologically important compounds. Heme Fe is present in hemoglobin, myoglobin, cytochromes, and in enzymes such as the catalases and peroxidases. Non-heme Fe containing enzymes include non-heme oxidases and oxygenases, Fe-sulfur proteins, superoxide dismutase, nitrogenases and ribonucleotide reductase. Fe exists in the ferrous (Fe^{2+}) and the ferric (Fe^{3+}) state and can readily undergo changes from one valence state to another. This is important, for example, in mitochondrial and bacterial electron transport chains for electron transfer. Fe is uniquely suited for electron transfer reactions as it can be poised at a

wide range of redox potentials. The Fe valence electrons have the ability to exist in low or high spin states. These properties enable it to catalyze many reactions involving oxygen reduction (Wrigglesworth and Baum, 1980).

Although Fe is the second most abundant metal and the fourth most abundant element in the earth's crust, in a well-aerated neutral or alkaline solution Fe is usually in the ferric form and readily forms insoluble complexes. Ferric salts at neutral pH form $Fe(OH)_3$ which has a solubility product of 4×10^{-36} (Free $[Fe]/[Fe(OH)_3]$. Thus any free Fe in excess of 2.5×10^{-18} M would be precipitated in the form of the hydroxide (Bullen, *et al.*, 1978). As cells require 0.4-4.0 uM environmental Fe for growth (Weinberg, 1981) organisms have evolved specialized Fe transport, storage and solubilizing systems. In vertebrates, most Fe is bound to proteins probably because free Fe could catalyze the production of highly destructive radicals, provide nutrition for invading parasites and not be efficiently stored, controlled or transported.

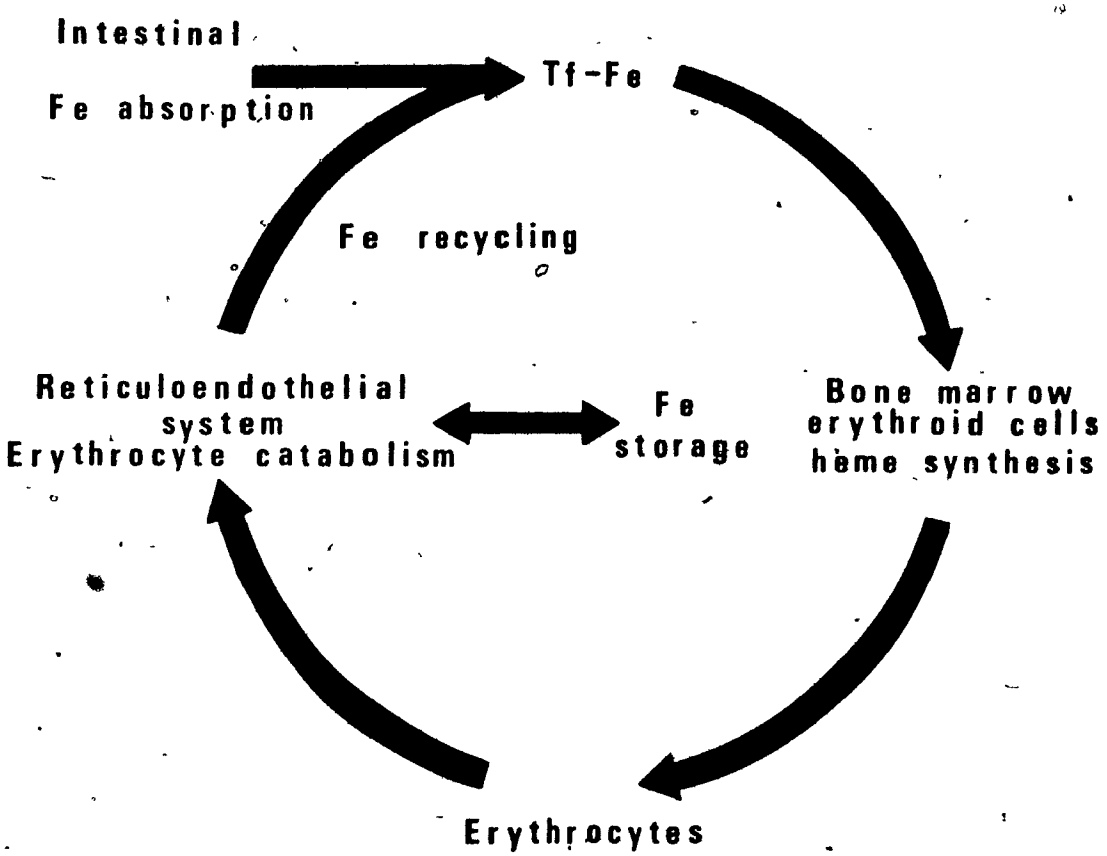
2.4 The role of Fe in metabolism

2.4.1 The vertebrate Fe cycle

The human body contains approximately 50 mg kg^{-1} Fe. Hemoglobin comprises 60-70% of the body Fe, myoglobin, cytochromes and enzymes 10% and the Fe-storage proteins ferritin and hemosiderin contain 20-30%. The transport protein Tf only contains 0.1-0.2% of body Fe (May and Williams, 1980). The Fe cycle in the body is dynamic and Fe metabolism is under

stringent control. An individual's daily dietary intake of Fe is approximately 10 mg of which only 1.0-1.5 mg is absorbed (Conrad and Barton, 1981). An equal amount of Fe is lost daily from the feçes, urine and sloughed skin, intestinal epithelial cells and body secretions. Absorption of Fe involves at least 2 steps: mucosal (villus cell brush border) uptake from the intestinal lumen and mucosal transfer (villus cell basolateral membrane export) to the plasma. Fe is solubilized by gastric acid and is absorbed in the upper duodenum. The mechanisms of Fe transit through the intestinal mucosa have not been elucidated (Finch and Huebers, 1986) but absorption of Fe is usually regulated according to the body's requirements. Fe in excess of body needs may be stored in the duodenal mucosa in the form of the storage compound ferritin. Some of this Fe may be subsequently lost when the intestinal villus cells are sloughed Heme Fe is absorbed more efficiently than inorganic Fe; however, the absorption of inorganic Fe is enhanced by ascorbic acid, amino acids and sugars. Absorbed Fe is bound by the transport protein Tf in the blood and lymph. An important role of this protein is to transport Fe from absorption sites to all cells which require it including erythroid cells in the bone marrow, placental cells to transfer Fe to the developing fetus, and to lymphocytes and other proliferating cells. Tf also transports Fe to Fe storage sites such as the liver. In immature erythroid cells Fe is incorporated into hemoglobin, the oxygen carrying protein of erythrocytes. At the end of their life span, approximately 120 days in man, red blood cells are degraded and removed by the reticuloendothelial (RE) system. The RE system consists of the total body pool of macrophages and includes all phagocytic cells except granulocytic leukocytes. The Fe from senescent erythrocytes enters a

Fig. 1 The vertebrate Fe cycle.


Intestinal
Fe absorption
Tf-Fe
Fe recycling
Reticuloendothelial system
Erythrocyte catabolism
Fe storage
Bone marrow
erythroid cells
heme synthesis
Erythrocytes

labile pool from whence it is either released again to the Tf pool (early release) or it enters the RE storage Fe pool (late release); the Fe storage compounds are ferritin and hemosiderin. In the dog, the early and late release Fe pools have half-lives of 30 min and 7 days respectively and the two pools contain equal amounts of Fe (Fillet *et al.*, 1974). In man, 70% of RE Fe is in the early and 30% is in the late system. With the return of Fe once again to Tf the cycle is complete (Fig. 1). Tf is thus the central component of the Fe cycle. The Tf's are actually a family of proteins divided into two groups: one group contains serum Tf and conalbumin (chicken ovotransferrin); the other group is comprised of the lactoferrins (Aisen, 1980).

2.4.2 The transferrins

2.4.2.1 Transferrin

Tf is found in the serum of vertebrates at a concentration of 2-3 mg ml^{-1} and is also found in the extravascular fluids such as lymph, cerebrospinal fluid and ascites fluid (Morgan, 1981). Tf is synthesized primarily by the liver hepatocytes but can be synthesized in small amounts by Sertoli cells (Skinner and Griswold, 1980), neuronal-glial cells (Markelonis *et al.*, 1982) and by lymphocytes (Soltys and Brody, 1970). Schade and Caroline (1946) first identified Tf in Cohn's fraction IV-3,4 of human plasma proteins and named it siderophilin. Holmberg and Laurell (1947) also discovered the protein in porcine serum and after elucidating the transport function of this protein called it transferrin. Tf is an

80,000 dalton, single chain polypeptide consisting of 679 amino acids (MacGillivray *et al.*, 1983). Tf is a glycoprotein; human Tf contains 6% carbohydrate as two identical chains (Aisen and Listowsky, 1980) attached to asparagine residues 413 and 611 (MacGillivray *et al* , 1983). The carbohydrate chain consists of two sialic acid, two galactose, three mannose, and four N-acetylglucosamine residues and the sequence was determined by Spik *et al.* (1975).

The complete amino acid sequence of human Tf has been determined from peptide analysis (MacGillivray *et al.*, 1982) and through sequence analysis of cDNA (Uzan *et al.*, 1984). This globular protein contains two homologous domains in which 40% of the amino acid residues are identical; each domain possesses one Fe-binding site (Aisen and Listowsky, 1980) Interestingly, it is believed that the ancestral Tf protein was approximately 40,000 daltons and contained one Fe-binding site and by a process of gene duplication and fusion the larger two-sited protein evolved (Greene and Feeney, 1968). *Pyura stolonifera*, an invertebrate which arose 200,000,000 years ago has an Fe-binding protein of approximately 41,000 (+/- 2,000) daltons with one Fe-binding site (Martin *et al.*, 1984) which can bind to the human Tf receptor and donate Fe for hemoglobin synthesis. It has been suggested that the ancestral molecule was duplicated after the development of a secretory kidney which would have excreted a molecule of 41,000 daltons (Williams *et al.*, 1982). Each molecule of Tf thus binds two molecules of Fe in the ferric state. The protein's affinity for Fe is extremely high; the association constant is approximately 10^{36} ([TfFe]/[Tf][Fe]) (Aasa *et al.*, 1963). The binding of Fe occurs with the concomitant binding of a carbonate or bicarbonate anion and the release of three protons (Aisen

and Listowsky, 1980). The simultaneous binding of the anion and metal is a unique biochemical feature of Tf. Tf can bind other transition metals but binds ferric Fe with the highest affinity (Tan and Woodworth, 1969). When Tf has bound Fe the complex forms a salmon-pink color with an absorbance maximum at 465 nm. In the N-terminal domain of human Tf the Fe binding ligands are thought to include: tyrosine residues 95 and 188, histidine residue 249 and aspartate 63. The arginine residue at position 124 has been suggested to be the position at which the anion binds (Chasteen, 1987).

Much controversy has arisen in the past concerning whether the two Fe-binding sites on Tf are identical in their Fe-binding properties and their functions. Fletcher and Huehns (1968) put forth the idea that Fe from the C-terminal or A site was donated preferentially to erythroid cells and to the placenta and that Fe from the N-terminal or B-site was given to storage or intestinal cells. It is currently believed, however, that there is not a functional difference between the two monoferric species of Tf in donating Fe to cells. This has been demonstrated by Delaney *et al.*, (1982) for rabbit reticulocytes and Van der Heul *et al.* (1984) in human lymphocytes, hepatocytes and bone marrow cells. *In vitro* studies, however, have revealed certain physicochemical differences between the two Fe binding sites. Studies by Princiotto and Zapolski (1975) and Lestas (1976) revealed that the two Fe binding sites differ with respect to the pH at which Fe is released. The C-terminal Fe-binding site is more acid stable and retains Fe longer with decreasing pH (Marx, 1984). At pH 6.7 the N-terminal site has less than one-twentieth the affinity of Fe that the C-terminal site has while at pH 7.4 the affinities differ by only a factor of

five or six (Aisen and Listowsky, 1980). In human serum more Fe is bound to the N-terminal than to the C-terminal binding site (Leibman and Aisen, 1979); 44% of Tf is present as apo-Tf, 24% is N-terminal monoferric Tf, 10% is C-terminal monoferric and 22% is diferric Tf (Williams, J., 1979).

The Tf gene in the human is located on the long arm of chromosome 3 (Yang *et al.*, 1984); it is 33.5 kb in length and composed of 17 exons and 16 introns. The DNA sequence of 620 bp of the 5' region of the human Tf gene was recently studied by Lucero *et al* (1986). Several putative regulatory regions were identified including metal binding regulatory elements, transcription factor binding sequences, a progesterone receptor element, a glucocorticoid regulatory sequence, a cAMP regulatory domain and several DNA enhancers. These authors point out that this study represents an interesting first step in the study of regulation of Tf gene expression.

Although each molecule of Tf can bind two molecules of Fe, Tf is normally only 30% saturated with Fe in the human (Bullen *et al* , 1978) and 50% saturated in the mouse (Letendre and Holbein, 1983). This degree of unsaturation is important as not only must Tf transport Fe to cells requiring it but this protein must also withhold Fe from pathogenic organisms.

2.4.2.2 Conalbumin

Conalbumin or ovotransferrin, first discovered in 1899 (Osborne and Campbell, 1900) is found in egg white and comprises 12% of egg white solids (Weinberg, 1984). The apparent function of conalbumin is to bind Fe and withhold it from bacteria which could infect the egg. The Fe-binding properties of this protein were discovered in 1943 by Schade and Caroline

(Schade and Caroline, 1944) who were using egg white to lyophilize *Shigella dysenteriae* and found that some component of the egg white prevented growth of the organism. These authors tested 10 vitamins and 31 elements but only Fe overcame the growth inhibition. Conalbumin is also a single chain polypeptide but it contains only one oligosaccharide chain composed of 4 residues of mannose and 8 of N-acetylglucosamine (Williams, 1968).

2.4.2.3 Lactoferrin

Lactoferrin (Lf), the other important member of the Tf family, was discovered in human milk in 1939 and was purified in 1958 by Johansson. Human milk contains 2-6 mg ml^{-1} Lf (Bullen *et al.*, 1972) which represents 20% of the protein content; the concentration of milk Lf, however, varies among the different mammalian species (Bezkorovainy, 1980). Lf is found in other exocrine secretions such as mucus, tears, saliva and also in the specific granules of polymorphonuclear leukocytes (Bezkorovainy, 1980) but its concentration in the serum is normally only 0.01 uM (0.8 ug ml^{-1}) (Rumke *et al.*, 1971). Lf, like Tf ,is a glycoprotein with a molecular weight of approximately 80,000 daltons and each molecule can bind two ferric Fe molecules but Lf has a higher affinity for Fe (Aisen and Leibman, 1972). Lf has some amino acid homology with Tf but as shown by Blanc and Isliker (1961) is antigenically distinct. Lf has an important antimicrobial role in the body; it is present in exocrine secretions and is well-situated to withhold Fe from potential pathogens. Lf continues to bind Fe even when the pH is below 4; it could thus bind Fe *in vivo* at sites where the pH has been lowered due to lactic acid release from bacteria or

leukocytes (Weinberg, 1984). Brock (1980) has also suggested that Lf might have a role in suppressing intestinal Fe absorption in infants. Lf in milk at an average concentration of 3 mg ml^{-1} contains 0.21 ug ml^{-1} Fe; this represents 5% Fe-saturation of the protein (Finkelstein *et al.*, 1983). Thus Lf is highly unsaturated accounting for its strong Fe-withholding and antimicrobial properties. The body's Fe-binding proteins Tf and Lf are only partially saturated with Fe and have very high association constants for Fe. The amount of free Fe in equilibrium with these proteins is about 10^{-18} M (Bullen *et al.*, 1978) well below the 0.4-4.0 uM level required for bacterial growth. Tf and Lf therefore have very important anti-bacterial roles. The importance of Fe in microbial infection and the bacteriostatic effects of Tf and Lf have been appreciated for many years. Numerous *in vitro* studies have demonstrated that serum is bacteriostatic but when enough Fe is added to saturate the Tf the bacteriostatic properties are lost. This has been shown using many organisms including *Clostridium perfringens* (Rogers, 1967), *Escherichia coli* (Fletcher, 1971) and *Mycobacterium tuberculosis* (Kochan, 1973). Similarly it has been shown that the injection of Fe compounds enhances bacterial virulence *in vivo*. Jackson and Burrows (1956) working with *Yersinia pestis* found that avirulent colony types could become virulent for mice by adding Fe to the inocula. Bullen *et al.* (1968) found that if guinea pigs were injected intraperitoneally with 2-7 X 10^4 *E. coli* O111, the organisms did not proliferate but if the inoculum was injected with enough ferric ammonium citrate to saturate circulating Tf, with hemoglobin, lysed erythrocytes or hematin hydrochloride the bacteria grew quickly and the animals died. In a later study, Bullen *et al.* (1974) investigating *Pseudomonas aeruginosa* infection in rabbits,

found that a sublethal infecting dose of bacteria led to fatal rapid growth if Fe compounds were injected. These authors also found that *Pseudomonas* grew slowly in peritoneal fluid or in media containing unsaturated Tf and twice as quickly when the Tf in peritoneal fluid or media was saturated with Fe. They observed that *Pseudomonas* infection caused a rapid drop in the percentage Fe saturation of Tf in the plasma and peritoneal fluids.

It has been suggested that breast feeding protects infants against *E. coli* enteritis and other infections due to the combination of antibodies, lysozyme and Lf found in human milk (Bullen *et al.*, 1978). Bullen *et al.* (1972) found that if *E. coli* O111 was inoculated into human milk to which bicarbonate had been added growth was inhibited. If enough Fe was added to saturate the Lf, *E. coli* was able to proliferate rapidly in the milk. The addition of bicarbonate allowed adventitious Fe to be bound to the Lf. If bicarbonate was not added, the citrate in the milk permitted *E. coli* to take up the non-Lf bound Fe as Fe-citrate (Bullen *et al.*, 1978).

2.4.3 Ferritin and metabolic Fe storage

Efficient Fe storage is very important as the body has a continuing need for Fe but free low molecular weight Fe could catalyze the production of free-radicals and increase the possibility of infection. In certain cases the body's demand for Fe may exceed the dietary intake and storage Fe must be mobilized. Ferritin and hemosiderin are the two compounds responsible for Fe storage. Ferritin can be synthesized by all body cells but the liver, spleen and bone marrow are especially ferritin-rich (Aisen and Listowsky, 1980). Interestingly, ferritin is not only synthesized by

vertebrates but is found in invertebrates, plants, fungi, and in certain bacteria (Crichton, 1984). Ferritin has a molecular weight of 450,000 daltons and is composed of 24 polypeptide chains (Crichton and Bryce, 1970, Bryce and Crichton, 1971) forming a shell in which Fe is deposited. The ferritin molecule is spherical and compact; the outside diameter is 124-130 A, the interior is hollow with the inside diameter being 70-80 A Six channels connect the hollow interior to the outside (Harrison, 1977). Each ferritin molecule can hold a maximum of 4500 Fe atoms but ferritin molecules are usually only two-thirds full. Fe enters ferritin in the ferrous form but is actually stored in the ferric form as $(FeOOH)_8$ $(FeO.OPO_3H_2)$ (Bezkorovainy, 1980). In the human there are two types of ferritin polypeptide chains designated H and L. H chains have a molecular weight of 21,000 daltons while L chains are 19,000 daltons (Arosio *et al.*, 1978). Tissue isoferritins have different ratios of H and L subunits. In heart and neoplastic tissue H chains predominate, while L chains predominate in liver and spleen (Drysdale, 1977). The amount of ferritin in the serum reflects the size of body Fe stores and is very small under normal conditions but may increase in certain disease states. Serum ferritin has a low Fe content and is glycosylated.

The entrance of Fe into cells causes enhanced ferritin synthesis which is not inhibited by actinomycin D (Drysdale and Munro, 1966). Ferritin m-RNA in the cell is regulated by an apoferritin subunit attached to it which prevents translation. When low molecular weight Fe enters the cell it can bind to the ferritin subunit removing it and allowing the m-RNA to be translated (Zahringer *et al.*, 1976). The half-life of ferritin in the rat is 3 days after which it is degraded by proteolytic enzymes in the

cytosol and secondary lysosomes. It is believed that degradation of ferritin results in the formation of the other Fe storage compound hemosiderin.

2.4.4 Ceruloplasmin

The last protein of Fe metabolism to be discussed is ceruloplasmin (Cp) the protein responsible for removing Fe from storage and returning it to the Tf pool. Cp is a single chain serum glycoprotein with a molecular weight of 132,000 daltons which contains 6 atoms of copper per molecule (Frieden, 1980). Seven biological functions have been ascribed to this protein the most pertinent being that it acts as a vehicle for transport of serum copper, it is an acute phase reactant protein and its serum concentration increases during the inflammatory response, and it has a role in mobilizing Fe from storage sites back into the Tf pool by virtue of its ferroxidase activity (Frieden, 1986). The serum ferroxidase activity of Cp was first postulated by Osaki *et al.* (1966) It is thought to behave as follows at least in RE cells: ferritin Fe, stored in the ferric state is reduced by ferritin reductase using flavin mononucleotide $FMNH_2$ as the reductant (Sirivech *et al.*, 1974) which is itself reduced by NADH + H^+. Fe (II) binds to certain sites on the RE cell membranes and when Cp binds to form a Cp Fe (II) complex Fe is oxidized and transferred to apo Tf (Frieden, 1983).

The importance of Cp in Fe metabolism has been demonstrated in several animal studies. When swine (Lee *et al.*, 1968; Roeser *et al.*, 1970) or mice (Letendre and Holbein, 1984a) were placed on copper-deficient diets

they became both hypoceruloplasminemic and hypoferremic displaying lowered serum levels of both Cp and Fe. When Cp was administered to these animals, plasma Fe levels increased immediately. Lowering of serum Fe occurred because the transfer of Fe from the RE cell to Tf was inhibited interrupting the Fe cycle. When Cp was again made available, Fe was released from storage with the resulting increase in serum Fe levels.

2.5 Changes in Fe metabolism during infection

It was emphasized in the above discussion that Fe metabolism is strictly regulated and is composed of various pools connected by the central transport component Tf. The Fe system is also dynamic; Fe is continually being turned over and moving through these interconnected pools. Because of the dynamic nature of the Fe system relatively rapid changes in Fe metabolism can occur during an inflammatory response In 1932 Locke *et al.* reported that pulmonary patients with tuberculosis had lower levels of serum Fe and horses injected with diphtheria toxin had reduced serum Fe levels. In a study by Pekarek *et al.* (1969) human volunteers were exposed to the bacterial pathogen *Francisella tularensis* and serum Fe levels were monitored. These levels were found to fall from an initial mean level on day 0 of 22.3 uM to 6.4 uM on day 5. In an experimental model of bacterial infection, Holbein (1980) injected C57 black mice intraperitoneally with 10^4 *Neisseria meningitidis* and monitored bacteria in the blood and plasma Fe levels over a 24 h period. Infection peaked at 6 h and disappeared after 9 h. The unsaturated iron binding capacity of the plasma increased immediately after injection with a corresponding drop in Tf-Fe saturation.

No Tf-Fe could be detected in the plasma at 18 or 24 h post infection demonstrating a very marked hypoferremic response. The decline in bacterial numbers corresponded to the decrease in plasma Fe. Injections of Fe as Fe-dextran (Holbein *et al.*, 1979; Holbein, 1980) or human Tf (Holbein, 1981) enhanced infection. Thus the hypoferremic response controlled the infection by denying the pathogen the Fe it required for growth

Hypoferremia can also be induced experimentally by injections of endotoxin (Kampschmidt *et al.*, 1965) or turpentine (Cartwright *et al.*, 1946; Kampschmidt and Upchurch, 1962; Hershko *et al.*, 1974, Beaumier *et al.*, 1984). Lee (1983) observed that two theories have been put forward concerning how the hypoferremic response occurs. Van Snick *et al.* (1974) proposed that hypoferremia occurs because Lf, when released from neutrophils during inflammation, removes Fe from Tf or competes with Tf for Fe released from macrophages. Lf then returns Fe to macrophages of the RE system and it is incorporated into Fe stores. Alternatively, Konijn and Hershko (1977) found that when hypoferremia was induced in rats by turpentine injection serum Fe levels began to fall at 6 h and were at the lowest level by 12-24 h post-injection. Enhanced ferritin synthesis, however, was maximal by 4-8 h post injection. Thus the decrease in serum Fe was preceded by an increase in ferritin synthesis. It was therefore postulated that incorporation of Fe into newly synthesized apoferritin prevents Fe from being immediately re-released into the Tf pool resulting in a decrease in the amount of Tf- bound Fe and in a hypoferremic response.

Letendre and Holbein (1983) investigated Fe kinetics in normal mice and in hypoferremic mice injected with *N. meningitidis*. Mice of both groups were injected with murine Tf labelled with ^{59}Fe and ^{125}I and the

temporal movement of the label through the body's Fe compartments was followed. Fe moved from Tf to the bone marrow then to erythrocytes. Fe kinetics in infected mice were similar to normal mice. There was no increased turnover of plasma Fe and no channelling of plasma Fe to storage compartments during hypoferremia. In a later study, (Letendre and Holbein, 1984) control and hypoferremic mice were injected with ^{59}Fe-labelled heat-damaged red blood cells and the fate of the label was followed in the two groups In the hypoferremic mice, the Fe from effete erythrocytes was not immediately returned to the Tf pool, as for control mice, but was stored in the RE system in ferritin. These authors (Letendre and Holbein, 1983) argued against a direct role for Lf in mediating hypoferremia as large quantities of Lf would be required to effect the decrease in plasma Fe observed. These authors, however, did not rule out an indirect role for Lf during hypoferremia by sequestering Fe at the site of inflammation in the peritoneal cavity. Alternatively, Lf might be involved in the impairment of Fe release from the RE system.

The mechanism triggering the hypoferremic response has not been elucidated. Many authors have presented evidence indicating that the compound known as endogenous pyrogen, leukocyte endogenous mediator (LEM) or interleukin-1 (IL-1) is involved in triggering the response (Lee, 1983) The first evidence for the existence of this compound was presented in 1948 (Beeson, 1948). It was discovered that a substance was liberated from polymorphonuclear leukocytes obtained from sterile peritoneal exudates of rabbits which could induce fever. Further work by Kampschmidt and Upchurch (1962; 1969) and Pekarek and Beisel (1971) elucidated the role of LEM in lowering serum Fe during inflammation.

LEM or IL-1 is a heat-labile protein of 13,000-16,000 daltons. It is synthesized and released by activated phagocytic cells (Bornstein, 1982) which can be stimulated by phagocytosis of foreign particles , endotoxin (Kampschmidt, 1978) or lymphokines produced during a mixed lymphocyte reaction (Dinarello, 1981). Three hours after activation of phagocytie cells, LEM is released (Dinarello and Wolff, 1978) by a process involving the synthesis of new m-RNA and protein. LEM or IL-1 is also responsible for the induction of fever and the enhanced synthesis of acute phase reactant proteins such as C-reactive protein, haptoglobin, serum amyloid A protein and ceruloplasmin (Kampschmidt, 1978). During infection and inflammation the host often develops a fever. This elevated body temperature may interfere with the ability of bacteria to synthesize siderophores (Weinberg, 1981). During the inflammation associated with infection serum concentrations of Cp may be increased. Letendre and Holbein (1984a) demonstrated that Cp levels rose dramatically during the recovery phase of murine meningococcal infection. Cp levels rose between 18 and 24 h post injection and were greater than 200% of control values at 24 h post inoculation. Beaumier *et al*. (1984) reported similar observations for turpentine-induced inflammation in mice and showed that the substantial increase in serum Cp was due to *de novo* Cp synthesis. It was suggested (Letendre and Holbein, 1984a) that Cp is synthesized during the recovery phase of infection in response to hypoferremia to help mobilize Fe from the RE system to re-establish normal serum Fe levels.

2.6 Changes in Fe metabolism during neoplastic disease

Many of the changes in Fe metabolism which occur during infection also occur during cancer. In an early study of Fe metabolism in malignant disease, Miller *et al.* (1956), found that cancer patients had serum Fe levels of 30-165 ug dl^{-1} compared to the normal range of 70-155 ug dl^{-1} These authors noted that the serum Fe response paralleled the disease state. As the neoplasm spread serum Fe levels decreased further; conversely, if the clinical state improved for example due to surgery, the serum Fe levels rose again. It was further observed that hypoferremia was usually but not always accompanied by anemia. Beamish *et al* (1972) compared serum Fe levels of normal controls with those of patients with Hodgkin's disease and non-Hodgkin's lymphoma Controls had a mean serum Fe level of 127 ug dl^{-1} (22.7 uM) and 35% Tf-Fe saturation; patients with stage 4 Hodgkin's disease had serum Fe levels of 39 ug dl^{-1} (6.97 uM) and Tf-Fe saturation of 16% Patients with non-Hodgkin's lymphoma had 60 ug dl^{-1} (10.7 uM) serum Fe and a 17% Tf-Fe saturation. Hypoferremia was found to result from impaired RE release of Fe.

In an experimental model of neoplastic disease, Chandler and Fletcher (1973) examined Fe metabolism in chickens injected with a lymphoid tumor. The chickens developed hypoferremia, anemia and had increased Fe stores in the spleen and liver. When tumor-bearing birds were injected with ^{59}Fe-Tf, the tumor incorporated label at three times the rate of spleen and three-quarters that of liver when uptake was related to organ weight 24 h post injection. The authors concluded that a combination of Fe uptake by the

tumor and a blockage in Fe re-utilization caused the decrease in serum Fe. These studies also imply that Fe is required for rapid tumor growth. Schade (1976) observed that mice injected with plasmacytoma cells had lowered levels of serum Fe and anemia. These plasmacytoma cells contained high levels of non-heme Fe in the form of ferritin while the livers of tumor-bearing mice contained lowered levels of Fe as compared to controls. When tumor-bearing mice were injected with excess Fe, the excess Fe was stored in the liver not in the tumor cells. The author concluded that plasmacytoma cells may deprive other cells of Fe to meet their own needs but do not store Fe over and above their needs. Tumor cells would require Fe as an enzyme co-factor for several Fe-containing enzymes including ribonucleotide reductase, which is necessary for DNA synthesis, catalase, peroxidase, tryptophan 2,3 dioxygenase and phenylalanine, tyrosine and tryptophan hydroxylases.

In contrast to the development of hypoferremia during neoplasia, Caroline *et al.* (1969) reported that 64% of patients with acute myeloblastic leukemia examined had elevated serum Fe levels. Several patients had Tf-Fe saturations of above 50% while others had 100% Fe-saturated Tf. This was correlated with the high incidence of *Candida* and other fungal infections in leukemia patients as *in vitro* experiments demonstrated that growth of *Candida albicans* was 1000 times greater in leukemic sera than in normal sera. In a later study by Hunter *et al.* (1984) it was again pointed out that acute leukemia differs from other forms of neoplasia as it is associated with hyperferremia. This condition is associated with low levels of serum Tf, high levels of serum Fe and an inability of the host to reduce Fe levels when an infection occurs. Sera from leukemic patients showed a

reduced ability to inhibit growth of *Pseudomonas aeruginosa*, a type of infection from which leukemic patients frequently die. Suppression of bone marrow function in this disease produces hyperferremia by decreasing Fe utilization by erythroid cells. Marrow suppression also leads to monocytopenia and neutropenia. Monocytes are responsible for the release of LEM (IL-1) and induction of hypoferremia and neutrophils release Lf which deprives bacteria of Fe at infection sites. This complex interaction of factors may well be responsible for the high rate of infection in these patients.

Abnormalities of Fe storage have been reported in certain cases of neoplastic disease. Dumont *et al.* (1976) reported that patients with untreated Hodgkin's disease had markedly elevated levels of hemosiderin in the lymph nodes. Patients did not have excessive Fe in their livers where the Kupffer cells normally account for 85% of the body RE activity. These authors further observed that patients with lymph nodes affected by non-Hodgkin's lymphoma and metastatic carcinoma had somewhat elevated lymph node Fe deposition. De Sousa *et al.* (1978) suggested that abnormal handling of Fe by monocytes of the spleen and lymph nodes may be linked to the pathogenesis of Hodgkin's disease.

Concentrations of ferritin in the serum have been observed to increase in several types of malignant disease. Jones *et al.* (1973) measured serum ferritin levels in patients with Hodgkin's disease and leukemia. The mean normal concentration for men and women respectively was 69 and 34 ng ml^{-1}. Patients with Hodgkin's disease had an average level of 215 ng ml^{-1}. Those with acute myeloblastic leukemia had a mean level of 589 ng ml^{-1}. Jacobs *et al.* (1976) found serum ferritin levels in Hodgkin's disease patients in-

creased at each advancing stage of the disease. In a further study, Jacobs *et al.* (1976a) found that women with early breast cancer had a mean serum ferritin level of 96.9 ug l^{-1} while the control mean concentration was 56.6 ug l^{-1}. Furthermore, patients with an initial level above 200 ug l^{-1} had a higher tumor recurrence rate over the next 4 years. In certain malignant diseases the increase in plasma ferritin may be related to an increased synthesis of this compound by the tumor cells (Sarcione *et al.*, 1977; White *et al* , 1974).

Neoplastic disease is often accompanied by what is termed the anemia of chronic disease. This is characterized by a decrease in serum Fe concentration and decrease in Tf saturation, decreased serum total Fe-binding capacity and normal or increased Fe stores. The pathogenesis of this anemia may be typified by: shortened red cell survival, impaired bone marrow response to the anemia and impaired flow of Fe from the RE cells to the serum and thus to the bone marrow for erythropoiesis (Cartwright and Lee, 1971; Lee, 1983). Zucker (1985) pointed out that anemia occurs with most cancer patients during the course of disease. Hemoglobin concentrations are generally 8-12 g dl^{-1} and the packed cell volume 26-38%. The survival of red blood cells is only 60-90 days instead of the usual 120 days. The bone marrow should be able to compensate for this but does not. Three possible reasons were suggested for this: (i) impaired flow of Fe from the RE system to the bone marrow (ii) inadequate production of erythropoietin and (iii) impaired marrow response to erythropoietin. Anemia may also occur due to bone marrow invasion by tumor cells. In this case, it has been suggested that anemia may be caused by crowding of the hematopoietic cells, production of toxins by the cancer cells, competition between bone marrow

and neoplastic cells for nutrients or lysis of erythroid cells by tumor cells. Furthermore, anemia is complicated during neoplastic disease by blood loss, nutritional malabsorption, infection and radio- or chemotherapy.

Along with these many changes in Fe metabolism which occur in neoplastic disease, several investigators have examined changes in serum Cp levels with a view to utilizing Cp as a disease marker Linder *et al.* (1979) in an experimental study, evaluated Cp activity in rats implanted with hepatic or mammary tumors and recorded a 50-200% increase in Cp oxidase activity. This enhanced activity was attributable to both increased synthesis of Cp and augmented enzyme activity. In a later clinical study, Linder *et al.* (1981) assayed Cp levels in sera of male patients with lung cancer and gastrointestinal cancer and women with breast cancer Cp levels were significantly elevated in patients with stage 1 lung cancer and even higher in patients with stage II and III lung cancer. Patients successfully treated for the disease had lower Cp levels while recurrent elevations or rapid increases appeared to be indicative of disease recurrence. Cp levels in sera of patients with non-malignant lung disease also were somewhat increased. Male patients with non-malignant gastrointestinal disease did not have significantly higher Cp levels while patients with gastrointestinal cancer did, especially in the case of colon cancer. However, sera of control women had higher Cp levels than control men of similar ages thus Cp levels in patients with breast cancer did not show as great an elevation in serum Cp. Also, patients with non-malignant breast disease had elevated Cp levels which would make it difficult to distinguish neoplastic from non-neoplastic breast disease on the basis of Cp analysis.

It was therefore concluded that Cp analysis would be useful only in certain situations for cancer diagnosis or prognosis.

2.7 Fe and carcinogenesis

It has been suggested that Fe, as well as being essential to life, in certain circumstances may have carcinogenic or cocarcinogenic properties. The mechanisms by which this might occur are, however, poorly understood. Richmond (1961) reported that when cells in culture were exposed to Fe-dextran, abnormalities in cells undergoing mitosis were evident. Willson (1977) proposed that if excessive decompartmentalization of Fe occurs free radicals may be produced and cancer may follow. Low levels of low molecular weight Fe have been shown in several cases to markedly increase the destruction of DNA caused by the production of hydroxyl free radicals from the active oxygen species superoxide (O_2^-) and hydrogen peroxide (H_2O_2). This was demonstrated by Brawn and Fridovich (1980) who showed that when the supercoiled *E. coli* plasmid Col E-1 was exposed to an enzymic flux of O_2^- and H_2O_2 nicking of the DNA occurred as evidenced by the conversion of supercoiled DNA to the open circular form. That this damage was caused by hydroxyl radicals was shown as protection was offered by superoxide dismutase and catalase and by benzoate, mannitol or histidine, known scavengers of hydroxyl radicals.

In another study, Robertson *et al.* (1971) reported on the incidence of cancer in gold mine workers in South Africa from 1964-1968. The cancer rate was surprisingly high and liver cancer was the most common type accounting for 486/923 (52.6%) of the cases. These patients were young males

and came primarily from Mozambique and South Africa. This study was later correlated with an earlier report of Bothwell *et al.* (1964) which discussed the cases of Fe overload in Bantu patients in South Africa. It was found that males consumed large amounts of a home-made alcoholic beverage which was brewed in Fe-containing pots. This drink contained an average of 3.8 mg Fe 100 ml^{-1} and the Fe intake was estimated to be at least 50 mg per day.

In a later study, Bomford and Williams (1976) evaluated venesection therapy in patients with idiopathic hemocromatosis. It was observed that 29% of treated and 19% of untreated patients died of hepatoma and another 22% of treated patients died of other forms of malignant disease. Nettesheim *et al.* (1975) found that inhalation of Fe_2O_3 particles enhanced diethylnitrosamine tumorigenesis in the lower respiratory tract of Syrian hamsters. In this case, Fe acted as a cocarcinogen. In 1977 Weinberg and Hibbs demonstrated that the tumoricidal activity of macrophages was inhibited by phagocytosis of red blood cells, hemoglobin, Fe salts or Fe-dextran. The authors therefore proposed that Fe could inhibit the tumor killing activity of macrophages thus decreasing the host resistance to tumor development.

The studies cited above imply that in particular situations Fe may have a role in causing cancer. How this occurs is unknown but it may involve the Fe-catalyzed production of free radicals or Fe may inhibit the function of macrophages responsible for surveillance against arising neoplastic cells.

2.8 Fe and *in vitro* animal cell culture

Numerous studies have examined the Fe requirements for *in vitro* culture of mammalian cells (Higuchi, 1970; Messmer, 1973; Rudland *et al.*, 1977) and in all cases requirements were found to be within the range of 0.4-4.0 uM suggested as the range of Fe concentrations permitting growth of all cellular forms (Weinberg, 1981). Several studies have also shown that Tf is required for the growth of mammalian cells in serum-free medium (Barnes and Sato, 1980; Hayashi *et al.*, 1978). Tf is also required for growth and DNA synthesis of mitogenically-stimulated lymphocytes (Tormey *et al.*, 1972; Tormey and Mueller, 1972) Tf has also been shown to be a more effective Fe donor to cultured cells than Fe salts (Messmer, 1973). Fe is required for tissue culture cell lines for a critical step in DNA synthesis (Robbins *et al.*, 1972; Fernandez-Pol, 1977) as cells deprived of Fe by Fe chelators become blocked in the G_1 or early S phase of the cell cycle (Fernandez-Pol, 1977; Basset *et al.*, 1985). It has been suggested that Fe is required for DNA synthesis and cell division as it is a necessary component of the enzyme ribonucleotide reductase which catalyzes the reduction of ribonucleosides to deoxyribonucleosides, an important step in DNA synthesis. Recently, Basset *et al.* (1985) showed that the antioxidant propyl gallate inhibited Tf enhanced growth of mouse L1210 leukemia cells and suggested that Fe could act via fatty acid hydroperoxide formation. It was further shown that hydroperoxides induced by Fe from unsaturated fatty acids could activate purified guanylate cyclase suggesting that cyclic GMP production may be associated with the mechanism by which Fe induces DNA synthesis.

There has been some speculation in the past that Tf may have another role in cell culture other than providing Fe to cells, for example, it might bind toxic cations or supply zinc to the cells (Brock, 1981). However, Titeux *et al.* (1984) demonstrated that Fe salts, hemin or hemoglobin could replace Tf for growth of human cell lines in serum-free media and Landschultz *et al.* (1984) found that the Fe chelator ferric pyridoxal isconicotinoyl hydrazone could replace Tf in defined media to stimulate proliferation of mouse embryonic kidney tissue. These studies suggest that the role of Tf in cell culture is solely as an Fe donor. However, an alternate hypothesis will be discussed later.

2.9 Cellular Fe acquisition

2.9.1 Microbial cells

As discussed in the above sections Fe in the host is well regulated. Tf and Lf have high association constants for Fe and are only partially saturated. Free Fe in equilibrium with Tf is 10^{-18} M and therefore is too low for bacterial growth. How then do bacterial pathogens multiply? Many bacteria and fungi synthesize and secrete Fe chelating compounds called siderophores. The word siderophore means "iron-bearer" and siderophores are defined as low molecular weight (500-1000 daltons), nonporphyrin, non-protein Fe-binding compounds produced by microorganisms (Neilands, 1981). There are two principal classes of siderophores, the phenolates or catechols and the hydroxamic acid siderophores. Siderophore Fe-binding ligands contain several sets of bidentate coordinating groups the donor

atoms of which are usually oxygens. Siderophores are considered to be virulence factors in disease.

When available Fe is below 1 uM, the genes for the synthesis of both siderophores and the corresponding outer membrane siderophore receptor proteins are derepressed. Siderophores are synthesized and secreted from the cell, ferric Fe is bound, the receptor binds the ferri-siderophore and brings it through the cell envelope and Fe is released by reduction (Neilands, 1981).

Enteric bacteria such as *Escherichia coli* and *Salmonella* synthesize the phenolate siderophore enterochelin; this molecule is a cyclic trimer of 2,3, dihydroxy-N-benzoyl-L-serine and is synthesized from chorismate from a branch of the pathway of aromatic acid biosynthesis. Three Fe-repressible outer membrane proteins of 81,000, 83,000 and 74,000 daltons are synthesized along with enterochelin. The 81,000 dalton protein is the enterochelin receptor protein Fep A. Under low Fe stress, *E. coli* also synthesizes the 78,000 dalton outer membrane receptor called Fhu A, this is the receptor for the siderophore ferrichrome which is produced by certain fungi (Neilands, 1981; 1982). Siderophores have high affinity for Fe and several studies have suggested that siderophores act as virulence factors by enabling pathogens to acquire Fe from Tf. For example, Tidmarsh and Rosenberg (1981) demonstrated that enterochelin-producing *Salmonella paratyphi* B could grow on Fe from bovine, rabbit or human Tf. Furthermore, Fe could be obtained by the organism when the Tf's were isolated in a dialysis bag. A mutant which was unable to produce enterochelin could not obtain Fe from Tf.

Griffiths and Humphreys (1980) isolated enterochelin from peritoneal

washings of guinea pigs lethally infected with *E. coli* O111 demonstrating that siderophores are synthesized *in vivo*. In a subsequent study (Griffiths *et al.*, 1983), it was found that the Fe-repressible *E. coli* outer membrane proteins observed *in vitro* were also synthesized by *E. coli* *in vivo* when injected into guinea pigs. These results suggested that pathogens are Fe-limited *in vivo*.

Enteric bacteria have been shown to have another interesting high affinity Fe acquisition system. P. H. Williams (1979) found that *E. coli* harbouring the plasmid ColV were more virulent for laboratory animals than plasmid-minus strains. Strains without the plasmid were growth inhibited by Tf while the plasmid-containing strains were not inhibited These results suggested that ColV had a role in obtaining Fe from Tf. It was later found that the ColV plasmid contained the genes for the siderophore aerobactin (Warner *et al.*, 1981; Braun, 1981). The aerobactin system actually consists of five contiguous genes, four for the synthesis of the siderophore and one for the 74,000 dalton receptor. Expression of the aerobactin genes has been found to be regulated by the fur (ferric uptake regulation) gene product, a 17,000 dalton protein. When Fe^{2+} is present intracellularly at a certain concentration it acts as a co-repressor with the Fur protein which binds to the operator of the aerobactin gene cluster to repress synthesis of aerobactin and its outer membrane receptor protein (Neilands, 1987).

E. coli strains may have the ability to synthesize both enterochelin and aerobactin; as enterochelin has a K_f of 10^{52} and aerobactin has a K_f of 10^{23} it might be asked why both systems would be required. Konopka *et al.* (1982) demonstrated that both enterochelin and aerobactin could remove Fe

from Tf and aerobactin did so less efficiently than enterochelin in media. In serum however, aerobactin transferred Fe to *E. coli* at a higher rate than did enterochelin. It has also been pointed out (Finkelstein *et al.*, 1983) that different siderophore systems might be active in different environments. Enterochelin is sensitive to oxidation and changes in pH while aerobactin is active over a wide range of environmental conditions.

Whether a pathogen obtains Fe *in vivo* depends on the number of molecules of siderophore produced and on the Fe saturation of Tf. This was illustrated in a study by Kochan *et al.* (1971) in which the number of generations of growth of *Mycobacterium tuberculosis* in tuberculostatic serum and tuberculostatic serum with 5 mg ml^{-1} 100% saturated Tf or 5 mg ml^{-1} apo-Tf to which various concentrations of the Fe chelator mycobactin had been added. It was found that it required approximately 1 ng of mycobactin ml^{-1} to achieve eight generations of growth in tuberculostatic serum containing 100% saturated Tf while it required 100 ng ml^{-1} mycobactin for six generations of division in tuberculostatic serum. One thousand ng ml^{-1} mycobactin were required for approximately five generations of growth in tuberculostatic serum to which 5 mg ml^{-1} apo-Tf had been added.

In section 2.2 Fe acquisition by *Neisseria meningitidis* was discussed. This organism does not produce siderophores but can obtain Fe from Tf by binding the protein directly Herrington and Sparling (1985) studying *Haemophilus influenzae* found that when cultured on media containing protoporphyrin IX and EDDA all of 33 *H. influenzae* type b isolates were able to grow on 30% Fe-saturated Tf but not on Lf. Twenty-eight of 35 nontypeable *H. influenzae* isolates examined could use Tf Fe but none of the *H. parainfluenzae* strains could use Tf Fe. Redhead *et al.* (1987) recently

demonstrated that *Bordetella pertussis* could utilize Fe from ovoTf (conalbumin) Lf and Tf with no evidence of siderophore production. Furthermore, radiolabelled protein binding studies indicated that the proteins were bound directly to the cells as was found for Tf utilization by *N. meningitidis*.

2.9.2 Eucaryotic cells and transferrin receptors

2.9.2.1 Properties of transferrin receptors

In 1959 Jandl *et al.* postulated that reticulocytes obtain Fe from Tf because they express receptors for Tf on their cell membrane. The first Tf receptor isolated and characterized was from rabbit reticulocytes (Ecarot-Charrier *et al.*, 1977). Tf receptors were subsequently isolated from human placenta (Seligman *et al* , 1979; Wada *et al.*, 1979). Two classifications of membrane receptors have been described: class I and class II. When ligands such as insulin bind to class 1 receptors information is transmitted to the cell causing modification of cell behavior or metabolism. Effects of ligand binding may be mediated through a second messenger such as cyclic AMP. Class II receptors transport ligands such as nutrients into the cell (Kaplan, 1981). The Tf receptor is an example of a class II receptor.

The Tf receptor is a transmembrane glycoprotein which has a molecular weight of 180,000 daltons consisting of two disulfide-bonded subunits each of 90,000 daltons (Seligman *et al.*, 1979; Wada *et al.*, 1979; Hamilton *et al.*, 1979; Omary *et al.*, 1980; Shindelman *et al.*, 1981; Sutherland *et al.*,

1981). Each subunit can bind one molecule of Tf. The human Tf receptor is 4.8% carbohydrate by weight (Seligman *et al.*, 1979) and has at least 3 N-asparagine-linked oligosaccharide chains of both high mannose and complex types (Omary and Trowbridge, 1981). The mature receptor has a covalently-linked palmitic acid molecule located close to the plasma membrane which may have a role in anchoring the receptor into the membrane (Omary and Trowbridge, 1981a) or in insertion of the receptor into the membrane (Koch and Hammerling, 1986) The external portion of the receptor is 70,000 daltons (Omary and Trowbridge, 1981a) while the intracellular part exposed to the cytoplasm is believed to be 5,000 daltons. In some cells, serine residues in this region of the molecule are phosphorylated (Schneider *et al.*, 1982). Schneider *et al.* (1984) and McClelland *et al.* (1984) determined the primary structure of the human Tf receptor from the m-RNA sequence and from a cDNA clone respectively. The receptor is a peptide of 760 amino acids which is unusual in not having an N-terminal signal peptide. It does have a membrane-spanning region 62 amino acids from the N-terminus. The molecule has a short N-terminal cytoplasmic domain and an extracellular C-terminal domain of 672 amino acids. The gene for the Tf receptor is located on chromosome 3 (Enns *et al.*, 1982) on the region 3q12 qter (van de Rijn *et al.*, 1983). In order to study Tf receptor gene regulation, Miskimins *et al.* (1986) subcloned and sequenced a 365-bp fragment from the 5' region of the human Tf receptor. This region possessed a TATA box, several GC-rich regions and was able to promote the bacterial chloramphenicol acetyltransferase gene system. Several high molecular weight proteins which bind to the Tf receptor gene promoter were discovered. Also the promoter region was found to contain protein binding

sites, one of which is thought to interact with Sp1, the cell transcription factor which has been shown to enhance transcription *in vitro*. Interestingly, the Tf receptor gene promoter demonstrated sequence homology to other gene promoters and it was suggested that the gene structure may allow constitutive low level gene expression but permit increased expression during the transition from the quiescent state to active growth.

The exact binding site where Tf binds to its receptor has not been located; however, Bost *et al.* (1985) wrote out m-RNA sequences complementary to the m-RNA sequences for the Tf receptor and compared them with m-RNA sequences for Tf. Significant amino acid and nucleotide homologies between Tf and Tf receptor complements were observed. It was suggested that these homologous sequences may represent the region where ligand and receptor interact.

It has been suggested that Tf receptors might be involved in natural killer (NK) cell recognition of target cells. NK cells are lymphocytes which spontaneously lyse tumor cells, hemopoietic stem cells, fetal thymus cells and virus-infected cells. As Tf receptors are associated with actively dividing cells they have been implicated in NK cell recognition. Baines *et al.* (1983) found that 100%-saturated Tf at physiological levels inhibited NK activity. Optimal inhibition occurred when the tumor target cells were pre-incubated with Fe-Tf. If target cells were pre-incubated with either diferric or apo-Tf before the NK assay, inhibition of effector-target cell conjugate formation occurred. Furthermore, if the effector cells were incubated with rabbit anti-human Tf antibody there was inhibition of target cell killing. From this data the following model was proposed. NK cells have Tf receptors which have Tf bound to them. *In*

vitro grown tumor cells have high numbers of Tf receptors. Tumor cell Tf receptors bind to the Tf bound to NK cells and lysis of the target cells follows conjugate formation. Studies by Vodinelich *et al.* (1983) also indicated that Tf receptors may have target function for NK cells. These authors found a relationship between NK sensitivity of human leukemia cell lines and the proportion of cells expressing Tf receptors. It was also shown that tryptic fragments of Tf receptors inhibited NK activity to target cells. Lazarus and Baines (1985) studied the correlation between Tf receptor expression and NK susceptibility. K562 cells were assayed for numbers of Tf receptors on days 1,3 and 5 of growth and were also assessed for NK lysis susceptibility. These cells displayed the highest number of Tf receptors on day 1 and the lowest number on day 5. This correlated well with the ability of these cells to compete with control K562 cells in NK assays. A good correlation was also found between Tf receptor numbers and NK susceptibility when K562 cells were treated with heat, trypsin, or sodium butyrate, a differentiation-inducing agent. Rabbit reticulocytes which possess high numbers of Tf receptors competed well with K562 cells in NK assays while rabbit erythrocytes, which lack Tf receptors, did not. These results further suggest that Tf receptors are involved in NK recognition of tumor cells.

2.9.2.2 Occurrence of transferrin receptors

As mentioned above, Tf receptors were first studied in erythroid and placental cells. Since that time Tf receptors have been examined on many different types of cells and tissues. This has generally been done either

through the use of fluorescent antibody techniques or by incubating cells with varying amounts of radiolabelled Tf at 4° or 37° C and performing Scatchard (1949) analysis to determine receptor numbers and receptor-ligand affinity (K_a) or the dissociation constant (K_d).

Van Bockxmeer and Morgan (1977) found that a Tf binding component was present on rabbit reticulocytes but not on mature erythrocytes. Generally, Tf receptors are expressed on immature erythroid cells from pronormoblasts to reticulocytes and are absent from erythrocytes. Larrick and Cresswell (1979) found Tf receptors on human B and T lymphoblastoid cell lines. In another study, Tf binding to lymphoblastoid cell lines in log phase and in cells approaching stationary phase was measured (Larrick and Cresswell, 1979a). In the latter growth phase a down regulation of Tf receptors was observed. Normal lymphocytes stimulated mitogenically or during a mixed lymphocyte culture had an increased number of Tf binding sites compared to resting lymphocytes. It was concluded that the number of cellular Tf binding sites is modulated by cellular growth, rapidly dividing cells having many Tf binding sites. Omary *et al.* (1980) and Trowbridge and Omary (1981) using the monoclonal antibody B3/25 to the Tf receptor found Tf receptors on cultured T-leukemic cell lines, several normal and malignant B-cell lines and several non-B, non-T cell lines. These receptors were not found on peripheral blood mononuclear cells, granulocytes, thymocytes or bone marrow cells. The antigen was expressed on non-induced HL-60 cells, a promyelocytic cell line, but expression of the antigen was reduced if the cells were treated with dimethylsulfoxide or sodium butyrate, agents which cause differentiation and growth arrest. The disappearance of the membrane antigen preceded the arrest of HL-60 cells in G_1 phase of the cell

cycle. Thus Tf receptors appeared to be expressed on actively proliferating cells and to be present in larger amounts on malignant cells than on non-malignant cells. Sutherland *et al.* (1981) performed similar studies with another monoclonal antibody to the Tf receptor, OKT9, which bound to many different leukemia and tumor cell lines. The antibody did not bind to normal lymphocytes or erythrocytes but did bind to lymphocytes stimulated by mitogens. If cells were induced to mature, differentiate and leave the cell cycle binding of the antibody was reduced. As in the papers discussed above, the presence of the Tf receptor was correlated with proliferation. It has also been found that treatment of Friend erythroleukemia cells with dimethylsulfoxide results in an induction of Tf receptors, hemoglobin synthesis and erythroid differentiation (Wilczynska and Schulman, 1980; Wilczynska *et al.*, 1984). The effect of dimethylsulfoxide on cellular Tf receptor expression appears to vary with cell type.

Trowbridge *et al.* (1982) looked for the presence of Tf receptors in murine cells and tissues. These receptors were detectable in all of 29 murine hematopoietic tumor cells lines but only in 5% of bone marrow cells and less than 1% of adult thymocytes and spleen cells. The murine Tf receptor was also shown to be a disulfide-bonded dimer of molecular weight similar to the human receptor. The murine Tf receptor was purified from murine myeloma cells by Van Driel *et al.* (1984). On a sodium dodecyl sulfate polyacrylamide gel under reducing conditions 2 bands of molecular weights 94,000 and 96,000 were observed. In a later study, (Van Driel and Goding, 1985) these 2 bands were found to correspond to receptor molecules with different numbers of N-linked oligosaccharides. In a subsequent study, Stearne *et al.*, (1985) reported the cloning of cDNA encoding the

murine Tf receptor. cDNA clones from the murine myeloma cells were isolated and partially sequenced. It was determined that receptor regions surrounding the membrane were highly conserved when compared to sequences in the human Tf receptor. It was noted that three-quarters of the cysteine residues were conserved; these may be involved in interchain disulfide bonding or covalent attachment of fatty acid residues. Finally, the results of these authors suggest that the murine receptor is a homodimer.

Faulk *et al.* (1980) using fluorescent antibody techniques examined normal and malignant breast tissue for the presence of Tf and Tf receptors. It was found that 16/22 breast carcinomas were positive for Tf whereas none of the normal breast tissue samples or benign mastopathies were positive. Similarly, Shindelman *et al.* (1981) found elevated binding of Tf to microsomes of malignant breast tissue as compared to non-neoplastic breast tissue. Gatter *et al.* (1983) examined normal and malignant tissues for the presence of Tf receptors. Tf receptors were found in most of the carcinomas examined, all of the sarcomas and all of the tissue samples from patients with Hodgkin's disease. Tf receptors in normal tissues were found only in certain sites: basal epidermis, endocrine pancreas, hepatocytes, Kupffer cells, testis and pituitary. The authors point out that several of these normal tissue sites are sites of potential Fe deposition in primary hemochromatosis. In a study by Yeh *et al.* (1984) it was found that Tf receptors were more prominent on peripheral blood monocytes from patients with lymphoma, myeloma or leukemia than on similar cells from normal controls. The authors suggested that measurement of Tf binding by such cells might be of diagnostic or prognostic value. These many studies cited above demonstrated an important point, that Tf receptors were prominent on

proliferating cells including malignant cells and much less evident on differentiated, resting or non-growing cells.

Stein and Sussman (1983) performed peptide mapping studies of the human Tf receptor from lymphocytes, human cell lines and placental tissue. It was determined that all cell types, normal or malignant, yielded identical peptide maps.

2.9.2.3 The intracellular transferrin cycle

Until relatively recently, although it was known that cells required Fe for growth and that this was obtained from Tf which bound to specific membrane receptors, the actual mechanism of Fe acquisition was uncertain. It was suggested (Octave *et al.*, 1981) that Fe could be obtained from Tf in two ways. Firstly, Tf could bind to the plasma membrane, release Fe at the membrane and then Fe could be transported via an intracellular molecule to sites in the cell which need it. The Fe could be separated from Tf by chelation, protonation, displacement of the bicarbonate anion, or by some reductive mechanism. The second proposal was that Tf could bind to membrane receptor sites and be internalized by endocytosis within a vesicle. Fe could be removed intracellularly, perhaps in the acidic lysosomes, and Tf could recycle to the plasma membrane and be released.

Hemmaplardh and Morgan (1977) using electron microscopy, autoradiography and ^{125}I-labelled Tf and Tf conjugated to ferritin or horseradish peroxidase demonstrated that rabbit reticulocytes take up Tf by endocytosis. When cells were incubated at 4° C, Tf was seen at the periphery of cells only; when cells were incubated at 37° C Tf was seen within the

cells. Larrick and Cresswell (1979) studying Tf binding to T and B lymphoblastoid cells at 37° C found that Tf binding was reversible, saturable and specific for Tf as only Tf could displace bound Tf. Larrick and Cresswell made a further important observation that 25 times as much apo-Tf as diferric Tf was required to give 50% inhibition of binding. Binding was inhibited by KCN and colchicine and decreased if cells were treated with trypsin or papain but not reduced if cells were treated with neuraminidase. Karin and Mintz (1981) using mouse teratocarcinoma cells demonstrated that at 4° C Tf binds to cells and at 37° C there is both binding and internalization of Tf. Fe was retained by the cells and apo-Tf released into the medium. Uptake was shown to be an energy-requiring process and Fe uptake was inhibited by the lysosomotropic agents NH_4Cl and chloroquine.

Since these early studies, much information has been obtained to elucidate the eucaryotic cellular Fe uptake system. It is now firmly established that Tf-Fe is taken up by receptor-mediated endocytosis (Karin and Mintz, 1981; Klausner *et al.*, 1983; Hopkins and Trowbridge, 1983; Harding *et al.*, 1983) a process by which other molecules including low density lipoprotein, insulin, and epidermal growth factor are also taken up.

Fe-bearing Tf has a high affinity for its receptor at a neutral extracellular pH (Dautry-Varsat *et al.*, 1983; Klausner *et al.*, 1983a) and binding takes place. Tf-receptor complexes cluster in membrane regions called coated pits. Coated pits are membrane invaginations which are covered on the cytoplasmic side by the protein clathrin. Clathrin is composed of three-armed complexes, triskelions, which consist of 180,000 dalton heavy chains and 35,000 dalton light chains. Coated pits invaginate

forming coated vesicles. These vesicles subsequently shed their clathrin coat and fuse with acidic endosomes which have a pH of 5.0-5.5 (Tycko and Maxfield, 1982). Fe is removed from Tf in these non-lysosomal acidic vesicles (Van Renswoude *et al* , 1982; Lamb *et al.*, 1983) but apo-Tf remains bound to the receptor as at low pH apo-Tf has high receptor affinity (Ecarot-Charrier *et al.*, 1977; 1980, Dautry-Varsat *et al.*, 1983; Klausner *et al.*, 1983a). Endosomes become acidic as protons are transported from the cytoplasm into the vesicles due to ATP-driven proton pumps (Galloway *et al.*,1983). Apo-Tf and the receptor are recycled to the cell surface and as apo-Tf is exposed to a neutral extracellular environment in which it has low affinity for the receptor it quickly dissociates from the receptor. Ciechanover *et al* (1983) demonstrated using HepG2 human hepatoma cells that this cycle takes an average of 15.8 min and the cycle has been estimated to be completed in three min in rabbit reticulocytes (Iacopetta and Morgan, 1983). It is not known after Fe is released from Tf in the endosome how it is incorporated into heme, ferritin or other proteins. The existence of low molecular weight intracellular Fe carriers has been suggested (Jacobs, 1977) but none have been isolated.

Several authors have found (Klausner *et al.*, 1983; Lamb *et al.*, 1983; Ciechanover *et al.*, 1983) that cells as well as possessing surface Tf receptors have a pool of internal Tf receptors which may represent 70-80% of total cellular receptors. When cells are incubated with Tf at 37° C this receptor population may be cycled to the surface of the cells and bind Tf.

The Tf cycle has been elucidated by both biochemical (Octave *et al.*, 1981; Karin and Mintz, 1981; Van Renswoude *et al.*, 1982; Lamb *et al.*, 1983;

Dautry-Varsat *et al.*, 1983; Klausner *et al.*, 1983, ~~1983~~a) and electron microscopy studies. For example, Harding *et al.* (1983) conjugated Tf to gold particles and followed the endocytic cycle in rat reticulocytes. After 5 min incubation of the cells at 37° gold-Tf particles were seen in coated pits and vesicles, inside small uncoated vesicles of varying size and inside tubular elements. After about 20 min the particles were seen in multivesicular endosomes. Intracellular Tf was not found to be associated with a lysosomal compartment Finally, gold-conjugated Tf was released from the cell. Hopkins and Trowbridge (1983) studied Tf endocytosis by conjugating a monoclonal antibody to the Tf receptor to gold particles and following the intracellular endocytic cycle of the Tf receptor in human epidermoid carcinoma cells.

2.9.2.4 Regulation of transferrin receptor expression

Cells can regulate Fe uptake to a certain extent by modulation of Tf receptor expression. As discussed above, receptor numbers are related to state of activation or differentiation. Receptor numbers also vary with the phase of the cell cycle; numbers are low in G_1 and increase in S and G_2 + M. Receptor numbers therefore increase prior to DNA synthesis It has been suggested that the number of cellular Tf receptors expressed is modulated by the concentration of intracellular heme (Ward *et al.*, 1984) or by a pool of intracellular chelatable Fe (Bottomley *et al.*, 1985). If cells are incubated with Fe chelators such as picolinic acid (Louache *et al.*, 1984; 1985) or desferrioxamine (Mattia *et al.*, 1984; Rao *et al.*, 1985) there is an increase in the number of Tf receptors expressed. Bomford *et*

al. (1986) suggested that desferrioxamine may increase Tf receptor expression by chelating an intracellular regulatory pool of Fe or by arresting cells in the S phase of the cell cycle when receptors are maximally expressed. Conversely, if cells are incubated with Fe salts (Ward *et al.*, 1982; Louache *et al.*, 1984; 1985; Rao *et al.*, 1985) or hemin (Louache, 1984) the number of cellular receptors decreases. These effects are directly due to an increase and a decrease respectively in receptor biosynthesis. Rouault *et al.* (1985) demonstrated by competition experiments that hemin and desferrioxamine affect the same intracellular pool of Fe. Because desferrioxamine cannot remove Fe from hemin it was suggested that hemin takes Fe to a chelatable pool and it is the level of Fe in this pool that regulates the expression of the Tf receptor gene.

Louache *et al.* (1985) found that in human erythroleukemia cells actinomycin D inhibited the effects of Fe salts or chelators on receptor biosynthesis but did not affect enhancement of cellular ferritin synthesis caused by Fe salts. It was suggested that in these cells Fe modulates the syntheses of Tf receptors and cellular ferritin at the levels of transcription and translation respectively.

May *et al.* (1984) reported that treatment of HL-60 human promyelocytic leukemia cells with phorbol diesters resulted in translocation of Tf receptors from the cell surface into the cell. This receptor internalization was associated with increased phosphorylation of the Tf receptor. Klausner *et al.* (1984) observed that both the binding of Tf and phorbol diesters led to internalization of Tf receptors in K562 cells. They proposed that ligand binding triggers internalization and since phorbol ester treatment of cells led to an increase in Tf receptor phosphorylation it was suggested

that phosphorylation of the Tf receptor was the signal for internalization. It remained to be determined whether ligand binding caused receptor phosphorylation In a subsequent study by May *et al* (1985) it was proposed that phorbol diesters stimulate protein kinase C which phosphorylates a serine residue on the Tf receptor. The Tf receptor is then internalized via a colchicine-sensitive pathway i.e one which involves microtubules. In contrast to these findings, Alcantara *et al*. (1986) studying lymphoblastoid cells found that phorbol diesters caused Tf receptor down regulation by a cytoskeleton-independent mechanism, whereas Tf caused down regulation of receptors via a cytoskeleton-dependent mechanism. These differing results suggest that there are different mechanisms for Tf receptor regulation in different types of cells. In 1986, Davis *et al* , using A431 human epidermoid carcinoma cells determined that phorbol diesters caused phosphorylation of the Tf receptor by protein kinase C and serine residue 24 was the site of phosphorylation However, the significance of the phosphorylation of the Tf receptor is not clear; it is not known if this does trigger internalization as it has also been reported that Tf receptors are internalized in the absence of bound ligand (Watts, 1985). Rothenberger *et al*. (1987) recently reported results of studies on the importance of the cytoplasmic domain and phosphorylation sites on Tf internalization Site-directed mutagenesis was used to obtain human Tf receptor cDNA's with a deletion of 36 of 65 amino acids of the cytoplasmic domain of the Tf receptor or to obtain point mutations of five cytoplasmic serine residues which could be phosphorylation sites. When the cDNA's were transfected into mouse L cells it was found that the deletion mutant receptor could not carry out endocytosis. In lines in which an alanine residue was sub-

stituted for serine 24 although there was no phosphorylation the receptors were still able to internalize Tf as efficiently as wild type receptors. These results indicate that all or part of the cytoplasmic domain of the Tf receptor is important for endocytosis but suggest that phosphorylation of the receptor is not necessary.

May and Cuatrecasas (1985) pointed out that in mitogenically-stimulated lymphocytes there is a sequential induction of the expression of interleukin-2 receptors, then Tf receptors and in a model originally proposed by Neckers (1984), transformed cells no longer have this sequential control of proliferation as they demonstrate constitutive expression of Tf receptors. This may partially explain the continued growth of malignant cells. This hypothesis suggests that Tf and its receptor have other growth-regulating properties in addition to Fe donation (May and Cuatrecasas, 1985). These authors further add that Tf binding may cause cellular signals such as production of a second messenger. Activation of a protein kinase and subsequent receptor phosphorylation may be important in this regard. This idea is interesting as other growth factor receptors such as epidermal growth factor and insulin have protein kinase activity and various oncogenes of transformed cells code for protein kinases.

2.10 Summary

The mammalian Fe cycle is a well-regulated and dynamic mechanism consisting of several integrated constituents including: the central transport component, Tf, hemoglobin of erythrocytes, the storage protein ferritin and the ferroxidase ceruloplasmin. Several changes occur in the

host Fe cycle during both infection and neoplastic disease. One important change is the reduction in the amount of Fe bound to Tf a process beneficial in limiting microbial Fe acquisition especially in the case of murine *Neisseria meningitidis* infection. Both pathogenic bacteria and tumor cells must obtain host Fe to proliferate. *N. meningitidis* is unusual in that it can obtain Fe by binding Tf directly as do tumor cells by expression of specific membrane Tf receptors. The meningococcal Tf receptor has not been characterized but the eucaryotic Tf receptor has been studied extensively as has the endocytic Tf cycle and the regulation of Tf receptor expression.

The purpose of this work was to study Fe acquisition in neoplastic cells using a murine ascites lymphoma model, in particular, to study the ability of the hypoferremic response to limit Fe acquisition by the lymphoma cells. Fe growth requirements by these cells was examined as was the lymphoma Tf-receptor binding specificity. Finally, Tf binding by neisserial and lymphoma cells was compared to discover the reason for the difference in the response of *N. meningitidis* and tumor cells to hypoferremia even though both cell types are capable of binding Tf directly.

3. MATERIALS AND METHODS

3.1 Mice

The mice used in this study were C57BL/6 males obtained from the Animal Resources Division , Health and Welfare, Ottawa, Canada or from Charles River Breeding Laboratories Inc., St. Constant, Quebec, Canada. They received feed (Purina Rat Chow) and water *ad libitum*. For experiments investigating the hypoferremic response, mice were 6-8 weeks of age and weighed 18-25 g at the outset of experiments.

3.2 Tumor cells

The tumor cell line used for *in vivo* studies was the EL4 lymphoma (ATCC TIB 39) line (originally a benzo(a)pyrene induced lymphoma) from C57 black mice. These cells were maintained by growing them alternately *in vivo* and *in vitro*. Mice were inoculated intraperitoneally (i.p.) with 1 X 10^5 tumor cells. Ten to fourteen days later, cells were removed and cultured for 48 h *in vitro*. Initially, cells were cultured in Dulbecco's Modified Eagle's Medium (Flow Laboratories, Mississauga, Ontario, Canada) but subsequently were cultured only in RPMI 1640 (RPMI, GIBCO Laboratories, Grand Island, U.S.A.) which contained no added iron salts. RPMI contained 10 mM HEPES buffer, 100 units ml^{-1} penicillin, 100 ug ml^{-1} streptomycin, 2 mM glutamine and 10% fetal bovine serum (F.B.S., Flow Laboratories). Cells were incubated at 37° C in a humidified atmosphere with 5% CO_2.

A subline of the original EL4 cell line was derived by culturing the original EL4 cells in RPMI supplemented with human transferrin (Tf, Sigma Chemical Co., St. Louis, Missouri, U.S.A) only. Continued passage of these cells in this medium gave rise to a subline which grew well *in vivo* and *in vitro*. This subline was designated EL4-1.

For one study, two other murine tumor cell lines were used: YAC-1, a Moloney virus induced lymphoma of A strain mice and P815, a methylcholanthrene induced mastocytoma of DBA/2 mice. These cells were obtained from the laboratory of Dr. M. G. Baines, Department of Microbiology and Immunology, McGill University. These two cell lines were maintained *in vitro* only in RPMI with 10% F.B.S.

For studies in which bone marrow cells were used, bone marrow cells were obtained aseptically from the femurs of C57 black mice by flushing the bone marrow cavity cells into sterile Dulbecco's phosphate buffered saline (PBS), pH 7.3, using a 23 gauge needle and a 1-ml syringe. A homogeneous cell suspension was obtained by resuspending the cells in PBS using a sterile pasteur pipette. Cells were then centrifuged (10 min, 300 X g, 20° C) and washed once in sterile PBS.

3.3 *In vivo* experiments

3.3.1 Measuring the hypoferremic response

In order to determine the appropriate inoculum of tumor cells for experiments, ten-fold dilutions of the EL4 cells were made in RPMI and groups of mice (5 per group) were injected i.p. with from 10^7 to 10 tumor

cells. These mice were observed for development of tumors and survival time. All mice injected with 10^3 to 10^7 tumor cells subsequently died, time to death being directly related to inoculum size. Sixty percent of mice injected with 10^2 tumor cells died. The remaining mice were alive and apparently healthy 4 months post inoculation Therefore, to ensure that all mice would develop tumors, 10^4 cells per mouse was chosen as the inoculum and used as follows. EL4 cells were removed from a mouse inoculated 10 days previously and diluted to 2 X 10^4 cells ml^{-1} in RPMI. Groups of mice were injected i.p. with 1 X 10^4 cells and at designated times, mice were sacrificed and tumor cells were removed by peritoneal lavage with 2 ml of RPMI and counted in a hemacytometer using trypan blue exclusion as a measure of cell viability. For determination of hematocrit, groups of 4 mice were bled from the tail into heparinized capillary tubes. These same 4 mice were then bled completely by cardiac puncture into 3 ml Vacutainer tubes (Becton Dickinson, Montreal, Quebec, Canada) for serum iron (Fe) determination. The blood was allowed to clot at room temperature for 30 min then refrigerated for 8 h. The serum was obtained by centrifugation (5 min, 15,000 X g, 20° C). Serum samples from 2 mice were pooled and serum total iron binding capacity (TIBC), unsaturated iron binding capacity (UIBC) and Tf Fe were determined using an ^{59}Fe radioassay kit (Becton Dickinson). This assay kit measures only Fe specifically bound to Tf it does not measure hemoglobin Fe or Fe present due to hemolyzed blood; the principles of the kit are described in Appendix I.

These same serum samples were used to perform ceruloplasmin (Cp) assays using the method of Ravin (1961). For the Cp assay the reaction mixture contained: 20 ul serum, 1.6 ml 0.4 M sodium acetate buffer pH 5.5,

and 200 ul 0.5% (w/v) para-phenylenediamine. The mixture was incubated at 37° C for 60 min when the reaction was terminated by the addition of 200 ul 0.5% (w/v) sodium azide at 4° C. The absorbance at 530 nm was then recorded.

Just prior to sacrifice, mice were weighed to determine total body weight and after sacrifice the weights of the liver, spleen and thymus were recorded. Control mice were injected at zero time with 0.5 ml saline (0.85% w/v NaCl) instead of tumor cells. Control mice were also weighed and bled to monitor hematocrit, serum Fe and Cp.

3.3.2 Assessment of the effect of transferrin Fe administration on tumor cell growth

To determine whether addition of Tf Fe enhanced tumor cell growth *in vivo*, mice were inoculated with EL4 cells as above and subsequently injected i.p. with 2 mg of 100% Fe-saturated Tf (2.8 ug Fe) on days 9, 11, 13, and 15 post tumor cell inoculation. Control mice were injected with saline instead of Tf. The preparation of Tf is detailed in section 3.5.1. Tumor cell growth rate was monitored as described in section 3.3.1 above.

3.3.3 Assessment of the effect of desferrioxamine on tumor cell growth

In an attempt to inhibit *in vivo* tumor cell growth by Fe deprivation, mice were injected i.p. with 1×10^4 tumor cells and then with 5 mg of the Fe chelator desferrioxamine (desferrioxamine methane sulfonate, Ciba-Geigy, Dorval, Quebec, Canada) in 0.5 ml PBS. Control mice were injected with

tumor cells and PBS. Mice were re-injected with desferrioxamine or PBS on days 2, 4, 6, 8, 10, and 12. On day 12 the desferrioxamine-treated group was divided into 2 groups: one group was given no further desferrioxamine treatment: the other was given further injections of desferrioxamine on days 14, 16, and 18 post tumor cell inoculation. Tumor cell growth was assessed at appropriate times.

3.3.4 Assessment of the effect of inflammation-induced hypoferremia on tumor cell growth

In order to create a model of hypoferremia in mice, turpentine was used as a sterile inflammatory agent. Mice were lightly anaesthetized with ether and injected subcutaneously in the back with 0.05 ml oil of turpentine (Fisher Scientific Co.) at 2 separate sites. To measure TIBC, UIBC, serum Fe and Cp levels, groups of 6 mice were bled by cardiac puncture at 0, 6, 12, 18, 24, 36, 48, 60, and 72 h as described in Section 3.3.1. Control mice were treated similarly but injected with saline only. In a later study, to assess the effects of an early hypoferremia on tumor growth, mice were injected with either saline or turpentine as above and then with 10^4 tumor cells one h later. Mice were bled to assess serum Fe levels at 0, 12, 24, 48 and 72 h and after 6, 8, 11, 14, 16 and 18 days. Four mice from each group were sacrificed at each time point. At the 6 latter time points tumor cell numbers were evaluated as well.

3.4 *In vitro* growth experiments

3.4.1 Cell culture preparation

EL4-1 cells were used for the majority of all *in vitro* growth experiments. These cells were removed from a mouse inoculated 10-14 days previously into RPMI and centrifuged (5 min, 270 x g, 20° C). Red blood cells present were then lysed by the addition of sterile distilled water for 15 seconds. An equivalent volume of 2 times concentrated RPMI was added to the cell suspension. Cells were washed twice in RPMI and finally incubated in RPMI with 0.3 uM 50% Fe-saturated human Tf for 48 h. The tumor cells were then washed and incubated in RPMI with no added Fe or Tf for 24 h to deplete the cells of endogenous Fe. Cells were centrifuged (5 min, 270 X g, 20° C), washed once in RPMI and inoculated into 10 ml RPMI in 30 ml tissue culture flasks containing various Fe sources. Initial cell numbers were 1 X 10^5 ml^{-1} (except for one set of experiments in which initial cell numbers were 1 X 10^4 cells ml^{-1}) and growth was assessed by performing daily cell counts in a hemacytometer using trypan blue exclusion. Initial growth experiments were performed in RPMI which was found to contain 0.019 +/- 0.009 ug ml^{-1} Fe (0.33 +/- 0.16 uM) as measured using a Perkin Elmer 703 atomic absorption spectrophotometer using the HGA-2200 graphite furnace and D_2 background correction. In subsequent experiments, to ensure that this low level of contaminating Fe did not alter the Tf Fe saturation during growth or uptake experiments, RPMI was deferrated by incubating the medium for approximately 20 h (37° C, 5% CO_2) with a two-fold molar excess of human apo-Tf. The medium was then filtered through a PM-10

Amicon filter (10,000 dalton exclusion limit) to remove the Tf and bound Fe. This medium was then designated Fe-extracted RPMI. Sources of Fe used in growth experiments included: human Tf, bovine Tf, murine Tf, conalbumin, lactoferrin (Lf), bovine hemoglobin, horse spleen ferritin and ferrous ammonium sulfate and all were obtained from Sigma Chemical Co. except murine Tf (Daymar Laboratories, Toronto, Ontario, Canada).

3.4.2 Fe uptake by the tumor cells

EL4-1 cells were removed from the peritoneum of a mouse following *in vivo* passage. After lysis of red blood cells and washing, cells were inoculated into Fe-extracted RPMI containing 0.3 uM 50% Fe-saturated human Tf at an initial density of 1 X 10^5 cells ml^{-1}. These cells were maintained in this medium at an approximate concentration of 5 X 10^5 cells ml^{-1} and used for uptake studies. For ^{59}Fe uptake studies, cells were washed once (5 min, 270 X g, 20° C) and inoculated into 10 ml Fe-extracted RPMI at a level of 1.5 X 10^6 ml^{-1}. At zero time, ^{59}Fe-Tf was added. Duplicate 1-ml samples were taken at 0, 1, 3 and 6 h. Cells were washed 3 times (2 min, 1430 X g, 20° C) in Fe-extracted RPMI. Supernatants, washes and pellets were counted in a Beckman 8000 gamma counter.

3.4.3 Transferrin binding experiments

All Tf binding experiments were performed at 4° C (except for time course studies which were carried out at both 4° C and 37° C) using 100% Fe-saturated human Tf. As described in section 3.4.2, cells were harvested

from mice and suspended in Fe-extracted RPMI. The red blood cells were lysed and cells were washed and inoculated into Fe-extracted RPMI containing 0.3 uM 100% Fe-saturated Tf at an initial level of 1×10^5 ml^{-1}. Cells were maintained as for uptake studies. Before a binding assay, cells were harvested, washed once (270 X g, 5 min, 20° C) then incubated for 90 min (37° C, 5% CO_2) in Fe-extracted RPMI to eliminate as much bound or internalized Tf as possible. Klausner *et al.* (1983) using K562 cells found that 50% of internalized Tf is released from the cells in the absence of added Tf. Tf release from the EL4-1 cells during this pre-incubation period was not measured in these studies. After incubation, cells were washed once (4° C) and used for binding assays.

3.4.3.1 Determination of receptor numbers

These assays were performed using a modification of the method of Lazarus and Baines (1985a). The wells of round-bottomed 96-well microtiter plates (Linbro, Flow Laboratories) were coated with a 10% (w/v) solution of bovine serum albumin (BSA, Sigma). One hundred ul of Fe-extracted RPMI containing 0.1% (w/v) BSA was added to all wells except the first well of a row in the microtiter plate. Ten ug of ^{125}I-labelled Tf in 200 ul of the above medium was added to the first well and serially diluted two-fold to well number 11. (After mixing the Tf in well 11, 100 ul was discarded.) Well 12 contained 100 ul media only. 1.5×10^6 cells in 100 ul of media were added to all 12 wells at 4° C. Cells and Tf were mixed and incubated for 90 min (4° C). After incubation, cells were removed from the wells into microcentrifuge tubes containing 600 ul RPMI with 0.1% BSA. Wells were

rinsed with 200 ul of this medium and this was added to the corresponding microcentrifuge tube. Cells were washed 4 times and supernatants, washes and pellets were counted as for uptake studies except that all manipulations were carried out at 4° C. Cells from well 12 were treated as for the other wells except that they were not incubated with Tf and at the end of the experiment, cells were counted to detect any cell lysis during the experiment. Results were plotted according to the method of Scatchard (1949) using the method of Chamness and McGuire (1975) to correct for non-specific binding.

3.4.3.2 Time course binding and uptake assays

EL4-1 cells were prepared as for receptor assays. 1.5 X 10^6 cells were incubated with 0.5 ug ^{125}I-labelled diferric human Tf in 200 ul RPMI containing 0.1% BSA in wells of microtiter plates. Cells were incubated with the labelled ligand for 2, 5, 30, 60 and 90 min at 4° C and 37° C. Duplicate wells were run for each time point. Non-specific binding was assessed by including wells which contained radiolabelled ligand and a 100-fold excess of unlabelled diferric Tf. Non-specific binding was subtracted from specific binding. Cells were centrifuged, washed 3 times (2 min, 1430 X g) in Fe-extracted RPMI with 0.1% BSA and supernatants, washes and pellets were counted.

3.4.3.3 Competitive binding assays

These assays were performed on the original EL4, EL4-1, YAC-1, P815 and murine bone marrow cells. All cell lines were grown and prepared as outlined in sections 3.2 and 3.4.3 respectively. Cells at 4° C were added to control microtiter wells containing 0.5 ug ^{125}I-labelled human Tf and to test wells which contained labelled Tf and 0.5, 5.0 or 25 ug of unlabelled competing ligands. Competing ligands included: human Tf, murine Tf, bovine Tf, human Lf and conalbumin prepared to 100% Fe-saturation. Binding was allowed to proceed for 60 min at 4° C then cells were harvested as described in 3.4.3.1. Duplicate wells were run for each condition tested. Non-specific binding was measured as outlined in 3.4.3.2.

3.5 Preparation of transferrin solutions

3.5.1 Preparation of 50% and 100% Fe-saturated unlabelled proteins

Human apo-Tf was loaded with Fe^{3+} to 50 or 100% saturation using ferric citrate (1:10 Fe:citrate ratio) prepared in 40 mM Tris (Trizma base, Sigma) 2 mM sodium bicarbonate buffer, pH 7.4. To achieve 50 and 100% saturation, 0.7 or 1.4 ug Fe mg^{-1} protein respectively was provided. Preparations were stirred for 20 min at room temperature and then allowed to sit at this temperature for a further 20 min Fe-loaded proteins were then dialyzed (4° C) twice against Tris-bicarbonate buffer, pH 7 4, and once against 5 mM Tris in saline, pH 7.4. Bovine Tf, human Lf and conalbumin were prepared in a similar manner. Murine Tf was dialyzed twice

against 10 mM sodium citrate, pH 5.0, and twice against Tris-bicarbonate buffer, pH 7.4, loaded with Fe to the desired level of saturation then dialyzed as for human Tf. The Fe saturation of Tf's was verified by measuring the ratio of optical densities at 465/280 nm. One hundred per cent Fe-saturated Tf at a concentration of 2 mg ml^{-1} gave an approximate ratio of 0.046 and this ratio for 50% Fe-saturated Tf was 0.023.

3.5.2 Preparation of ^{59}Fe-radiolabelled transferrin

Human apo-Tf was dissolved in 40 mM Tris, 2 mM bicarbonate buffer pH 7.4 and dialyzed overnight. Tf was ferrated from ^{59}Fe citrate by mixing acidic $^{59}FeCl_3$ (New England Nuclear, Lachine, Quebec, Canada, specific activity 2-40 Ci g^{-1} Fe) with ^{56}Fe ferric citrate in Tris bicarbonate buffer, pH 7.4. To load 4.5 mg Tf to 50% Fe saturation, 2.2 uCi ^{59}Fe ug^{-1} total Fe was used. The apo-Tf was added to the ^{59}Fe citrate and stirred at room temperature for one h. The preparation was then dialyzed for 24 h against two changes of Tris-bicarbonate buffer, pH 7.4, and once against 5 mM Tris in saline, pH 7.4, to remove unbound Fe. Tf was loaded to 50% saturation by addition of 0.7 ug Fe mg^{-1} protein. The specific activity was approximately 3.33 X 10^3 cpm ug^{-1} protein.

3.5.3 Preparation of ^{125}I-radiolabelled transferrin

Iodinated Tf was prepared using the chloramine T method as described by Lazarus and Baines (1985a). Diferric Tf was prepared at a concentration of 25 mg ml^{-1}. Two hundred ul (5 mg) was transferred to a microcentrifuge

tube, 1 mCi ^{125}I (Amersham) in a 10 ul volume was added to the Tf. Chloramine T (100 ug in 10 ul) was then added to start the reaction which proceeded at room temperature for 15 min. The iodination was terminated with the addition of 125 ug of sodium metabisulfate in a 25 ul volume. The labelled Tf was then separated from unbound ^{125}I on a Sephadex G-25 (medium, dry bead diameter 50-150 um) column (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with PBS, pH 7.3. Fractions of 0.5 ml were collected and the fractions that were pink in colour were pooled. To determine the percentage of the label bound to the Tf a trichloroacetic acid precipitation was performed. Five ul of labelled protein was mixed with 200 ul 1% (w/v) BSA in PBS and 200 ul of 20% (w/v) trichloroacetic acid. This mixture was centrifuged (10 min, 16,000 x g, 20° C). An aliquot of the supernatant and the pellet were counted in a Beckman 8000 gamma counter and the percentage of label bound to the protein was determined. The labelled Tf was then dialyzed for 24 h The concentration of the labelled protein was determined using the Bio-Rad protein assay (Bio-Rad dye reagent, Bio-Rad, Mississauga, Ontario, Canada) using diferric human Tf as the standard. Finally, the labelled protein was mixed with an equivalent volume of 2% (w/v) BSA, filter sterilized, dispensed into aliquots and stored at 4° C. ^{125}I-labelled human Tf was prepared to a specific activity of approximately 2×10^5 cpm ug^{-1}.

3.5.4 Deglycosylation of transferrin

N-glycanase was purchased from Genzyme Corp., Boston, Massachusetts, U.S.A. Human and murine Fe-Tf's were prepared to 100% Fe-saturation at a concentration of 2 mg ml^{-1}. The following were mixed aseptically: 55 ug human Tf or 50 ug murine Tf, 30 ul 0 55 M sodium phosphate buffer pH 8.6, 8.4 ul 100 mM 1,10-phenanthroline hydrate and 20 ul N-glycanase (5 units) This mixture was incubated at 37° C in a water bath for 17 h. The volume of this mixture was diluted to 1.5 ml with sterile 5 mM Tris in saline, pH 7.4 and the mixture was dialyzed for 2 h at 4° C against the same sterile buffer. The deglycosylated protein was then aseptically dispensed in aliquots and immediately stored at -80° C. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was run on the native and deglycosylated proteins and stained using the silver staining method. The removal of carbohydrate was verified by a Western blotting procedure performed by Pardo Pannunzio of this department. Briefly, native and deglycosylated Tf's were run on SDS-PAGE, and blotted onto nitrocellulose paper. This was subsequently treated with concanavalin A conjugated with horseradish peroxidase. Competitive binding experiments were performed to compare the binding of native and deglycosylated proteins.

3.6 Preparation of bovine hemoglobin and ferritin

Bovine hemoglobin (0.34 g) was dissolved in 5 ml distilled water and dialyzed against citrate-phosphate buffer, pH 7.0 for 24 h then against distilled water. The solution was analyzed by atomic absorption

spectrophotometry to determine the Fe concentration. Ferritin (horse spleen ferritin) was dialyzed overnight against 10 mM sodium citrate, pH 6.5 to remove any traces of cadmium which is used in the purification procedure. The Fe concentration of the solution was measured as for hemoglobin by atomic absorption spectrophotometry.

3.7 Detection of transferrin on EL4 cells

Tumor cells were removed from mice between 12-18 days post inoculation into cold serum-free medium containing 0.02% sodium azide. To these cells was added 0.1 ml of a 1:9 dilution of rabbit anti-mouse Tf at 4° C and cells were incubated (4° C) for 30 min. The cells were washed 3 times with azide-containing medium at 4° C. Then 0.1 ml of a 1:9 dilution of fluorescein-labelled goat anti-rabbit globulin (GIBCO) was added to the cells which were subsequently incubated for 30 min in the dark at 4° C. Cells were then washed as before. Two drops of glycerol mounting fluid, pH 8.4, were added to the preparation. Cells were placed in a hemacytometer, observed and photographed under both ultra-violet (fluorescence) and bright field microscopy using a Leitz Dialux 20 EB microscope at a magnification of 400 Control cells were treated in a similar manner but normal rabbit serum was used instead of antiserum.

3.8 Characterization of cells

3.8.1 Determination of cellular Fe

The Fe content of the tumor cells was determined by atomic absorption spectrophotometry using the graphite furnace mode. Cells were homogenized in saline using a glass tissue homogenizer which had been pre-rinsed with 6 N HCl to remove any traces of Fe. Protein determinations were performed using the method of Lowry *et al.* (1951).

In order to detect heme in EL4 cells, homogenates were scanned spectrophotometrically from 450 to 350 nm using a Perkin Elmer 555 UV-VIS spectrophotometer. Horse heart cytochrome c (Sigma, type III) was used as a standard and the heme was detected by its Soret peak absorption in the 405-420 nm region. For both Fe and heme analysis of *in vivo* grown cells, mice were bled completely from the retro-orbital sinus before the tumor cells were harvested. After tumor cells were removed, any contaminating red blood cells were lysed. The tumor cells were then washed twice, resuspended in saline, homogenized and assayed.

3.8.2 Respiration studies

Respiration studies were performed on EL4 cells grown both *in vitro* and *in vivo* using a Clark polarographic O_2 electrode. Assays were done on 3 ml samples in serum-free media containing 1 X 10^6 cells ml^{-1}. Initial O_2 uptake rates were established by following uptake for 10 min then 10 ul of 300 mM potassium cyanide was added and O_2 uptake was monitored for an ad-

ditional 20 min.

3.8.3 Glucose and lactic acid assays

The glucose concentration of spent Fe-extracted RPMI in which EL4-1 cells had been cultured with 0.3 uM 50% Fe-saturated human Tf for 72 h was measured by the phenol-sulfuric acid method of Dubois *et al.* (1956). Standards containing 20-100 ug glucose and samples containing control and spent RPMI were assayed. Sample volumes were made up to 1 ml with distilled water, 1 ml of 5% (w/v) phenol and 5 ml of concentrated H_2SO_4 (96%, reagent grade) were added. After 10 min, tubes were vortexed then incubated 15 min at 27° C in a water bath and the absorbance at 490 nm was read on a Pye-Unicam SP8-400 spectrophotometer.

Lactic acid in the spent culture supernatant was measured using the assay of Rosenberg and Rush (1966). 0.2 ml distilled water blanks, lactic acid standards (prepared to contain 0.5-2 umol ml^{-1} lactic acid, Sigma) or supernatants were mixed with 2.8 ml glycine-semicarbazide hydrochloride buffer, pH 10.0, and 0.6 ml NAD solution, pH 6.0, and the absorbance at 340 nm was read for all tubes Beef heart lactate dehydrogenase (Sigma) was diluted to a concentration of 2 mg protein ml^{-1} and 0 4 ml was added to the tubes which were incubated one h at 37° C. After incubation, the absorbance at 340 nm was read again. $\hat{}E$ was determined as $E_2-0.9E_1-B_2-0.9B_1$ where E_1 and E_2 are the absorbance readings before and after the addition of enzyme and incubation respectively and B_1 and B_2 are the initial and final blank readings. A standard curve of $\hat{}E$ was plotted from which the concentration of lactate in the supernatant was determined.

3.8.4 API ZYM enzyme assays

The enzyme assays on EL4-1 cells were performed using the API ZYM system from Analytab Products Division of Ayerst Laboratories Inc. A description of the API ZYM kit is found in Appendix II. EL4-1 cells were removed from the peritoneal cavity of a mouse and cultured *in vitro* in RPMI containing 75 nM 50% Fe-saturated human Tf. After 48 h, cells were washed and divided into two sets: one set was grown as described above, the other was grown in the absence of Tf or Fe for 40 h. Cells were washed in saline and resuspended in 3 ml saline at 7.6 X 10^6 cells ml^{-1}. The API ZYM strips were inoculated with Fe-sufficient and Fe-deprived cells and incubated at 37° C for 4 h. Reagents A and B, provided in the API kit, were added and the strips were read by comparing the colour reactions to those in the colour chart provided. The colour intensity was estimated on a scale from 0-5.

3.9 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels were run to assess the purity of proteins and to look for a decrease in molecular weight due to deglycosylation of Tf. Gels were run in slabs of 1.5 mm thickness using the system of Laemmli (1970). Running gels were 12 cm in length and were 10% (w/v) acrylamide and 0.26% (w/v) bisacrylamide while the stacking gels were 4% (w/v) acrylamide and 0.11% (w/v) bisacrylamide. Gels were run at 25 mA and 35 mA for migration through the stacking and running gels respectively in a buffer system con-

sisting of 0.025 M Tris, pH 8.3, 0.19 M glycine and 0.1% (w/v) SDS. Samples were boiled for 5 min in sample buffer containing 0.08 M Tris, pH 6.8, with 2% (w/v) SDS, 10% (w/v) glycerol, 0.04% (w/v) bromophenol blue and 5% (v/v) beta-mercaptoethanol. Low molecular weight markers (Pharmacia Fine Chemicals) were run along with the proteins of interest. Gels were stained with Coomassie Blue R-250 (0.125% Coomassie Blue, 50% methanol, 10% acetic acid) overnight and then destained with a destaining solution of 50% methanol, 10% acetic acid. Alternatively, gels were stained by the silver staining method. For the latter method, gels were fixed overnight in 5% trichloroacetic acid-5% sulfosalicylic acid and transferred to 5% methanol: 7% acetic acid: 88% deionized water for 3 h. Gels were then fixed for 30 min in 10% glutaraldehyde, rinsed, reduced with dithiothreitol, rinsed, stained with $AgNO_3$ (1 ml of a 20% (w/v) solution of $AgNO_3$ in 199 ml distilled water) for 30 min and rinsed with water. A Na_2CO_3 developer solution was added until the desired intensity of staining was achieved then staining was stopped by addition of citric acid.

3.10 Bacterial studies

3.10.1 Bacterial cultures

Neisseria meningitidis SD1C, a serogroup B strain, was obtained from the Neisseria Repository, NAMRU, School of Public Health, University of California, Berkeley. *Neisseria subflava* was the ATCC 10555 strain. The *E. coli* strain used was AB2463pDT1-2 which was obtained from D. Touati. Frozen stock cultures were prepared in Mueller-Hinton broth (MHB, Oxoid,

Basingstoke, England) containing 20% glycerol and stored at -80° C.

3.10.2 Culture conditions

Frozen cultures were thawed at 37° C and streaked on to MHA plates; MHA is MHB with the addition of 1.5% agar (Difco Laboratories, Detroit, Michigan). Plates were incubated at 37° C with 5% CO_2. After approximately 18 h a colony was subcultured to a new plate. To obtain working cultures for Tf binding experiments, 3 isolated colonies were inoculated into 20 ml MHB and incubated for 6 h at 37° C with shaking (100 rpm). This starter culture was used to inoculate a second MHB culture with a one percent (v/v) inoculum which was incubated for 16 h when cells were harvested (4,080 X g, 5 min, 4° C). At the time of harvest, cells were examined using phase contrast microscopy, a TMPD oxidase test was performed and the optical density of the culture was recorded. Cells were washed once (5,900 X g, 5 min, 4° C) in Fe-free Neisseria Defined Medium (NDM,
Archibald and DeVoe, 1978) containing 0.1% BSA and then were resuspended to the desired concentration in this medium.

3.10.3 Binding assays

Binding assays were performed in microtiter plates as for tumor cell studies. Four mg unlabelled diferric Tf was added to well one and serially diluted two-fold in NDM with BSA to well 12; 0.5 ug of ^{125}I-labelled diferric Tf was added to each well and the well contents were mixed. Bacteria

were added to each well at 4° C at a concentration of 5 X 10^9 cells per well. After 30 min incubation, cells were transferred to microcentrifuge tubes containing 600 ul of media. Wells were rinsed with 200 ul of media and this was also added to the corresponding microcentrifuge tubes. Cells were centrifuged (1 min, 15,000 X g, 4° C) and washed twice by resuspension in 1 ml NDM with BSA. Supernatants, washes and pellets were counted.

To examine Tf binding to Fe-deprived cells, MHB was prepared containing 18 ug ml^{-1} ethylene-diamine-di-ortho-hydroxyphenyl acetic acid (EDDA) (MHB-EDDA) A 50-ml culture was grown for 16 h in MHB; 10-ml volumes of culture were then transferred to each of two centrifuge tubes. Cells were centrifuged, washed once with either MHB or MHB-EDDA and inoculated into MHB or MHB-EDDA, incubated for 4 h and harvested.

Time course binding studies were performed using *Neisseria meningitidis* SD1C in wells containing 70 ug Tf (69.5 ug unlabelled diferric Tf and 0.5 ug ^{125}I-labelled Tf) and 5 X 10^9 cells. Cells were harvested after 2, 5, 10, 30 and 60 min

Competition studies were performed on Fe-sufficient and Fe-deprived cells. Control wells contained 0.5 ug labelled and 69.5 ug unlabelled Tf. Competitors included the following unlabelled proteins: human Tf, human Lf and conalbumin. Competitors were added at an equivalent concentration (70 ug) or at a ten-fold concentration (700 ug). For time course and competition studies, non-specific binding was assessed by including wells which contained 0.5 ug labelled Tf and 3500 ug unlabelled Tf.

3.10.4 Plate Assays

The Fe concentration of MHB and Difco agar were measured by atomic absorption spectrophotometry. MHB was found to contain 0.96 ug Fe ml^{-1} and the agar was found to contain 0.015 ug Fe mg^{-1}. Fe-limited MHA plates were prepared to contain 2.0, 2.5 and 3.0 umol EDDA $umol^{-1}$ Fe. *Neisseria meningitidis* and *Neisseria subflava 10555* were plated using the spread plate method. After plates were dry, wells were punched in the agar. The desired amounts of the Tf proteins under study were added to the wells. Plates were incubated at 37° C with 5% CO_2. Zones of growth exhibition (SD1C) or inhibition (*Neisseria subflava*) were measured and plates were photographed.

4. RESULTS AND DISCUSSION

4.1 *In Vivo* Experiments

4.1.1 Measuring the hypoferremic response

Initially, experiments were conducted to observe the growth pattern of EL4 cells *in vivo* and to determine whether the proliferation of these cells elicited a hypoferremic response in C57 mice. Fig. 2A, a photograph of tumor cells removed from the peritoneal cavity, demonstrates the morphology of *in vivo* grown cells. It can be seen that cells are fairly round and uniform in size and shape compared to *in vitro* grown cells which are pleomorphic (Fig. 2C). The two cells photographed in Fig. 2C are representative of the morphology of *in vitro* grown cells. The presence of Tf on the surface of these cells was revealed by fluorescent antibody studies (Fig. 2B).

The tumor cells grew exponentially until day 10 (Fig 3) when the growth rate became slower. Although this change in growth rate did occur during the hypoferremic response (Fig. 4), many factors other than Fe limitation could have been involved including: crowding of the cells in the peritoneal cavity resulting in a limiting nutrient other than Fe, production of one or more toxic metabolites, activation of the host inflammatory response or other facets of the immune system.

Ruddon (1981) pointed out that the growth kinetics of some tumor cells in experimental animals resemble the growth kinetics of cells in culture

Fig.2 Photomicrographs of murine lymphoma cells EL4 cells were harvested from the peritoneal cavity of tumor-bearing mice 18 days post inoculation. Cells were incubated with rabbit anti-mouse Tf for 30 min at 4°C then washed and incubated with fluorescein-labelled goat anti-rabbit globulin for 30 min at 4°C in the dark. This temperature was used and 0 02% NaN_3 was added to the medium in order to see surface-bound not internalized Tf. After a final washing, cells were observed and photographed under (A) bright field and (B) UV (fluorescence) illumination. The final magnification is 576X. (C) EL4 cells were cultured *in vitro* for 48 h in RPMI 1640 containing 10% F.B.S., washed once and photographed under bright field microscopy; the final magnification is 822.4X.

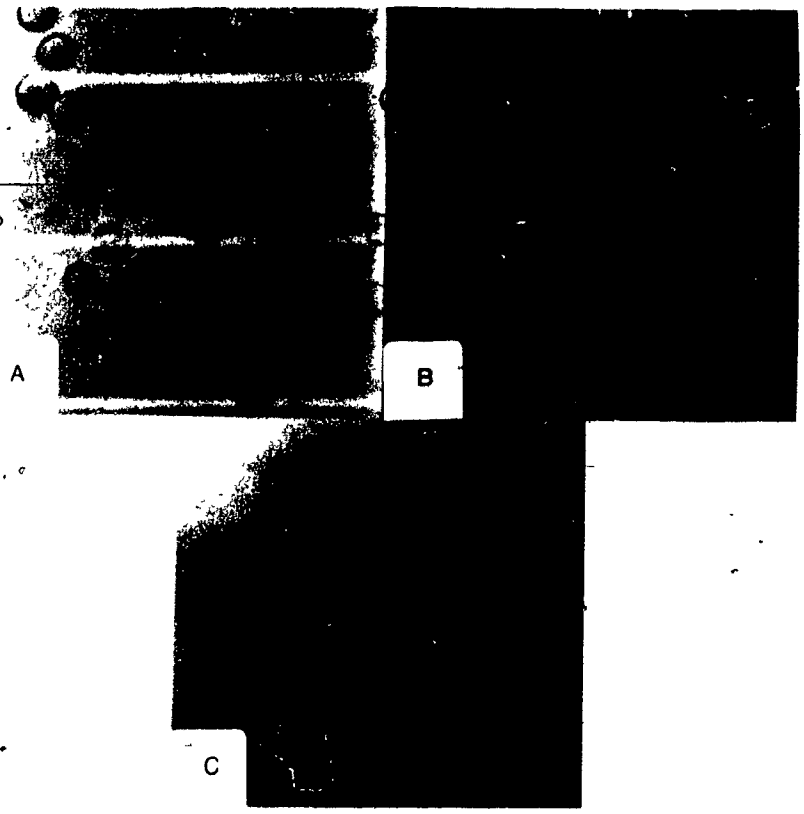

A
B
C

Fig. 3 Growth of EL4 cells in C57 mice. Each mouse was injected i.p. with $1,0^4$ EL4 cells. At the times indicated, mice were sacrificed and tumor cells were removed by peritoneal lavage and counted in a hemacytometer with trypan blue exclusion. Points represent the average number of tumor cells from 4 experiments; 4 mice were sacrificed per time point per experiment.

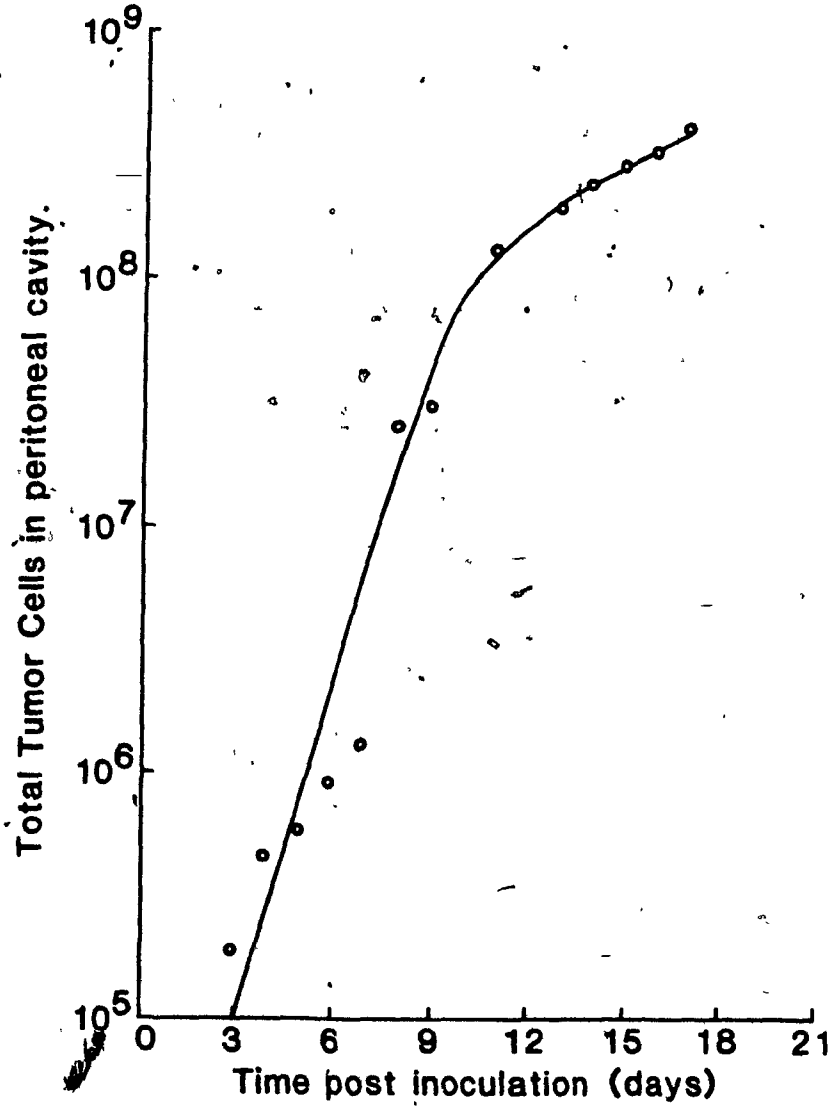
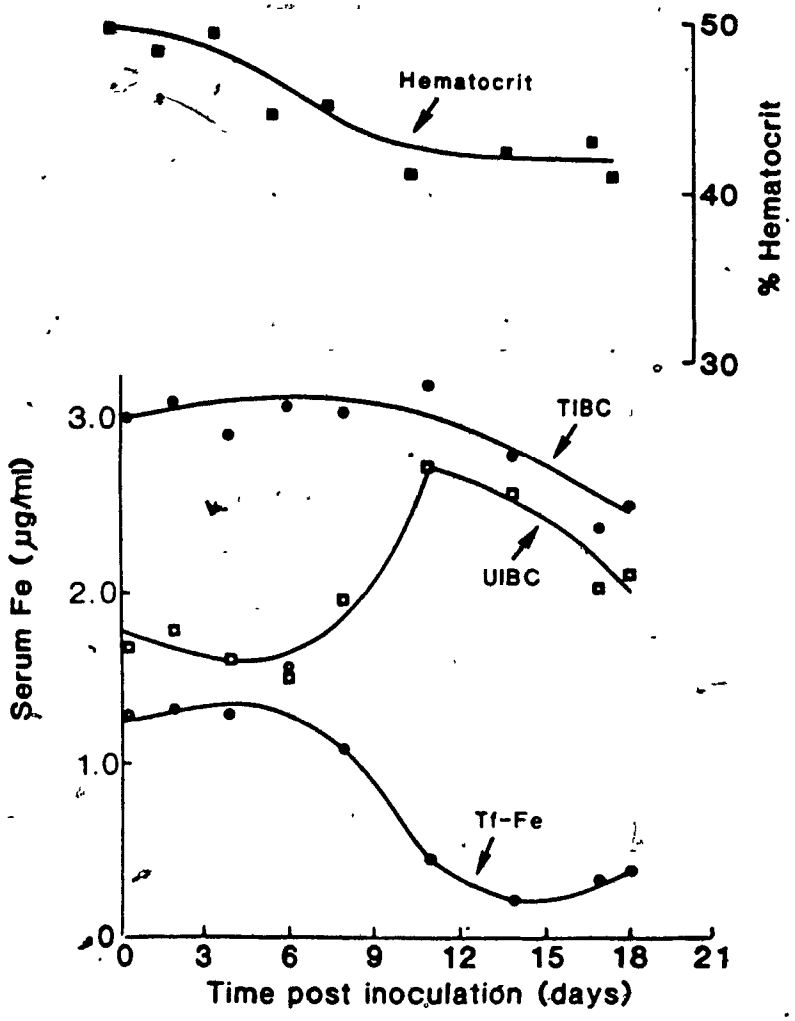

Total Tumor Cells in peritoneal cavity.
10^9
10^8
10^7
10^6
10^5
0
3
6
9
12
15
18
21
Time post inoculation (days)

Fig. 4 Changes in Fe metabolism during EL4 ascites tumor growth in mice. C57 mice were injected with 10^4 EL4 cells and at the times indicated, groups of mice were tail bled for hematocrit determinations (■) and then sacrificed by cardiac puncture to obtain blood for determination of serum total Fe-binding capacity (TIBC) (●), unsaturated Fe-binding capacity (UIBC), (□), and Tf-Fe (○).


Hematocrit
50
40
30
% Hematocrit
TIBC
UIBC
Tf-Fe
3.0
2.0
1.0
0
Serum Fe (µg/ml)
0
3
6
9
12
15
18
21
Time post inoculation (days)

with an initial lag phase of slow growth, an exponential phase of rapid growth and then another phase of slow growth reaching a steady state of cell growth and cell loss. In many types of human cancers, however, this type of growth pattern does not occur as there may be significant cell loss or death due to exfoliation from the tumor and nutrient limitation in the central less well vascularized areas of solid tumors Also there is wide variation in the doubling times of different types of both solid tumors and leukemias and lymphomas (Ruddon, 1981). Growth of EL4 cells *in vivo* did not demonstrate a lag phase (Fig. 3), but grew exponentially from day 0 to day 11 and subsequently the growth rate slowed down

Shortly after inoculation, the mice had serum Fe levels (Fig. 4) of 1.13 ug ml^{-1} (20.17 uM) representing 44% Fe saturation of the serum Tf. By day 14 post injection, these levels had fallen to 0 22 ug ml^{-1} (3 93 uM) corresponding to 8.1% Fe saturation of Tf, revealing a marked hypoferremic response The serum Fe levels for normal control mice which remained relatively constant over an equivalent time period are found in Table 1 The drop in serum Fe in the tumor-bearing mice was more pronounced than that found in one study of human disease (Beamish *et al* , 1972) in which controls had a serum Fe level of 22 7 uM (35% Fe saturation). Patients with stage 4 Hodgkin's disease had serum Fe levels of 6.97 uM (16% saturation) and patients with non-Hodgkin's lymphoma were observed to have serum Fe levels of 10.7 uM (17% saturation)

The fall in hematocrit of the C57 mice from an initial value of 49 6 (+/- 0.76) % to 40.6 (+/- 0 87) % on day 16 (Fig. 4) reveals the development of an anemia following the onset of the hypoferremic response Anemia might be expected to follow a prolonged hypoferremic response as the Tf Fe

TABLE 1.

Serum Fe levels in normal control mice.[1]

Time (Days post inoculation)	TIBC[2] (ug ml^{-1})	UIBC[3] (ug ml^{-1})	Tf-bound Fe (ug ml^{-1})	% Saturation	Hematocrit
0	3.04 (0.05)[4]	1.65 (0.03)	1.39 (0.08)	45.6 (1.7)	46.7 (1.0)
4	3.41 (0.05)	1.85 (0.14)	1.56 (0.10)	45.8 (3.6)	45.7 (1.0)
8	3.22 (0.02)	1.52 (0.11)	1.71 (0.12)	52.9 (3.5)	47.9 (1.2)
12	3.22 (0.03)	1.90 (0.10)	1.31 (0.11)	40.8 (3.3)	49.1 (0.8)
16	3.35 (0.03)	1.84 (0.05)	1.52 (0.06)	45.3 (1.6)	47.8 (1.1)
20	3.29 (0.08)	2.03 (0.06)	1.26 (0.08)	38.2 (2.0)	52.0 (0.8)

[1]Mice were injected i.p. with 0.5 ml saline and at the indicated times were tail bled for hematocrit determination and bled by cardiac puncture for determination of serum [2]TIBC (total iron-binding capacity), [3]UIBC (unsaturated iron-binding capacity) and serum Fe. These latter determinations were performed using a Becton Dickinson radioassay kit. Nine mice were sacrificed per point; the sera from 3 mice were pooled giving 3 values per point which were then averaged.

[4]Numbers in parentheses represent the standard error of the mean.

supplied to the developing erythroid cells in the bone marrow would be decreased. As discussed in section 2.6 however, the anemia which occurs during neoplasia may be more complex as a decreased red cell survival time and an impaired marrow response to erythropoietin may also be involved (Lee, 1983; Zucker, 1985). The occurrence of hypoferremia and the fall in hematocrit during neoplasia have been shown in other experimental systems Chandler and Fletcher (1973) found that chickens injected with a lymphoid tumor had serum Fe levels of 0.8 ug ml^{-1} while controls had serum Fe levels of 1.5 ug ml^{-1}. The hematocrit values were approximately 13% lower in tumor-bearing birds by day 12 post inoculation. Schade (1976) injected mice with 2 X 10^5 plasmacytoma cells and recorded that control mice had serum Fe levels of 2.87 ug ml^{-1} while mice bearing plasmacytomas had 1.16 ug ml^{-1} serum Fe three weeks after inoculation. Hematocrit values were 47.4% in normal mice and 30% in tumor-bearing mice.

The reduction in serum Fe levels in the mice in the present studies began relatively late in the course of the disease, at approximately day 8. By this time the peritoneal tumor burden was quite high (an average of 2 3 X 10^7 cells per mouse) with death of the mice occurring 16-20 days after inoculation. This is in sharp contrast to the time course of hypoferremia during bacterial infection. In one study, (Holbein, 1980) when C57 black mice were injected with 10^4 *Neisseria meningitidis*, serum Fe levels decreased immediately and continuously until no Tf Fe could be detected at 18-24 h post infection. Infection peaked at 6 h and then disappeared rapidly after 9 h in concert with the hypoferremic response. It is not known why the onset of hypoferremia occurred late in the course of tumor growth. Hypoferremia is just one component of the inflammatory response

which includes fever, synthesis of acute phase proteins and activation of the host immune system. It would seem that the host anti-tumor defense is only stimulated when the EL4 tumor burden has reached a very high level

In this study the EL4 cells were growing intraperitoneally and would thus obtain Tf Fe from the ascites fluid which is part of the extravascular Tf pool. The Tf Fe saturation of the ascites fluid was not measured in these studies as it would have been difficult to obtain sufficient ascites fluid from the mice. However, the extravascular Tf pool is in equilibrium with the serum Tf pool and a reduction in the Tf Fe saturation in the serum Tf pool would be reflected in the Tf Fe saturation of the ascites fluid

It has been shown in the case of murine infection with *Neisseria meningitidis* (Letendre and Holbein, 1984) that hypoferremia occurs because Fe from senescent red blood cells is not returned to the Tf pool but is stored in the RE system as ferritin. It is believed that this same mechanism operates during malignancy (Weinberg, 1984) and there may also be a reduction in intestinal Fe absorption (Lee, 1983).

The weights of the mice and of the liver, spleen and thymus were recorded and compared to that of normal controls (Tables 2 and 3). The liver and spleen were of interest as they are organs involved in Tf and Fe metabolism. The thymus was of interest as the tumor cell line used was originally a thymoma. Over a period of 20 days, the tumor-bearing mice gained an average of 8.3 (+/- 1.0) g while control mice gained 1.3 (+/- 0.8) g. A substantial portion of the weight gain in the tumor-bearing mice can therefore be attributed to the tumor burden and to the ascites fluid. Organ weights remained relatively constant in the control group. In the tumor group, the liver weight increased with body weight until day 11.

TABLE 2.

A comparison of whole body and liver weights of normal control and tumor-bearing mice over a 20-day experimental period.[1]

Time (Days)	Whole Mouse				Liver			
	Tumor-bearing		Control		Tumor-bearing		Control	
0	20.8	(1.0)[2] g	25.1	(0.4) g	1.14	(0.08) g	1.41	(0.02) g
2	21.9	(0.4)			1.30	(0.03)		
4	21.7	(0.8)	25.6	(0.6)	1.23	(0.04)	1.41	(0.05)
6	21.9	(1.0)			1.25	(0.04)		
8	21.8	(0.6)	24.3	(0.5)	1.26	(0.06)	1.39	(0.05)
11	24.5	(1.3)			1.39	(0 07)		
12			26.5	(0.5)			1.43	(0.04)
14	25.0	(1 2)			1.28	(0.05)		
16	27.2	(1.4)	26.9	(0.6)	1 23	(0.06)	1.48	(0.03)
18	28.1	(1.8)			1.17	(0.05)		
20	29.1	(1.7)	26.4	(0.8)	1.00	(0.12)	1.40	(0.05)

[1]Mice were inoculated (day 0) with 10^4 EL4 cells or 0.5 ml saline At the indicated times, the whole body, liver, spleen and thymus were weighed. Values represent the mean of 4 experiments (4 mice per point per experiment) for tumor-bearing mice and the mean of 9 mice for the control group. [2]Numbers in parentheses represent the standard error of the mean.

TABLE 3.

A comparison of the spleen and thymus weights of tumor-bearing and normal control mice.[1]

Time (Days)	Spleen		Thymus	
	Tumor-bearing	Control	Tumor-bearing	Control
0	0.08 (0.02)[2] g	0.08 (0.003) g	0.05 (0.01) g	0.05 (0.002) g
2	0.09 (0.007)		0.06 (0.006)	
4	0.08 (0.002)	0.10 (0.006)	0.05 (0.005)	0.05 (0.003)
6	0.08 (0 008)		0.06 (0.008)	
8	0.08 (0.004)	0.09 (0.004)	0.05 (0.009)	0.05 (0.005)
11	0.1 (0.008)		0.05 (0.005)	
12		0.10 (0.004)		0.05 (0.005)
14	0.09 (0.01)		0.05 (0.007)	
16	0.09 (0.008)	0.08 (0.003)	0.03 (0.006)	0.04 (0.003)
18	0.08 (0.012)		0.03 (0.005)	
20	0.07 (0.017)	0.09 (0.004)	0.03 (0.003)	0.05 (0.003)

[1]The experimental details were as described in Table 2.

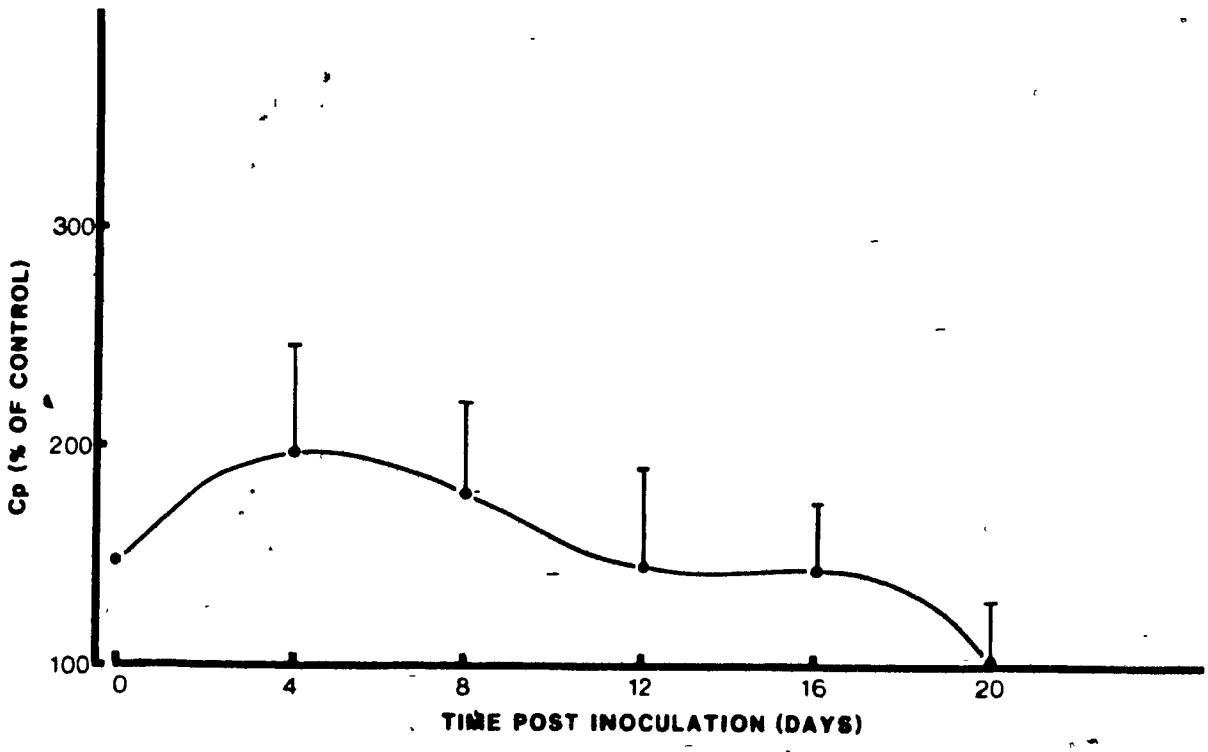
[2]Numbers in parentheses represent the standard error of the mean.

Subsequently, the liver weights decreased in the terminal stages of disease In Schade's study (1976), at three weeks post tumor cell inoculation, normal mice weighing an average of 18.9 g had an average liver weight of 0.80 g and tumor-bearing mice with an average weight of 22.0 g had an average liver weight of 1.1 g. During the acute phase response, there are many structural and biosynthetic changes which occur in the liver. The overall weight of the liver increases, the ribosomal RNA increases and the amount of smooth endoplasmic reticulum increases, also the membranes of the Golgi apparatus proliferate and there is an increase in microtubules (Lee, 1983). This might account for some of the increase in liver weight during the middle phase of the experiment Spleen and thymus weights also showed a decrease at the end of the experimental period Weight reduction may be a reflection of tissue destruction which accompanies tumor growth and tissue invasion. It is not known how this would affect organ function or synthesis of proteins by the liver

The serum ceruloplasmin (Cp) response was also followed in tumor-bearing and normal mice (Fig. 5). Serum Cp activity of tumor-bearing mice is expressed as a percentage of control values Cp is of interest for two reasons: it is involved in Fe metabolism by virtue of its ferroxidase activity and serum levels have been found to increase in patients with lymphomas and other neoplastic diseases (Shah *et al.*, 1984; Margerison and Mann, 1985; Linder *et al.*, 1981). Work has been done to evaluate the use of Cp as a marker to monitor the course of malignant disease (Linder *et al.*, 1981; Shah *et al.*, 1984; Margerison and Mann, 1985, Mercer and Talamo, 1985).

In the present study, there was a slight rise in Cp levels on days 4

Fig 5 Serum Cp activity in tumor-bearing mice. C57 mice were injected with 10^4 EL4 cells or saline and were sacrificed by cardiac puncture at designated times and Cp oxidase levels were determined on the sera by the method of Ravin (1961). The Cp values, averaged from 3 experiments, are calculated as percentage of control values (+ the standard error or the mean).


300
200
100
Cp (% OF CONTROL)
0
4
8
12
16
20
TIME POST INOCULATION (DAYS)

and 8, however, this was not found to be statistically significant ($P < 0.05$, Student's t-test). In another study, (Linder *et al.*, 1979) in rats with transplantable mammary or hepatic tumors, Cp oxidase activity increased 50-200% during neoplastic growth. This was found to be due to both increased synthesis of Cp and increased oxidase activity Capel and Thornley (1982) found that the plasma Cp oxidase levels were not significantly increased in C57 black mice injected with Lewis lung carcinoma. It is therefore likely that Cp response to tumor growth varies with the species of animal and the type of neoplasm studied. As discussed in section 2.5 it was found that when C57 mice were injected with *N. meningitidis* serum Cp levels rose to greater than 200% of control values at 24 h post infection (Letendre and Holbein, 1984a). These authors postulated that the rise in serum Cp levels which occurs during the convalescent phase of infection may be a response which follows hypoferremia to re-establish normal serum Fe levels. In this tumor model, in contrast to the murine meningococcal infection model, the disease state worsens, the serum Fe levels become lower and the animals subsequently die. As there is no recovery phase to either the disease process or the hypoferremic response there may be no response on the part of the host to re-establish normal serum Fe levels. This may provide at least a partial explanation for the limited rise in Cp activity.

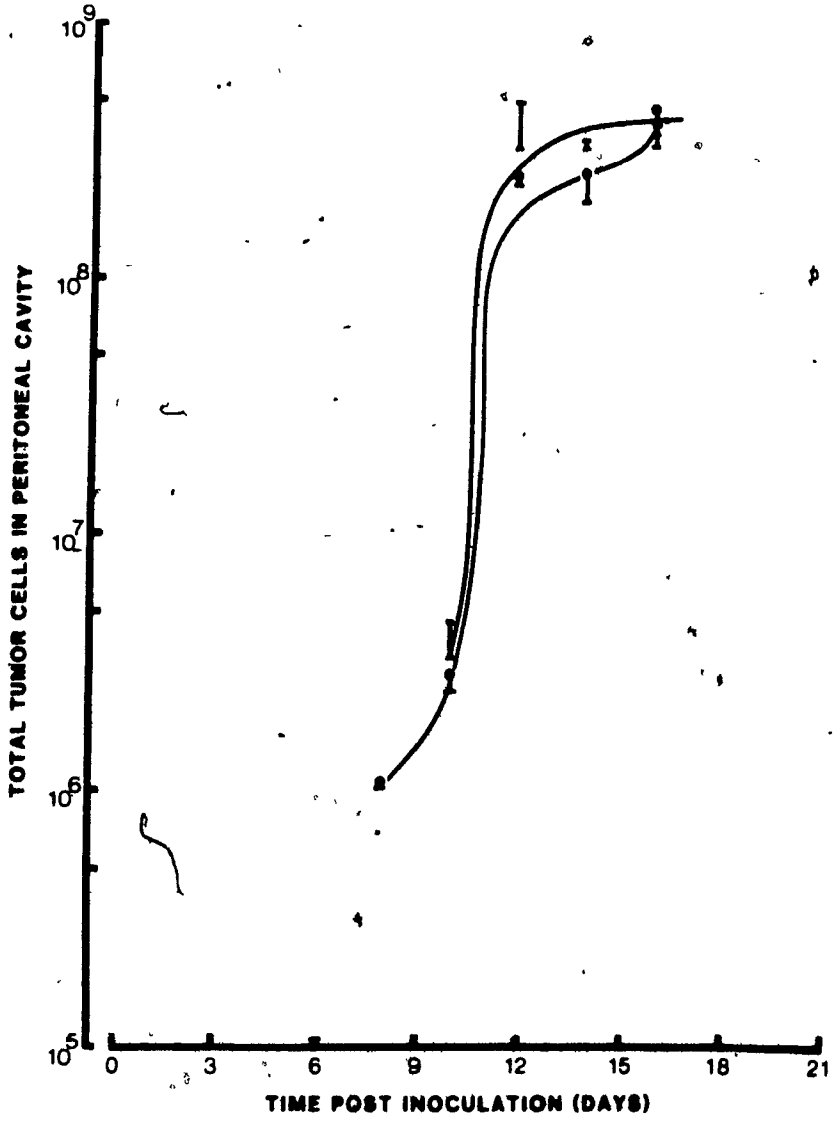
4.1.2 The effect of transferrin Fe administration on tumor cell growth

In the next phase of the study, an attempt was made to manipulate tumor cell growth *in vivo* by either supplying more Fe to the tumor cells or

depriving the cells of Fe. In an initial study (Fig. 6), mice were injected with 2 mg 100% saturated human Tf containing 2.8 ug Fe on several days during the logarithmic phase of EL4 cell growth. This extra Fe did not enhance growth compared to growth in control mice treated with saline It could be argued that the murine tumor cells would be unable to utilize human Tf. By this point in the work, however, the EL4-1 subline had been established *in vitro* and was growing well with human Tf as the Fe source, demonstrating that murine cells can efficiently utilize human Tf Fe Furthermore, *in vitro* studies demonstrated that 1 X 10^5 EL4-1 cells could proliferate when provided with 6 ug ml^{-1} 10% Fe-saturated human Tf providing 0.00084 ug ml^{-1} Fe (Fig. 13). Therefore 2 mg 100% Fe-saturated Tf carrying 2.8 ug Fe should be adequate to provide additional Fe for the larger number of cells growing *in vivo* Holbein (1981) found that human Tf stimulated *Neisseria meningitidis* infection in mice. Injection of 17 5 mg of Tf containing 22.7 ug Fe (93% saturated) caused 100% mortality compared to no deaths among the control infected mice. A lower dose, 5 mg Tf bearing 6.5 ug Fe (93% saturated), stimulated and prolonged infection In the case of *Neisseria* infection, the hypoferremic response has been shown to be inhibitory (Holbein, 1980) to *N. meningitidis* growth, injections of Tf-Fe therefore made Fe available and enhanced growth.

In the *Neisseria meningitidis* system, Fe-dextran was also found to enhance infection (Holbein *et al.*, 1979; Holbein, 1980). Dosages higher than 15 mg kg^{-1} prolonged and exacerbated infection, while dosages above 125 mg kg^{-1} produced fatal infections (Holbein, 1980).

Fig. 6 The effect of added exogenous transferrin Fe on EL4 tumor cell growth in mice. Mice were inoculated with 10^4 tumor cells. Mice treated with Tf (▲) were given 2 mg of 100% saturated Fe-Tf (2.8 ug of Fe) in 0.4 ml saline i.p. on days 9, 11, 13, 15 and 17 post tumor cell inoculation. Control mice were treated with an equivalent volume of saline (●). Points represent the average number of tumor cells from 2 mice in one experiment.


TOTAL TUMOR CELLS IN PERITONEAL CAVITY
10^9
10^8
10^7
10^6
10^5
0
3
6
9
12
15
18
21
TIME POST INOCULATION (DAYS)

4.1.3 The effect of desferrioxamine on tumor cell growth

An attempt was made to deprive tumor cells growing *in vivo* of Fe by injecting mice i.p with the Fe-chelating drug desferrioxamine. Desferrioxamine is a hydroxamic acid type of microbial siderophore which binds Fe with high affinity in a 1:1 ratio Its methane sulfonate derivative is more water-soluble and is used clinically (under the Ciba-Geigy trade name Desferal) to treat patients for Fe-overload by the intramuscular, intravenous, or subcutaneous routes (Hoffbrand, 1980). Desferal was used in this study. Desferrioxamine is believed to remove Fe from the RE system and from hepatocytes by chelating Fe from the low molecular weight labile Fe pool. Ferrioxamine (Fe^{+3}) is then excreted in the urine and bile (Hoffbrand, 1980). During hypoferremia, Fe from senescent red blood cells is stored in the RE system and this recently acquired Fe appears to be more easily chelated than the remainder of the RE Fe (Hershko and Rachmilewitz, 1978). It was thought that desferrioxamine injections might reinforce the hypoferremic response by removing Fe from the RE system allowing for more Fe storage and a greater hypoferremic response. It is not known how much Fe could be removed from the mice using this protocol as this compound has a very short half-life *in vivo* (5-10 minutes for an intravenous injection, Summers, *et al.*, 1979).

Initially, (days 5-11, Fig. 7) the growth rate of tumor cells appeared to be slower in desferrioxamine-treated mice. Tumor cell numbers reached the same level in the later stages of the disease, however, and the treatment did not increase the survival time of the mice. Serum Fe levels were measured in the two groups of mice in one of the two studies (Table 4)

Fig. 7 The effect of desferrioxamine injections on the *in vivo* growth of EL4 cells. Mice were inoculated with 10^4 cells. Desferrioxamine-treated mice (■) were injected i.p with 5 mg of desferrioxamine on days 0, 2, 4, 6, 8, 10, 12, 14, 16, and 18 or were given no further desferrioxamine treatment after day 12 (▲). Control mice (●) were injected with PBS. The points represent the average cell numbers harvested from 3 mice in each of 2 separate experiments (+/- the standard error of the mean).

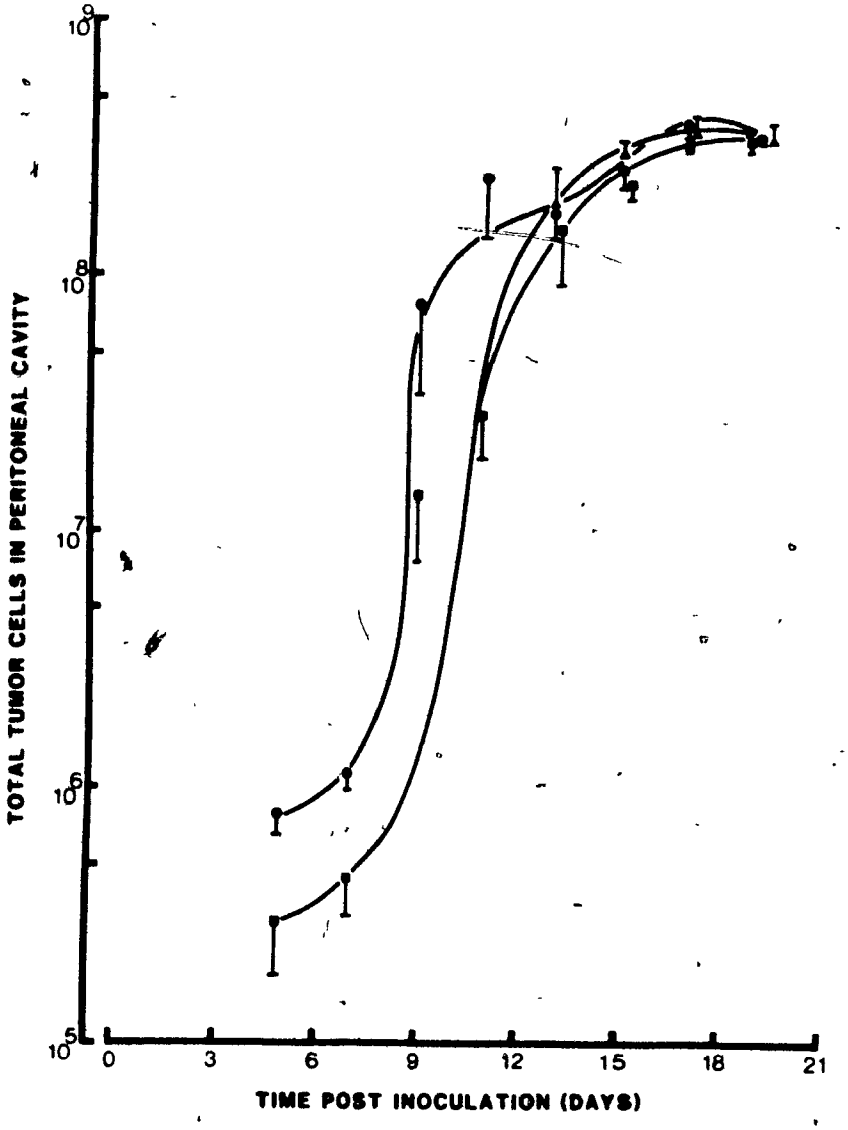

TOTAL TUMOR CELLS IN PERITONEAL CAVITY
10^9
10^8
10^7
10^6
10^5
0
3
6
9
12
15
18
21
TIME POST INOCULATION (DAYS)

TABLE 4.

Serum Fe response in control and desferrioxamine-treated mice.[1]

Time (Days)	Control Serum Fe (ug ml^{-1})	Control % saturation of Tf	Desferrioxamine-Treated Serum Fe (ug ml^{-1})	Desferrioxamine-Treated % saturation of Tf
0	1.13	44.2		
3	1.86	55.6	2.30	64.1
5	1.72	54.1	2.03	64.1
7	1.40	45.5	1.85	56.6
9	1.40	46.2	1.87	60.4
11	0.95	29.1	1.43	42.4
13	0.54	15.7	0.42	11.3
15	0.41	12.3	0.31	9.3
17	0.34	10.3	0.17	5.5

[1]Mice were inoculated with 10^4 EL4 cells. Desferrioxamine-treated mice were injected i.p. with 5 mg of desferrioxamine on days 0, 2, 4, 6, 8, 10, 12, 14, 16, and 18. Control mice were injected i p. with 0 5 ml phosphate-buffered saline. Four mice were bled by cardiac puncture for serum Fe determination at each of the times indicated. The serum samples from the four mice were pooled and serum iron was determined using a Becton Dickinson radioassay kit.

There was no augmentation of hypoferremia due to desferrioxamine treatment in the early stages of the experiment, in fact serum Fe levels were higher in the desferrioxamine group. On days 13, 15 and 17 serum Fe and Tf saturation levels were lower in the desferrioxamine-treated group. On the basis of the serum Fe levels, the slower tumor cell growth rate cannot be attributed to an initial enhanced hypoferremic response. Perhaps desferrioxamine itself injected into the peritoneal cavity had an antiproliferative effect on the EL4 cells. This might be due to desferrioxamine entering the tumor cells and chelating Fe required as an enzyme co-factor for ribonucleotide reductase or any of the numerous other Fe-requiring enzymes. Lederman *et al.* (1984) studied the effect of desferrioxamine in lymphocytes *in vitro* and found it inhibited DNA synthesis; this drug decreased intracellular levels of deoxyribonucleoside triphosphates and blocked cells in the S phase of the cell cycle The short half-life of the drug *in vivo* due to urinary excretion might, however, argue against this idea.

In certain other disease states desferrioxamine treatment has been found to be beneficial. Holbein *et al.* (1979) found that fatal Fe-dextran enhanced infections could be reversed if mice were injected with 1,250 mg kg^{-1} desferrioxamine up to 3 h after infection with 10^3 or fewer *Neisseria meningitidis* cells. Lalonde and Holbein (1984), studying the role of Fe in *Trypanosoma cruzi* infection in mice, found that a combination of desferrioxamine treatment and an Fe-deficient diet reduced the pathogenicity of this intracellular pathogen of the RE system in both susceptible C_3H mice and moderately resistant B_6 mice.

4.1.4 The effect of inflammation-induced hypoferremia on tumor cell growth

Another attempt was made to decrease *in vivo* growth of EL4 cells by Fe deprivation. In the initial *in vivo* studies (Fig. 3 and Fig. 4), it was noted that the onset of the hypoferremic response occurred late in the disease when the tumor burden was high. It appeared that the response occurred too late to be of benefit to the host. The question then arose, would an early hypoferremic response lower serum Fe levels sufficiently to inhibit tumor growth? This could be answered using turpentine to create a model of hypoferremia. Mice were injected subcutaneously in the back with turpentine and this induced a marked hypoferremic response (Fig 8). Serum Fe levels had fallen by 12 h and reached their lowest level (0.65 ug ml^{-1}, 19.6% Fe saturation of Tf) by 24 h. The reduction in serum Fe levels between 12 and 36 h was found to be highly significant ($P < 0.001$, Student's t-test). Between 48 and 60 h levels of serum Fe had returned to normal Twenty-four h after turpentine injection serum TIBC had risen dramatically

If at 48-60 h post inoculation serum Fe levels had returned to normal but the amount of Tf in the pool was increasing (shown by increase in the TIBC values) this would in effect result in a prolonged reduction in Tf Fe saturation. Therefore in the next study, mice were injected subcutaneously with either turpentine or saline and injected one h later with tumor cells. In the early hypoferremic response (Fig. 9), serum Fe levels dropped to 0.62 ug ml^{-1} (16.9% saturation) at 24 h post injection, the Fe kinetics being somewhat different than in the initial turpentine studies (Fig. 8). Serum Fe had increased by day 6 and there was then a second

Fig. 8 The hypoferremic response during turpentine-induced inflammation in mice. Mice were lightly anaesthetized with ether and injected subcutaneously in the back in two separate sites with 0.05 ml turpentine. At the indicated time points, 6 mice were bled by cardiac puncture for determination of TIBC (▲), UIBC (●), Tf-Fe (■), and ~~Cp~~ oxidase activity (○). Values shown represent the average data from two experiments.

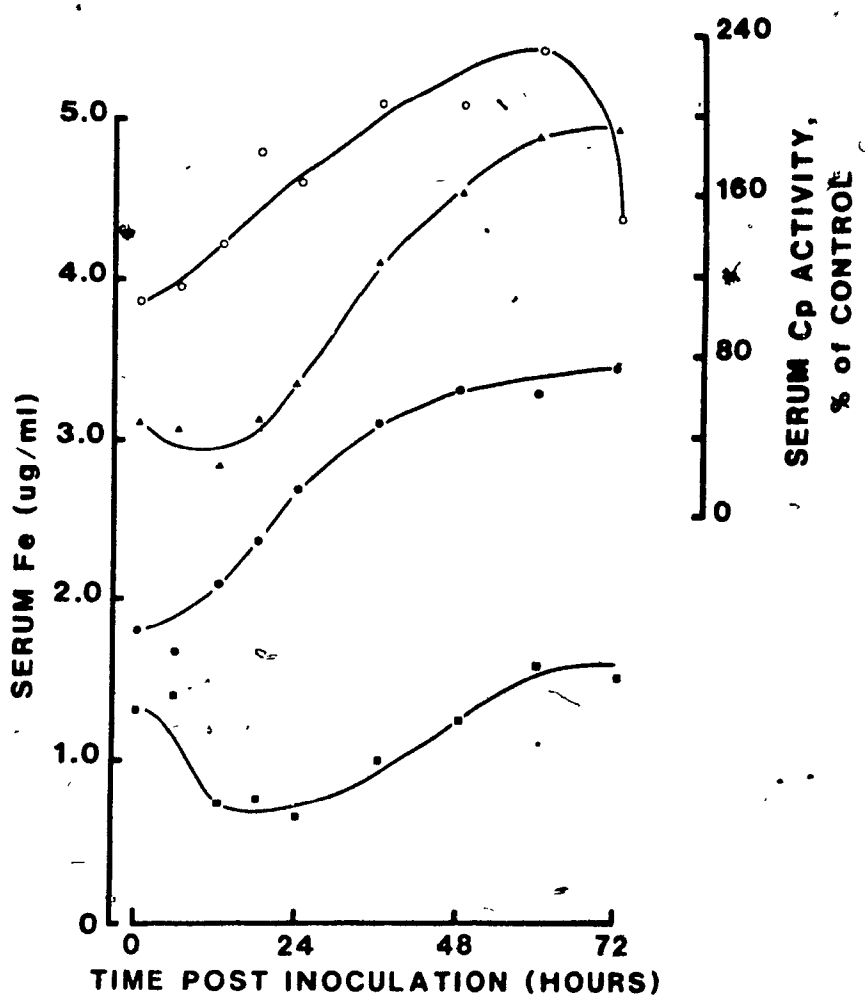

5.0
4.0
3.0
2.0
1.0
0
SERUM Fe (ug/ml)
240
160
80
0
SERUM Cp ACTIVITY, % of CONTROL
0
24
48
72
TIME POST INOCULATION (HOURS)

Fig. 9 Changes in Fe metabolism due to turpentine-induced inflammation and EL4 tumor cell growth in mice. Mice were injected subcutaneously in the back with 0.05 ml turpentine in two separate sites. One h later, mice were injected with 10^4 tumor cells. At the indicated times, 4 mice were bled by cardiac puncture to determine TIBC (●), UIBC (□), and Tf-Fe (○).

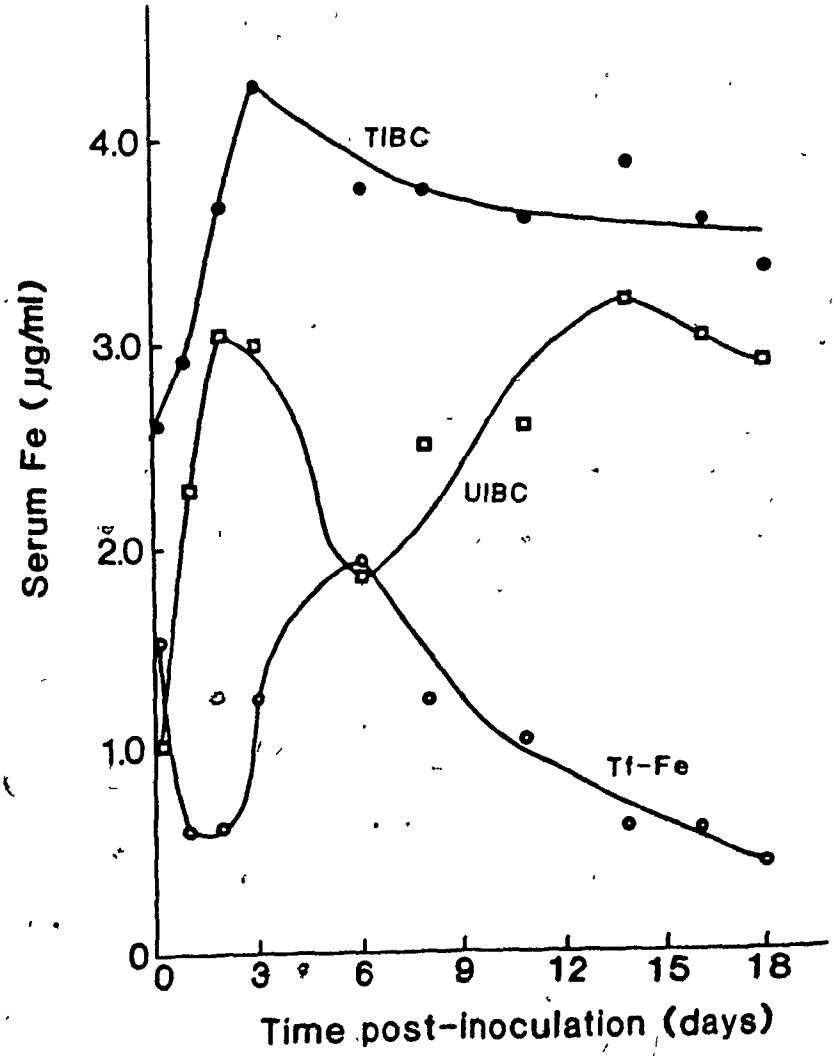
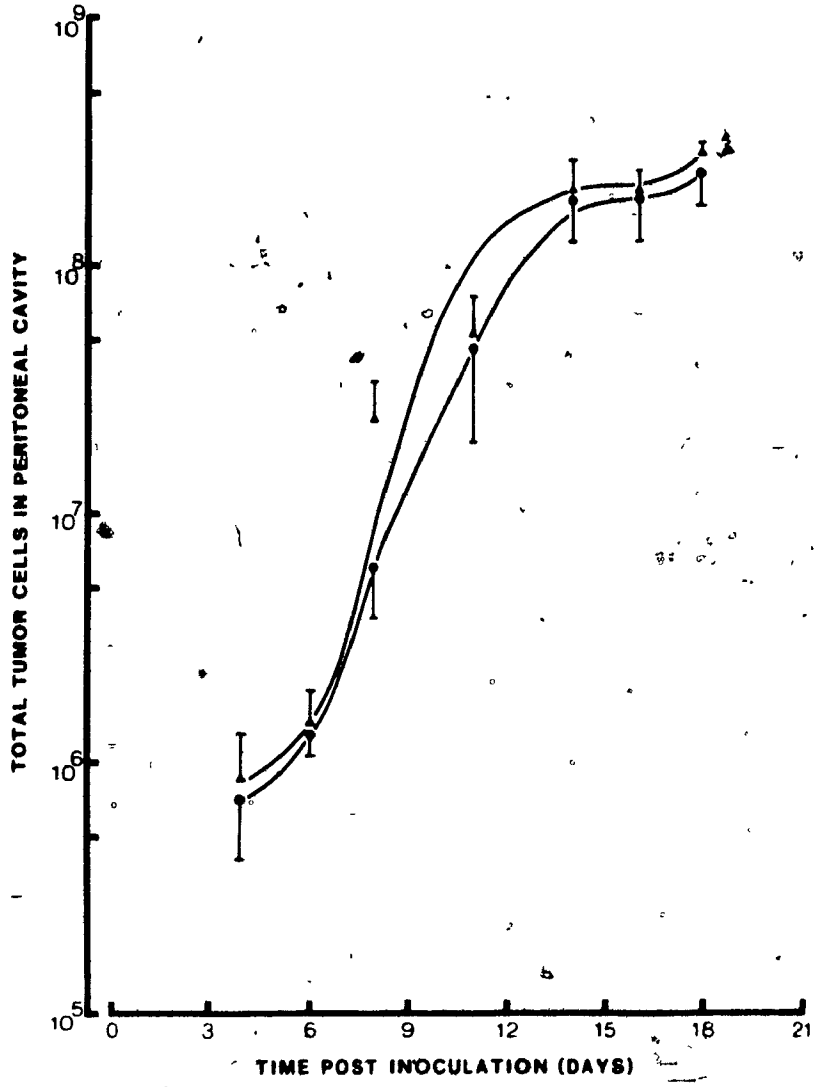

TIBC
UIBC
Tf-Fe
Serum Fe (µg/ml)
4.0
3.0
2.0
1.0
0
0
3
6
9
12
15
18
Time post-inoculation (days)

Fig. 10 The influence of turpentine inflammation-induced hypoferremia on the growth of EL4 tumor cells in mice. Mice were injected subcutaneously in the back in two sites with 0.05 ml of either saline (●) or turpentine (▲). One h later they were injected with 10^4 tumor cells. Cells counts were performed at the appropriate times. The indicated values represent average tumor cell numbers recovered from 6 mice in two experiments.


TOTAL TUMOR CELLS IN PERITONEAL CAVITY
10^9
10^8
10^7
10^6
10^5
0
3
6
9
12
15
18
21
TIME POST INOCULATION (DAYS)

hypoferremia due to the proliferation of tumor cells. After turpentine administration, the TIBC rose and remained high throughout the experiment. Although Tf Fe saturation levels remained low throughout most of the experiment, no inhibition of tumor cell growth was observed (Fig. 10).

In contrast to this, a similar study was carried out with *Neisseria meningitidis* infection in C57 mice (Beaumier *et al.*, 1984). Twelve h after mice were induced to become hypoferremic with turpentine injections, they were inoculated with *Neisseria meningitidis*. In the hypoferremic group, the peak of infection was significantly lower. Also, the infection was cleared in 5.2 h in turpentine-treated mice as opposed to 10.4 h in saline control mice

When turpentine is injected hypoferremia is induced and the whole inflammatory process is stimulated, resulting in increased synthesis of acute phase proteins. This is demonstrated in the turpentine studies by enhancement of Cp activity (Fig. 8). Cp activity had increased by 12 h post turpentine inoculation and reached 235% of control levels at 60 h, a much greater stimulation of Cp activity than that observed due to tumor cell growth (Fig. 5). It is interesting to note that lowering of serum Fe in combination with other facets of the inflammatory response induced by turpentine injections had no inhibitory effect on *in vivo* EL4 tumor cell growth.

Both *Neisseria meningitidis* and EL4 cells must acquire Tf Fe from the host in order to proliferate. However, the hypoferremic response has a much different effect in the two systems. In the case of murine infection with the meningococcus, infection can be Fe enhanced with Fe-dextran or Fe-Tf or can be Fe-controlled e.g. by subcutaneous turpentine injection or merely by

the naturally-occurring hypoferremic response. The onset of the hypoferremic response is immediate and beneficial to the host. In the case of EL4 cells the hypoferremic response occurs late in the disease and is of no real benefit to the host. With the protocols used in these studies the *in vivo* growth of tumor cells could not be influenced by Fe addition or deprivation. It would seem that EL4 cells have very low Fe requirements perhaps due to a primarily glycolytic metabolism and/or are equipped to obtain sufficient Fe for all their needs under the adverse conditions of low Tf Fe saturation.

In order to define the Fe requirements of these tumor cells for growth and to assess the effect of a reduction in Tf Fe saturation on Fe acquisition and growth, we turned to an *in vitro* system using the EL4-1 cells.

4.2 *In Vitro* Growth Experiments

4.2.1 The effect of transferrin Fe saturation on *in vitro* growth of EL4-1 cells

The first *in vitro* experiment was designed to demonstrate that EL4 cells do require Fe for growth. When EL4 cells were grown in RPMI 1640 with 10% fetal bovine serum (Fig. 11) cells grew rapidly reaching their maximal number by day 3 (1.7×10^6 cells ml^{-1}). With the addition of 50 ug ml^{-1} desferrioxamine growth was significantly inhibited. When the desferrioxamine was saturated with Fe, growth was identical to that of the control indicating that the growth inhibition was directly due to Fe deprivation.

All further *in vitro* work was carried out using the EL4-1 subline; these cells were adapted to growth in RPMI 1640 synthetic medium and human Tf. The subline was developed by culturing the original EL4 cells in RPMI containing human Tf only and repeatedly passaging the cells in this medium. The growth of the cells in the absence of serum was initially poor with a cell doubling time of 72 h but repeated subculture in this medium yielded the EL4-1 subline with an average doubling time of 24 h. The original EL4 cells growing in serum containing medium demonstrated an average doubling time of 12 h. The two cell lines showed an identical time course of tumorigenesis in mice. Even after passage through a mouse, the EL4-1 cells retained their ability to grow in the absence of serum. These cells grew well (Fig. 12) on 100% saturated human Tf (50 ug ml^{-1}) It was found that the addition of 50 ug ml^{-1} apo-Tf enhanced growth by binding medium Fe

Fig. 11 The effect of desferrioxamine on the *in vitro* growth of EL4 cells. EL4 cells were inoculated into RPMI 1640 containing 10% F.B.S. at an initial level of 1 X 10^5 cells ml^{-1} with no further additions (▲) or with the addition of 50 ug ml^{-1} desferrioxamine (●) or with 50 ug ml^{-1} Fe-saturated desferrioxamine (■). Cell counts were performed at the times indicated in a hemacytometer using trypan blue exclusion.

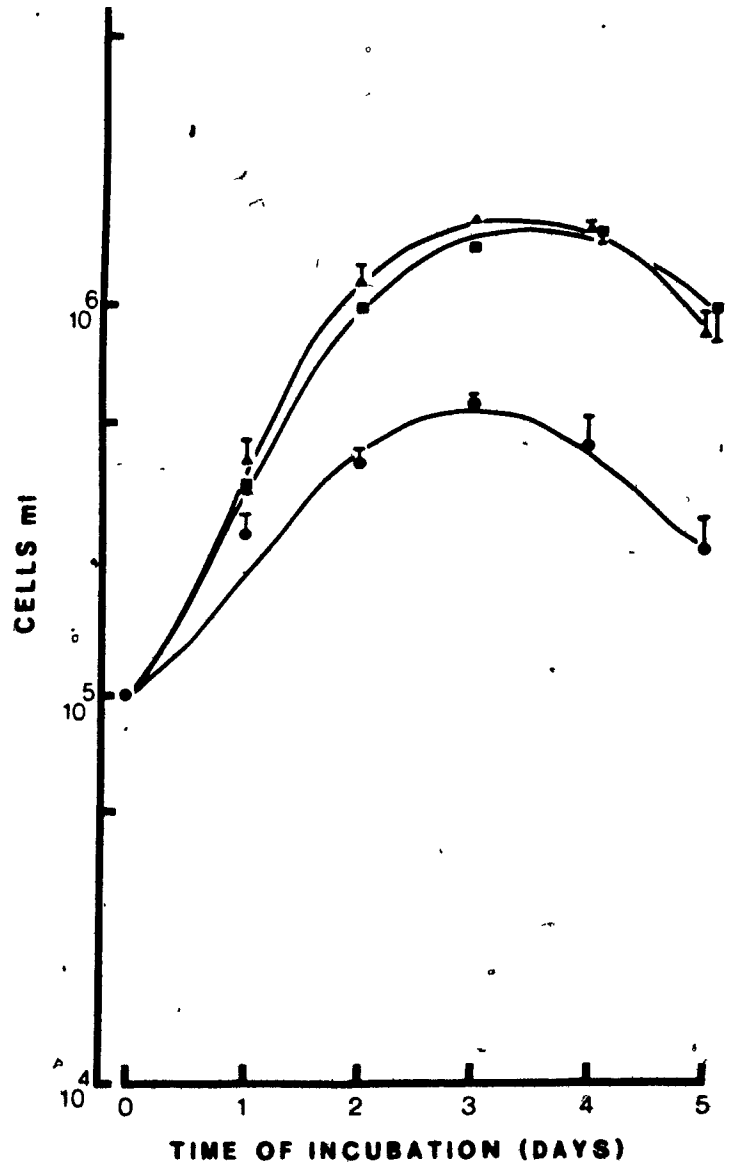
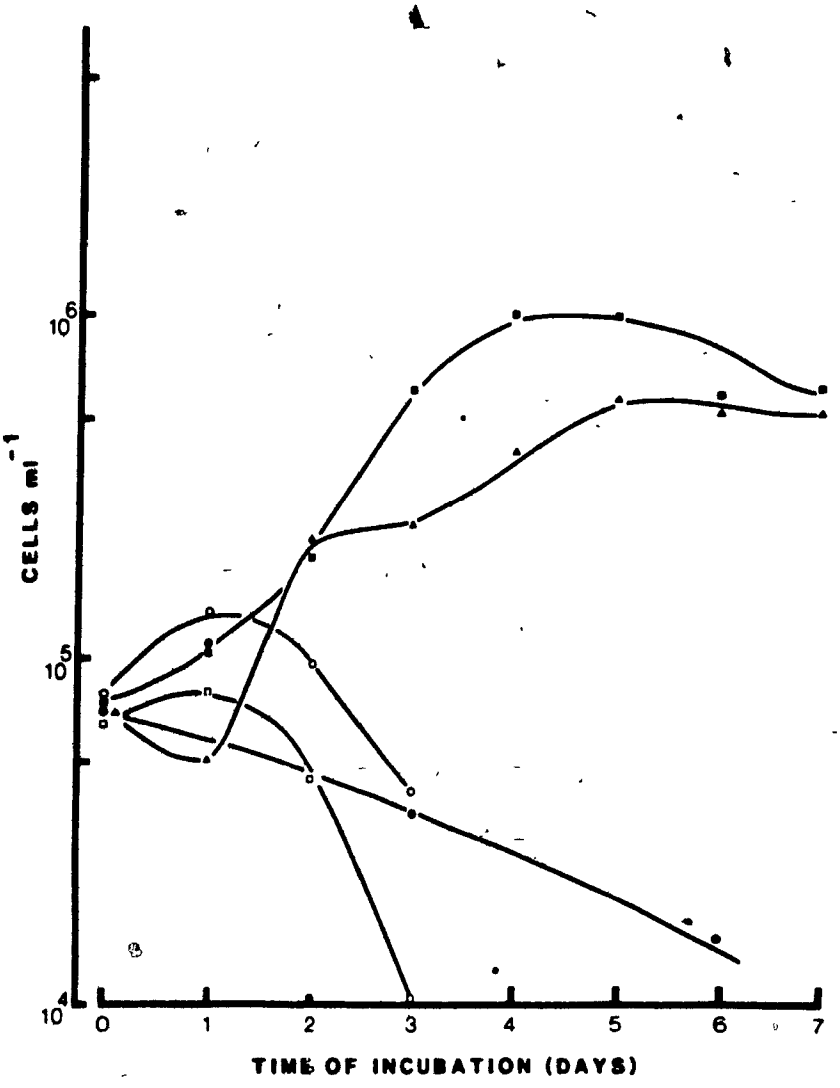

CELLS ml⁻¹
10⁶
10⁵
10⁴
0
1
2
3
4
5
TIME OF INCUBATION (DAYS)

Fig. 12 Growth of EL4-1 cells *in vitro* on human transferrin. EL4-1 cells were inoculated into RPMI containing 50 ug ml^{-1} 100% saturated human Tf (■), 50 ug ml^{-1} apo-Tf (Δ), 50 ug ml^{-1} boiled apo-Tf (□), 50 ug ml^{-1} human serum albumin (O) or into RPMI containing no additions (●). Cells were enumerated at the appropriate times as described in Fig. 11.


CELLS ml^{-1}
10^6
10^5
10^4
0
1
2
3
4
5
6
7
TIME OF INCUBATION (DAYS)

while boiled apo-Tf or human serum albumin had no growth-enhancing effects.

It was found using atomic absorption spectrophotometry, that RPMI 1640 contained 0.019 +/- 0.009 ug ml^{-1} (0 33 +/- 0 16 uM) total Fe Theoretically, this is enough Fe to 27% Fe saturate 50 ug ml^{-1} apo-Tf. Therefore for all subsequent *in vitro* studies RPMI was Fe extracted by incubating it in the presence of a two-fold molar excess of apo-Tf for 20 h at 37°, 5% CO_2. Ferrated Tf was then removed by Amicon ultra-filtration as discussed in section 3.4.1.

The question of the effect of a decrease in Tf Fe saturation on EL4-1 cell growth was then directly addressed. Cells were grown in RPMI containing 6 ug ml^{-1} (75 nM) human Tf at 100%, 50% and 10% saturation and with no Tf (Fig. 13). The media thus contained 0.0084 (0 15 uM), 0 0042 (0 075 uM), 0.00084 (0.015 uM) and 0 ug ml^{-1} Fe respectively Fig. 13 demonstrates that the cells do not proliferate in the absence of Tf-Fe and that cells can grow with very little Tf-borne Fe present. In this experiment, the cells did grow better on 100% and 50% saturated Tf than on 10% saturated Tf. However, this may have been a function of the low concentration of Fe available at the 10% saturation level rather than a function of Fe availability due to low percent saturation. In fact in this experiment, at the low level of Tf Fe provided, at each saturation level peak growth of the cells was directly proportional to the amount of Tf Fe available. In the next experiment, the Tf concentrations were raised to 50 ug ml^{-1} (Fig. 14). Growth was good in all cases with virtually no difference between 100% and 50% Fe saturation (1.25 and 0.625 uM Fe respectively). By day 4 when peak growth was reached at both these saturation levels cell density was 1 X 10^6 cells ml^{-1} representing the maximal

Fig. 13 Growth of EL4-1 lymphoma cells on serum-free RPMI 1640 with 75 nM human transferrin at 100%, 50% and 10% saturation. EL4-1 cells were removed from the peritoneal cavity of a mouse and grown for 48 h in Fe-extracted RPMI containing 0.3 uM 50% Fe-saturated human Tf. Cells were washed and incubated for 24 h (37° C, 5% CO_2) in RPMI containing no Fe or Tf. Cells were then washed and inoculated at an initial level of 1 X 10^5 cells ml^{-1} into Fe-extracted RPMI containing 75 nM (6 ug ml^{-1}) human Tf of 100% (■), 50% (●) and 10% saturation (▲) or into media containing no Tf (□). These conditions represent 0.15, 0.075, 0.015 and 0.0 uM total Tf Fe respectively. Growth was assessed as in Fig. 11.

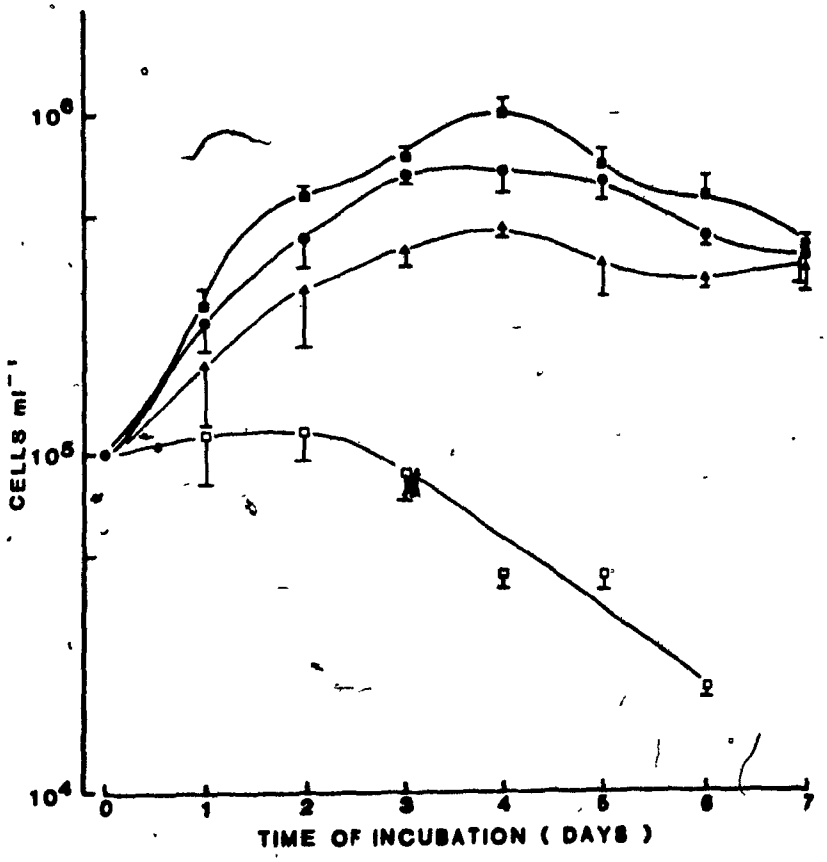
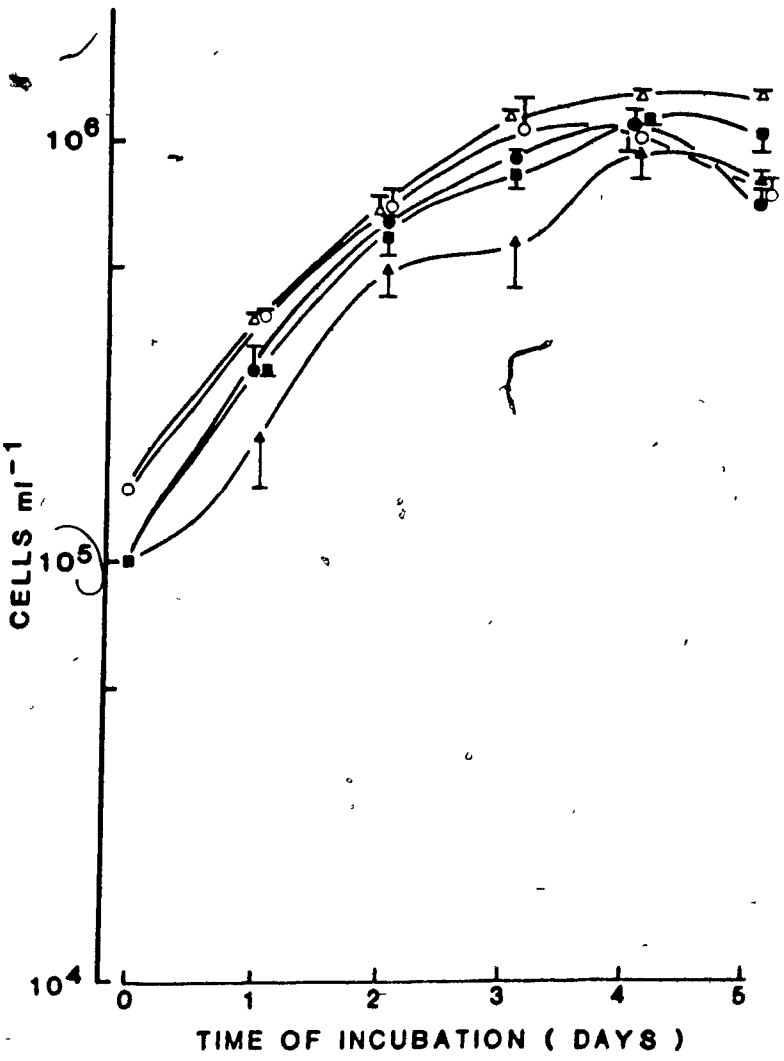

10^6
10^5
10^4
CELLS ml^{-1}
0
1
2
3
4
5
6
7
TIME OF INCUBATION (DAYS)

Fig 14 Growth of EL4-1 lymphoma cells on serum-free RPMI with 0 625 uM or 37.5 uM human transferrin at various saturation levels. The experiments were performed as described in Fig. 13 but media contained 0.625 uM (50 ug ml^{-1}) Tf at 100% (■), 50% (●) and 10% (▲) saturation or 37 5 uM (3.0 mg ml^{-1}) Tf at 50% (O) and 10% (Δ) saturation. These trials contained 1.25, 0.625, 0 125, 37 5 and 7.5 uM Fe respectively.


10^6
10^5
10^4
CELLS ml^{-1}
0
1
2
3
4
5
TIME OF INCUBATION (DAYS)

saturation density for these cells. Thus growth was not Fe limited. Growth at 10% saturation (0.125 uM Fe) was good but slightly lower. If available Tf concentrations were raised to the physiological level of 3 mg ml^{-1} (37.5 uM Tf) there was no difference in growth between cells grown with 50% saturated Tf (2 1 ug ml^{-1} or 37.5 uM Fe) and cells grown with 10% Fe saturated Tf (0.42 ug ml^{-1} or 7.5 uM Fe). This decrease in Fe saturation from 50% to 10% mimics the decrease in Tf Fe saturation which would occur during the hypoferremic response *in vivo*. At 10% saturation and physiological Tf levels cells demonstrated excellent growth, at 10% saturation and Tf concentrations 1/60 of physiological levels, cells demonstrated good growth indicating that even at very low saturation levels Tf Fe is readily available for tumor cell proliferation.

When cells were inoculated at a lower initial level of 1 X 10^4 cells ml^{-1} cells grew only slightly more slowly on 10% saturated Tf than 50% saturated Tf at physiological Tf levels and cells reached the same final saturation density (Fig. 15). This figure also demonstrates that these cells can grow on bovine hemoglobin providing 17 9 uM Fe although growth is noticeably poorer than growth on 10% saturated Tf providing 7.5 uM Fe.

4.2.2 Growth of EL4-1 cells on non-transferrin Fe sources

Figure 16 shows that EL4-1 cells can grow on bovine hemoglobin, horse spleen ferritin and the inorganic Fe salt ferrous ammonium sulfate. The cells grew better on this latter source of Fe when 50 ug ml^{-1} apo-Tf was added to the medium as apo-Tf is able to bind the Fe and render it directly available to the cells. The cells grew better on bovine

Fig. 15 Growth of EL4-1 cells on physiological transferrin levels and bovine hemoglobin. EL4-1 cells, prepared as described in Fig. 13, were inoculated at an initial level of 1 X 10^4 cells ml^{-1} into RPMI containing 37.5 uM (3.0 mg ml^{-1}) human Tf at 50% (▲) and 10% saturation (■) and containing no Tf (O). Cells were also inoculated into a flask containing bovine hemoglobin containing 17.9 uM Fe (●).

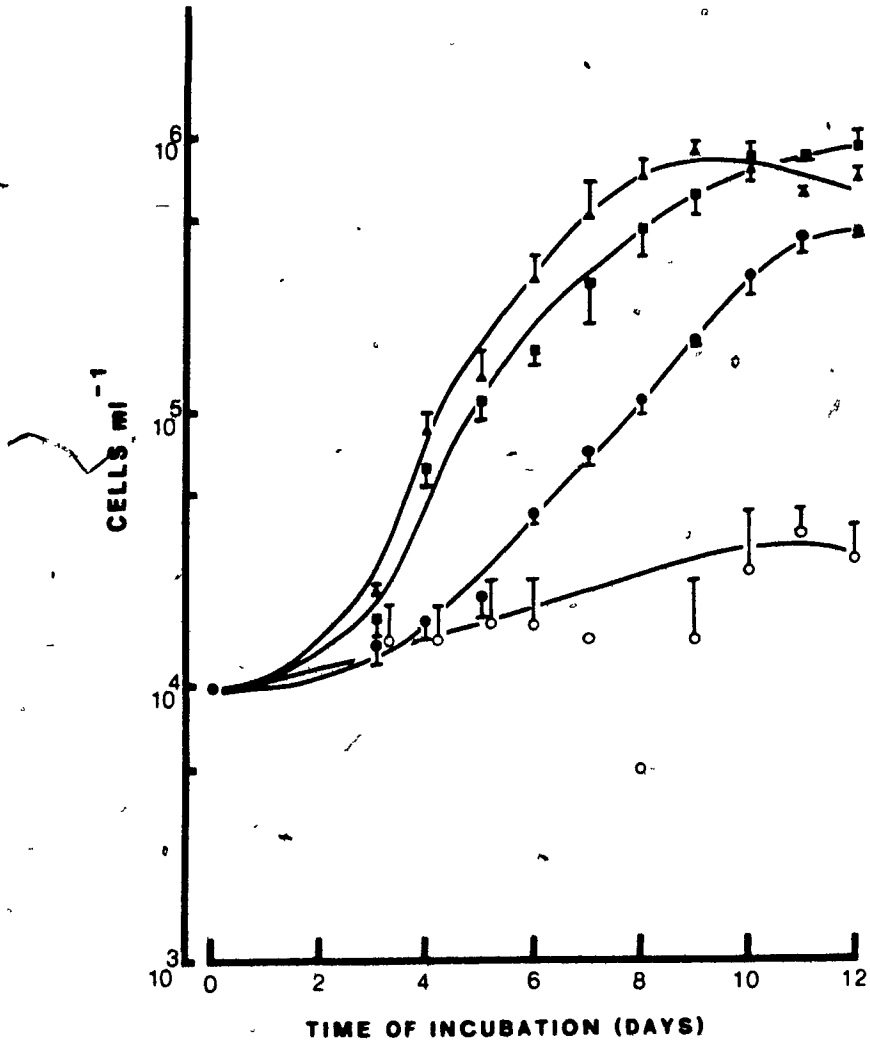
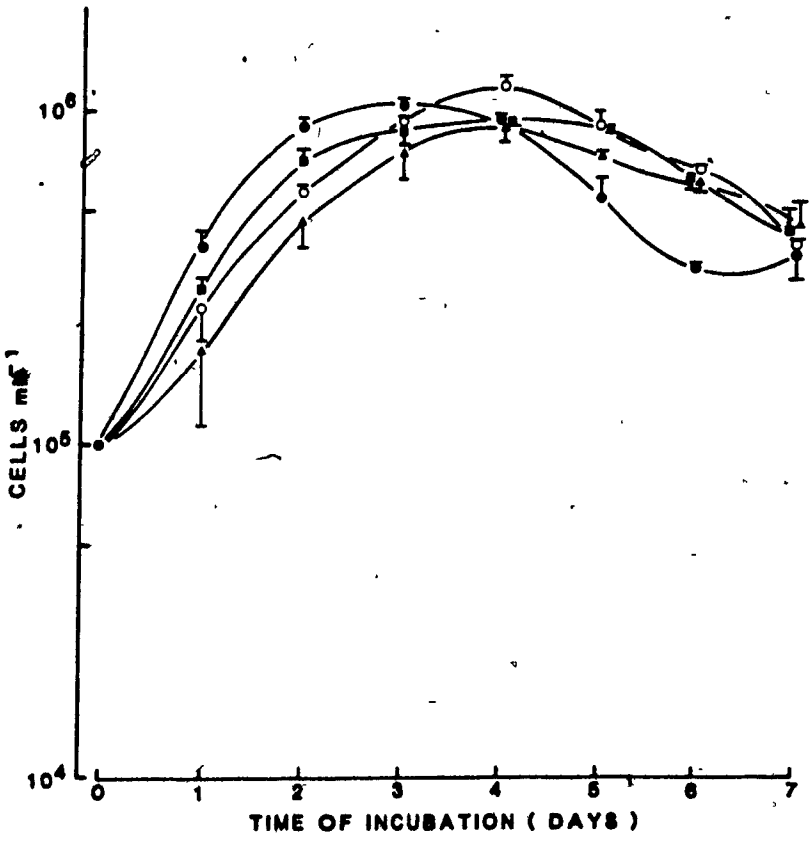

CELLS ml^{-1}
10^6
10^5
10^4
10^3
0
2
4
6
8
10
12
TIME OF INCUBATION (DAYS)

Fig. 16 Growth of EL4-1 cells on various Fe complexes. EL4-1 cells, prepared as described in Fig. 13, were inoculated at a starting level of 1 X 10^5 cells ml^{-1} into RPMI containing bovine hemoglobin (●), horse spleen ferritin (○), ferrous ammonium sulfate (▲) and ferrous ammonium sulfate with 50 ug ml^{-1} human apo-Tf (■). All Fe sources were added to give a final Fe concentration of 17.9 uM (1 ug ml^{-1}).


10^6
10^5
10^4
CELLS ml^{-1}
0
1
2
3
4
5
6
7
TIME OF INCUBATION (DAYS)

hemoglobin when started at a higher initial number of 1 X 10^5 cells ml^{-1} than when they were started at 1 X 10^4 cells ml^{-1} (Fig. 15) It is not known whether inorganic Fe was directly available to the cells or whether it was made available by traces of Tf which could have been transferred with the cells. Attempts were made to minimize this by washing cells and incubating them in Fe and Tf-free media for 24 h then washing the cells again before using them in an experiment.

These results are interesting for two reasons. First, the results demonstrate that EL4-1 cells can utilize Fe sources other than Tf for growth. Titeux *et al.* (1984) also found that Fe salts, hemin or hemoglobin could replace Tf for serum-free culture of 3 human leukemic cell lines Hemoglobin would probably not normally be available *in vivo* as free heme or hemoglobin from lysed erythrocytes is normally bound by hemopexin or haptoglobin and removed from the circulation. During the tissue destruction which may accompany neoplastic disease, however, hemoglobin Fe might become available to the tumor cells Secondly, these studies demonstrate that Tf is a much more efficient Fe donor to the cells than the other Fe sources studied. If Fig. 14 and Fig. 16 are compared it can be seen that the lymphoma cells grew almost as well on 50 ug ml^{-1} 50% saturated Tf (0 035 ug ml^{-1} Fe) as on hemoglobin providing 1 ug ml^{-1} Fe and better than on ferritin or inorganic Fe donating 1 ug ml^{-1} Fe.

4.2.3 Determination of cellular Fe, respiration rate, cellular heme, lactic acid production and enzyme profiles of the lymphoma cells

The fact that the lymphoma cells could grow on such low levels of Tf-

Fe as 6 ug ml^{-1} at 10% saturation providing 0.00084 ug ml^{-1} Fe suggested that the cells have a primarily glycolytic metabolism as in this case little Fe would be required for the synthesis of cytochromes and the other Fe-containing compounds of the respiratory chain. In order to investigate this, various aspects of the cellular physiology of the lymphoma cells were examined. Cellular Fe levels were measured by atomic absorption spectrophotometry; the presence of cellular hemes was investigated by looking for a heme Soret peak at approximately 417 nm when scanning a homogenized cell preparation spectrophotometrically. The cellular respiration rate was also measured (Table 5). The presence of hemes was not detectable (Table 5); the cells must, however, possess low levels of hemes as the cells demonstrated a low level of cyanide-sensitive respiration. The addition of cyanide eliminated respiration completely.

The Fe content of EL4-1 cells (Table 5) can alternatively be expressed as 22 ng Fe per 10^6 cells or 39.3 nmol per 10^8 cells. Smit *et al.* (1982) measured the Fe content of several tissue culture cells and found that DON Chinese hamster fibroblasts, Chinese hamster ovary cells (CHO) and BW 1 mouse hepatoma cells contained 35.2, 42.6 and 38.6 nmol Fe per 10^8 cells, levels which are very similar to those found for EL4-1 cells. It is interesting to note that normal and malignant cultured cells such as the EL4 and mouse hepatoma cells contain relatively equivalent amounts of cellular Fe This implies that there is no enhanced storage of Fe by malignant cells grown *in vitro*. A similar observation was made by Schade (1976) in *in vivo* studies in plasmacytoma-bearing mice.

To further examine the glycolytic capacity of EL4-1 cells, these cells were grown in RPMI with 0.3 uM 50% saturated human Tf for 72 h after which

TABLE 5.

Cellular Fe, O_2 consumption and heme Fe of EL4 cells.

Cells Grown	Cellular Fe [1] ug Fe mg^{-1} protein[2]	O_2 Consumption[3] nmol/min/10^6 cells (+ cyanide)	Heme Fe[4] OD^{417}/ 10^6 cells
EL4 *in vitro*	0.108 (0.042)[5]	0.62 (cyanide sensitive)	not detectable
EL4 *in vivo*	0.075 (0.021)	0.52 (cyanide sensitive)	not detectable
EL4-1 *in vitro*	0.168 (0.016)	not done	not done

[1]Cellular Fe was determined on homogenized cell preparations using atomic absorption spectrophotometry in the graphite furnace mode.

[2]Protein assays were performed according to the method of Lowry *et al.* (1951).

[3]Respiration studies were performed using a Clark polarographic O_2 electrode. Determinations were done with 3 ml samples of cells (1 X 10^6 cells ml^{-1}). After initial O_2 uptake rates were established (approximately 10 min) 10 ul of 300 mM KCN was added and uptake was followed for 20 min.

[4]To detect hemes, cell homogenates were scanned from 450-350 nm (Perkin El-

mer 555 UV-Vis spectrophotometer). Horse heart cytochrome c was used as a standard. For Fe and heme analysis of *in vivo* cells, mice were bled completely from the retro-orbital sinus before the tumor cells were harvested. Any contaminating erythrocytes were lysed. The cell suspension was washed twice and resuspended in saline.

[5]Numbers in parentheses represent the standard deviation.

time both the residual glucose and the lactic acid concentration of the medium were measured. It was determined that of the original 11 1 umol ml^{-1} glucose present, 4.45 umol of glucose was utilized. This could theoretically give rise to 8.91 umol ml^{-1} lactic acid. The actual value of lactic acid measured was 6.63 umol ml^{-1}, 74.4% of the maximal theoretical value. Thus a substantial proportion of the glucose utilized was metabolized to lactic acid suggesting that these cells do indeed have a highly glycolytic metabolism and this may at least in part account for the low Fe requirements for growth.

Finally, in this phase of the study, the enzyme profiles of Fe-deprived and Fe-sufficient cells were compared (Table 6) The only qualitative differences in the enzyme contents of the cells were that the Fe-deprived cells demonstrated a very slight positive reaction for alpha-mannosidase and alpha-fucosidase. There were also several quantitative differences. Quantitation was, however, crude being by visual comparison of control and test color development. (The API ZYM system used for the enzyme analysis is fully described in appendix II.)

4.2.4 Growth of EL4-1 cells on various transferrins

The next aspect of the study involved performing growth experiments to determine whether cells could grow on other forms of Tf besides human Tf; these included murine Tf, bovine Tf, lactoferrin (Lf) and conalbumin Figure 17 demonstrates that EL4-1 cells grow equally well on 50% Fe-saturated mouse or human Tf at the low levels of 3 and 6 ug ml^{-1} (37.5 and 75 nM Tf and Fe respectively). Figure 18 reveals that the cells grew less

TABLE 6.

Enzyme profiles[1] of Fe-sufficient[2] and Fe deprived[3] EL4-1 cells.

Number	Enzyme	Fe-sufficient	Fe-deprived
1	alkaline phosphatase	1/2[4]	1
2	esterase (C4)	1	2
3	esterase lipase (C8)	2	3
4	lipase (C14)	1/2	1/2
5	leucine aminopeptidase	5	3
6	valine aminopeptidase	1	1
7	cystine aminopeptidase	2	2
8	trypsin	-	-
9	chymotrypsin	-	-
10	acid phosphatase	2	4
11	phosphoamidase	2	2.5
12	alpha-galactosidase	-	-
13	beta-galactosidase	1	2
14	beta-glucuronidase	2	3
15	alpha-glucosidase	1/2	1
16	beta-glucosidase	1/2	1
17	N-acetyl-beta-glucosaminidase	1	2
18	alpha-mannosidase	-	1/2
19	alpha-fucosidase	-	1/4

[1]The enzyme profiles were done using the API ZYM system from Analytab Products Division of Ayerst Laboratories. Cells were washed in saline and resuspended in 3 ml saline at 7.6 X 10^6 cells ml^{-1}. The API ZYM strips were inoculated and incubated at 37° C for 4 h. Reagents A and B were added and the strips were read by comparing the colour reactions to those in the colour chart provided.

[2,3]EL4-1 cells were removed from a mouse and cultured *in vitro* with 75 nM 50% Fe-saturated human Tf. After 48 h cells were washed and split into 2 sets: one set was grown as above, the other was grown without Tf or Fe for 40 h.

[4]The colour intensity was estimated on a scale from 0-5 as determined by comparison to a colour chart provided with the API ZYM system.

Fig. 17 A comparison of growth of EL4-1 cells [redacted]uman and murine transferrin. The lymphoma cells, prepared as in Fig. 13, were inoculated into media containing 37.5 (3 ug ml^{-1}) (■) and 75 (□) nM (6 ug ml^{-1}) murine Tf or 37.5 (●) and 75 (○) nM human Tf at 50% Fe saturation.

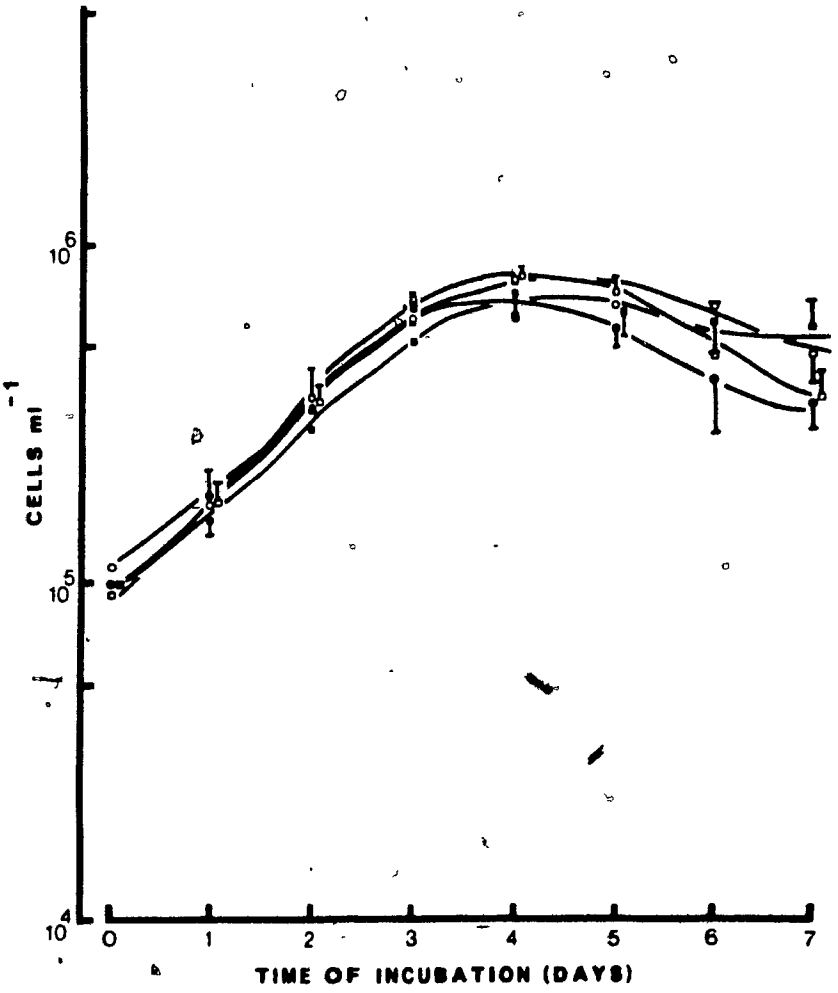
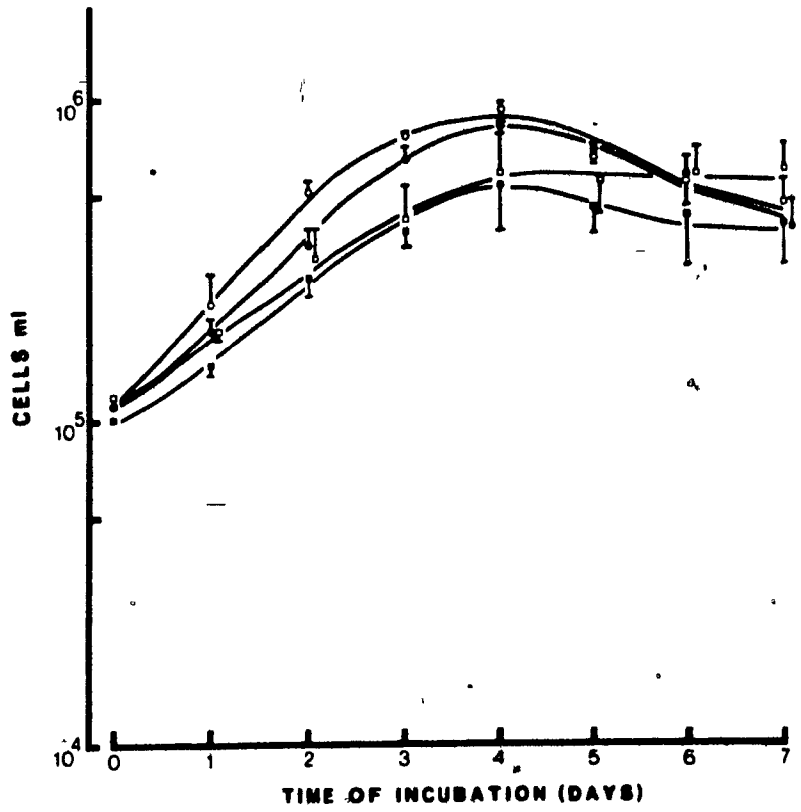
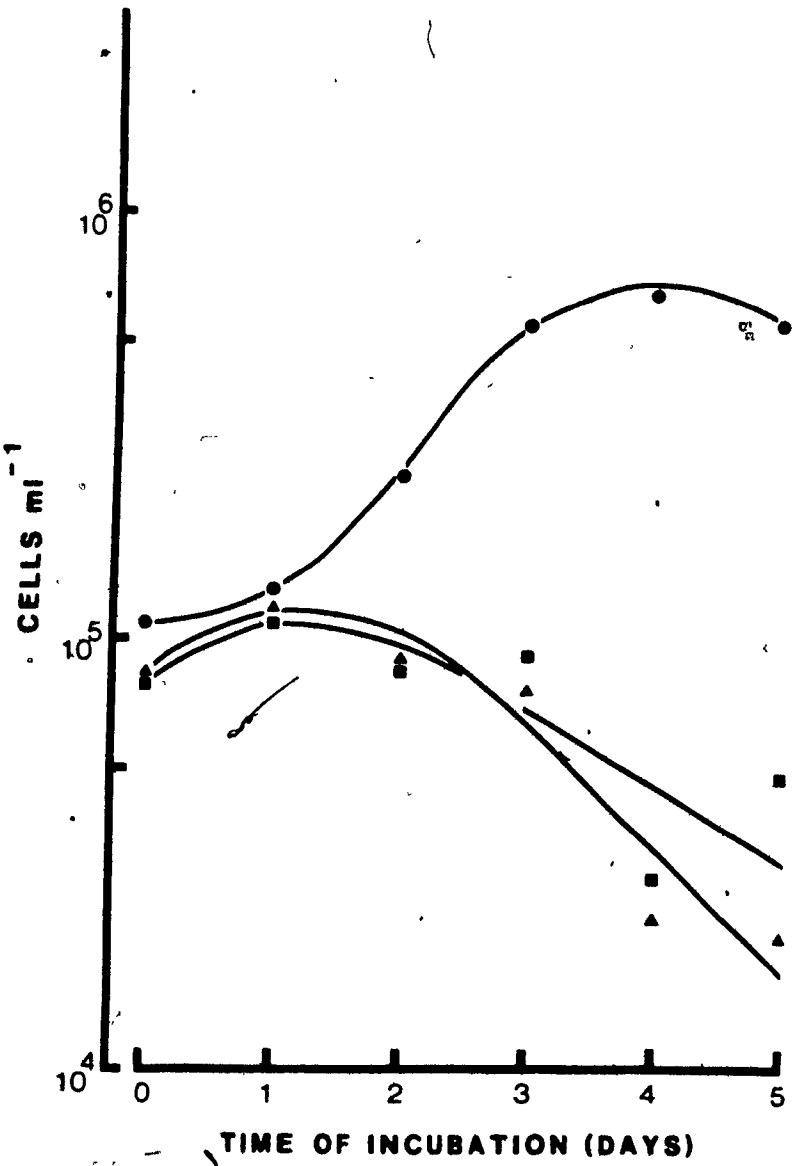

CELLS ml^{-1}
10^6
10^5
10^4
0
1
2
3
4
5
6
7
TIME OF INCUBATION (DAYS)

Fig. 18 A comparison of growth of EL4-1 cells on human and bovine transferrin. Lymphoma cells, cultured and prepared as described for Fig. 13, were inoculated into media containing 75 nM (6 ug ml^{-1}) (●) or 250 nM (20 ug ml^{-1}) (○) human Tf and 75 nM (■) or 250 nM (□) bovine Tf at 50% Fe saturation.


10^6
10^5
10^4
CELLS ml^{-1}
0
1
2
3
4
5
6
7
TIME OF INCUBATION (DAYS)

well on 50% Fe-saturated bovine Tf at both 6 and 20 ug ml^{-1} than on human Tf (this represents 75 and 250 nM Tf and Fe respectively). Figure 19 shows that these lymphoma cells could not utilize Fe from 50% Fe-saturated conalbumin or Lf. These results are similar to the findings of others using different cell lines. For example, Messmer (1973) showed that Chinese hamster V79 cells grew well with human and rabbit Tf's but poorly with porcine and bovine Tf's and conalbumin. Furthermore, Chinese or Syrian hamster serum and human serum were found to be better Fe sources for the cells than were rat or mouse sera. Bartek *et al.* (1985) found that MOLT-3 human T-leukemic cells could grow on human, porcine or rabbit Tf at a concentration of 5 ug ml^{-1} but could not grow on bovine Tf at this concentration. Tsavaler *et al.* (1986) recently found that human K562 cells grew equally well on bovine or human Tf at the relatively high concentration of 300 ug ml^{-1}. Penhallow *et al.* (1986) found that for *in vitro* culture of HeLa cells a higher concentration of bovine Tf was required than of human Tf. Thus, as is particularly apparent at low Tf concentrations, cells have Tf species preferences for growth. EL4-1 cells grow better on human or murine Tf than on bovine Tf and cannot use Lf or conalbumin Fe. Lf, however, would not be expected to be a good Fe source as it does not release Fe at low pH as Tf does; therefore it would not relinquish Fe intracellularly within an acidic endocytic vesicle.

Fig. 19 Growth of EL4-1 cells on conalbumin and lactoferrin. EL4-1 cells, prepared as in Fig. 13, were inoculated into media containing 75 nM (6 ug ml^{-1}) human Tf (●), 1000 nM (80 ug ml^{-1}) conalbumin (■) and 1000 nM Lf (▲). All proteins were prepared to 50% Fe saturation.


CELLS ml^{-1}
10^6
10^5
10^4
0
1
2
3
4
5
TIME OF INCUBATION (DAYS)

4.2.5 Uptake of ^{59}Fe from 50% Fe-saturated human transferrin by EL4-1 cells and the effect of the hypoferremic response

The rate of Fe acquisition by EL4-1 cells was determined by measuring uptake of ^{59}Fe from 50% saturated Tf at concentrations from 12.5 to 600 nM Tf and Fe. A plot of uptake velocity (Fig 20A) as a function of medium Fe concentration at a constant Tf concentration demonstrated that uptake velocity was approaching maximal at 150 nM Fe and Tf Saturation of the Fe uptake system occurred at 1/250 of the physiological Tf levels Subsequently, the effect of the hypoferremic response on Fe uptake was examined. Cells were incubated in aliquots with 75 nM 50% saturated ^{59}Fe- Tf plus sufficient added apo-Tf to reduce the per cent Fe saturation from 50% to 27, 15, 8 and 4% (Fig. 20C). It is evident that this substantial decrease in saturation does not effect a proportional decrease in Fe uptake by the cells. This same data was plotted as uptake velocity versus increase in medium UIBC (Fig. 20B). The gradual increase in UIBC from 75 to 1635 nM mimics the decrease in Tf Fe saturation and corresponding rise in UIBC which would occur *in vivo* during the hypoferremic response. The results demonstrate that the Fe uptake velocity is relatively insensitive to large increases in UIBC.

In a subsequent experiment, uptake of ^{59}Fe from Tf was measured by cells in the presence of 24 ug ml^{-1} (300 nM) 50% Fe-saturated Tf and by cells in which this effective Tf saturation was reduced to 10% by the addition of apo-Tf (Fig. 21). With this five-fold reduction in Tf Fe saturation only a 34% decrease in Fe uptake was observed In contrast, when studies were performed in which unlabelled 50% Fe-saturated Tf was added to

Fig. 20 Evaluation of Fe uptake from transferrin under various conditions. (A) The relationship between the steady state velocity of Fe uptake by EL4-1 cells and the concentration of 50% Fe-saturated Tf was determined. ^{59}Fe-labelled 50% saturated Tf was added to EL4-1 cells (1.5 X 10^6 ml^{-1}) at the indicated concentrations at zero time. Uptake is calculated from total Fe taken up after one h incubation. Data are averages of 4 replicates. (B) An examination of the relationship between the steady state uptake velocity and the per cent Fe saturation of Tf at a constant Fe concentration of 75 nM. Uptake is calculated from total Fe taken up after one h incubation of EL4-1 cells (1.5 X 10^6 ml^{-1}) with ^{59}Fe-labelled Tf. In each case, the 75 nM Fe was added as monoferric Tf and the UIBC progressively increased by addition of apo-Tf. Tf Fe saturation values for UIBC levels of 75, 198, 415, 816 and 1635 nM are 50, 27, 15, 8 and 4% respectively. The results are determined from data obtained in Fig. 20(C).

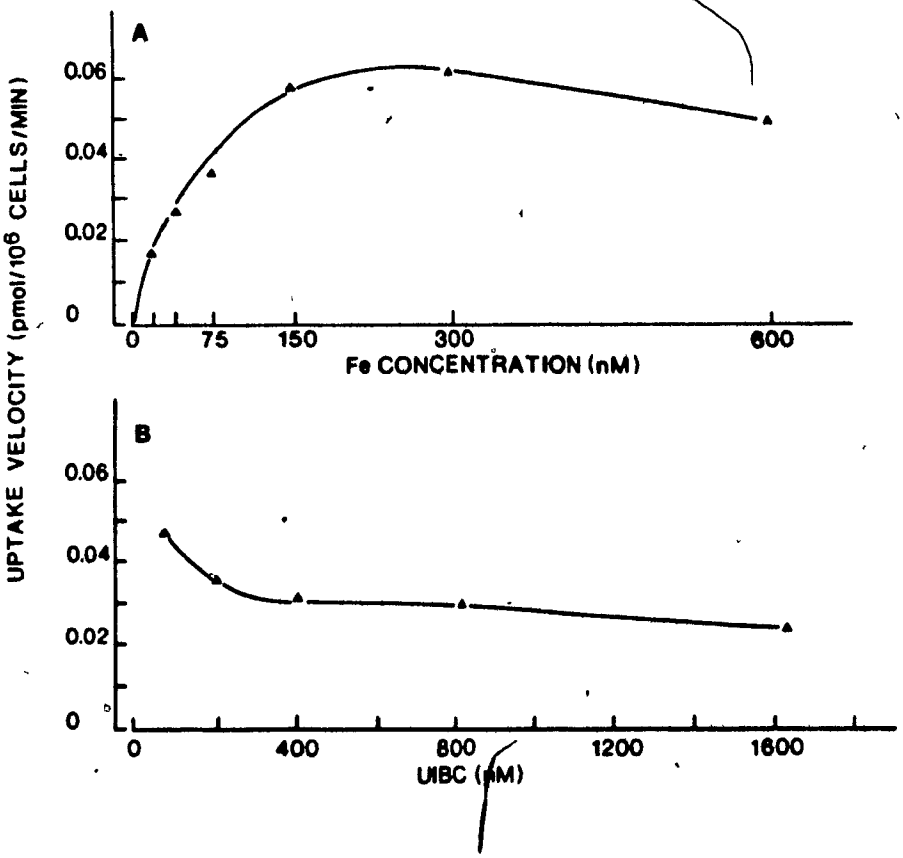

A
0.06
0.04
0.02
0
0
75
150
300
600
Fe CONCENTRATION (nM)
B
0.06
0.04
0.02
0
0
400
800
1200
1600
UIBC (nM)
UPTAKE VELOCITY (pmol/10^6 CELLS/MIN)

Fig. 20C The effect of the addition of increasing concentrations of apo-Tf on Fe uptake was examined. Seventy-five nM (6 ug ml^{-1}) 50% Fe-saturated ^{59}Fe-labelled Tf was added to each of 5 flasks containing 1 5 X 10^6 cells ml^{-1}. The control flask contained no further additions (●). To the remaining flasks increasing concentrations of apo-Tf were added to give the final total Tf concentrations 136 nM (▲), 240 nM (■), 445 nM (○) and 855 nM (△). Duplicate one-ml samples were removed at the specified times and Fe uptake was measured. Data points represent the average of 4 experiments.

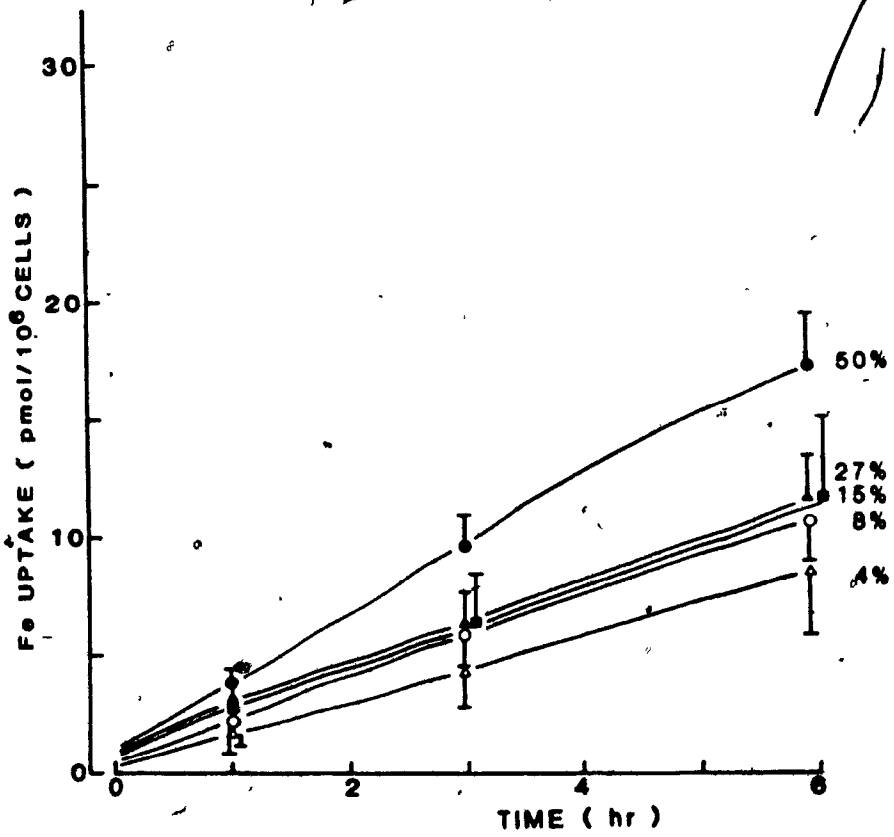
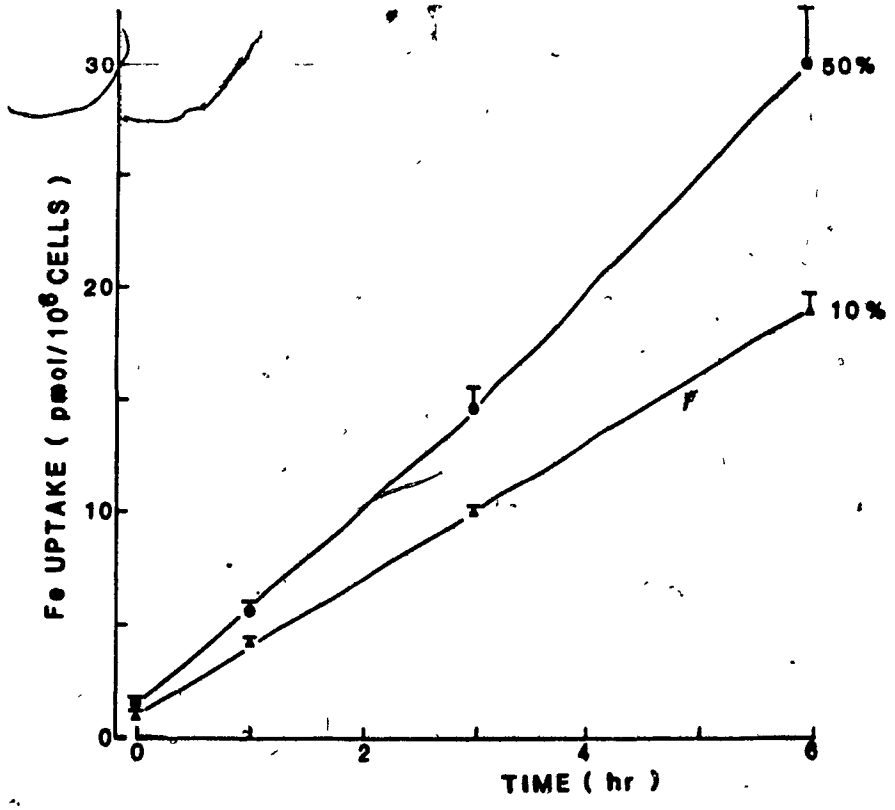

30
20
10
0
Fe UPTAKE (pmol/10⁶ CELLS)
0
2
4
6
TIME (hr)
50%
27%
15%
8%
4%

Fig. 21 The effect of increasing the concentration of apotransferrin (raising the UIBC) on the Fe uptake rate by EL4-1 cells. The lymphoma cells (1.5 X 10^6 ml^{-1}) were incubated with ^{59}Fe-Tf at 50% saturation (300 nM Tf, 300 nM Fe) (●) and at 10% saturation (1500 nM Tf, 300 nM Fe) (▲). Duplicate one-ml samples were removed at the indicated times and Fe uptake was measured. Data points represent the average of two experiments.

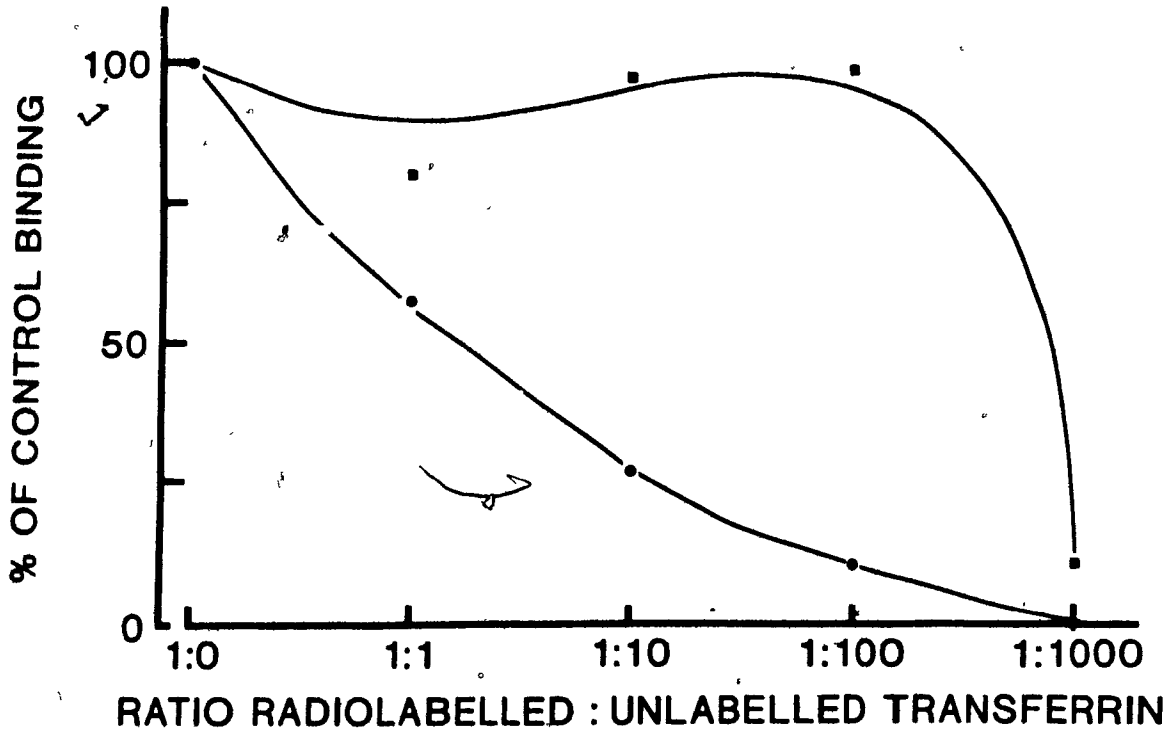

30
20
10
0
0
2
4
6
Fe UPTAKE (pmol/10^6 CELLS)
TIME (hr)
50%
10%

assays in ratios of 1:1 and 1:3 with radiolabelled Tf, uptake of labelled Fe was reduced by 50% and 62% respectively at 1, 3 and 6 h. Fe-containing Tf has a much greater inhibitory effect on uptake than apo-Tf.

In order to explore the reason for this slight inhibition by apo-Tf on Fe uptake, control microtiter wells of EL4-1 cells were incubated with 24 ug·ml^{-1} 50% Fe-saturated ^{59}Fe-Tf at 4° C; test wells also contained unlabelled 50%-saturated Tf or apo-Tf at ratios of 1·1, 1.10, 1:100 and 1:1000 to radiolabelled Tf This assay was performed at 4° C in order to examine binding of Tf to cells as opposed to uptake of Fe. It can be seen (Fig. 22) that relative to ferrated Tf, apo-Tf offered very little competition to labelled Tf for binding to the cells. As apo-Tf has very little binding affinity for the cellular Tf receptors the addition of apo-Tf to uptake assays, to mimic the hypoferremic response, caused little inhibition of Fe uptake from Tf. These results concur with those of Dautry-Varsat *et al.* (1983) and Klausner *et al.* (1983a) who found that eucaryotic Tf receptors have high affinity for ferri-Tf and a lower affinity for apo-Tf at neutral pH. At acidic pH, however, the Tf receptor has a high affinity for apo-Tf. It is this differential response of the receptor to ferri- and apo-Tf which enables the cellular Tf cycle to function.

In Fig. 21 it was demonstrated that a 1·5 dilution of 50% Fe-saturated Tf with apo-Tf decreased Fe uptake by 34%. In Fig. 22 apo-Tf added at a ratio of 1:100 to radiolabelled ferri-Tf did not affect binding of ferri-Tf at 4° C. It should be pointed out, however, that in one case uptake at 37° C over a 6 h period was being measured and in the other case binding at 4° C after 60 min was being examined. Fig. 21 demonstrates that the difference in uptake at 50% and 10% saturation after 60 min was very

Fig. 22 Binding of 50% saturated ^{59}Fe-labelled human transferrin to EL4-1 cells in the presence of unlabelled 50%-saturated transferrin or apotransferrin. EL4-1 cells were added at a level of 3×10^6 ml^{-1} to 24-well microtiter plates containing 24 ug ml^{-1} ^{59}Fe-labelled 50%-saturated human Tf (control wells). Test wells also contained unlabelled 50%-saturated human Tf (●) or apo-Tf (■) in ratios of 1:1, 1:10, 1:100 and 1:1000 to labelled Tf. Binding proceeded at 4° C for 60 min.


% OF CONTROL BINDING
100
50
0
1:0
1:1
1:10
1:100
1:1000
RATIO RADIOLABELLED : UNLABELLED TRANSFERRIN

slight. The apparent difference between the data in Fig. 21 and Fig. 22 do not suggest that some of the ^{59}Fe was non-specifically bound to the Tf as the Tf was Fe loaded with ferric citrate which delivers Fe specifically to the Tf Fe binding site. Also, Tf preparations were checked for specific Fe binding by determining the absorbance ratio at 465/280 nm (section 3.5.1) which demonstrates that Fe is correctly bound to the Fe binding site of Tf.

4.3 Transferrin binding studies

4.3.1 Enumeration of surface transferrin binding sites on EL4-1 cells

The results of section 4.2.4 were interesting as they suggested that as the EL4-1 cells grew better on specific types of Tf, the cellular Tf receptor might preferentially bind certain types of Tf over others. Before examining Tf-receptor binding specificity, several other aspects of Tf binding to EL4-1 cells were examined. Initially, binding studies at 4° C were performed in which EL4-1 cells were incubated with various concentrations of ^{125}I-labelled Tf in RPMI 1640 media containing 0.1% BSA to minimize non-specific binding. The amount of Tf bound at each concentration was measured (Fig. 23). From this data, Scatchard plots were drawn and the number of surface binding sites were determined from the abscissa intercept (Inset, Fig. 23). In these studies non-specific binding was corrected for using the method of Chamness and McGuire (1975). The cells were found to have an average of 1.1 X 10^5 surface binding sites per cell. The dissociation constant (K_d), determined from the slope of this graph, was 5.9 X 10^{-9} M representing the ligand concentration at which half the receptors are occupied. This concentration is far lower than the mean physiological Tf concentration in the serum, 37.5 uM, indicating a very high receptor-ligand affinity. Many similar binding studies have been performed on human, mouse and rat cells and a few of these studies have been summarized in Table 7 for a comparison with EL4-1 cells. It can be seen that EL4-1 cells are very similar to other cultured cell lines in both the number of binding sites and the K_d. Ciechanover *et al.* (1983) found that HepG2 human

Fig. 23 Binding of radiolabelled diferric human transferrin to EL4-1 cells at 4° C as a function of medium transferrin concentration. EL4-1 cells were cultured *in vitro* in RPMI with 0.3 uM human diferric Tf. Cells were harvested and incubated (37° C, 90 min) to eliminate internalized Tf. The cells were washed and 1.5 X 10^6 cells were added to microtiter wells prepared as follows. Ten ug of ^{125}I-labelled Tf in RPMI with 0.1% BSA was added to well one of the microtiter plate then diluted serially two-fold to well 11. The final volume in each well was 200 ul. Binding proceeded for 90 min at 4° C. Data points represent the average of 4 experiments. Inset: A Scatchard plot of the above data.

BSA (0.1%) was added to the medium to minimize non-specific binding. For the Scatchard analysis, any non-specific binding which occurred was corrected for using the method of Chamness and McGuire (1975).

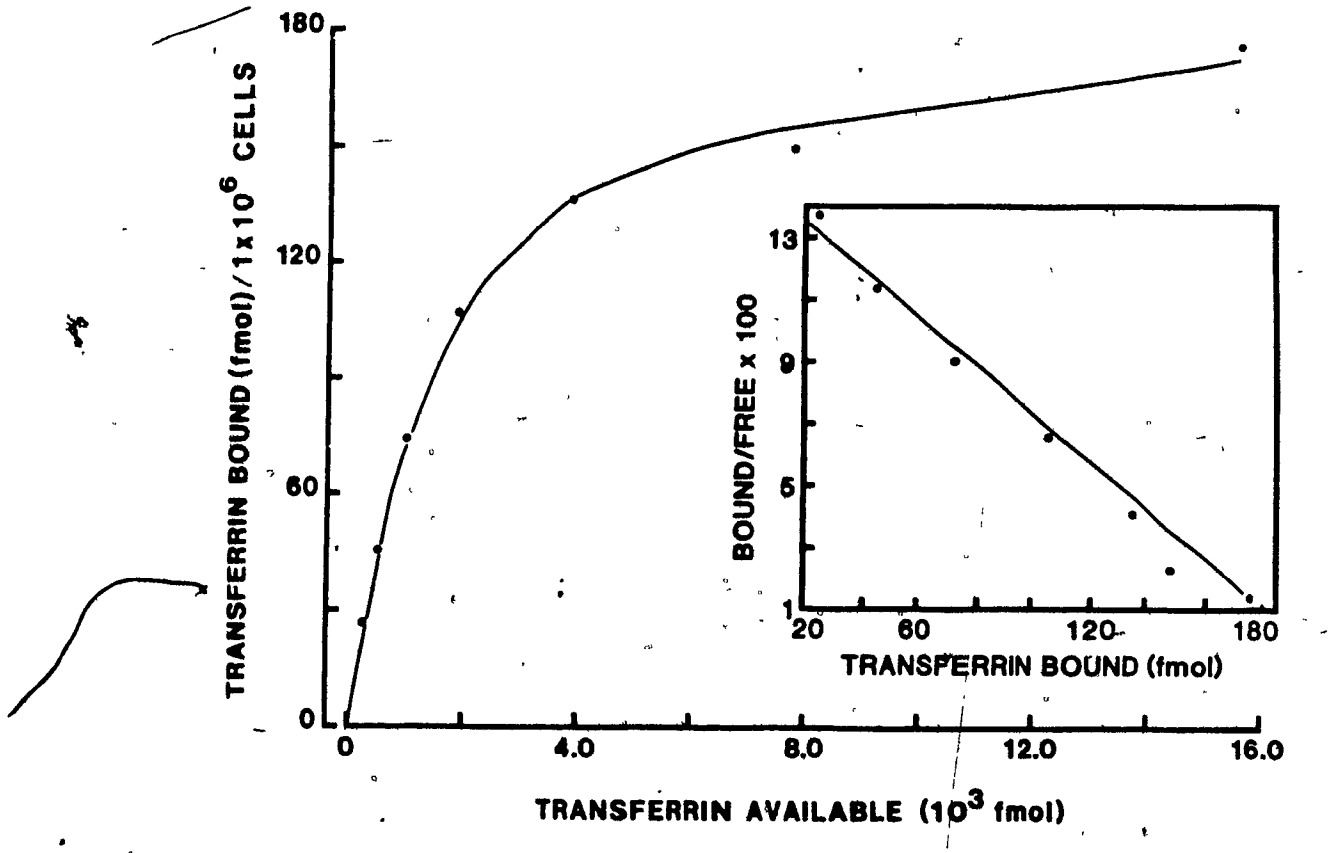

TRANSFERRIN BOUND (fmol)/1x10^6 CELLS
180
120
60
0
0
4.0
8.0
12.0
16.0
TRANSFERRIN AVAILABLE (10^3 fmol)
BOUND/FREE x 100
13
9
5
1
20
60
120
180
TRANSFERRIN BOUND (fmol)

TABLE 7.

A comparison of cell lines with respect to number of transferrin binding sites per cell and transferrin binding affinity.

Cell line and type (Reference)	Temp. (° C)	Binding sites per cell	K_a-M^{-1} K_d-M
K562-human erythroleukemia(1)	4	1.5×10^5	K_d-2.1×10^{-9}
HGC-25-human pancreatic carcinoma(2)	37	0.37×10^5	K_a-2.58×10^{7}
Human fibroblasts(3)	37	3.9×10^5	K_d-5.1×10^{-9}
A431-human epidermoid carcinoma(4)	5	1.2×10^5	-
HepG2-human hepatoma(5)	4	5.1×10^4	K_d-4.4×10^{-9}
L1210-mouse leukemia(6)	37	6.4×10^5	-
Mouse teratocarcinoma(7)	4	5.7×10^3	K_d-6.7×10^{-9}
RILQ-mouse thymic lymphoma(8)	15	1.2×10^5	K_a-5.1×10^{8}
Mouse spleen(8)	15	5.5×10^4	K_a-4.3×10^{8}
Mouse thymus(8)	15	$<5.0 \times 10^3$	-
Mouse reticulocytes(8)	15	6.1×10^4	K_a-5.9×10^{8}
Rat erythroblasts(9)	0	5.0×10^5	K_a-2.5×10^{7}

References:

(1) Klausner *et al.* (1983a)

(2) Urushizaki *et al.* (1984)

(3) Ward *et al.* (1982)

(4) Hopkins and Trowbridge (1983)

(5) Ciechanover *et al.* (1983a)

(6) Takahashi and Tavassoli (1982)

(7) Karin and Mintz (1981)

(8) De Abrew (1981)

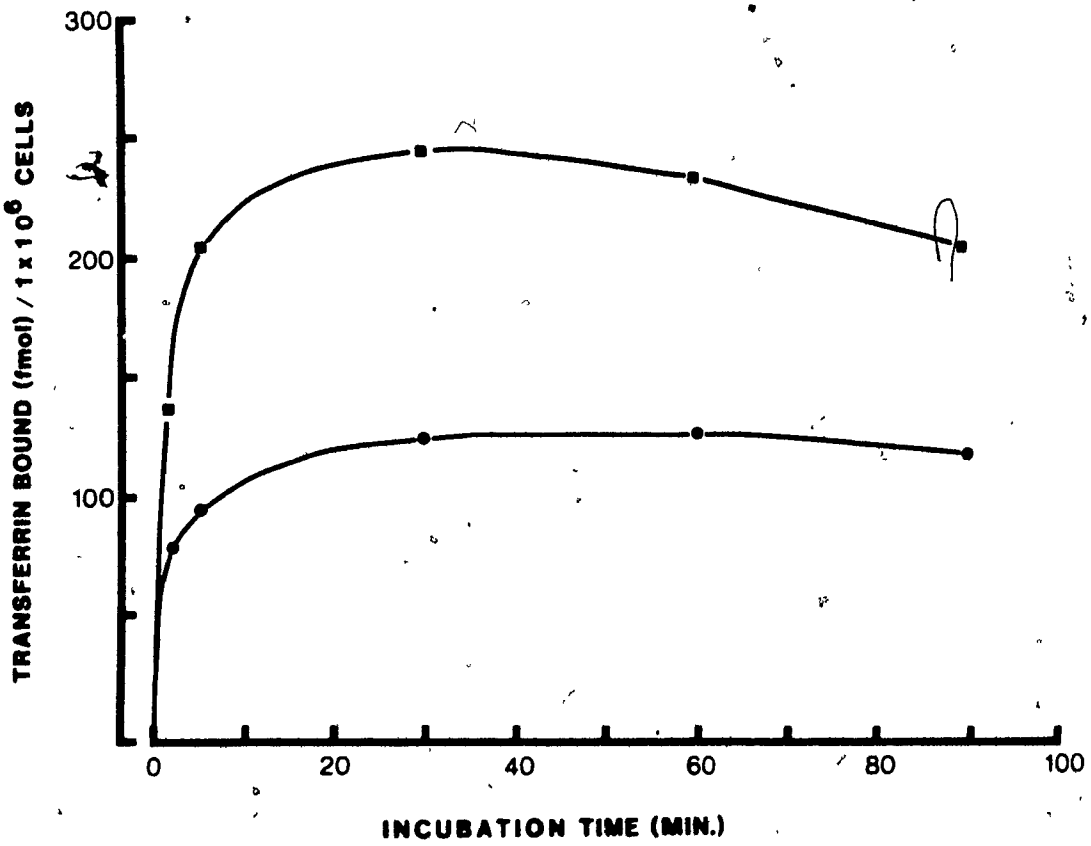
(9) Octave *et al.* (1982)

hepatoma cells contain 5 X 10^4 surface Tf binding sites and 1 X 10^5 internal sites. Thus the majority of Tf binding sites are internal. The total number of binding sites can be determined by performing binding studies at 37° C but this was not done in this study. The majority of experiments were performed at 4° C in order to examine binding as opposed to uptake of Tf.

4.3.2 Time course binding and uptake studies

The time course of the binding and uptake of ^{125}I-Tf by EL4-1 cells was performed at 4° C and at 37° C respectively (Fig. 24). Binding was rapid and a plateau was reached by 30 min at both 37° C and at 4° C The curve at 37° C represents both binding and uptake of Tf. Numerous reports indicate that at 37° C bound Tf is internalized and the internal pool of receptors is recycled to the surface of the cells for more binding to occur (section 2.9.2.3). In a subsequent experiment, cells were incubated at 37° C for one h with 0.5 ug ^{125}I-Tf. They were then centrifuged, washed 3 times and bound Tf was determined. Cells were then re-incubated at 37° C with 50 ug of unlabelled diferric Tf for 10 min and 40 min. After 10 min incubation, 34% of the label had been released into the medium; after 40 min, 82% of the label had been released indicating that Tf is taken up, presumably the Fe is removed and subsequently the Tf is released from the cells. Karin and Mintz (1981) studying murine teratocarcinoma cells found that 80% of cell-associated ^{125}I-Tf was released after one h of incubation and was trichloroacetic acid insoluble indicating that there had been no gross degradation of ligand.

Fig. 24 Time course of transferrin binding by EL4-1 cells. EL4-1 cells were cultured and prepared as described in Fig. 23. Cells were added to microtiter wells containing 0.5 ug radiolabelled human Tf in RPMI with 0.1% BSA at 37° C (■) and 4° C (●). Binding was terminated at the indicated times.


300
200
100
TRANSFERRIN BOUND (fmol) / 1 x 10^6 CELLS
0
20
40
60
80
100
INCUBATION TIME (MIN.)

4.3.3 Competitive binding assays

In section 4.2.4, growth of EL4-1 cells on several different types of Tf was examined and it was found that of the Tf's tested human and murine Tf were the optimal Fe sources. Conalbumin and Lf could not be used and bovine Tf was not as good as human or murine Tf. In order to determine if this was because these compounds had a lower binding affinity, competitive binding studies were performed at 4° C (Table 8). In these studies, cells were added to control wells containing 0.5 ug of ^{125}I-labelled Tf and test wells containing labelled Tf and 0.5, 5, or 25 ug of a competing unlabelled protein. It can be seen (Table 8) that when unlabelled human Tf was added to cells in an amount equal to radiolabelled Tf approximately 50% inhibition of binding occurred demonstrating that the iodination procedure did not alter the binding sites on the Tf or Tf binding to the receptors. Lf, conalbumin, and bovine Tf were not competitive with human Tf even when present at 50 times greater concentration. Zapolski and Princiotto (1976) found that human Lf and conalbumin could not supply rabbit reticulocytes with Fe for heme synthesis. Moreover, neither Lf nor conalbumin interfered with reticulocyte uptake of Fe from Tf. Messmer (1973a) studied the binding of human ^{125}I-Tf to V79 Chinese hamster fibroblasts and found that it required 120 times as much bovine Tf as human Tf to achieve 50% inhibition of labelled human Tf binding. It was also found in these studies that conalbumin did not compete significantly with human Tf for binding to these cells. The EL4-1 cells used in this assay had been grown *in vitro* in human Tf. To determine whether cells grown in fetal bovine serum and thus on bovine Tf responded in a similar manner, binding studies were performed

TABLE 8.

Effect of competition with unlabelled human, murine and bovine transferrin, lactoferrin and conalbumin on the binding of radiolabelled human transferrin to EL4-1 cells.[1]

Ratio of labelled human to unlabelled Tf	1:1	1:10	1:50
Competing Ligand[2]			
Human Tf	53.9 (3.1)[3]	13.7 (3.0)	1.0
Murine Tf	94.5 (1.5)	82.0 (2.0)	64.0 (8.0)
Bovine Tf	90.5 (2.1)	97.3 (2.8)	90.2 (2.1)
Lactoferrin	119.9 (3.4)	96.9 (3.9)	94.0 (12.0)
Conalbumin	127.4 (25.5)	96.0 (0.0)	101.0 (5.0)

[1]EL4-1 cells were cultured *in vitro* in Fe-extracted RPMI with 0.3 uM diferric human Tf. Cells were harvested by centrifugation, washed once and then incubated for 90 min in Tf-free RPMI at 37° C to eliminate any internalized Tf. Cells were harvested and washed again at 4° C and added to wells containing 0.5 ug radiolabelled human Tf or labelled Tf and 0.5, 5 or 25 ug of an unlabelled ligand. Binding (4° C) was terminated after 60 min.

[2]Numbers represent labelled Tf bound in the presence of unlabelled Tf as a percent of labelled Tf bound in the absence of unlabelled Tf.

[3]Numbers in parentheses represent the standard error of the mean.

using the original EL4 cell line which had been cultured in RPMI 1640 with 10% F.B.S for two months (Table 9) Similar results were obtained Thus even though cells had been cultured in bovine serum for a prolonged period of time the cells preferentially bound human Tf over bovine Tf. This demonstrates that this Tf receptor preference is not unique to the EL4-1 line or produced by the selection for a serum-independent cell line. Schneider *et al.* (1986) performed 4° binding studies with K562 erythroleukemia cells and found that it required 2000 times as much bovine Tf to obtain 50% inhibition of binding as for human Tf. Penhallow *et al.* (1986) reported that a ten-fold excess of ovoTf (conalbumin) or bovine Tf did not compete with human Tf for binding to human HeLa cells. These authors also performed a soluble receptor assay using 5 nM labelled human Tf and various competing levels of homologous and heterologous sera. With human serum, one-half maximal binding occurred at 6.5 nM Tf and with fetal calf serum one-half maximal binding occurred at 3000 nM Tf.

An interesting finding was that murine Tf did not compete as well as human Tf for binding to the receptors of these mouse cells although the cells grew well on murine Tf (section 4.2.4) The cells used for these initial binding studies were grown *in vitro* with human Tf. To rule out the fact that this might have had some effect on binding, cells were removed from the peritoneal cavity of a mouse and then cultured in either human or mouse Tf before binding studies were performed (Table 10). Virtually the same results were obtained as in Table 8; therefore the apparent preferential binding of human Tf was not due to pre-growth in media containing human Tf.

EL4-1 cells had originally been selected because they could grow in

TABLE 9.

Effect of human, murine and bovine transferrin on the binding of radiolabelled human transferrin to the original EL4 cells.[1]

Ratio of labelled to unlabelled Tf	1:1	1:10
Competing Ligand[2]		
Human Tf	49.5 (4.0)[3]	5.1 (1.8)
Murine Tf	100.1 (0.5)	88.8 (0.4)
Bovine Tf	104.2 (2.4)	109.0 (0.5)

[1]EL4 cells were cultured *in vitro* for two months in RPMI 1640 with 10% F.B.S. Cells were prepared and binding assays performed as in Table 8.

[2]Numbers represent labelled Tf bound in the presence of competing Tf as a percent of labelled Tf bound in the absence of competing ligand.

[3]Numbers in parentheses represent the standard error of the mean.

TABLE 10.

Effect of human and murine transferrin on the binding of radiolabelled human transferrin to EL4-1 cells cultured with human or murine transferrin as their Fe source.[1]

	Murine Tf grown cells		Human Tf grown cells	
Ratio of labelled to unlabelled Tf	1:1	1:10	1:1	1:10
Competing Ligand[2]				
Human Tf	48.2 (2.2)[3]	13.6 (1.8)	52.1 (1.5)	9.1 (1.1)
Murine Tf	101.8 (0.9)	84.9 (4.0)	100.6 (0.2)	81.7 (1.3)

[1]EL4-1 cells were removed from the peritoneal cavity of a mouse and were cultured *in vitro* in RPMI 1640 containing 0.3 uM diferric human or mouse Tf for 48 h prior to the binding assay. The growth during this time represented 2.5 doublings. Details for the binding experiment were as in Table 8.

[2]Numbers represent labelled Tf bound in the presence of unlabelled (competing) Tf as a percent of labelled Tf bound in the absence of unlabelled Tf.

[3]Numbers in parentheses represent the standard error of the mean.

RPMI synthetic medium and human Tf *in vitro*. For the results to be generalized it was necessary to know if the receptors on these cells were somehow different from other murine cells. Therefore competitive binding experiments were also carried out using YAC-1 cells, P815 cells and normal bone marrow cells from adult C57 black mice (Table 11). The relative response of all these cell types to murine and human Tf was similar.

Rich *et al.* (1981) compared the effect of human and mouse Tf's on *in vitro* growth of early BFU-E and late CFU-E erythropoietic precursors in murine bone marrow. They found that mouse bone marrow cells have greater specificity for mouse Tf than human Tf as 950 CFU-E colonies/10^5 cells were obtained using mouse Tf and 650 CFU-E colonies/10^5 cells were obtained with human Tf. Lappin *et al.* (1985) also found that mouse Tf was more stimulatory than human Tf in the mouse spleen cell microassay for estimation of erythropoietin. Rich *et al.* (1981) suggested that mouse Tf is better able to bind cell receptors and transfer Fe to proliferating and differentiating murine erythroid cells than human Tf. It is difficult, however, to compare growth studies in which only one type of Tf is present over a relatively long period of time with competition studies at 4° C in which two types of Tf are present simultaneously for a short period of time. In the present studies, EL4-1 cells grew equally well on human and murine Tf but when both were present in binding studies human Tf was preferentially bound. Also, it is difficult to compare tumor cells with erythroid cells as erythroid cells have a much higher Fe requirement for hemoglobin synthesis. Lane (1972) found that only half as much human Tf was bound to rabbit reticulocytes as rabbit Tf and suggested that fewer receptors can bind human Tf because the molecule is larger and when bound

TABLE 11.

Effect of unlabelled human and murine transferrin on the binding of radiolabelled human transferrin to EL4-1, P815, YAC-1 and normal murine bone marrow cells.[1]

	Human Tf as the competing ligand		
Ratio of labelled to unlabelled Tf	1:1	1:10	1:50
Cell type			
EL4-1 [2]	49.4 (1.5)[3]	10.9 (2.5)	2.0 (0.6)
P815	45.6 (2.0)	10.9 (0.8)	2.4 (0.5)
YAC-1	60.2 (1.8)	15.5 (2.3)	3.8
Bone Marrow	48.1 (3.0)	19.1 (2.9)	-

	Murine Tf as the competing ligand		
Ratio of labelled to unlabelled Tf	1:1	1:10	1:50
Cell type			
EL4-1	101.8 (3.0)	91.0 (2.6)	62.9 (4.2)
P815	93.8 (1.4)	84.9 (5.2)	63.9 (6.1)
YAC-1	100.0 (2.0)	91.3 (1.8)	71.2 (2.5)
Bone Marrow	98.5 (2.5)	100.6 (4.4)	65.7 (6.6)

[1]EL4-1 cells were cultured as described in Table 8. P815 and YAC-1 cells were maintained in RPMI 1640 with 10% F.B.S Murine bone marrow cells were obtained from adult C57 mice as described in section 3.2. Cells were prepared for the assay and the assays performed as described for Table 8.

[2]Numbers represent labelled Tf bound in the presence of unlabelled (competing) Tf as a percent of labelled Tf bound in the absence of unlabelled Tf.

[3]Numbers in parentheses represent the standard error of the mean.

inhibits binding of other molecules to adjacent binding sites. The results from the present study might be unique to the particular mouse Tf used i.e. to its murine source or purification procedure. However, the protein was pure as determined by SDS-polyacrylamide gel electrophoresis, bound Fe like human Tf, as determined by absorbance at 465 nm, and the EL4-1 cells were able to grow well on it. Perhaps human Tf binds more readily to murine receptors because of its molecular weight and/or conformation but does not release Fe as readily intracellularly. Thus mouse Tf might remain a better Fe source for growth and differentiation of murine erythroid cells with their high Fe requirements. There is at present no evidence for this.

4.3.4 The effect of deglycosylation of transferrin on binding

Transferrin is a glycoprotein and human Tf contains 6% carbohydrate. The molecule contains two branched carbohydrate chains which terminate in sialic acid residues; the carbohydrate chains are found in the C-terminal half of the molecule attached to asparagine residues 413 and 611 (MacGillivray *et al.*, 1983). The proportion of carbohydrate varies, however, among the different species of Tf's. Hatton *et al.* (1974) determined the carbohydrate content of bovine, porcine, rabbit and dog Tf's and found that human Tf has twice the amount of carbohydrate as these species. Conalbumin has only one carbohydrate chain consisting of 4 residues of mannose and 8 of N-acetylglucosamine. Chicken serotransferrin, on the other hand also contains one carbohydrate chain but consists of two galactose, two mannose, 3 N-acetylglucosamine and one or two residues of sialic acid (Williams, 1968). Carbohydrates on proteins are postulated to have several

functions including: labelling of secreted proteins for export, recognition of proteins by target cells or protection against proteolytic damage (Olden *et al.* 1982).

Since the carbohydrate content of Tf varies among species and since there are definite differences in the binding of various species of Tf to cell receptors the importance of the carbohydrate chain of the Tf molecule in receptor recognition of the ligand was examined. Human Tf was deglycosylated using the enzyme N-glycanase, obtained from Genzyme Corp and the deglycosylated Tf was used in competition binding studies (Table 12A) That the carbohydrate had been removed was ascertained by running native and deglycosylated Tf's on an SDS-polyacrylamide gel, silver staining the gel and looking for a single band of decreased molecular weight (Fig 25A). Examination of the gel revealed that the majority of the protein was deglycosylated. On the gel in the lanes in which deglycosylated Tf was run three bands are apparent The faint band of highest molecular weight is believed to be a small quantity of native non-deglycosylated Tf The two lower molecular weight bands are thought to represent mono and di-deglycosylated Tf, that is, Tf from which one and two oligosaccharide chains have been removed. Deglycosylation was further examined by Pardo Pannunzio of this department using a Western blotting procedure. The Tf's were run on a SDS-polyacrylamide gel, were blotted onto nitrocellulose paper and probed with concanavalin A coupled to horseradish peroxidase The results in Fig. 25B, confirm that the enzymatic deglycosylation was successful. The deglycosylated protein was as competitive as native human Tf when added in concentrations equal to labelled ligand but was somewhat less competitive when a 10-fold excess was used. The results of the 1:1

Fig 25A A comparison of native and deglycosylated human transferrin by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Native and deglycosylated human Tf were run on SDS-PAGE as described in section 3.9. The gel was subsequently stained using the silver staining method.

Lane 1-deglycosylated human Tf (2.6 ug)

Lane 2-deglycosylated human Tf (1.3 ug)

Lane 3-native human Tf (2 ug)

Lane 4-native human Tf (1 ug)

Lane M-low molecular weight markers.

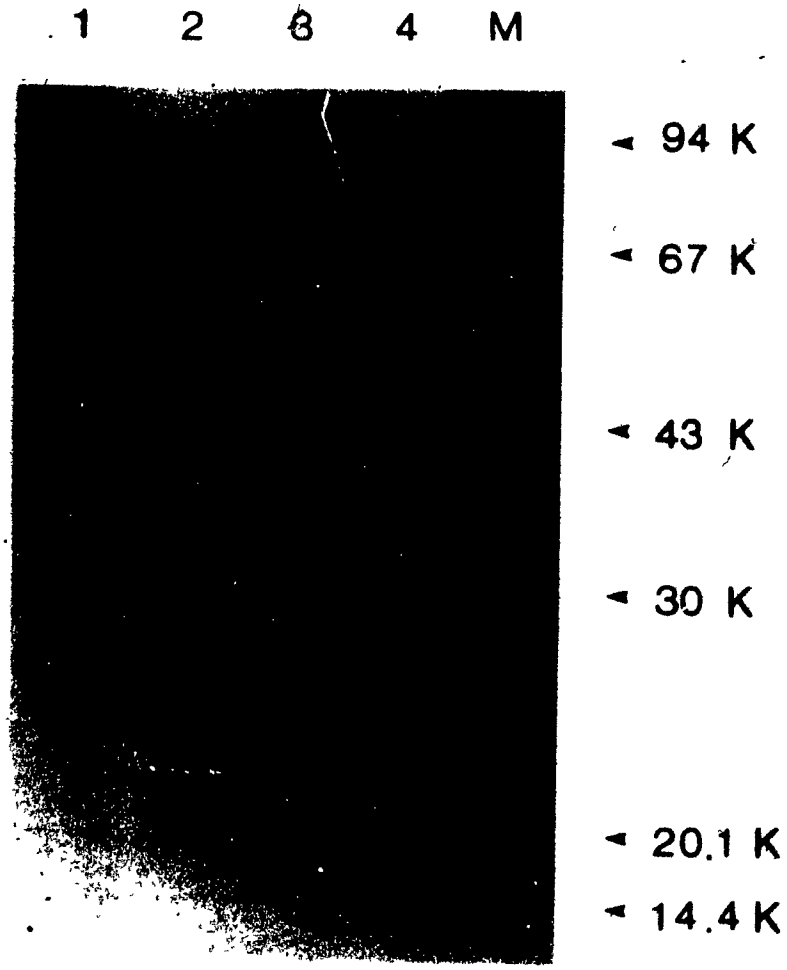

1
2
3
4
M
94 K
67 K
43 K
30 K
20.1 K
14.4 K

Fig. 25B A comparison of native and deglycosylated human and murine transferrins by Western blotting. The Tf's were run on SDS-PAGE, blotted onto nitrocellulose paper and probed with concanavalin A coupled to horseradish peroxidase.

Lane 1-deglycosylated murine Tf

Lane 2-native murine Tf

Lane 3-deglycosylated human Tf

Lane 4-native human Tf.

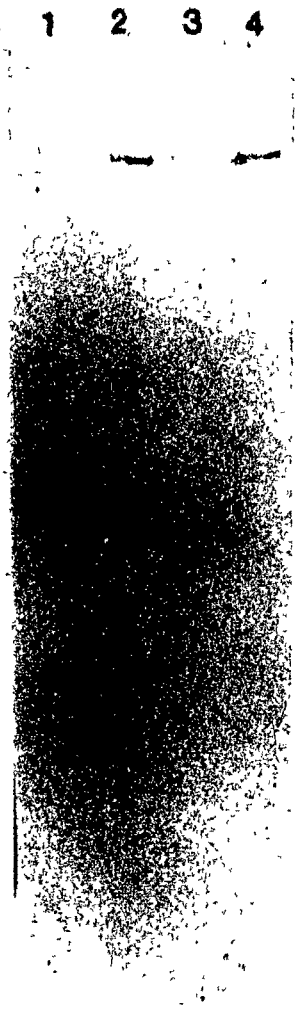

1 2 3 4

TABLE 12A.

Effect of native human and deglycosylated human transferrin on the binding of radiolabelled human transferrin to EL4-1 cells[1].

Ratio of labelled to unlabelled Tf	1:1	1:10
Competing Ligand[2]		
Human Tf	61.5 (2.5)[3]	14.8 (0.4)
Deglycosylated human Tf	67.4 (2.4)	35.3 (3.5)

[1]EL4-1 cells were cultured and prepared for the assay as described in Table 8. Control wells contained 0.5 ug labelled Tf, test wells contained labelled Tf and 0.5 or 5 ug of either native or deglycosylated Tf. Binding proceeded for 60 min at 4° C.

[2]Values represent labelled Tf bound in the presence of competing Tf as a percent of labelled Tf bound in the absence of competing ligand.

[3]Numbers in parentheses represent the standard error of the mean.

competition suggest that the deglycosylated Tf binds as well as native Tf. At a 1:10 ratio the deglycosylated Tf demonstrates good competition but is not as competitive as native Tf. The six per cent decrease in molecular weight may make it somewhat less competitive with the larger native molecule, this being more evident at higher competitive ratios. Alternatively, removing the carbohydrate from the molecule may affect the charge of the molecule causing it to adhere to non-receptor sites or other protein molecules. Similar studies were performed using murine Tf (Table 12B). There appeared to be no difference in molecular weight between native and deglycosylated murine Tf on an SDS-polyacrylamide gel (data not shown). This suggests that murine Tf has a lower carbohydrate content and therefore the decrease in molecular weight due to deglycosylation was not enough to be seen on a gel. Western blotting, however (Fig. 25B), revealed that murine Tf was successfully deglycosylated. Deglycosylation of the mouse Tf did not improve its binding to EL4-1 cells; it was still not competitive with human Tf in competition assays (Table 12B).

These results suggest that the carbohydrate chain does not have a role in either receptor recognition or binding of Tf to the tumor cell receptors. The carbohydrate chain may have a role in labelling Tf for secretion from hepatocytes or in protecting the molecule from proteolytic degradation. Urushizaki *et al.* (1984) found using Scatchard plot analysis that there was no difference in binding of sialo- and asialo-Tf to the Tf receptors of K562 cells. Kornfeld (1968) removed all the sialic acid residues from human Tf and found that this treatment did not affect the uptake of Fe by rabbit reticulocytes. The carbohydrate was further removed enzymatically using beta-galactosidase, alpha-mannosidase, and beta-N-

TABLE 12B.

Effect of native murine and deglycosylated murine transferrin on the binding of radiolabelled human transferrin to EL4-1 cells[1].

Ratio of labelled to unlabelled Tf	1:1	1:10
Competing Ligand[2]		
Native human Tf	65.9 (0.9)[3]	14.9 (1.8)
Native murine Tf	103.0 (1.5)	92.4 (0.4)
Deglycosylated murine Tf	102.2 (0.7)	102.3 (1.5)

[1]EL4-1 cells were cultured and prepared for the assay and the assay was carried out as described in Table 12A.

[2]Values represent labelled Tf bound in the presence of competing Tf as a percent of labelled Tf bound in the absence of competing ligand.

[3]Numbers in parentheses represent the standard error of the mean.

acetylglucosaminidase. Tf preparations which had 39 and 47% of their remaining sugar residues removed were able to donate Fe to reticulocytes in a normal fashion. It was also found that isolated heterosaccharide chains did not interfere with the ability of Tf to donate Fe to reticulocytes. Hemmaplardh and Morgan (1976) treated rabbit reticulocytes with neuraminidase in order to release 30-50% of the membrane sialic acid. This treatment had no effect on Fe or Tf uptake or incorporation of Fe into heme. The authors concluded if sialic acid is present on the Tf receptor it is not important in its interaction with the Tf protein.

In very recent studies, Reckhow and Enns (1987) incubated A431 human carcinoma cells with tunicamycin, an antibiotic which inhibits glycosylation; this prevented formation of disulfide bonds between the two receptor subunits. The resulting monomeric unglycosylated receptor could not bind Tf and was not transported to the cell surface. Thus removal of receptor carbohydrate from receptors already *in situ* and preventing initial glycosylation have very different effects. The results of Reckhow and Enns suggest that the carbohydrate labels the receptors for translocation to the cell surface and that the carbohydrate chains are important in inserting the receptor in the membrane in the correct orientation. The disulfide bonds are probably important in stabilizing the proper receptor conformation.

4.4 Binding of transferrin to *Neisseria meningitidis*

4.4.1 Rationale for binding studies

The pathogen *Neisseria meningitidis* can obtain Fe from Tf, not by the production of siderophores and outer membrane siderophore receptor proteins but by binding Tf directly (Archibald and DeVoe, 1979). Holbein (1980), in the mouse model of meningococcal infection, demonstrated that the hypoferremic response was important in controlling *N. meningitidis* infection. Subsequently, Simonson *et al.* (1982) demonstrated that when *N. meningitidis* was presented with 30% saturated Tf or apo-Tf at physiological concentrations the same number of Tf molecules were bound. These authors also showed that a twenty-fold excess of apo-Tf inhibited Fe uptake from 30%-saturated Tf by over 90%.

The results of these authors together with the results obtained in this work which suggest that the hypoferremic response has little inhibitory effect on tumor cell Fe acquisition led to the following hypothesis. The hypoferremic response is effective in limiting infection with *N. meningitidis* and has little effect on the growth of eucaryotic tumor cells because of the difference in the ability of the two receptor systems to distinguish between ferric and apo-Tf. This ability is very important in the case of tumor cells which have a very high affinity for diferric Tf and a much lower affinity for apo-Tf and it is suggested that the procaryotic neisserial receptors are less able to discriminate between Fe-bearing and Fe-free Tf. Thus in the case of meningococcal infection host hypoferremia is beneficial in opposing the pathogen.

In the next phase of the work, several aspects of the binding of Tf to the pathogen *N. meningitidis* were examined.

4.4.2 Plate assays

Initially, it was verified that this bacterium could utilize Fe from Tf when Fe-starved and that the non-pathogen *Neisseria subflava* could not. This was done using plate assays in Mueller-Hinton agar (MHA) containing ethylene-diamine-di-ortho-hydroxyphenyl acetic acid (EDDA) to sequester medium Fe. The cultures were plated by the spread plate method. Wells were made in the agar and charged with various amounts of Tf having 100%, 50%, 25% or 0% Fe saturation and then plates were incubated. The results are shown in Fig. 26A and B and Table 13. In Table 13, results are expressed as the area of the zone of growth or growth inhibition. *N. meningitidis* was able to grow on unsaturated Tf (50%, 25%) while these unsaturated Tf's caused growth inhibition of the non-pathogen *N. subflava*, the area of growth inhibition being proportional to the degree of unsaturation of the protein. It was also found, using similar plate assays, that the meningococcus could obtain Fe from 50% saturated Lf but not from 50% saturated conalbumin. On the other hand, *N. subflava* was growth inhibited by both unsaturated proteins. *N. meningitidis* could utilize bovine Tf Fe but only at higher Tf levels than for human Tf suggesting that *N. meningitidis*, a human pathogen, has a preference for human Tf.

Mickelsen and Sparling (1981) examined 21 strains of *N. meningitidis*, 29 strains of *N. gonorrhoeae* and 45 commensal neisserial strains and found that all of the strains of pathogenic neisseriae could obtain Fe from 25%-

Fig. 26A Photographs of plate assays demonstrating the nutritional availability of Fe from various transferrins for *N. meningitidis*. Mueller-Hinton agar plates contained 3 umol EDDA $umol^{-1}$ Fe.

Top:

Well 1-286 ug human Tf 100% saturated

Well 2-571 ug human Tf 100% saturated

Well 3-571 ug human Tf 50% saturated

Well 4-571 ug human Tf 0% saturated

Well 5-571 ug human Tf 25% saturated

Bottom:

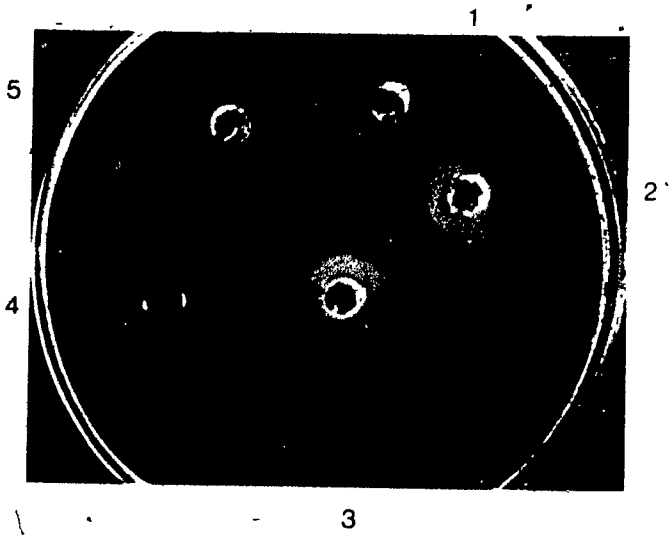
Well 1-286 ug human Tf 50% saturated

Well 2-286 ug conalbumin 50% saturated

Well 3-143 ug conalbumin 100% saturated

Well 4-286 ug human Lf 50% saturated

Well 5-143 ug human Lf 100% saturated


1
2
3
4
5

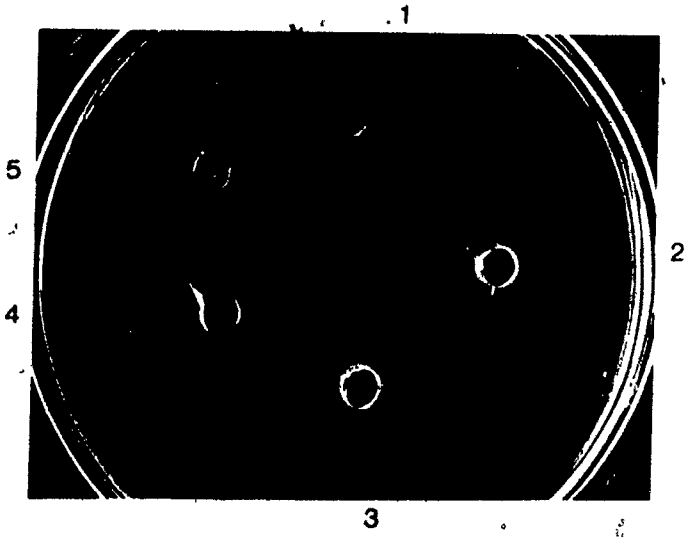

1
2
3
4
5

Fig. 26B Photographs of plate assays demonstrating the nutritional availability of Fe from various transferrins for *N. subflava*. Mueller-Hinton agar plates contained 2 umol EDDA $umol^{-1}$ Fe.

Top:

Well 1-286 ug human Tf 50% saturated

Well 2-143 ug human Lf 100% saturated

Well 3-286 ug human Lf 50% saturated

Well 4-143 ug conalbumin 100% saturated

Well 5-286 ug conalbumin 50% saturated

Bottom:

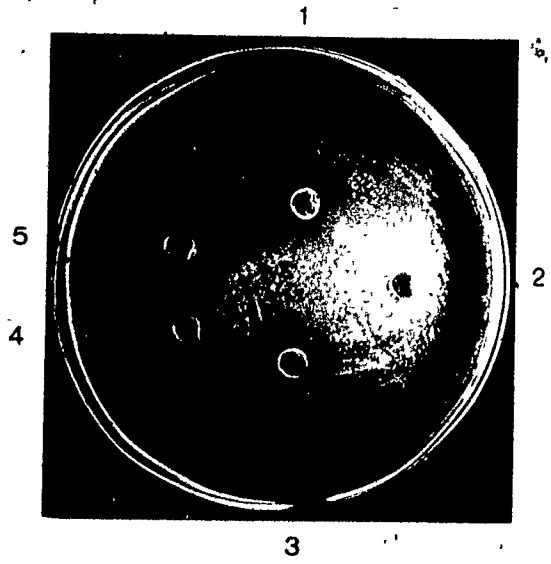
Well 1-286 ug human Tf 100% saturated

Well 2-571 ug human Tf 100% saturated

Well 3-571 ug human Tf 0% saturated

Well 4-571 ug human Tf 25% saturated

Well 5-571 ug human Tf 50% saturated


1
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3
4
5

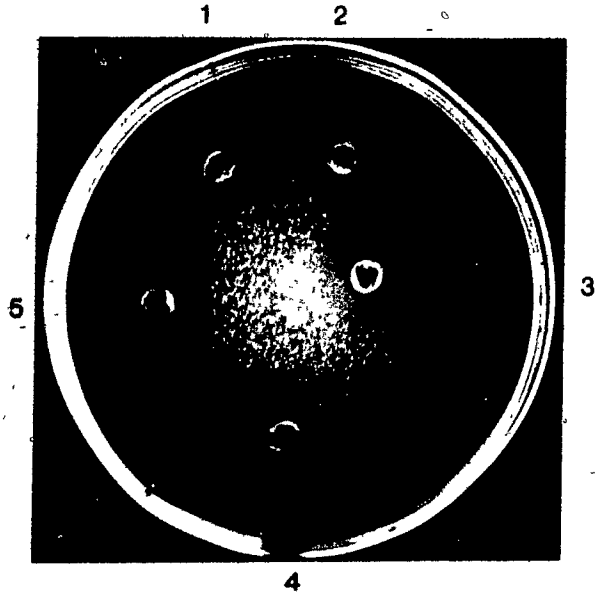

1
2
3
4
5

TABLE 13.

Nutritional availability of Fe bound to various transferrin proteins to *N. meningitidis* and *N. subflava*.

Type of Tf used	total ug protein per well	% Fe saturation	total ug Fe per well	growth response (area mm^2) *N. meningitidis*	*N. subflava*
Human Tf	286	100	0.4	170-E[2]	0
Human Tf	571	100	0.8	200-E	0
Human Tf	571	50	0.4	200-E	90-I[3]
Human Tf	571	25	0.2	200-E	180-I
Human Tf	571	0	0.0	0	240-I
Human Tf	286	50	0.2	140-E	240-I
Human Lf	143	100	0.2	40-E	0
Human Lf	286	50	0.2	40-E	160-I
Conalbumin	143	100	0.2	80-E	0
Conalbumin	286	50	0.2	0	240-I
Human Tf	286	50	0.2	260-E	
Bovine Tf	286	50	0.2	0	
Bovine Tf	571	50	0.4	100-E	
Bovine Tf	1142	50	0.8	130-E	

[1]Cells from cultures grown on MHA plates were suspended in MHB containing 7

ug ml^{-1} EDDA and plated on MHA plates containing 2.0 umol EDDA $umol^{-1}$ Fe for *N. subflava* and 3.0 umol EDDA $umol^{-1}$ Fe for *N. meningitidis*. Cultures were plated by the spread plate method. Wells of approximately 5 mm in diameter were made in the agar, then the desired amount of Tf was added. Plates were incubated at 37° C with 5% CO_2. *N. meningitidis* plates were incubated for 18 h, except for the plate with bovine Tf which was incubated 46 h, *N. subflava* plates were incubated 30 h.

[2]Growth enhancement.

[3]Growth inhibition.

saturated Tf but only 22% of the non-pathogenic neisseriae could do so. Simonson *et al.* (1982) found that *N. sicca* and *N. flava* could not take up Fe from 30%-saturated ^{59}Fe-Tf even when these cultures were starved for Fe. Mickelsen and Sparling (1981) also found that none of the neisseriae strains tested could obtain Fe from 25% Fe-saturated conalbumin. Mickelsen *et al.* (1982) found that all *N. meningitidis* strains tested could utilize Lf Fe for growth but only 53% of *N. gonorrhoeae* strains and 24% of commensal strains tested could do so. An important question arose from the results of the plate assays: can *Neisseria subflava* not utilize Tf-Fe because it cannot bind to Tf or because it is unable to remove Fe from the protein?

4.4.3 Enumeration of *Neisseria meningitidis* transferrin binding sites

In this phase of the work, 4° C binding studies were performed with *N. meningitidis* in order to determine the number of cellular Tf binding sites and their Tf dissociation constant (K_d) as was done for the EL4-1 cells (section 4.3.1). In an initial experiment, this was done in a manner identical to that used for the EL4-1 cells using very low ligand concentrations. A Scatchard plot revealed 14 molecules of Tf bound per cell (Fig. 27). This value for the number of binding sites seemed extremely low If the affinity of the neisserial receptor is not as high as in the eucaryotic system perhaps higher concentrations of Tf are required to obtain significant binding. The experiment was repeated using higher available Tf concentrations (Fig. 28). The average linear range for these Scatchard plots was from 2 to 63 ug. The number of binding sites per cell was 252

Fig. 27 A Scatchard plot of binding of radiolabelled human diferric transferrin to *N. meningitidis* cells at 4° C. *N. meningitidis* cells were grown in MHB then harvested and resuspended in RPMI with 0.1% BSA. Cells (2.6×10^9) were added to microtiter wells containing various levels of ^{125}I-labelled human Tf as described in Fig 23 for EL4-1 cells. Binding proceeded for 30 min at 4° C.

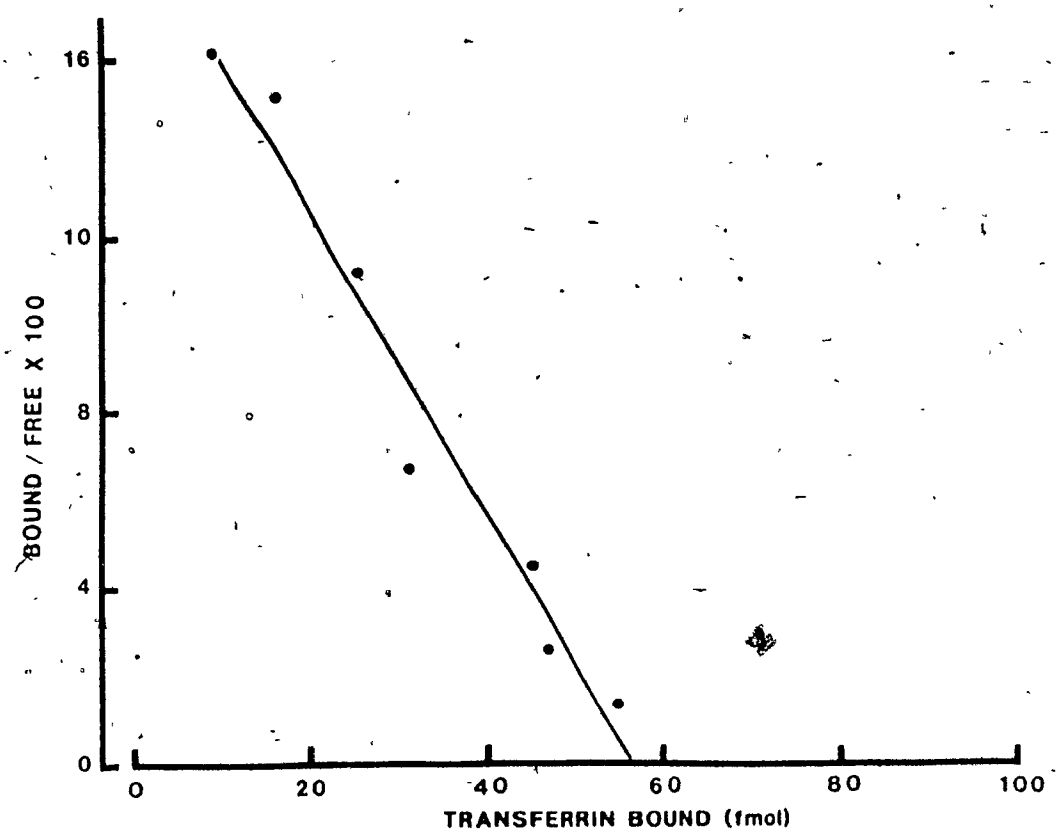
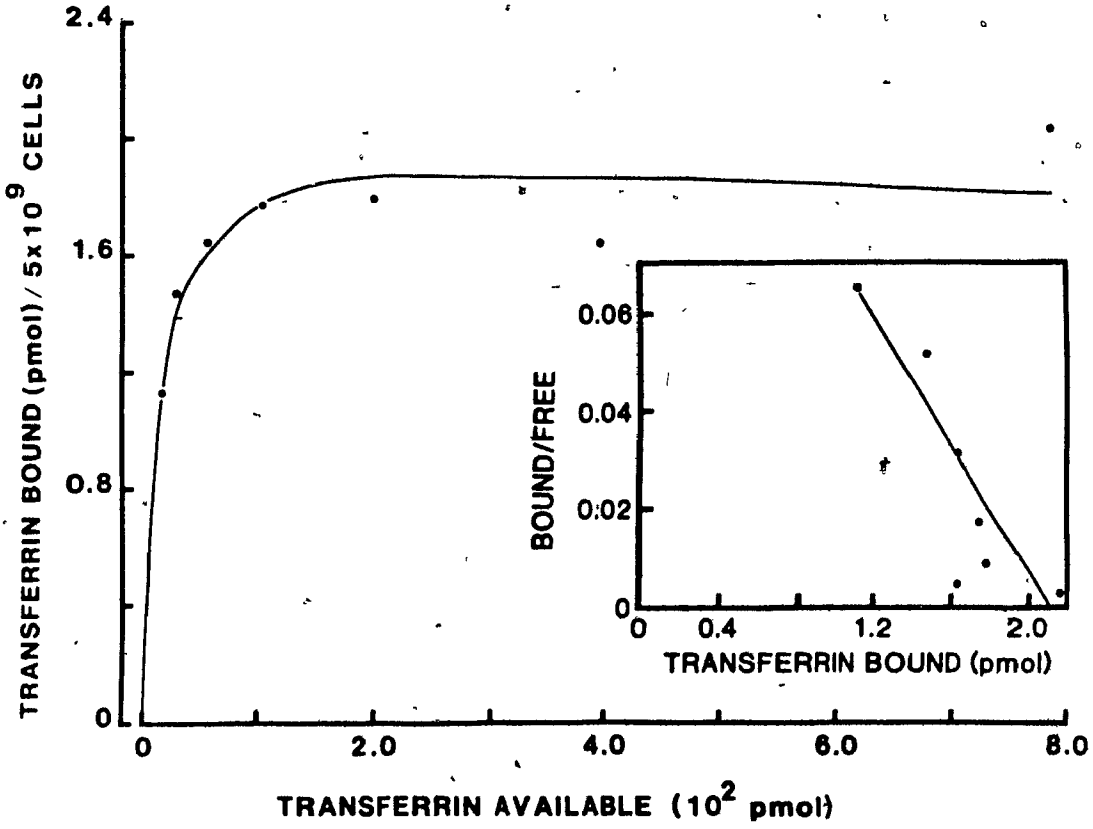

16
10
8
4
0
BOUND / FREE X 100
0
20
40
60
80
100
TRANSFERRIN BOUND (fmol)

Fig. 28 Binding of radiolabelled diferric human transferrin to *N. meningitidis* cells at 4° C as a function of available transferrin concentration *N. meningitidis* cells were grown in MHB, harvested and washed in NDM with 0.1% BSA. Cells (5×10^9) were added to microtiter wells containing different amounts of labelled Tf in NDM with BSA at 4° C. Binding proceeded for 30 min at this temperature. Data points represent the average of 4 experiments. The Tf concentration on the X-axis represents Tf available in pmol in a final volume of 200 ul. This would represent 0-4000 nM available Tf. Inset: A Scatchard plot of the above data.


2.4
1.6
0.8
0
TRANSFERRIN BOUND (pmol) / 5x10^9 CELLS
0
2.0
4.0
6.0
8.0
TRANSFERRIN AVAILABLE (10^2 pmol)
0.06
0.04
0.02
0
BOUND/FREE
0
0.4
1.2
2.0
TRANSFERRIN BOUND (pmol)

and the K_d was 7.5 X 10^{-8} M. Most of the experiments with *N. meningitidis* were performed at 4° C in order that comparisons with the tumor cell work could be made. Also in these studies a relatively high cell number was used (5 X 10^9 cells per well); by performing assays at 4° C problems due to cellular autolysis and production of metabolic acid by the cells were prevented.

In order to evaluate the effect of Fe deprivation on Tf binding to the meningococcal cells, similar studies were carried out comparing Fe-sufficient and Fe-deprived cells grown in MH-EDDA for 4 h (Table 14 and Fig. 29). It was found that the number of binding sites was significantly higher in Fe-deprived as compared to Fe-sufficient cells (Student's t-test $t=-3.54$, $p < 0.05$). The K_d's of the two groups were not, however, significantly different.

Although 356 Tf binding sites is significantly higher than 136 it might be questioned whether 356 is significantly higher than 252 binding sites. There is a certain degree of variation in the number of Tf binding sites per cell from experiment to experiment. This results from some variation in the cells themselves and amount of binding varies with the age of the labelled Tf used. Thus only Fe-deprived and Fe-sufficient cells which have been prepared and assayed at the same time can be compared. This was done in the above experiments and it was demonstrated that there was a significant difference in the number of Tf binding sites between Fe-deprived and Fe-sufficient meningococcal cells.

Simonson *et al.* (1982) measured binding of ^{125}I-labelled 30% Fe-saturated Tf at a concentration of 2.5 mg ml^{-1} under Fe-sufficient and Fe-deprived conditions and found that 4.5 X 10^4 and 4.6 X 10^4 molecules of Tf

TABLE 14.

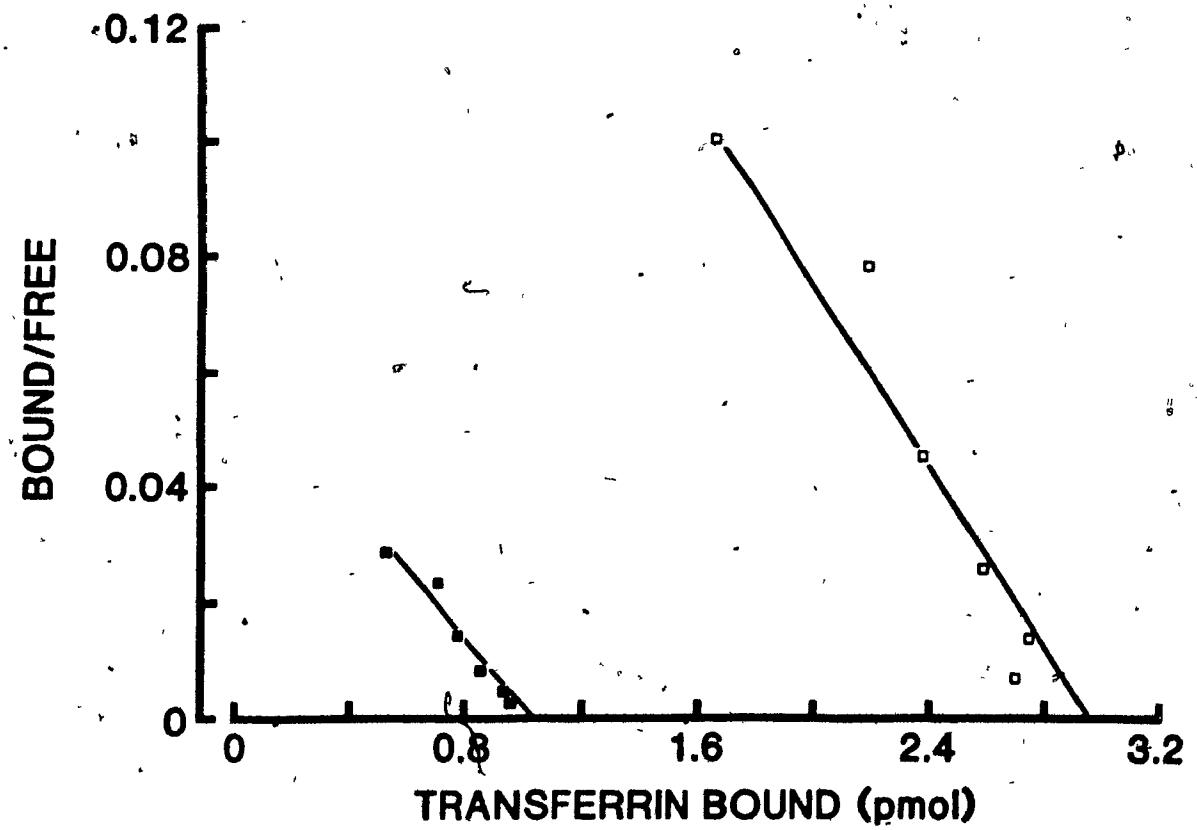
Binding of human transferrin to Fe-sufficient and Fe-deprived *N. meningitidis*[1].

Fe-Sufficient Cells		Fe-Deprived Cells	
Binding Sites per Cell	K_d	Binding Sites per Cell	K_d
136 (11.5)[2]	9.7×10^{-8}	356 (61.0)	7.0×10^{-8}

[1] *N. meningitidis* was cultured in MHB for 16 h. Aliquots of this culture were centrifuged and washed once in either MHB or MHB with 3.0 umol EDDA $umol^{-1}$ Fe before inoculation into these same media. Cells were incubated for 4 h at 37° C (5% CO_2, 100 rpm) then harvested by centrifugation. Subsequently, the cells were washed in NDM with 0.1% BSA (4° C) and resuspended in this medium at the same temperature. Cells were added to microtiter wells prepared as follows. Four mg unlabelled diferric Tf was added to well one in NDM containing 0.1% BSA. This was serially diluted two-fold in this same medium to well 12. 0.5 ug ^{125}I-labelled diferric Tf was then added to each well and the contents were mixed. Binding proceeded at 4° C for 30 min; cells were harvested as described in section 3.10.3.

[2] Numbers in parentheses represent the standard error of the mean. Values are the average of three separate experiments.

Fig. 29 Scatchard plots of binding data for Fe-sufficient and Fe-deprived *N. meningitidis* cells. *N. meningitidis* was cultured for 4 h in MHB or MHB containing 3 umol EDDA per umol Fe to obtain Fe-sufficient (■) and Fe-deprived (□) cells respectively. Cells were harvested and binding was performed as in Fig. 28. The abscissa intercepts represent pmol Tf bound per 5 X 10^9 cells. The data is averaged from 3 experiments.


0.12
0.08
0.04
0
BOUND/FREE
0
0.8
1.6
2.4
3.2
TRANSFERRIN BOUND (pmol)

respectively were bound per cell. This is a much higher level of binding than obtained in the present studies but the linear Tf concentration range for the Scatchard plot was much lower 3.9-0.13 uM as opposed to 31.25 uM. This further suggests that the amount of Tf bound is dependent on the available Tf concentration. There are two possible explanations for the differing results concerning Fe-deprivation in these two studies. Perhaps the enhanced effect on binding is evident only at the lower ligand concentrations used in the present studies. Also in these studies, 18 ug ml^{-1} EDDA was added to the medium while 7 ug ml^{-1} were used in the studies of Simonson *et al.* (1982). The higher concentration of EDDA used in the present studies may have caused increased Fe-deprivation which resulted in enhanced binding.

Tf binding to the meningococcus was subsequently examined at physiological Tf levels in a microassay at both 4° C and 37° C to ascertain whether temperature had an effect on the amount of Tf bound. Cells (1.2 X 10^9) were added to either 200 ul NDM or 200 ul NDM with 0.1% BSA containing 500 ug Tf. Binding was terminated after 30 min incubation of cells (100 rpm) by adding the cells to a 20-fold excess of unlabelled diferric Tf (Table 15) Fewer molecules of Tf were bound at 37° C than at 4° C; perhaps at the higher temperature Tf leaves the receptor more rapidly. The addition of 0.1% BSA to the medium resulted in a substantial decrease in the number of molecules of Tf binding to the cells. This suggests that in the absence of BSA there is a component of non-specific binding of Tf to the cells. Compared to the results obtained with Scatchard analysis, roughly ten times more Tf was bound using the higher physiological levels of Tf. This higher level of total binding probably represents the sum of

TABLE 15.

Binding of transferrin to *N. meningitidis* at 4° C and at 37° C[1].

	4° C		37° C	
	pmol Tf bound per 1.2 X 10^9 cells	binding sites per cell	pmol Tf bound per 1.2 X 10^9 cells	binding sites per cell
NDM	8.6 (0.4)[2]	4330 (221)	6.7 (0.4)	3512 (175)
NDM	8.5 (0.9)	4228 (439)	5.7 (0.4)	2860 (192)
NDM+BSA	5.2 (0.7)	2662 (353)	3.8 (1.6)	1879 (822)

[1]*N. meningitidis* was cultured in MHB for 16 h. Cells were harvested and washed as described in section 3.10.2 and resuspended in either NDM or NDM with 0.1% BSA. At zero time 1.2 X 10^9 cells were added to vials containing physiological levels of radiolabelled human Tf in a final volume of 200 ul of NDM or NDM with BSA. Cells were incubated for 30 min at either 37° C or 4° C (100 rpm). Binding was terminated by adding the cells to micro-centrifuge tubes containing a 20-fold excess of unlabelled diferric Tf.

[2]Numbers in parentheses represent the standard error of the mean.

high affinity binding sites revealed by Scatchard analysis and perhaps a population of lower affinity binding sites. It is possible that Tf may bind with a lower affinity to other cellular binding sites, for example, there might be a certain degree of cross-reactivity with the cellular Lf binding sites (see Table 16). This would only be evident at higher Tf concentrations as Scatchard analysis reveals only high affinity binding sites. In performing the Scatchard analysis (Fig. 28) non-specific binding was corrected for using the method of Chamness and McGuire (1975). However, with physiological Tf levels even in the presence of 0.1% BSA there is probably some degree of non-specific binding of Tf to the cells in the absence of the many other serum proteins found *in vivo* which may further compete for non-specific binding to the cells. This number of binding sites is still lower than that found by Simonson *et al.* However, several aspects of the present experiments and those of Simonson *et al.* were different and might account for the differences in the number of binding sites. The degree of Tf Fe saturation was 100% in these studies and 30% in the earlier studies of Simonson *et al.* The method of iodination was different in the two studies and the iodinated proteins were prepared to different specific activities. In the studies of Simonson *et al.*, BSA or other serum proteins were not included in the medium to minimize non-specific binding. Therefore some of the binding observed may have been non-specific. The possibility that the organism itself had changed somewhat by subsequent *in vitro* passage also cannot be ruled out. The important point is that both studies indeed demonstrate that *Neisseria meningitidis* does directly bind Tf and this is how Fe is obtained.

Similar binding studies were performed with both *N. subflava* and *E.*

coli. In both cases there was no specific binding of Tf to the cells as there was for *N. meningitidis*. With both *E. coli* and *N. subflava* binding studies were performed as for *N. meningitidis* in microtiter wells in NDM containing 0.1% BSA by adding 4 mg unlabelled diferric Tf to well one and performing serial two-fold dilutions to well 12. Subsequently, 0.5 ug ^{125}I-labelled diferric Tf was added to each well and mixed with the unlabelled Tf. With both *E. coli* and *N. subflava* the number of counts bound to the cells in each well varied randomly from well to well. There was no increase in binding of radiolabelled Tf with decreasing concentration of unlabelled ligand suggesting no competition for a common binding site as there was for *N. meningitidis*. In the case of *N. subflava*, the number of counts per well ranged from 44 to 82 cpm above the counter background (approximately 30 cpm). For *E. coli*, counts varied from 58 to 250 cpm above background. With both these organisms this small degree of binding was interpreted to represent non-specific binding to the cells. It would not be expected that *E. coli* would be able to bind Tf as this organism has evolved other high-affinity Fe acquisition mechanisms involving the synthesis of siderophores and corresponding outer membrane siderophore-receptor proteins. As discussed in section 2.9.1, siderophores have a high affinity for Fe and are thus able to remove Fe from Tf and transport it to the bacterial cell.

These findings are interesting as they reveal a major difference between the pathogenic *Neisseria meningitidis* and the commensal *Neisseria subflava*, the ability to bind and obtain Fe from the serum protein Tf. Presumably, this is because the pathogen has the ability to synthesize outer membrane Tf receptor binding sites and the non-pathogen lacks this

ability. This might therefore be an important characteristic of virulence for *N. meningitidis*.

4.4.4 Time course binding

Time course binding studies were performed with the meningococcus at 4° C only (Fig. 30). Binding was found to be rapid as maximal binding was achieved after 5 min. A zero time point was not included; it was not possible to accurately measure zero time binding as cells had to be removed from the wells into microcentrifuge tubes, centrifuged and washed. The earliest time point that could accurately be measured was two min.

4.4.5 Competitive binding assays

Competitive binding assays were performed on the meningococcus in a manner similar to that used for the tumor cells except that in the control wells 0.5 ug of labelled diferric Tf was mixed with 69.5 ug of unlabelled diferric Tf. This concentration of ligand was used to ensure saturation of binding sites. Competing proteins in test wells included diferric and apo human Tf, human Lf and conalbumin added in concentrations of 70 or 700 ug. Competition assays were performed on both Fe-sufficient and Fe-deprived cells (Table 16) and similar results were obtained in both cases. The results for human diferric Tf were as expected, the addition of an equivalent amount of unlabelled diferric Tf resulted in close to 50% inhibition of binding. Apo-Tf offered as much inhibition as did diferric Tf. This suggests that neisserial receptors do not discriminate between ferri and

Fig. 30 Time course binding of diferric human transferrin to *N. meningitidis* cells at 4° C. Cultures of *N. meningitidis* cells were prepared as described in Fig. 28. Cells were added to wells containing 70 ug ^{125}I-labelled human Tf in NDM containing BSA in a final volume of 200 ul at 4° C. The Tf concentration was therefore 350 ug ml^{-1} or 4.4 uM. Binding was terminated at the indicated times.

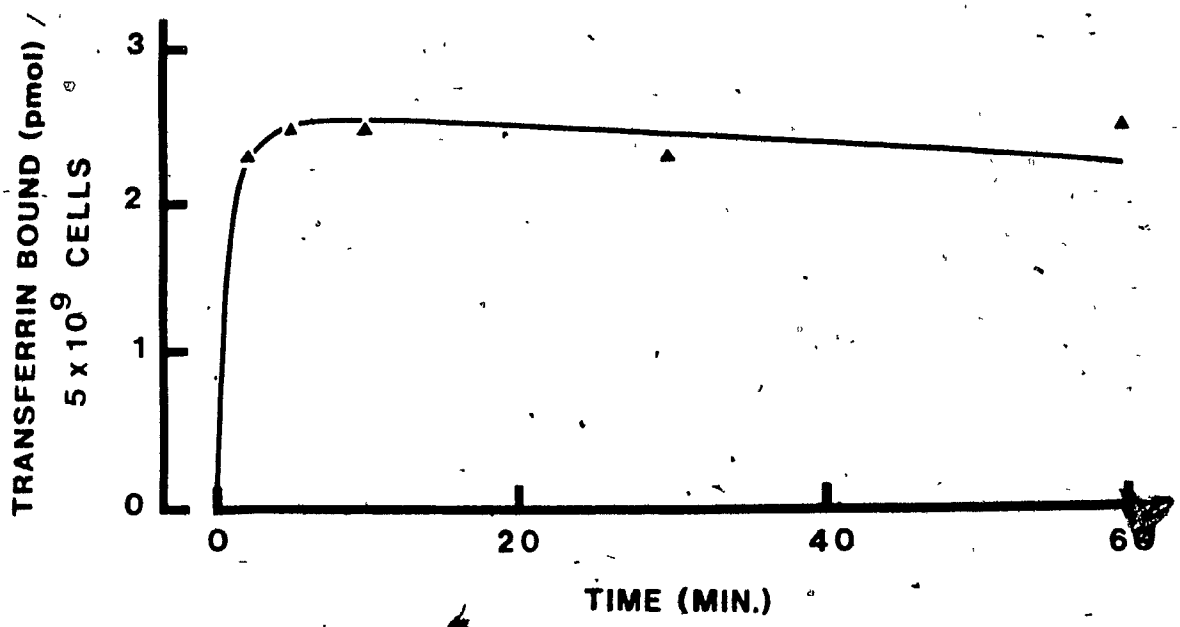

TRANSFERRIN BOUND (pmol) / 5 x 10^9 CELLS
3
2
1
0
0
20
40
60
TIME (MIN.)

TABLE 16.

Effect of human transferrin, lactoferrin and conalbumin on the binding of radiolabelled human transferrin to *N. meningitidis* grown under Fe-sufficient and Fe-deprived conditions.[1]

Ratio of labelled to unlabelled Tf	Fe-sufficient Cells	Fe-deprived Cells
Competing Ligand[2]		
1:1 Human Tf	52.8 (3.1)[3]	52.6 (4.7)
1:1 Human Apo-Tf	50.9 (10.1)	
1:10 Human Tf	13.2 (3.2)	8.9 (1.3)
1:1 Human Lf	85.7 (5.9)	86.6 (2.1)
1:10 Human Lf	83.7 (10.3)	85.4 (2.7)
1:1 Conalbumin	73.4 (3.9)	
1:10 Conalbumin	86.6 (1.9)	

[1]Cultures were grown and prepared as described in section 3.10.3 and Table 14. Control wells contained 70 ug labelled-Tf in a final volume of 200 ul (350 ug ml^{-1} or 4.4 uM Tf); this concentration represents 11.6% of the physiological Tf concentration. Test wells contained labelled Tf and either an equivalent amount of unlabelled competing protein or a ten-fold excess. Binding was carried out at 4° C for 30 min. With the exception of apo-Tf, all proteins including radiolabelled Tf were prepared to 100% saturation.

[2]Values represent labelled Tf bound in the presence of unlabelled (competing) Tf as a percent of labelled Tf bound in the absence of unlabelled Tf. [3]Numbers in parentheses represent the standard error of the mean.

apo-Tf to the same degree as eucaryotic Tf receptors.

Lf, when added to cells at a concentration equal to that of human Tf, offered about 15% inhibition. A ten-fold greater concentration, however, offered no further competition. The results of the plate assays demonstrated that these bacteria are able to utilize Lf Fe. The binding results suggest that there are two separate sites or binding systems for Tf and Lf. Since Lf did offer 15% inhibition to Tf binding there must be a certain degree of cross-reactivity i.e. Lf can bind to some of the Tf binding sites but further addition of Lf caused no more binding to these few sites. Alternatively, when Lf is bound to its specific sites on the cell this may cause steric inhibition of Tf binding to a small proportion of its binding sites. Mickelsen *et al.* (1982) examined Fe acquisition in *N. gonorrhoeae* and found that all gonococcal strains studied could obtain Fe from Tf but only certain ones could obtain Fe from Lf. These authors therefore also suggested that two independent Fe acquisition systems are involved in obtaining Fe from Tf and Lf. Conalbumin also appeared to be competitive in our assays although according to results from our plate assays this bacterium cannot obtain Fe from this protein. Conalbumin may have caused inhibition of Tf binding by some form of steric hindrance or inhibition rather than by specific occupation of cellular binding sites. It is also possible that the binding of conalbumin represents a lack of specificity in the binding of Tf to neisserial receptors.

5.GENERAL DISCUSSION, SUMMARY AND CONCLUSIONS

5.1 Introduction

In any disease process such as infection, cancer, or other form of chronic disease, the response or lack of response on the part of the host is of great importance. Profound changes in host Fe metabolism occur due to inflammation, infection, and malignancy. One of the major changes is the occurrence of the hypoferremic response. It has been established for some time that this response is beneficial during infection and has been regarded as a host response to deny invading pathogens necessary Fe, a concept termed nutritional immunity. This form of nutritional immunity is very effective in helping to control *Neisseria meningitidis* infection in mice. As this organism obtains Fe by binding Tf directly as tumor cells do, it had been suggested that hypoferremia might also withhold Fe from tumor cells (Weinberg, 1981; 1984). It was postulated that if this was the case that hypoferremia could be created or augmented as an adjunct to other forms of neoplastic therapy. For example, BCG or *Corynebacterium parvum* might have been useful agents in causing or increasing hypoferremia in an experimental model. Alternatively, as Fe is required for growth, perhaps tumor cell Fe acquisition could be specifically interfered with in some manner.

The EL4 tumor model proved to be an appropriate one for this study. This is a tumor of C57 black mice and Fe metabolism in these mice has been well-characterized in previous studies (Holbein *et al.*, 1979; Holbein, 1980; 1981; Letendre and Holbein 1983; 1984; 1984a). Several manifesta-

tions of clinical neoplastic disease were exhibited in mice bearing this tumor including: hypoferremia, anemia and a decrease in organ weight late in the disease process. As the tumor was an ascites tumor it was convenient to count peritoneal tumor cells to monitor *in vivo* growth and also to transfer cells to tissue culture for *in vitro* growth. The ability of the cells to grow *in vitro* in tissue culture media with human Tf instead of serum was a distinct advantage as cells could be cultured and growth assessed in a simple, well-defined system.

5.2 Initial studies

Initial experiments showed that although there was a marked hypoferremia, its onset was late in the course of disease after much tumor cell proliferation had occurred. If LEM or IL-1 is the triggering agent for the hypoferremic response, stimulation of peritoneal macrophages would be required for the synthesis and release of this compound and the results suggest that this did not occur until the tumor burden was very high. It would appear that it is only when the peritoneal cavity is extremely crowded with tumor cells that an inflammatory response occurs in the peritoneal cavity. As hypoferremia is only one aspect of the entire host inflammatory response this suggests that other aspects of the host response may also be suppressed during neoplasia. Numerous mechanisms have been postulated whereby tumor cells evade the host immune response. Tumor cells, although they do possess distinct tumor-associated antigens, arise from host tissue and may be antigenically similar to other host cells. Tumor cells may shed antigens which can form blocking immune complexes or

malignant cells may release immunosuppressive factors. Invasion of the bone marrow by tumor cells may cause inhibition of production of macrophages and other cells of the immune system. It is not known in this murine ascites cell system which factor(s) account for the late onset of the inflammatory response.

The mechanism of the occurrence of hypoferremia was not examined in these studies. It was assumed that as in infection and inflammation it occurred due to impaired re-entry of senescent erythrocyte-derived Fe into the Tf pool. The cause of anemia in this tumor model was also not explored but was most likely due to the decrease in available Fe delivered to the bone marrow although as stated in section 2.6 there are several other factors which may contribute to anemia during neoplastic disease.

A considerable amount of work has been done to explore the use of monitoring serum Cp levels as a marker for malignant disease. Letendre and Holbein (1984a) suggested that serum Cp levels increase during the convalescent phase of infection following hypoferremia to re-establish normal serum Fe levels. In this tumor model, serum Cp levels rose somewhat early in the disease before the onset of hypoferremia; the reason for this is unknown. The Cp levels in tumor-bearing mice were not significantly different from those of control mice at any of the time points examined. There was no recovery phase to this disease process as there was for meningococcal infection in mice; the serum Fe levels continued to decline until the death of the mice. One could postulate in terms of Letendre and Holbein's theory that as there was no recovery phase there was no host response to increase serum Fe levels and therefore no increase in Cp levels. To further evaluate the role of Cp in Fe metabolism it would be

interesting to monitor serum Cp and Fe levels in an experimental system in which the animals' condition was improved by removal of the tumor or by radio- or chemotherapy. A rise in serum Cp and Fe levels might then be expected to occur.

5.3 A comparison between the EL4 tumor and *Neisseria meningitidis* systems

Further early studies demonstrated that it was difficult to manipulate tumor cell growth *in vivo* by withholding or supplying additional Fe. It was initially believed that it was the late onset of hypoferremia which was important and that if this response could be induced earlier tumor cell growth would be inhibited by Fe deprivation. Elicitation of an early hypoferremia with turpentine, however, had no increased inhibitory effect on tumor cell proliferation. All of the *in vivo* results in this study were much different from those observed with murine meningococcemia (Holbein *et al.*, 1979; Holbein, 1980; 1981; Beaumier *et al.*, 1984) even though in both systems Fe is obtained by direct binding of Tf. In the neisserial system, hypoferremia was much more severe as serum Tf Fe levels were undetectable at 18-24 h post injection (Holbein, 1980) and hypoferremia could sufficiently deprive cells of Fe to cause inhibition of growth. Furthermore, *in vivo* growth of *N. meningitidis* could be enhanced by addition of Fe or inhibited by desferrioxamine or turpentine-induced hypoferremia. In the bacterial system, the onset of hypoferremia is rapid. This suggests that the inflammatory response is elicited immediately. This could be because of the much faster growth rate of the bacterial cells compared to tumor cells

and the fact that the bacterial antigens are immediately recognized by the host as foreign. Although the time of onset of hypoferremia does not appear to be important as far as inhibiting tumor growth is concerned, its late onset emphasizes the point discussed above that during neoplastic disease the host response does not function as it should.

The results observed in the tumor cell and meningococcal systems suggested that there are important fundamental differences in the response of the two cellular systems to Tf. It appeared as if the tumor cells had a unique ability to obtain sufficient Fe under conditions of extremely low Tf saturation. This was verified by subsequent *in vitro* work. These tumor cells could grow on the relatively minute Fe level provided by 6 ug ml^{-1} 10% Fe-saturated human Tf. Fe uptake from 50%-saturated Tf was saturated at 150 nM Tf and Fe which is approximately 1/250 of the normal serum Tf concentration. Furthermore, addition of apo-Tf to mimic hypoferremia did not decrease Fe uptake proportionally to the amount of apo-Tf added. It was therefore demonstrated in three ways: by *in vivo* studies and by both growth and uptake studies that the hypoferremic response is not effective in limiting Fe acquisition by tumor cells.

These results are entirely reasonable in light of what is now known about the eucaryotic Tf cycle. Dautry-Varsat *et al.* (1983) and Klausner *et al.* (1983a) demonstrated that there is a difference in the affinity of the Tf receptor for ferri-Tf and apo-Tf at physiological pH. As the Tf receptor has very little affinity for apo-Tf at this pH it offers very little competition to ferri-Tf for receptor binding. Binding studies with EL4-1 cells demonstrated that the K_d (5.9 nM) for Tf binding was 1/6356 of the concentration of Tf in the serum or 1/100,000 of the concentration of

serum proteins (Dautry-Varsat and Lodish, 1984) revealing an extremely high ligand-receptor affinity. For neisserial receptors to be half occupied 7.5 X 10^{-8} M diferric Tf was required indicating a somewhat lower receptor affinity. Diferric and apo-Tf appeared to be equally competitive in *N. meningitidis* binding studies. Based on the results of these studies and those of Simonson *et al.* (1982) the following hypothesis was suggested to explain the difference in effectiveness of hypoferremia in the two systems. The hypoferremic response is effective in the case of *N. meningitidis* and ineffective in the case of tumor cells because of the difference in the ability of the two receptor systems to distinguish between Fe-bearing and Fe-free Tf. This ability is high in the case of tumor cells and lower in the case of *N. meningitidis*. Therefore when tumor cells are growing *in vivo* and hypoferremia occurs although there is an increase in the number of molecules of apo-Tf present in the serum or ascites fluid the tumor cell receptors, having relatively little affinity for apo-Tf, preferentially bind ferri-Tf. When *N. meningitidis* is growing *in vivo* and hypoferremia occurs the cell receptors, being less discriminating, recognize the protein not the Fe moiety and apo-Tf is bound inhibiting the binding of ferri-Tf.

Fe-sufficient *N. meningitidis* were found to have 252 Tf binding sites per cell which appears extremely low when compared to the number of binding sites found on EL4-1 (1.1 X 10^5 surface binding sites) and other tumor cells. However, the relative sizes of the two types of cells must be considered as meningococcal cells are much smaller than the eucaryotic tumor cells. If the number of binding sites per um^2 of cell surface for the two types of cells is compared, Fe-sufficient meningococci have 164 binding sites per um^2 of surface area and EL4-1 have 126 sites per um^2 of surface

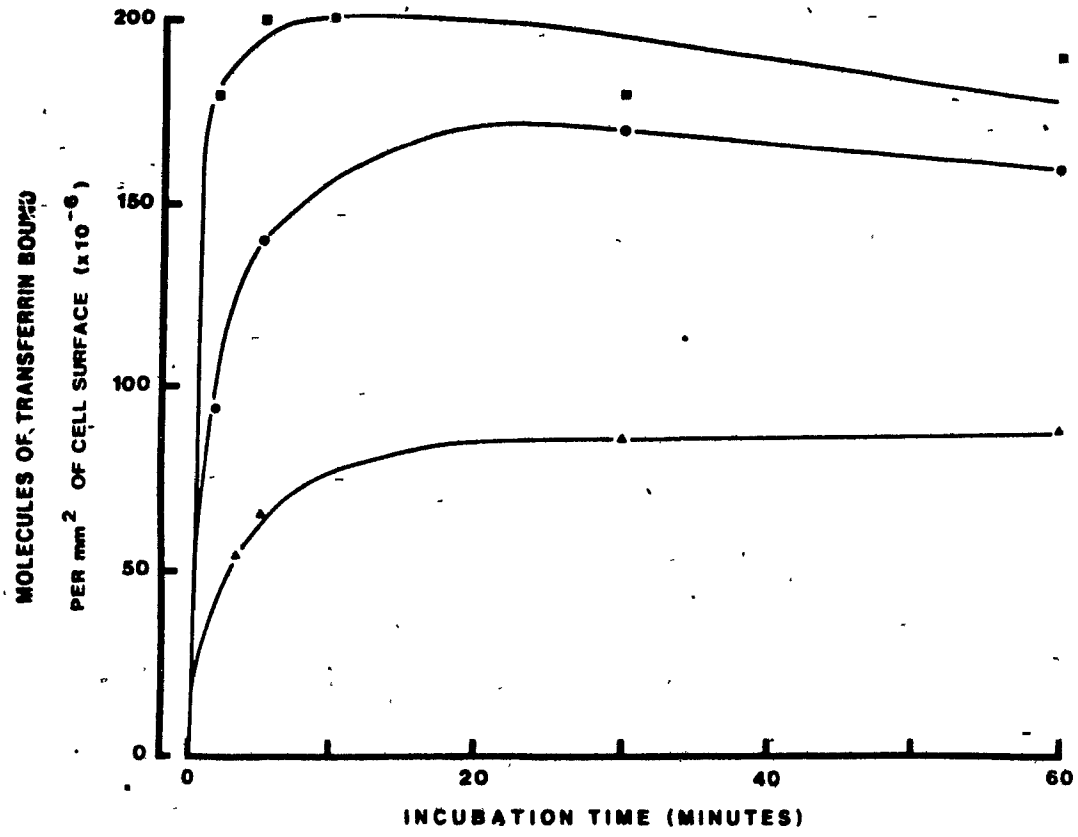
area. Therefore *N. meningitidis* has 30% more binding sites per um^2. To illustrate this, the binding of Tf molecules on the basis of cellular surface area for the two cell types is compared in Fig. 31. The data for this figure were taken from Figs. 24 and 30. A comparison of these binding data for the meningococcus and EL4-1 cells at 4° C in these particular experiments suggests that *N. meningitidis* is binding 113% more molecules of Tf per mm^2 of cell surface area.

5.4 A discussion of the EL4 work and implications for future research

It could be asked whether similar results concerning the hypoferremic response might be expected in other tumor models, for example, in a solid tumor model of neoplastic disease with entirely different growth kinetics. However, in several tumor cell lines studied by others (Dautry-Varsat *et al.*, 1983; Klausner *et al.*, 1983a; Urushizaki *et al.*, 1984) the same principles apply; that is, the Tf receptor has a very high affinity for ferri-Tf and a much lower affinity for apo-Tf. On this basis it could be predicted that similar results would be obtained in other tumor systems.

The fact that tumor cells can utilize other sources of Fe such as hemoglobin and ferritin make it more unlikely that tumor cells growing *in vivo* would be Fe-deprived. Normally, neither hemoglobin nor appreciable ferritin would be available. During malignant disease, especially in the later stages in which both hemolysis of red blood cells and tissue invasion and destruction occur, these sources of Fe would likely become more available. The mechanism of Fe uptake from these two Fe sources was not investigated in this study. It is not known whether these cells possess recep-

Fig. 31 A comparison of the time course of binding of diferric human transferrin to EL4-1 and *N. meningitidis* cells. Time course binding results were taken from Fig. 24 and Fig. 30 for comparison, *N. meningitidis* cells at 4° C (■), EL4-1 cells at 37° C (●) and at 4° C (▲).


200
150
100
50
0
0
20
40
60
INCUBATION TIME (MINUTES)
PER mm^2 OF CELL SURFACE (1x10^{-6})

tors for hemoglobin or whether this compound is taken up by non-specific or fluid phase endocytosis. Ferritin, a large molecule, may have to be broken down at the cell surface before Fe uptake occurs. It was recently reported (Fargion *et al.*, 1987) that certain cell lines, including the human erythroleukemic K562 line, possess specific receptors for the ferritin H subunit. This suggests a mechanism whereby Fe could be obtained from ferritin and it would be interesting to determine whether the murine tumor cells also exhibit ferritin subunit receptors. It is also possible that binding of ferritin to cells may serve a purpose other than Fe transfer such as some form of immune regulation as serum ferritin is generally Fe-poor.

The present studies and those of others have demonstrated clearly that eucaryotic cells have distinct Tf species preferences for growth. Competition binding studies determined that in most cases growth preferences were directly correlated with the binding affinity of a particular Tf for Tf receptors. In general, cells of a particular species have been found to prefer the homologous Tf. The murine tumor cells used in these studies and several human lines examined by others have little affinity for bovine Tf and higher concentrations are required in media for growth. Cells are generally cultured in media containing fetal bovine serum at a concentration of 10% which would provide 300 ug ml^{-1} Tf, a relatively high concentration, which has been shown to be adequate for two human lines (Tsavaler *et al.*, 1986; Penhallow *et al.*, 1986) and would be sufficient for EL4-1 cells.

Conalbumin and Lf do not compete for binding to Tf receptor sites.

Specific Lf receptors, distinct from Tf binding sites, have been reported on phytohemagglutinin-stimulated human peripheral blood lymphocytes (Mazurier *et al.*, 1987). It cannot be ruled out that EL4 cells which are lymphoma cells may also possess specific Lf binding sites; alternatively, these sites may have been lost during the transformation process. Determination of specific Lf binding sites on EL4-1 cells could be made by radiolabelling Lf and measuring binding, as was done for Tf, and then by performing competition studies with unlabelled Lf. It would be interesting to see whether Lf binding sites were present on the EL4-1 cells as well as on mitogen-stimulated murine lymphocytes. If Lf is acting as an Fe source it would have to undergo a different intracellular mechanism for Fe release than Tf as Lf does not release Fe at low pH. Lf may be degraded in the lysosomes and the Fe released in this way. It has been suggested in the case of lymphocytes that Lf may possess some function apart from Fe transfer, for example it may have an immuno-regulatory role (Mazurier *et al.*, 1987). It would be interesting to determine whether malignant lymphocytes still possess these receptors as this could be a possible immune mechanism to which malignant lymphocytes are no longer susceptible but which may control proliferation of normal lymphocytes.

The exception to the rule that cells prefer their homologous Tf in these studies was murine Tf which did not bind as readily as human Tf to EL4-1 and to the other murine cells examined. As mentioned in section 4.3.3, the murine Tf used for these studies appeared to be pure and functional. Ideally, the studies should be repeated with another type of murine Tf to verify the observed murine receptor preference for human Tf. On the basis of the present studies it appears that the three-dimensional

conformation of human Tf has a better "receptor fit" than murine Tf and in competition studies it binds to a greater degree than mouse Tf. Elucidation of the tertiary structure of both Tf's and of the receptor and determination of the sites where ligand and receptor interact would demonstrate why the murine receptor preferentially binds human Tf.

These and other studies indicate that although the carbohydrate moiety is one portion of the Tf molecule that differs from species to species it is not the variation in carbohydrate that causes differences in Tf binding affinities. As the various species of Tf evolved certain critical residues were conserved such as those involved with Fe binding and those forming stabilizing disulfide bridges (Williams, 1982). The differences in the binding affinities of the various Tf's suggest that there have been amino acid changes in the regions of the molecule involved in binding to the receptor. The primary amino acid sequence determines the three dimensional conformation of the molecule and this is what is recognized by the receptor. Determination of the receptor binding domain on the Tf molecule is an important problem in the area of Tf research. The amino acid sequences of human Tf, conalbumin and Lf are now known as is the sequence of the human Tf receptor. The elucidation of the amino acid sequences of other species of Tf and the tertiary structures of the various Tf's would be important steps in answering the question of how ligand and receptor interact. This problem has also been approached in studies in which Tf's have been enzymatically cleaved into N and C terminal halves. Monoclonal antibodies have been made to the entire molecule and to the N and C-terminal moieties and binding behaviour in the presence and absence of the

monoclonal antibodies examined. Work by Brown-Mason and Woodworth (1984) using ovoTf (conalbumin) and chick embryo erythrocytes suggests that both N and C terminal domains of the protein are necessary for binding to occur indicating that both domains contain sequences necessary for receptor recognition.

The results in this thesis which indicate the difficulty in manipulating tumor cell growth *in vivo* by supplying or withholding Fe do not necessarily mean that certain principles of Fe acquisition cannot be used in tumor therapy. Several interesting studies have been performed using monoclonal antibodies to tumor cell Tf receptors. As pointed out by Ward (1987), a serious problem in cancer therapy is directing the therapeutic agent solely to the tumor cells and not to normal tissue and monoclonal antibodies to tumor antigens might be an appropriate means of doing this. For example, Trowbridge and Domingo (1981) demonstrated that monoclonal antibodies to Tf receptors could be coupled to ricin or diphtheria toxins and these conjugates were toxic to tumor cells *in vitro*. It was also found that the monoclonal antibodies alone and the ricin-antibody conjugates inhibited the growth of melanoma cells in nude mice. In another study (Trowbridge *et al.*, 1984), i.v. or i.p. injection of the Tf receptor monoclonal antibody R17 208 had a significant effect on *in vivo* growth of SL-2 murine T-cell leukemia cells. Mice treated with the monoclonal antibody demonstrated enhanced survival and inhibition of the primary tumor at the inoculation site. It has, however, been pointed out that other cells including erythroid cells also have Tf receptors and would be susceptible to these antibodies or antibody-conjugates perhaps resulting in

anemia. Ward (1987) suggested that such anemia might be relatively easy to treat and this form of therapy might involve less toxicity than other forms of anti-neoplastic therapy.

The results presented in section 4.1.3 suggested that initially desferrioxamine injections had an inhibitory effect on EL4 tumor cell proliferation *in vivo*. Lederman *et al.* (1984) demonstrated that when lymphocytes are exposed to desferrioxamine *in vitro* this Fe chelator affects cellular DNA synthesis, it decreases intracellular pools of deoxyribonucleoside triphosphates and blocks cells in early S phase. As the enzyme ribonucleotide reductase requires Fe as a co-factor, these inhibitory effects can be directly attributed to Fe deprivation. This suggests possible applications for use of this drug in neoplastic therapy. Perhaps if continuous infusions of this drug were given, as for Fe overload therapy, tumor cell proliferation could be inhibited by Fe deprivation or tumor cells could be synchronized in early S phase and then other cytotoxic drugs which act on S phase cells could be given to kill this fraction of tumor cells.

During the past several years, much information has been gained concerning eucaryotic cellular Fe acquisition and the endocytic Tf cycle. Several interesting and challenging questions, however, remain to be answered. The problem of elucidating the relative sites of ligand and receptor interaction has already been discussed. Also, it is still not known what triggers Tf-Tf receptor internalization. Binding of ligand appears not to be the trigger as evidence suggests that receptors are internalized in the absence of bound ligand (Watts, 1985). Receptor phosphorylation had been suggested as a possible signal for internalization but

more recent evidence suggests that this is not the case (Rothenberger *et al.*, 1987). It is known that the cytoplasmic domain is required for Tf internalization; perhaps conformational changes in this region of the receptor may be involved. Alternatively there may be intracellular signals or binding factors which act on this domain which cause internalization. Further deletion mutagenesis studies should pin-point the actual part of the cytoplasmic domain needed for internalization. If phosphorylation is not an internalization signal, what is its role, if any, in endocytosis and does its role vary with cellular type or between transformed and non-transformed cells? It has been suggested that Tf-receptor interaction may have a role other than Fe acquisition such as a role in cellular growth regulation (May and Cuatrecasas, 1985). Phosphorylation of the Tf receptor may provide an intracellular signal which is important in this regard. Whatever the role of receptor phosphorylation it is an interesting question to be answered.

Several questions need to be resolved concerning what becomes of Fe intracellularly once it has been released from Tf. Does Fe initially become part of a labile pool; is it bound to a low molecular weight carrier (Jacobs, 1977)? What is the nature of this carrier and is it involved in intracellular Fe transport for example in transporting Fe to the mitochondria for heme synthesis?

Finally, an interesting problem is how different ligands and receptors are sorted intracellularly; what directs certain ligands and /or receptors to the lysosomes to be degraded while Tf and its receptor are kept within distinct vesicles and recycled to the cell surface? The fate of ligands and receptors seems to depend on the role of the ligand. Some provide in-

formation or signals to the cell and these are generally degraded. Other ligands such as Tf provide a necessary nutrient (although as suggested above Tf may have a dual role) and it is certainly an advantage to an organism to have both the receptor and ligand recycled. Information obtained concerning intracellular sorting will not only further elucidate the Tf cycle but will also yield important information about cellular growth and physiology in general.

5.5 A discussion of *Neisseria meningitidis* work and implications for future research

The findings in this thesis confirmed previous findings of others that *Neisseria meningitidis* can utilize Tf and Lf Fe but not conalbumin Fe. It was also observed that this organism had a preference for human over bovine Tf. These studies also confirmed that *N. meningitidis* directly binds Tf (Archibald and DeVoe, 1979; Simonson *et al*, 1982) as it possesses surface binding sites. This distinguishes the pathogenic from the commensal neisseriae as *N. subflava* cannot utilize Tf Fe as it does not possess receptor binding sites. This indicates that possession of these binding sites and the ability to remove Fe from Tf is an important characteristic of virulence. It was also found that *E. coli*, a siderophore-producing bacterium, does not have Tf binding sites.

When one considers bacterial mechanisms of Fe acquisition *in vivo*, it is interesting that the majority of bacteria have evolved siderophores while a minority, such as *N. meningitidis*, *Haemophilus influenzae* and *Bordetella pertussis* (Redhead *et al.*, 1987) have not. This difference can

probably be related to the environment in which the organisms are found. Bacteria such as *E. coli* and *Salmonella* are enteric bacteria and are found in the vertebrate intestine where under certain circumstances they may cause disease. They are also hardy organisms and can survive in soil, sewage and other environments for some time. The ability to synthesize siderophores would enable the organism to obtain Fe *in vivo* or in these external environments. *N. meningitidis*, *Haemophilus* and *Bordetella* are neither ubiquitous nor resistant organisms and cannot live for long outside the human host and therefore are evolved to obtain Fe *in vivo* only. To be able to obtain Fe by binding Tf directly is a definite advantage for a pathogen over the production of siderophores. Siderophores must be released, bind Fe and return it to the cell. This mechanism of Fe acquisition may not be as secure as direct Tf binding as siderophores are diluted by secretion into the blood and obtaining Fe will be directly dependent on the concentration of siderophore attained.

The fact that *Neisseria* and *Bordetella* can utilize Lf Fe presumably because this protein also binds to surface binding sites would provide these pathogens with a another advantage. Lf is found in the mucosal secretions of the naso- and oro-pharynx where these pathogens would initially gain entrance to the body. The function of Lf in this location is to bind Fe and withhold it from invading pathogens. Those bacteria which are able to bind and deferrate this protein would not be inhibited and would be at an advantage in the initial stage of infection. The binding of human Tf or Lf to the bacterial cell might also serve to mask or cover up bacterial surface antigens and reduce the host immune response.

The studies of Redhead *et al.* (1987) demonstrated that *Bordetella per-*

tussis could utilize Tf, Lf and conalbumin for growth. Competitive binding studies suggested that all three proteins bound to the same receptor site. In this respect *Bordetella* differs from *N. meningitidis* as the latter organism cannot utilize conalbumin Fe for growth and the present results and those of Mickelsen *et al.* (1982) with *N. gonorrhoeae* suggest that utilization of Lf Fe involves binding sites distinct from the Tf binding sites. Competition binding studies with *N. meningitidis* indicated that the Tf binding sites do not have the same degree of specificity that the eucaryotic sites have as conalbumin and Lf interfered with Tf binding to a certain extent.

There are numerous intriguing questions which remain to be answered concerning the mechanism whereby *N. meningitidis* removes Fe from Tf. It is interesting that this pathogen can bind Tf when Fe-sufficient but that more binding sites are induced under conditions of Fe deprivation; furthermore, this bacterium reportedly cannot remove Fe from Tf unless it is Fe-deprived (Simonson *et al.*, 1982). Therefore at least two Fe-acquisition components are induced by Fe deprivation: additional Tf binding sites and an Fe-removal mechanism. It is not known what is induced in the cell by Fe-deprivation that enables the cell to remove Fe from Tf. It is not known whether an outer membrane protein apart from the Tf binding site is induced, which is involved in Fe removal or whether periplasmic binding proteins or cytoplasmic membrane components are involved as well. It remains to be determined how the pathogen removes Fe from Tf after binding; perhaps the newly-induced surface component has an affinity for Fe similar to Tf and can remove Fe from this protein or alternatively, a reductive mechanism may be involved. It is not known how Fe acts intracellularly to

cause derepression of this Fe-removal function; perhaps Fe acts as a co-repressor with a regulatory protein to repress this mode of Fe acquisition under Fe-sufficient conditions in a manner analogous to that which occurs during siderophore synthesis (section 2.9.1).

Neisseria gonorrhoeae can also utilize Tf Fe for growth presumably also by directly binding Tf. This could easily be verified by performing binding studies with radiolabelled Tf. It would be interesting to enumerate cellular Tf binding sites and compare this to the number found on *N. meningitidis*.

Herrington and Sparling (1985) found that the human pathogen *Haemophilus influenzae* could also utilize Tf Fe but that the non-pathogenic *H. parainfluenzae* could not, a situation similar to the neisseriae. It could be determined whether this also holds true for other pathogenic and non-pathogenic haemophili. These studies could then be carried further, as was done with *N. meningitidis*, by performing binding studies and enumerating Tf binding sites.

The neisserial Tf binding site has never been isolated or characterized and this is an important area which needs to be explored. To approach this problem, initially outer membrane fractions of Fe-deprived and Fe-sufficient *N. meningitidis* and a non-pathogenic neisseria should be prepared and run on SDS-PAGE to determine which Fe-repressible proteins are present. These proteins could be transferred to nitrocellulose paper by immunoblotting, probed with ^{125}I-Tf and subjected to autoradiography. This procedure might localize the Tf binding protein. Fe-deprived cells, which have more binding sites, could be incubated with radiolabelled Tf and a

cross-linking reagent could be used to bind ligand to receptor. The use of a cross-linking reagent would prevent SDS-mediated dissociation of the Tf and its receptor. Subsequently, the outer membrane fraction could be isolated and run on SDS-PAGE followed by autoradiography. From this an estimate of the receptor molecular weight could be obtained. By running this same material on two-dimensional electrophoresis the components might be further resolved and characterized. Similar studies could be performed with *Bordetella* and *Haemophilus*. Affinity chromatography using Tf coupled to Sepharose might also be useful in isolating the Tf binding site from radiolabelled bacterial outer membrane fractions.

The genetics of bacterial acquisition of Fe from Tf is another important area to be explored. In order to do this, the *N. meningitidis* culture would have to be mutagenized and mutants incapable of utilizing or binding Tf isolated. Using the meningococcal wild type strain, chromosomal DNA could be digested by restriction enzymes, a library of fragments isolated and inserted into suitable *E. coli* compatible cosmids. These could subsequently be inserted into *E. coli* or non-pathogenic neisserial hosts and expression of the genes of interest screened for by growth on Tf *in vitro*. Another approach would be to subject *N. meningitidis* chromosomal DNA to restriction enzyme digestion and insert the fragments into a bi-directional shuttle vector, such as the pLES2 vector described by Stein *et al.* (1983), which can function in both *E. coli* and *N. gonorrhoeae*. The resulting plasmids could then be inserted into an *E. coli* mutant defective in high affinity Fe acquisition systems (i.e. defective in enterochelin biosynthesis and lacking the ColV plasmid carrying the aerobactin genes). The presence of functioning Tf Fe acquisition genes could be screened for by determin-

ing whether the bacteria could grow in Fe-deficient media with Tf as the sole Fe source. (The media must also be deficient in citrate and any siderophore-like compounds.) The presence of novel Fe-repressible outer membrane proteins could be ascertained using SDS-PAGE. Binding of labelled Tf to the cells followed by treatment with a cross-linking reagent could determine which protein is able to bind Tf. Deletion analysis could further map the areas of the fragments required for Tf binding and Fe uptake. Subsequent work including *in vitro* transcription, S1 nuclease mapping and DNA and RNA sequence analysis could be useful for promoter mapping. Use of the S1 nuclease mapping assay could be used to measure the specific RNA levels under conditions of Fe-sufficiency and Fe limitation to investigate transcriptional regulation by Fe. Regulation could also be studied by constructing a protein fusion of the putative promoter region and a promoterless lacZ gene. Beta-galactosidase activity could then be measured under Fe-deficient and Fe-replete conditions.

The use of *E. coli* for this aspect of the work may be appropriate as both the genome and Fe regulation are well-characterized in this organism and mutants defective in high affinity Fe uptake are available. Once Tf Fe acquisition has been characterized in the *E. coli* system, the vector plasmid could be inserted into an *N. meningitidis* mutant defective in Tf Fe utilization to determine whether the genes are still functional in *Neisseria*. This may necessitate insertion of neisserial chromosomal sequences (uptake sequences) into the plasmid in order that the DNA will be taken up by the *Neisseria* (Stein *et al.*, 1983).

It may be difficult, however, to insert the neisserial chromosomal fragments into the vector and secondly, the genes of interest may not be

expressed in the *E. coli* mutant. Alternatively, the gene(s) for the Tf binding protein might be expressed and those for Fe removal from Tf not expressed. Binding in the absence of Fe removal could be studied through the use of fluorescent antibody studies, covalent cross-linkers or radiolabelled Tf binding studies. Preliminary work must, however, be done to determine the feasibility of this approach.

As discussed in section 2.2, Black *et al.* (1986) have isolated a mutant which does not produce a 70,000 dalton Fe-repressible outer membrane protein and cannot grow on Tf or Lf or remove ^{55}Fe from these proteins. It is not known whether this Fe-repressible protein is the Tf binding site or if another protein is involved in Fe removal from Tf and Lf. Further work has been done in laboratory of P. F. Sparling on this aspect of Fe acquisition. The 70,000 dalton protein has been isolated but, as mentioned in section 2.2, the *N. meningitidis* mutant does not appear to be transformation competent and the genetic analysis has not proceeded (D. Dyer, personal communication). The present results indicate that Tf and Lf have separate binding sites, however, another inducible protein such as the 70,000 dalton protein might be involved in Fe removal from both proteins.

The future possibilities for this work are potentially quite important. As has been pointed out by both Black *et al.* (1986) and Redhead *et al.* (1987) these outer membrane Fe-acquisition proteins might prove to be useful in the development of vaccines. It has been extremely difficult in the past to produce an effective vaccine for group B meningococci and if the organism's Fe-binding proteins could be isolated and purified they may be suitable proteins for use as immunizing agents. Many questions remain to be answered concerning how organisms such as the pathogens *Neisseria*,

Bordetella and *Haemophilus* obtain Fe from Tf *in vivo*. These many questions, however, provide scope for exciting future research.

5.6 Summary and conclusions

This study was begun to examine the effect of the hypoferremic response on tumor cell Fe acquisition using a lymphoma of C57 black mice as a model. Although a marked hypoferremic response was elicited in the host during tumor cell growth the response occurred late in the disease and did not appear to benefit the host. Attempts to manipulate tumor cell growth *in vivo* by providing more Fe or withholding Fe were unsuccessful, suggesting that these tumor cells were capable of obtaining sufficient Fe under adverse strongly hypoferremic conditions. Subsequent *in vitro* growth and uptake studies revealed that the lymphoma cells could obtain Fe from Tf when the Fe saturation was lower than would occur *in vivo* during hypoferremia.

Fe acquisition by the lymphoma cells was further examined in terms of Fe requirements for growth, Tf preferences for growth and types of Fe utilized by the cells. Cellular Tf binding to the cells was studied to enumerate Tf binding sites and examine Tf-Tf receptor specificity. Binding of Tf to *Neisseria meningitidis* was also examined to determine why the hypoferremic response is effective in inhibiting Fe acquisition and growth in the neisserial system but not in the tumor cell system. Taken together, the present results and those of Simonson *et al.* (1982) strongly suggest

the following hypothesis to be true. Hypoferremia is not effective in the tumor cell system as the more sophisticated eucaryotic Tf receptors can discriminate between ferri- and apo-Tf as they have high affinity for the former and low affinity for the latter at neutral pH thus the presence of apo-Tf does not interfere with binding. In contrast, the neisserial receptor cannot as readily discern between Fe-bearing and Fe-free Tf and apo-Tf is bound by the receptor inhibiting the binding of Fe-containing Tf thus causing inhibition of growth by Fe deprivation.

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APPENDIX I

Becton Dickinson ^{59}Fe Radioassay Kit

The kit contains an acidic ^{59}Fe reaction solution, sodium carbonate solution, resin tablets and UIBC reference serum. Serum samples are set up in duplicate tubes labelled TIBC and UIBC; 200 ul of serum are added to each tube. Control total and blank tubes are included to which 200 ul of water is added. UIBC reference tubes are also included and 200 ul of reference serum is added to them. Initially 500 ul ^{59}Fe reaction solution is added to all TIBC tubes. As this is an acidic solution any unlabelled Fe bound to serum Tf will be removed. After 10 min, 500 ul of sodium carbonate solution is added to all TIBC tubes, then UIBC tubes then all total and blank tubes. Because of the excess of radiolabelled Fe present in the TIBC tubes, all available Fe binding sites will be occupied with labelled Fe. Subsequently, 500 ul of ^{59}Fe reaction solution is added to all UIBC, blank and total count tubes. Radiolabelled Fe will then bind to all available Fe sites on the serum Tf in the UIBC tubes. After 10 min, resin tablets are added to TIBC and UIBC tubes; this removes any unbound Fe. Tubes are incubated, vortexed and re-incubated then centrifuged in an Eppendorf centrifuge for 10 min. Supernatants are counted for one min in a gamma counter. Calculation of Fe binding capacity is based on the known concentration of exogenous radioactive Fe and the dilution factor. The UIBC reference serum is used to standardize the results. The assigned UIBC reference value supplied with the kit and the counts obtained from the UIBC reference tubes are used to obtain a calculation constant K for each assay. The constant K is used to calculate the UIBC and TIBC for each serum

sample. Serum Fe is the difference between TIBC and UIBC. The percent Fe saturation of Tf is obtained by serum Fe/TIBC X 100%.

APPENDIX II

The API ZYM System

The API ZYM system is a semi-quantitative micromethod for detection of enzyme activities. The system consists of a series of microcupules containing chromogenic enzyme substrates. The addition of the aqueous sample starts the reactions. The EL4-1 cell suspensions were adjusted to contain 7.6×10^6 cells ml^{-1} With a pasteur pipette, 2 drops of cell suspension were added to each cupule. The strips were incubated for 4 h at 37^{o} C. After incubation, one drop of reagent A and one drop of reagent B were added to each cupule and the strip was exposed to light. A colour reaction of 0-5 was assigned by comparison to the colour chart provided. In each reaction, the enzyme substrate is bound to beta-naphthol or beta-naphthylamine. The enzyme, if present, reacts with substrate to form the released product and beta-naphthol or beta-naphthylamine. These 2 latter compounds react with the detector reagents to produce a colour.

Number	Enzyme	Substrate	Colour
1	alkaline phosphatase	2-naphthylphosphate	violet
2	esterase (C4)	2-naphthylbutyrate	violet
3	esterase lipase (C8)	2-naphthylcaprylate	violet
4	lipase (C14)	2-naphthylmyristate	violet
5	leucine aminopeptidase	L-leucyl-2-naphthylamide	orange

6	valine aminopeptidase	L-valyl-2-naphthylamide	orange
7	cystine aminopeptidase	L-cystyl-2-naphthylamide	orange
8	trypsin	N-benzoyl-DL-arginine-2-naphthylamide	orange
9	chymotrypsin	N-glutaryl-phenylalanine 2-naphthylamine	orange
10	acid phosphatase	2-naphthyl phosphate	violet
11	phosphoamidase	Naphthyl AS-Bl-phosphodiamide	blue
12	alpha-galactosidase	6-Br-2-naphthyl-alpha D-galactopyranoside	violet
13	beta-galactosidase	2-naphthyl-beta-D-galactopyranoside	violet
14	beta-glucuronidase	Naphthyl-AS-Bl-beta D-glucuronide	blue
15	alpha-glucosidase	2-naphthyl, 2-D-glucopyranoside	violet
16	beta-glucosidase	6-bromo-2-naphthol-beta-D-gluco-pyranoside	violet
17	N-acetyl-beta-glucosaminidase	1-naphthyl-N-acetyl-beta-D-glucosaminide	brown

18	alpha-mannosidase	6-bromo-2-naphthyl-2-D-mannopyranoside	violet
19	alpha-fucosidase	2-naphthyl-alpha-L-fucopyranoside	violet

Reagent A

Tris (Hydroxymethyl) aminomethane		250	g
Hydrochloric acid (37%)		110	ml
Laurylsulfate (sodium dodecyl sulfate		100	g
Distilled water	q.s.	1000	ml

Reagent B

Fast Blue BB		3.5	g
2-methoxyethanol	q.s.	1000	ml

APPENDIX III

List of Abbreviations

Fe	iron
Tf	transferrin
TMPD	tetramethyl-p-phenylenediamine
RE	reticuloendothelial
Lf	lactoferrin
Cp	ceruloplasmin
LEM	leucocyte endogenous mediator
IL-1	interleukin-1
F.B.S.	fetal bovine serum
PBS	phosphate buffered saline
TIBC	total iron binding capacity
UIBC	unsaturated iron binding capacity
BSA	bovine serum albumin
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
MHB	Mueller-Hinton Broth
MHA	Mueller-Hinton Agar
EDDA	ethylene-diamine-di-ortho-hydroxyphenyl acetic acid
MHB-EDDA	Mueller-Hinton Broth containing EDDA